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/[1] The term 'project' used in this template equates to an 'action' in certain other Horizon 2020 documentation

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1. Explanation of the work and overview of the progress

1.1 Objectives

Syn2Psy is an Innovative Training Network (ITN) that provides PhD-level training to 14 Early Stage Researchers (ESRs), to integrate studies across different levels, to better understand and treat neuropsychiatric disorders. Their scientific contributions will inform novel therapeutic approaches for alleviating this leading cause of disability worldwide.

Syn2Psy scientific aims are to:

1) Identify synaptic mechanisms that are dysfunctional in neuropsychiatric disorders;

2) Generate and characterize human cell-derived and animal models of disease that will inform on neuronal pathways that are disrupted and may be targets for therapies;

3) Test rational therapies and understand how life stress impacts on disease pathways.

Furthermore, by combining the strong scientific know-how of this consortium with the skills of the non-academic partners, our aim was to train new generation of high achieving ESRs and provide them with the transferable skills necessary for thriving careers in a flourishing area. This includes providing experience on drug, clinical trials and technology development, expose the ESRs to clinical research and training in societal awareness and outreach. The combination of interdisciplinary and intersectoral expertise is particularly suited for addressing synapse pathology in neuropsychiatric disorders and creating competitive training to answer a critical societal challenge.

Syn2Psy's objectives were divided in seven Work Packages (WP):

WP1	New models of neuropsychiatric disorders
WP2	Testing the Excitation/Inhibition imbalance hypothesis in neuropsychiatric disorders
WP3	Modulators of neuropsychiatric disorders
WP4	Training and Doctoral Programme plan
WP5	Dissemination & Communication and Exploitation
WP6	Management
WP7	Ethics requirements

The objectives for each WP and a description of the work carried out during the life-time of the project towards the achievement of each listed objective and task within each WP are described in 1.2. This includes an overview of the project results towards the objective of the action in line with Annex 1 to the Grant Agreement, a summary of deliverables and milestones, as well as the details of the work carried out by each beneficiary and partner.

Comment about COVID-19-related impact on Syn2Psy results and achievements

This Final Technical Report refers to the lifetime of the project, from March 2019 to August 2023. The year 2020, from March 2020 up to February 2021, was strongly affected by the SARS-CoV-2 pandemic and subsequent confinement, restrictive measures and inability to travel. This led to the closure of the laboratories, which represented a major disruption in the ESRs' projects at the initial stage (the first 6 to 18 months) of their PhD projects and led to a substantial delay of the individual projects, delay in training, postponement of network-wide events, postponement of deliverables, milestones and secondments, as well as uncertainty regarding the possibility of rescheduling secondments. For more details of the direct impact of the

confinement on the research projects and training in 2020, refer to the document "Confinement period I: Confinement impact on research projects and training", submitted via formal notification on the EC Portal in August 2020.

The consequences of this forced pause were mitigated by adapting the individual projects and modifying the time-line, which led to a substantial recovery of the delay. The implementation of the action was not affected, nevertheless a few of the project objectives (thesis defences, article publication) will be fulfilled with delay.

1.2 Explanation of the work carried out per WP

1.2.1 Work Package 1 – New models of neuropsychiatric disorders

One of the goals of WP1 was the characterization of new genetic models of Intellectual Disability (ID), Schizophrenia (SCZ) and Autism Spectrum Disorder (ASD) at the neurophysiological, morphological and behavioural levels, using models available at CNC and EPFL, which were built based on human genetic data or on the implication of the candidate proteins in crucial pathophysiological pathways. Moreover, this WP aimed to test human-derived neurons and brain organoids as a model for neurodevelopmental disorders, as well as to identify shared pathways and neuronal circuits that are implicated in neuropsychiatric disorders. To accomplish this, studies were conducted to establish the role of Stargazin in psychiatric disorders; to identify the role of specific mitochondrial genes in the development of psychopathologies and to develop and characterize human-derived neurons and brain organoids as models of neurodevelopmental disorders.

The description of the work carried out during the life time of the project towards the achievement of each task defined for WP1 is described below. A detailed description of the experiments and results can be found in Annex 1, including a summary of the exploitable results for each project and how they can be exploited (when applicable).

To establish the role of Stargazin in psychiatric disorders (**Task 1.1**), **ESR1**, **Orsolya Antal**, under the supervision of Ana Luísa Carvalho at CNC, generated a knock-in mouse model carrying the S148N variant of Stargazin, identified in a patient suffering from schizophrenia.

The focus of this project was to establish behavioural phenotypes in this model and to investigate some of the potential underlying neural correlates through the assessment of neuronal morphology and function, as well as some features of metabolism and bioenergetics in the prefrontal cortex (PFC).

The behavioural phenotypes were accessed through a battery of tests aimed at evaluating locomotor activity and some mood-related phenotypes, as well as cognitive changes. Results suggest impairments in associative learning in a contextual fear conditioning and extinction paradigm, in spatial memory in an object displacement test and in spatial working memory in a T-maze test relying on spontaneous alternation.

Subsequently, the morphological and functional characterisation of PFC neurons were analysed. Pyramidal neurons in the PFC were sparsely labelled and overall dendritic complexity was assessed, revealing no gross alterations in neuronal structure. The analysis of dendritic spines has revealed no changes in total spine density, but an enrichment of more immature spine subtypes over mature spines.

Given the essential role of mitochondria in maintaining synaptic activity, their crucial role in signal integration and that mitochondrial alterations are emerging as a common feature of neuropsychiatric disorders, changes in mitochondrial structure and function were checked for their contribution to the phenotypes observed. To evaluate this, mitochondrial function was characterized by assessing mitochondrial respiration using the Seahorse XF extracellular flux analyser in mitochondrial extracts from the PFC. Through this assay, a tendency for hyperactivity in these isolated mitochondria was observed. In addition, potential differences in mitochondrial structure were evaluated using electron microscopy (EM), revealing subtle alterations in mitochondrial size and complexity. This analysis was carried out within the framework of a secondment in collaboration with Professor Carmen Sandi at EPFL.

Full description of the achievements: Annex 1 – ESR1 Orsolya Antal.

To study the role of Mitofusins in neurodevelopmental trajectories of psychopathology (**Task 1.2**), **ESR2, Alessandro Chioino**, supervised by Carmen Sandi and Simone Astori at BMI-EPFL, dissected the participation of GTPase mitofusin 2 (Mfn2) in mitochondrial, morphological and synaptic alterations in the nucleus accumbens (NAc) of medium spiny neurons (MSNs) expressing dopamine receptor 1 (D1-MSNs) in mice, as well as its impact thereof on effort-related behavior.

Indeed, accruing evidence points to the contribution of mitochondria in the aetiology of psychiatric conditions (Andreazza et al., 2018; Pei, Wallace., 2018), but the underlying molecular mechanisms are not fully identified. Previous results from the lab of Professor Carmen Sandi demonstrated that the low expression of the mitochondrial GTPase Mfn2 in the nucleus NAc is associated with anxiety-like and depressive-like behaviors in rats, altered mitochondrial function and reduced synaptic inputs in MSNs (Gebara et al., 2021).

The downregulation of Mnf2 in D1-MSNs promoted the adoption of passive coping in the forced swim test, as indicated by a tendency to increase immobility. cFos mapping of neuronal activation during this behavioral task revealed a significantly decreased engagement of NAc neurons and a different activation pattern in brain areas involve in motivation and stress response. Electrophysiological recordings in mice with Mfn2 downregulation in D1-MSNs indicated altered E/I balance and reduced dendritic complexity. RNA sequencing of translationally active mRNA from D1-MSNs revealed altered gene expression for intrinsic excitability (Kcnj3), spine complexity, glutamatergic receptors (GluA3, mGlur2) and microtubule transport. Qualitative mitochondria evaluation using electron microscopy showed that Mfn2 downregulation altered mitochondrial structure, as indicated by increased organelles circularity. Full description of the achievements: Annex 1 – ESR2 Alessandro Chioino.

ESR3, Giuseppe Cammarata, supervised by João Peça at CNC, implemented a contingency plan to generate human-derived neurons and brain organoids (**Task 1.3**): instead of directly differentiating dental stem cells, Giuseppe used a reprogramming approach involving dental stem cells and fibroblasts.

It has been demonstrated that human-induced pluripotent stem cell (hiPSC)-derived human unguided neural organoids offer greater stability and consistency in generating neurons compared to direct differentiation of dental stem cells.

Biological samples were collected from patients and control subjects. Giuseppe contributed to the reprogramming of three cell lines from the ProTeAN biobank into hiPSCs. These lines have been characterized to assess their pluripotency (Oliveira AR, et al., 2022) and they will be used as a source for developing three-dimensional neural organoids.

In addition to this, Giuseppe conducted a study which involved the chronic exposure of human unguided neural organoids derived from hiPSCs from the control pool to valproic acid (VPA). The neurophysiological, morphological, and network activity properties of both control organoids and those treated with VPA at different concentrations were tested. Data obtained so far indicates that chronic exposure to VPA has various effects on the development and physiology of neural organoids. Specifically, VPA enhances neuronal differentiation, influences the renewal of neural progenitor cells (NPCs), increases the formation of excitatory synapses over inhibitory synapses, affects network physiology by increasing activity (hyperactivity), and heightens the excitability of exposed neurons.

These findings suggest a potential impact of VPA on the inhibitory system within the treated organoids, indicating an excitatory/inhibitory imbalance. Further investigations are needed to understand the underlying cause of this imbalance. Full description of the achievements: Annex 1 – ESR3 Giuseppe Cammarata.

1.2.2 Work Package 2 – Testing the Excitation/Inhibition imbalance hypothesis in neuropsychiatric disorders

The goal of WP2 was to dissect nanostructural changes in synapse dynamics in models of neuropsychiatric disorders, as well as to explore the mechanisms underlying excitation/inhibition (E/I) imbalance in models of neuropsychiatric disease and to monitor E/I balance at the population level. For this, WP2 focused on the identification of the link between synaptic nanoscale alterations and behavioural defects in animal and human models of Shank3-related Autism Spectrum Disorder (ASD); on the characterization of cortical E/I imbalance in vivo in models of synaptic dysfunction using mesoscopic methods; on the identification of defects in single

N-methyl-D-aspartate (NMDA) receptor dynamics in Psychosis; on the identification of presynaptic defects and alterations in glutamate receptor dynamics in Fragile X Syndrome (FXS); on the depiction of synapses and functional networks in a new paradigm to model Down's Syndrome (DS); and on the characterization of the synaptic and neural correlates of spatial memory deficits in a glycine receptor knockout model of ASD.

The description of the work carried out during the life time of the project towards the achievement of each task defined for WP2 is described below. A detailed description of the experiments and results can be found in Annex 1, including a summary of the exploitable results for each project and how they can be exploited (when applicable).

To link autism related mutations in *Shank3* gene over synaptic, network and behaviour defects (**Task 2.1**), **ESR4, Diogo Soares**, supervised by Daniel Choquet and Eric Hosy at IINS-CNRS, started the development of a pathological model with the use of a short hairpin RNA (shRNA), which decreases Shank3 expression of approximately 40%. Preliminary experiments did not reveal a noticeable modification of AMPA receptor organization nor currents after shRNA expression, thus, the development of two new models was planned: human pluripotent cells (hPS) and *Shank3* knock-out (ko) mouse. In October 2020, after Diogo Soares resigned his position at CNRS, and while the new ESR4 fellow (Veronica Villeri) was being recruited, the ko *Shank3* mouse model was established and hPS was implemented on another pathological model. Full description of the achievements: Annex 1 – Diogo Soares.

As soon as the new fellow was recruited, **ESR4, Veronica Villeri**, also supervised by Daniel Choquet and Eric Hosy at IINS-CNRS, started investigating the nanoscale organization of glutamate receptors in in vitro models of monogenic autism by means of d-Storm super resolution microscopy. More specifically, Veronica carried out four lines of experiments:

1) It is known that long-term potentiation (LTP) causes a reduction of SYNGAP1 at the spine, however, little information is available on the effects of long-term depression (LTD) on synaptic SYNGAP organization, as compared to long-term potentiation (LTP), which is known to cause a reduction of SYNGAP1 at the spine. Veronica investigated if and how LTD stimulation determines a change in the number of synaptic SynGAP1 and its colocalization with either AMPA receptors or PSD95 in WT rat hippocampal cultures. Preliminary data showed that there is 30% ca. reduction in Synaptic SynGAP1 following NMDA-mediated LTD.

2) SynGAP1+/- primary hippocampal cell cultures are characterized by an LTP-like basal state. Evidences from the Lab of Daniel Choquet showed that this may be caused by an increase in calcium-permeable AMPA receptors. Veronica investigated whether there is a parallel increase in synaptic NMDA receptors. Preliminary experiments showed no significant differences in NMDA levels.

3) Veronica started the optimization of the cell culture conditions and main line of investigation in Shank3 in vitro models, by studying whether changes could be observed in synaptic NMDA or AMPA receptors' density in primary culture of either cortical or hippocampal neurons of Shank3+/-, Shank3-/- and WT mice at DIV10 and DIV14.

4) Finally, Veronica optimized the culture and super-resolution imaging conditions of I3 human neurons in order to study changes in glutamate receptors organization in Syngap1+/- and Syngap1-/- human I3 neurons.

To monitor the cortical E/I balance *in vivo*, (**Task 2.2**), **ESR5, Marcos Sintes**, formerly under the supervision of Thomas Knöpfel and under the supervision of Vincenzo De Paola at ICL since March 2021, generated a suitable mouse model for ASD and a method to individually assess ASD-related traits to select animals for *in vivo* imaging experiments. As a first step towards the completion of this task, the three-chamber test was optimised to characterise social behaviours in mice. Then, animals prenatally treated with valproic acid (VPA) were used as ASD model mice. These animals were tested along with the control mice, using the three-chamber test to assess potential deficits in sociability and social memory. The obtained videos were analysed using tracking software to obtain travel distance during the test, time in each chamber and time of approach of the test mice to each cub mouse. To study 5HT neuromodulatory effects on E/I balance during sensory processing, Marcos characterised the behavioural effects of psilocybin in the VPA mouse model of ASD. Psilocybin was used as a 5HT2AR agonist and its effects in control and ASD model mice social behaviours were tested using the three-chamber test. In a first set of tests, ESR5 performed three chamber tests on treated and untreated VPA and control animals and analysed the results using tracking software. The results of these experiments were made public as a BioRxiv preprint in September 2020: https://doi.org/10.1101/2020.09.09.289348.

After the modification of Marco's project to "human cortical circuit wiring in ASD and the role of psychedelics", under the supervision of Prof. Vincenzo de Paola (more information regarding the change in project and supervisor in the report "ESR5 Transfer of Marcos Sintes to Vincenzo De Paola's group", submitted via formal notification on the EC Portal on 21 March 2022), Marcos generated neurons from control donors and developed both an *in vitro* and *in vivo* system for the characterisation of the short and long-term effects of psychedelic drugs on human cortical wiring. Marcos produced novel results in the field and optimised a new cortical transplantation model that offers unique advantages over the *existing in vitro* and *in vivo* models for the study of the effects of these compounds at the cellular and molecular level.

First, using different approaches, Marcos showed that the cells express 5HT2A receptors, which acts as the main target of psychedelic drugs, at a level comparable to the adult human cortex and optimised an *in vitro* live-cell imaging technique to study the acute effects of 5HT2A receptor activation on neuronal activity. Using this approach, it was confirmed that the cells are excited by a selective agonist and inhibited by a selective antagonist, which indicates the presence of a functional G protein signalling pathway associated to the serotonergic receptors. qPCR was used to compare the expression of markers of neuronal activity and plasticity after the treatment with different drugs both acutely and subacutely, observing an increase in the expression of these markers with a moderate dose of the psychedelic drug TCB-2.

Finally, human control cortical progenitors were transplanted to the cortex of mice and generated cortical grafts expressing GFP. Two-photon *in vivo* microscopy was used to measure the growth of individual axonal projections from the human neurons before and after treatment with the psychedelic drug psilocybin to study its effects on structural plasticity, producing preliminary data that helped optimising our current experiments with ASD patient derived cells. Description of the achievements: Annex 1 – ESR5 Marcos Sintes.

To study the physiopathological effects of altered NMDA receptor dynamics in Psychosis (**Task 2.3**), **ESR6, Daniel Hunter**, supervised by Laurent Groc at IINS-CNRS started by examining the synaptic disturbance induced by indirect actions of both NMDAR- and GABAaR-autoantibodies, encompassing dysfunction at excitatory and inhibitory inputs to hippocampal cells, using a range of patch-clamp electrophysiological techniques. Further, Daniel has broadened the model of disease pathogenesis to include the altered functionality of GABAergic interneurons in hippocampal networks, by characterising shifts in intrinsic cellular excitability of discreet hippocampal cell populations.

Whole-cell voltage-clamp electrophysiological recording of CA1 pyramidal neurons was performed in organotypic hippocampal slices to investigate the spontaneous neurotransmission at excitatory and inhibitory synapses. It was demonstrated that exposure to both NMDAR- and GABAaR- autoantibodies reduces the amplitude of both AMPAR-mediated and GABAaR-mediated postsynaptic currents (sEPSC and sIPSC, respectively). Intriguingly, the relative reductions observed in sIPSC amplitudes from cells exposed to NMDAR- and GABAaR-autoantibodies were greater than those in observed in sEPSCs. The more pronounced deficits in inhibitory neurotransmission suggest that, on balance, the synaptic inputs onto pyramidal cells becomes unbalanced in favour of hyper- excitation. The rise and decay kinetics of the remaining sEPSC and sIPSCs was further examined, revealing no alterations in the waveform of synaptic currents, suggesting a loss of both AMPA- and GABAaRs from their respective postsynaptic compartments.

To understand how these aberrant synaptic inputs are functionally integrated into hippocampal network activity, hippocampal cell populations were subsequently investigated for alterations in intrinsic excitability. Here, a current-clamp technique was employed to characterise possible alterations across a range of intrinsic cellular properties on both excitatory and inhibitory populations. It was shown that GABAaR-autoantibodies uniquely disrupt the excitatory cell population, by driving an increase in the resting membrane potential, yielding an increased propensity to fire action potentials in response to current injection. This increase in resting potential may be underpinned by the loss of tonic GABAergic inhibition on these cells, known to contribute to dynamic intrinsic excitability, which we have also observed to be abolished after exposure to GABAaR-autoantibodies. Moreover, Daniel demonstrated a unique impact of NMDAR-autoantibodies on the inhibitory cell populations, whereby intrinsic excitability is significantly reduced. In this case, exposure to NMDAR-autoantibodies appears to hyperpolarise the resting membrane potential of GABAergic interneurons, in turn reducing their action potential output. This is in line with a classical disinhibitory model, in which potent inhibitory control over hippocampal networks is abolished, leading to destabilised hyper-excitable

activity in the excitatory cell population. Together, these findings are indicative of two discreet disease models whereby synaptic inputs are dysregulated and differentially gated by intrinsic cellular properties at the scale of hippocampal network function. Full description of the achievements: Annex 1 – ESR6 Daniel Hunter.

To examine alterations of the synaptic properties in Fragile X Syndrome (FXS) (**Task 2.4**), **ESR7, Elisa Corti**, supervised by Carlos Duarte, Ramiro Almeida and Paulo Pinheiro at CNC, dissected the contribution of fragile X messenger ribonucleoparticle (FMRP) - an RNA binding protein involved in the trafficking and translational control of a wide variety of mRNAs - in maintaining a proper balance of the physiology of the synapse.

Among FMRP targets, there are mRNAs that encode for essential proteins for a proper synaptic function and physiology. Due to the vast heterogeneity of FMRP target mRNAs, it is crucial to understand the contribution of FMRP in maintaining a proper balance of the physiology of the synapse. ESR7 investigated the role of FMRP in:

i) The number and formation of presynaptic sites. Elisa characterised the development of presynaptic terminals in hippocampal cultures from WT and Fmr1 KO mice and observed that the lack of FMRP impairs the accumulation of presynaptic proteins in the axonal shaft, in a way that depends on the protein of interest and the stage of neuronal development. Moreover, the regulation of local protein synthesis is unbalanced in axons lacking FMRP, and this also seems to be dependent on the developmental stage of the neurons.

ii) Synaptic plasticity. Elisa studied LTP in acute WT and Fmr1 KO hippocampal slices and found that preventing TrkB activation and blocking GluN2B-containing NMDAR dampen LTP in WT but not in KO slices. Elisa also prepared synaptoneurosomes from the hippocampi of WT and Fmr1 KO mice and observed that the GluN2A synaptic content of Fmr1 KO hippocampi is higher than the one of WT hippocampi. To better dissect the contribution of FMRP in synaptic homeostasis, Elisa knocked down Fmr1 expression in primary hippocampal cultures using a shRNA. This manipulation impairs BDNF-induced synaptic accumulation of GluN2A- and GluN2B-containing NMDAR, as well as their lateral diffusion.

Full description of the achievements: Annex 1 – ESR7 Elisa Corti.

To illuminate the mechanisms of synaptic and neural network dysfunction in a new in vivo model of Down's Syndrome (DS) (**Task 2.5**), **ESR8, Elizabeth Brockman**, supervised by Vincenzo De Paola at ICL, investigated he mechanisms behind the cellular alterations observed after transplanting induced pluripotent stem cells (iPSCs) and using multiphoton microscopy (previously described by Vincenzo De Paola's group), in which DS grafts were shown to have increased synaptic structural stability and decreased neuronal oscillatory activity. Indeed, human cortical circuit assembly is critical to neurodevelopment, but the understanding of this process in DS remains incomplete.

This project focused on the effect of major cortical cell types and microenvironment on axonal bouton stability and strength. Elizabeth differentiated patient-derived iPSCs and isogenic controls into neural progenitors and injected into the cortex of immunodeficient mice with cranial windows. The differentiation protocol was altered to normalize graft size differences and GFAP+ and SATB2+ cell numbers across genotypes, as these were overproduced and underproduced, respectively, in previous DS grafts compared to controls. 2-photon images were taken of axonal en passant boutons (EPB) and of somatic calcium signals over time. EPB turnover rate (TOR) and EPB size (strength) changes were quantified using EPBscore software. Calcium imaging was analyzed via MATLAB/Fiji scripts to determine event frequency, integral, and amplitude. No significant difference was found in EPB TOR and EPB strength or somatic calcium signals between DS and control grafts. Therefore, differences in synaptic stability and somatic activity outcomes have been normalized across genotypes. Correlative analysis was used to see if the normalization of SATB2+ and GFAP+ cell levels contributed to this rescue. SATB2+ cell densities were not found to be significantly correlated to any outcomes. GFAP+ cell numbers significantly correlate with synaptic stability outcomes, but not with neural activity outcomes. Overall, Elizabeth concluded that trisomy 21-related dysregulation is not sufficient to alter axonal bouton stability and neuronal activity compared to control neural networks of similar scale and cellular composition. Full description of the achievements: Annex 1 – Elizabeth Brockman.

To understand the role of glycinergic signalling in ASD (**Task 2.6**), **ESR9, Laura Upton**, supervised by Catalina Betancur at IBPS-CNRS, investigated the cellular and functional properties underlying the behavioural and synaptic defects observed in Glra2-deficient male (Glra2Y/-) mice. Indeed, pathogenic variants in the GLRA2 gene, which encodes the glycine receptor alpha-2 subunit, have been implicated as a novel cause of autism

spectrum disorder (ASD) and intellectual disability. Professor Catalina Betancur's group previously showed that Glra2-deficient male (Glra2Y/-) mice present with deficits in object recognition and altered synaptic plasticity in the prefrontal cortex (PFC).

Glra2-/Y mice show many of the hallmarks of neurodevelopmental brain dysfunction observed in other rodent models of ASD. In the PFC of adult mice, Laura observed decreased inhibitory synapses, increased spine density and dendritic complexity of pyramidal neurons, increased cortical somatostatin interneurons, and in P14-P21 mice, an increased frequency of miniature excitatory post-synaptic currents in layer V pyramidal neurons. The electrophysiology experiments were performed during Laura's secondment at the University of Edinburgh, where she had the opportunity of learning to patch. Overall, these findings point to excitatory hyperconnectivity in the PFC and suggest an imbalance of excitatory and inhibitory transmission in Glra2-/Y mice.

In addition, Laura assessed c-Fos expression as a marker of neuronal activation following novel object recognition and found that the rostral infralimbic PFC was hypoactivated in Glra2-/Y mice following this task. To further assess the altered PFC activity in Glra2-/Y mice during cognition, Laura is currently recording calcium transients from infralimbic glutamatergic neurons during this task using fiber photometry. Finally, as part of a collaboration during the secondment at the University of Edinburgh, the possibility of sub-threshold seizure activity and sleep dysfunction in Glra2-/Y mice was assessed, since both epilepsy and sleep disorders are common features in ASD and rodent models of autism. To do this, 24-48 h multi-electrode EEG recordings were obtained from Glra2-/Y mice and wild-type littermates, and the analysis of this experiment is ongoing. Full description of the achievements: Annex 1 – Laura Upton.

1.2.3 Work Package 3 – Modulators of neuropsychiatric disorders

WP3 had the goal of testing the endocannabinoid system, fast spiking interneurons manipulation and proteastasis as modulatory targets in neuropsychiatric disorders, as well as assessing the interplay between prenatal stress and genetic risk in the development of disease and evaluating the time window of opportunity for intervention in models of neuropsychiatric disorders. With this purpose, WP3 focused on the study of the therapeutic potential of the endocannabinoid system in an Autism Spectrum Disorder (ASD) model; on the identification of aberrant synaptic mRNA translation as a point of convergence for multiple genetic causes of ASD/ID; on the establishment of the impact of the corticotropic releasing hormone system on Schizophrenia (SCZ)- and ASD- related endophenotype; on the identification of circuit deficits associated with ASD; as well as on the identification of the role of the excitation/inhibition (E/I) imbalance in the corticothalamic system in the development of neuropsychiatric disorders.

The description of the work carried out during the life time of the project towards the achievement of each task defined for WP3 is described below. A detailed description of the experiments and results can be found in Annex 1, including a summary of the exploitable results for each project and how they can be exploited (when applicable).

To assess the endocannabinoid system as a therapeutic target in the mouse model of autism Shank3-deficient mice and study the consequences of Shank3 deficiency on the endocannabinoid system (**Task 3.1**), **ESR10**, **Flavio Tomasi**, supervised by Catalina Betancur at IBPS-CNRS, studied the effects of administration of endocannabinoid modulators on the phenotype of a Shank3-deficient mouse model.

Flavio showed that the inhibition of the cannabinoid CB1 receptor with rimonabant (an orthosteric antagonist) or AEF0217 (a novel CB1 receptor signalling-specific inhibitor developed by Aelis Farma for clinical use) is beneficial, improving a subset of core behavioural deficits in this mouse model. While rimonabant can't be used in patients due to serious side effects, AEF0217 is safe and devoid of side effects. AEF0217 also normalized cortical hyperexcitability in pyramidal neurons in the medial prefrontal cortex of knock-out (KO) mice. The electrophysiological study was performed during the secondment in the laboratory of João Peça in Coimbra, where Flavio explored the effects of repeated administration of AEF0217 on pyramidal neurons in the medial prefrontal cortex of WT and KO mice. Flavio also explored the effects of Shank3 deficiency on the endocannabinoid system and found no significant changes in the levels of various endocannabinoids in four brain regions, i.e. prefrontal cortex, hippocampus, striatum and cerebellum, and in plasma, or in the levels of CB1 receptors in the same brain regions. Finally, the effects of AEF0217 on possible alterations in the

morphology of prefrontal pyramidal neurons in KO mice and on LTP in the hippocampus are currently being investigating. Full description of the achievements: Annex 1 – Flavio Tomasi.

To investigate the excitation/inhibition imbalance hypothesis of neuropsychiatric disorders in the corticothalamic system (**Task 3.2**), **ESR11**, **Ágata Silván**, supervised Kjartan Herrik and Charlotte Hougaard at Lundbeck, studied how pharmacological modulation of small conductance Ca2+–activated K+ (SK) channels affects neuronal firing and intrinsic properties in the thalamic reticular nucleus (TRN). Two SK positive allosteric modulators (PAMs) and one negative allosteric modulator (NAM) were used: CyPPA, NS309 and NS8593, respectively. By acting on the burst afterhyperpolarization (AHP), negative modulation of SK channels resulted in increased AP firing, increased burst duration and decreased intervals between bursts. Conversely, both CyPPA and NS309 increased the afterburst AHP, prolonging the interburst interval, which additionally resulted in reduced AP firing in case of NS309. Alterations in SK channel activity would be expected to alter functioning of thalamocortical circuits. Targeting SK channels could be promising in treating disorders involving thalamic reticular dysfunction such as psychiatric and neurodevelopmental disorders. Full description of the achievements: Annex 1 – Ágata Silván.

Insights from genetic forms of ASD and ID identify protein synthesis as a common dysregulated cellular pathway underlying pathophysiology. To identify convergent dysregulation of mRNA translation in multiple models of autism (**Task 3.3**), **ESR12**, **Manuela Rizzi**, supervised by Emily Osterweil at CIP-UEDIN, explored the translation fraction in two models of autism (respectively Fragile X Syndrome and Syngap Haploinsufficiency) by means of Translating Ribosome Affinity Purification and RNA-seq (TRAP-seq). The emphasis was on two key brain regions implicated in learning and cognition, functions disrupted in patients with these disorders and phenocopied in animal models. More extensively increased protein synthesis is deemed responsible of the exaggerated mGlu1/5-induced long-term synaptic depression(mGluR-LTD) observed in CA1 pyramidal neurons from Fmr1-/y and Syngap+/- mice. Moreover, the observed deficits in learning and context discrimination would also suggest a disrupted functioning of the amygdala. Protein synthesis is key in the first phase of acquisition and for subsequent maintenance of long-term memories. Thus, aberrant protein synthesis may represent a convergent phenotype in different brain regions as well as define as a powerful therapeutic option to target multiple disorders from the complex and highly heterogenous spectrum of autism. To this end retrieval of a commonly misregulated fraction of transcripts in the two models of disease was expected and would inform on key molecular mechanisms of functions. While exploration of the CA1 hippocampal region relied on analysis of previously collected datasets for the two models of disease and interrogation of whether hypothetical mechanism of function envisaged already for Fmr1-/y would also apply to Syngap+/- dataset, dissection of basolateral amygdala regions was preceded by optimization steps to yield the cell-specific translated fraction under naïve and activity-induced state. Full description of the achievements: Annex 1 – Manuela Rizzi.

To establish the impact of the corticotropic releasing hormone system on schizophrenia and ASD related endophenotype (**Task 3.4**), **ESR13**, **Loredana Cumpana**, supervised by Carmen Sandi and Simone Astori at BMI-EPFL, investigated the neuromodulation of NREM sleep by the corticotropin-releasing hormone (CRH) through the activation of its receptor in the thalamic reticular nucleus (TRN). CRH is a peptide associated with stress and anxiety that acts as a potent modulator throughout the nervous system. The CRH receptor 1 (CRHR1) has a high expression in the TRN neurons, key generators of sleep rhythmogenesis that sustain sleep spindles during NREM sleep through low-threshold burst discharges. As part of the secondment with CTI, ESR13 reviewed the literature on the effects of extra-hypothalamic sources of CRH on sleep rhythms, which revealed the lack of information on this topic and the need to investigate it with region selectivity.
Using viral mapping, diverse CRH afferents to the TRN were identified, such as the basolateral amygdala, zona incerta and subnuclei within the geniculate nucleus.
Combined EEG and photometry recordings showed that the endogenous release of CRH at the TRN during NREM sleep exhibits an oscillatory pattern that anticorrelates with the sigma infraslow oscillation, representing clusters of sleep spindles, similar to the reported dynamics of noradrenaline. This suggests a relationship between CRH release and sleep spindles.
Selectively manipulating CRH release during NREM sleep using optogenetics affected the consolidation of

NREM sleep bouts and the sigma power surge at transitions to REM sleep.
In ex-vivo electrophysiology recordings, CRHR1 activation decreased TRN neurons' low-threshold bursting, and increased the amplitude of cortical excitatory postsynaptic potentials when combined with noradrenaline release.
Altogether, the data indicate that CRH may act as a limiting factor in the homeostatic process for NREM sleep by modulating the excitability of TRN neurons. These mechanisms can provide a novel target to stabilize sleep impairments associated with stress and anxiety, which has been shown to improve cognitive processing or symptoms of various psychiatric disorders. Full description of the achievements: Annex 1 – Loredana Cumpana.

To study the circuit deficits associated with ASD/ID (**Task 3.5**), **ESR14**, **Vanesa Salazar**, supervised by Peter Kind at the CBDS-UEDIN, addressed social and circuit deficits in Autism Spectrum Disorder (ASD) and Intellectual Disability (ID), with a focus on SYNGAP1 haploinsufficiency. One of the main findings of this project was that both rat SYNGAP1 lines (deletion of GAP domain in heterozygosis and a null heterozygous of SYNGAP1 gene) exhibit lower levels of social interaction and a general lack of interest while their ability to habituate and discriminate another conspecific are unaltered. This discovery sheds light on the social behaviour deficits observed in individuals with SYNGAP1 mutations and provides a basis for further investigation into potential therapeutic interventions.
Furthermore, rearing the rat models from weaning until adulthood in a highly enriched environment and socially stimulating environment might prevent the expression of the previously observed lack of interest. This result suggests that intervention through social stimulation during critical periods can be an effective strategy to address social deficits in SYNGAP1 haploinsufficiency. Additionally, a a wireless set-up was implemented to record functional connectivity using in vivo local field potential (LFP) during social interaction. Currently, the data collected from these experiments is being analysed, and the findings are expected to provide insights into the circuit-level alterations in reward-related brain areas associated with social behaviour. Full description of the achievements: Annex 1 – Vanesa Salazar.

ESR15, **Veronica Villeri**, supervised by Emily Osterweil, after being transfered to UEDIN, studied whether Fmr1-/- and Syngap1 +/- mice show differences at the level of neuronal activation in the basolateral amygdala after encoding for a fear-related memory. Indeed, both mouse models have shown an impaired fear-related memory, tested via the fear conditioning paradigm. If there is a defect at the level of memory encoding, a reduced activation of BLA cells and/or a change in their response can be hypothesised. To test the first hypothesis, mutant and WT mice were subjected to fear conditioning and then sacrificed in order to assess c-Fos expression by imaging. Veronica performed confocal imaging of brains stained for c-Fos, Neun and a basolateral amygdala (BLA) marker and the relative tridimensional image analysis and showed that in both mutants, as in the respective wild-type, there is a significant increase in the number of c-Fos+ cells, suggesting the formation of a fear-related memory engram. However, while there was no difference between the level of neuronal activation between Syngap1+/- and the respective wild-type, a reduced c-Fos expression in Fmr1+/- was observed, as compared to the respective wild-type. Full description of the achievements: Annex 1 – Veronica Villeri. Justification to the deviation to Annex 1 of **Task 3.6**, see ESR15 – Veronica Villeri on section "Deviations from Annex 1 and Annex 2").

1.2.4 Work Package 4 – Training and Doctoral Programme plan

WP4 had the goal of implementing secondments and the network-wide training activities, network schools, common courses and workshops in the fields of synapse biology and neuropsychiatric disorders, as well as to provide all ESRs with a complete set of capabilities for their future careers, focusing on research, communication, impact skills, as well as on bridging the science-business and/or the science-society gaps. This WP synchronized all training activities (local and networkwide), including doctoral studies, short courses, workshops, network schools and secondments, and integrates the training activities into the career plan for each ESR.

All 14 Syn2Psy fellows had the opportunity to enrol in **courses** offered by the host laboratory PhD program (**Task 4.1**). Due to COVID-19-related restrictions, some fellows had their doctoral courses either postponed or transferred to an online format. Nevertheless, all ESRs that were required to attain a certain amount of credits per year to comply with the requirements for the doctoral school, were able to fulfil the requirements.

Table I. List of courses taken by the ESRs throughout the life-time of the project.

ESR	Courses
ESR1 Orsolya Antal	Laboratory animal science; Core technologies; Connecting researchers with the society; Portuguese language course; Laboratory rotation module. Syn2Psy Network Schools 1, 2, 3 and 4; Science Communication workshop.
ESR2 Alessandro Chioino	State-of-the-Art Topics in Neuroscience VIII. Fear Learning: from neuronal circuits to translation; Introductory course in laboratory animal science; Responsible Conduct in Biomedical Research; French course A1/A2; Image processing for Life Sciences; Focus on FIJI program language. Syn2Psy Network Schools 1, 2, 3 and 4; Science Communication workshop.
ESR3 Giuseppe Cammarata	Cellular and molecular biology; Experimental Biology and Biomedicine 1; Portuguese language course; Science Communication course; Core technologies; Laboratory Rotations module; Data Science: R Basics. Syn2Psy Network Schools 1, 2, 3 and 4; Science Communication workshop.
ESR4 Diogo Soares	Level B French classes.
ESR5 Marcos Sintes	Ensuring Integrity 1: Plagiarism Awareness; Data Science; Introduction to Sampling & Hypothesis Testing; Data Science: Further Hypothesis Testing; Advanced Academic Writing; Animal training course at National University of Singapore Syn2Psy Network Schools 1, 2, 3 and 4; Science Communication workshop.
ESR6 Daniel Hunter	Research integrity in scientific professions; Regulatory training for staff developing experimental procedures and projects; Surgery training course. Syn2Psy Network Schools 1, 2, 3 and 4.
ESR7 Elisa Corti	Core technologies; Animal handling course; Portuguese language course; Laboratory Rotations module; Synapse Biology course. Syn2Psy Network Schools 1, 2, 3 and 4; Science Communication workshop.
ESR8 Elizabeth Brockman	Introduction to Learning & Teaching; Introduction to Assessment & Feedback for Learning; Research Computing: Introduction to LaTeX; Designated trainer for craniotomy surgeries. Syn2Psy Network Schools 1, 2 and 3; Science Communication workshop.
ESR9 Laura Upton	Regulatory course for project designers (rodents & lagomorphs); Python Initiation course; Initiation course for experimental surgery in rodents. Syn2Psy Network Schools 1, 2, 3 and 4; Science Communication workshop.
ESR10 Flavio Tomasi	Regulatory course for project designers (rodents & lagomorphs). Syn2Psy Network Schools 1, 2, 3 and 4; Science Communication workshop.
ESR11 Ágata Silván	Neurograd Winter School; Introduction to Matlab; Responsible conduct of research 2: getting ready for submission; Creative techniques for thesis writing. Syn2Psy Network Schools 1, 2, 3 and 4.
ESR12 Manuela Rizzi	Autumn school for PhD students; Animal course; Tick@lab; Bioinformatic for Genomics course. Syn2Psy Network Schools 1, 2, 3 and 4; Science Communication workshop.
ESR13 Loredana Cumpana	Animal experimentation course Module 1; State-of-the-art topics in Neuroscience VIII: Fear leaning: from neuronal circuits to translation; Research Data Management Plan Training workshop; Stay away from plagiarism Training workshop; Understanding statistics and experimental design; Introduction to Applied Data Science. Syn2Psy Network Schools 1, 2, 3 and 4; Science Communication workshop.

ESR14 Vanesa Salazar	SCOTPIL ABC Training and examination; Rodent (mouse and rat) handling training; PGR Time management (online) workshop; Writing a PhD First Year Report for International students; The Multi-scale brain; Introduction to coding through MATLAB; Python - Learn to programming; Python - Learn to programming workshop; The Virtual Brain Cloud workshop; A primer on using Neo and Elephant to analyse electrophysiological data workshop; Data sharing on EBRAINS; Practical training on rat surgeries. Syn2Psy Network Schools 1, 2, 3 and 4; Science Communication workshop.
ESR15 Veronica Villeri	Syn2Psy Network Schools 2, 3 and 4; Science Communication workshop.

More information regarding the PhD courses attended by the fellows during the first year of the PhD programme, refer to the document "Report on PhD courses" (D4.8 D37), submitted via continuous reporting on the EC Portal in September 2020.

Four **wide-ranging Network Schools** (NS), were planned for the lifetime of the project (**Task 4.2**). The initial plan included four in-person Network Schools (theoretical and hands-on): NS1 - Basic concepts on imaging tools and data analysis in Coimbra, Portugal; NS2 - Genetic approaches for the study of neuropsychiatric disorders in Paris, France; NS3 - Drug discovery and development: from idea to product in Copenhagen, Denmark; and NS4 - Advanced methods for imaging the brain: from nanoscopic to mesoscopic perspectives in Bordeaux, France.

NS1 was held in Coimbra in September 30 to October 4, 2019. This network school was a one-week intensive microscopy course focused on the principles and high-end applications of quantitative fluorescence microscopy. Participants gained a theoretical understanding and hands-on experience on state-of-the-art equipment such as widefield microscopy, laser scanning and spinning disk confocal microscopy, multi-photon microscopy, deconvolution methods, and digital image processing and analysis. NS1 was organized by Luísa Cortes (CNC – Head of Microscopy Unit MICC) and Monika Marx (ZEISS, Syn2PSy partner). The training was complemented by talks from invited speakers with expertise in microscopy facilities management and/or expertise in specific microscopy techniques.
This course was also attended by six external researchers.



Figure 1 – Group photography taken at CNC, Coimbra University, during the Syn2Psy Network School 1.

For more information regarding Network School 1, including details of the programme, attendees and course satisfaction, refer to the document "Report on Network School 1" (D4.2 D24), submitted via continuous reporting on the EC Portal in October 2019.

The restrictions and confinement measures imposed by COVID-19, which included a strict lockdown for several months and impossibility to travel, led to the postponement of Network Schools 2 and 4 and the modification of the format of Network School 3 to an online mode. These modifications were included in the amendment to the Grant Agreement, approved in March 2021.

NS3 was held online from Lundbeck on May 5 to 7, 2021, under the topic "Drug discovery and development: from Idea to Patient". This NS was a three-day intensive course that focused on the principles of drug development. The ESRs gained theoretical understanding on how Lundbeck and other pharmaceutical companies develop scientific ideas into treatments to help patients with neuropsychiatric disorders. Through the seminars on Pre-Clinical, Clinical and Business Development and Licensing, given by Lundbeck's specialists, the ESRs were taken through the steps from generating ideas to treating patients. This course also provided the ESRs with information and skills on **bridging the science-business gap**. This NS was organized by Kjartan Herrik (Principle Scientist – Lundbeck).
NS3 was complemented with a workshop on the early signs and clinical variability of Autism Spectrum Disorders, given by Carla Almeida - PIN (Syn2Psy partner) and with seminars about the pros and cons of working in academia and industry, as well as a round table discussion about this topic.



Figure 2 - Early Stage Researchers during the workshop with PIN – NS3.

For more information regarding Network School 3, including details of the programme, attendees and course satisfaction, refer to the document "Report on Network School 3" (D4.3 D25), submitted via continuous reporting on the EC Portal in July 2021.

NS4 was held in Bordeaux from March 28 to April 1, 2022, at the Bordeaux Imaging Center (BIC), Centre Broca Nouvelle-Aquitaine. Under the topic "Advanced methods for imaging the brain: from nanoscopic to mesoscopic perspectives", NS4 was a one-week intensive course focused on advanced microscopy methods. ESRs gained understanding of the theory and applications of Stimulated Emission Depletion (STED) microscopy, Single Molecule Localization microscopy (SMLM), Structured Illumination microscopy (SIM) and Expansion microscopy. ESRs learned the challenges and solutions to perform single molecule localization microscopy in depth within complex tissues, labelling strategies, pixel reassignement as an alternative to super resolution techniques, imaging of structural dynamics of brain microstructure and how to apply single particle techniques to neuroscience questions. Furthermore, during the practical sessions, the ESRs had the opportunity to learn, hands-on, technical aspects of Confocal, STED, SMLM, Expansion, Airy Scan, Pixel

Reassignement, as well as data analysis. This training was organised by Daniel Choquet (Principal Investigator at IINS-Interdisciplinary Institute for Neuroscience), Laurent Groc (Principal investigator at IINS), Eric Hosy (researcher at IINS) and Christel Poujol (manager at BIC), with the participation of Julien Kissenberger (ZEISS, Syn2Psy partner).
NS4 was complemented with a workshop on career development provided by Nicolas Bourg, founder of Abbelight, under the topic "From research to a global recognized company, from PhD student to CTO and founder of Abbelight?"

For more information regarding Network School 4, including details of the programme, attendees and course satisfaction, refer to the document "Report on Network School 4" (D4.5 D27), submitted via continuous reporting on the EC Portal in May 2022.



Figure 3 – ESRs during Network School 4 in Bordeaux.



Figure 4 – ESRs during Network School 2 in Paris: (Top left) All 14 ESRs with Network School 2 organiser Catalina Betancur. (Top right) ESRs at the Museum of Natural History in Paris. (Bottom) ESRs during the visit to Paris by boat.

NS2, under the title "Genetic approaches for the study of neuropsychiatric disorders" was held in Paris, at the Institute of Biology Paris-Seine (IBPS), Campus Marie et Pierre Currie on April 4 – 7, 2022.
NS2 was a four-day intensive course focused on the application of genetics to neuropsychiatric disorders. Participants gained theoretical understanding on the concepts and approaches of complex genetics, including genetics of autism spectrum disorder, schizophrenia and intellectual disability. ESRs learned how to leverage population resources to improve rare variant interpretation in developmental disorder patients and how to model neurodevelopmental disorders in a dish. Furthermore, the ESRs had the opportunity to participate in a workshop about case studies and critical thinking in neuropsychiatric genetics. This school was organised by Catalina Betancur (Principal Investigator at CNRS-IBPS).
NS2 was complemented with a full-day workshop on career development provided by career consultant Sarah Blackford under the topic "Career planning and the job market". During this workshop the ESRs had the opportunity to investigate the employment market, learn about the variety of opportunities and review job seeking strategies to be more knowledgeable about the world of scientific employment and how to secure a position.

For more information regarding Network School 2, including details of the programme, attendees and course satisfaction, refer to the document "Report on Network School 2" (D4.3 D25), submitted via continuous reporting on the EC Portal in May 2022.



Figure 5 – ESRs during Network School 2 in Paris: (Left and centre) ESRs during Career Development workshop exercise. (Right) Poster of SWAT exercise during Career Development workshop with Sarah Blackford.

To measure the overall satisfaction towards the Network Schools, ESRs and PIs were asked to rate the impact of Syn2Psy 4 Network Schools in the ESRs' training. On a scale from 1 to 5, where five is the highest score, ESRs and PIs rated the Network Schools with 4.15/5 and 4.2/5, respectively.

Syn2Psy is committed to provide training opportunities that lead to the development of the ESRs professional careers. As such, Syn2Psy provided **advanced training aiming at transferable skills** throughout 2021 and 2022. As described above, the ESRs participated in a workshop with career consultant Sarah Blackford under the topic "Career planning and the job market" during NS2. Furthermore, online training was provided by Chaperone, a specialised company that offers personalized career development for scientists, in the form of 7 group workshops (topics were selected based on the ESRs' choice) and one individual session per ESR.
Online group workshops held in 2021:

- Project Management;
- How to Write a Scientific Article;
- How to Prepare for a Scientific Conference;
- Wellbeing/Work-Life Balance.

(Post-workshop report provided by Chaperone, with satisfaction information on Annex 2)

Online group workshops held in 2022:

- Thesis Writing and Thesis Defence
- CV and Cover Letter Writing
- Interview Preparation

(Post-workshop report provided by Chaperone, with satisfaction information on Annex 3)

For the choice of the individual workshops, the ESRs received assistance from Chaperone staff to better understand their specific individual career needs. The choice of topics fell into two main domains: career options and how to apply for specific roles; and time management and wellbeing skills. (Post-workshop report provided by Chaperone, with satisfaction information on Annex 4)

The majority of the ESRs found the workshops to be relevant to their future and reported that it contributed to enhance their skills, rating the overall training sessions with 4/5 (satisfaction survey conducted by Chaperone). Of note, the "Wellbeing and work-life balance" was the workshop most appreciated by the students.

Based on this, and on the fact that COVID-19 pandemic had a major effect on mental health globally (early career scientists having been considered particularly vulnerable within academia), Syn2Psy Supervisory Board decided to provide the ESRs with a series of informative online **psychology webinars** to help the ESRs identify and prevent mental health issues and, ultimately, to be more resilient when facing the challenges of being a PhD student in the post-pandemic circumstances.

The webinars were provided by the clinical psychologist and former researcher in Neuroscience, Desiree Dickerson, and the topics chosen by the ESRs were as follows:

- Strategies for managing our well-being in research;
- Navigating uncertainty in research;
- Motivation and procrastination;
- Perfectionism and imposter syndrome;
- Rethinking productivity and Navigating supervision.

On a scale from 1 to 5, where 5 is the highest, the ESRs rated with 3.8/5 the contribution of the tools provided during the sessions in the enhancement of their overall well-being and the increase of their resilience to stress and anxiety.

(Report from Desiree Dickerson on Annex 5 and complete satisfaction survey results on Annex 6)

Furthermore, within Syn2Psy, the ESRs had the opportunity to develop transferable skills in outreach, communication and public engagement, as well as in **bridging the science-society gaps** through the several initiatives (discussed in WP5 - Dissemination & Communication and Exploitation), including the Science Communication workshop held in Coimbra, on October 18th, 2022 (included in the 3rd PhD day programme - see complete programme on Annex 7). This training was organised by Sara Amaral (head of the Science Communication Office at CNC-Center for Neuroscience and Cell Biology) with the participation of Marta Costa and Karine Paniza (Communication Division at University of Coimbra), with the participation of Mariana Alves (co-founder of Cartas com Ciência and selected for the Euclid Network in 2021, which highlights 100 women in Europe in Social Entrepreneurship).
The Science Communication workshop was a one-afternoon intensive course where the ESRs gained understanding in the importance and impact of communicating science for all publics. ESRs participated in a debate about knowledge democratization, followed by practical storytelling and media-training (how to speak to a camera) exercises. The workshop ended with the creation of a video (Figure 6) where the ESRs summarize what they learned in the media-training part of the course.



Figure 6. Media-training video. Click on the image for the full episode.

To expand the ESRs' international, intersectoral and interdisciplinary research and training, and to assist in building networks of working relationships to aid them in their future careers (**Task 4.3**), all ESRs except one (see ESR12 – Manuela Rizzi on section Deviations from Annex 1 and Annex 2) undertook **secondments** in other beneficiaries and/or partner organisations for a duration of up to 30% of the recruitment period:

Table II. All secondments performed by ESRs performed A) within and B) beyond the 36-months of contract.

Fellow Id	Name	Sending Organisation	Sector of Sending Org.	Sending Country	Secondment Organisation	Sector of Secondment Org.	Secondment Country	Start Date	End Date	Working Time Commitment	Working Time (%)	Duration (months)
A) Secondments performed before the end of the 36-months fellows' contracts												
11	Orsolya Antal	CNC	Academic	Portugal	CTI	Non-Academic	Portugal	02/11/2021	15/01/2022	Full Time	100%	2.47
9	Elisa Corti	CNC	Academic	Portugal	Lundbeck	Non-Academic	Denmark	25/10/2021	24/11/2021	Full Time	100%	1
10	Giuseppe Cammarata	CNC	Academic	Portugal	CHUC	Non-Academic	Portugal	01/11/2021	30/01/2022	Full Time	100%	3
11	Orsolya Antal	CNC	Academic	Portugal	EPFL	Academic	Switzerland	21/02/2022	30/06/2022	Full Time	100%	4.33
3	Alessandro	EPFL	Academic	Switzerland	CNRS	Academic	France	16/05/2022	16/08/2022	Full time	100%	3.07
10	Giuseppe Cammarata	CNC	Academic	Portugal	UEDIN	Academic	United Kingdom - hybrid	02/11/2022	10/02/2023	Full time	100%	3.33
17	Veronica Villeri	CNRS	Academic	France	CTI	Non-academic	Portugal - remote	01/08/2022	30/09/2022	Full time	100%	2.00
17	Veronica Villeri	CNRS	Academic	France	UEDIN	Academic	United Kingdom	18/07/2022	30/09/2022	Full time	100%	2.47
13	Marcos Sintes	ICL	Academic	UK	CHUC	Non-academic	Portugal	27/03/2023	31/03/2023	Full time	100%	0.13
16	Daniel Hunter	CNRS	Academic	France	UEDIN	Academic	United Kingdom	28/08/2022	03/01/2023	Full time	100%	4.27
14	Elizabeth Brockman	ICL	Academic	UK	CHUC	Non-academic	Portugal	13/11/2022	17/11/2022	Full time	100%	0.13
6	Laura Upton	CNRS	Academic	France	UEDIN	Academic	UK	16/09/2022	20/12/2022	Full time	100%	3.17
6	Laura Upton	CNRS	Academic	France	UEDIN	Academic	UK	06/04/2023	05/05/2023	Full time	100%	0.97
5	Flavio Tomasi	CNRS	Academic	France	CTI	Non-academic	Portugal - remote	08/10/2021	07/12/2021	Full time	100%	2.00
5	Flavio Tomasi	CNRS	Academic	France	CNC	Academic	Portugal	12/09/2022	23/12/2022	Full time	100%	3.40
8	Ágata Silván	Lundbeck	Non-academic	Denmark	EPFL	Academic	Switzerland - remote	09/03/2020	30/06/2020	Full time	100%	3.77
8	Ágata Silván	Lundbeck	Non-academic	Denmark	EPFL	Academic	UK - remote	01/03/2022	30/04/2022	Part time	50%	1.00
4	Loredana Cumpana	EPFL	Academic	Switzerland	CTI	Non-academic	Portugal - remote	24/01/2022	27/03/2022	Full time	100%	2.07
12	Vanesa Salazar	UEDIN	Academic	UK	CTI	Non-academic	Portugal - remote	05/01/2022	28/02/2022	Full time	100%	1.80
12	Vanesa Salazar	UEDIN	Academic	UK	CNRS	Academic	France	08/04/2022	08/07/2022	Full time	100%	3.03
B) Secondments performed during the project extended period, after the end of the 36-months fellows' contracts												
13	Marcos Sintes	ICL	Academic	UK	CHUC	Non-academic	Portugal	27/03/2023	31/03/2023	Full time	100%	0.13
16	Daniel Hunter	CNRS	Academic	France	UEDIN	Academic	UK	28/08/2022	03/01/2023	Full time	100%	4.27
14	Elizabeth Brockman	ICL	Academic	UK	CHUC	Non-academic	Portugal	13/11/2022	17/11/2022	Full time	100%	0.13
6	Laura Upton	CNRS	Academic	France	UEDIN	Academic	UK	16/09/2022	20/12/2022	Full time	100%	3.17
6	Laura Upton	CNRS	Academic	France	UEDIN	Academic	UK	06/04/2023	05/05/2023	Full time	100%	0.97
5	Flavio Tomasi	CNRS	Academic	France	CNC	Academic	Portugal	12/09/2022	23/12/2022	Full time	100%	3.40
8	Ágata Silván	Lundbeck	Non-academic	Denmark	EPFL	Academic	UK - remote	01/03/2022	30/04/2022	Part time	50%	1.00

Note: Of these secondments, only four are listed in the "Project Secondments Periods" table on the "Researchers" tab on the EC Portal, due to a technical issue that led to the inability to edit the table. The secondments presented here include all secondments performed by the ESRs.

To measure the satisfaction towards the secondments, ESRs and PIs were asked to rate role of the secondment(s) in the overall PhD training/PhD project. On a scale from 1 to 5, where five is the highest score, ESRs and PIs rated the impact of the secondments with 3.31/5 and 3.6/5, respectively.
For the detailed information regarding each of the secondments, including the objectives and tasks performed, refer to the document "Secondment Reports" (D4.7 D35), submitted via continuous reporting on the EC Portal on August 31st, 2023.

A **Personal Career Development plan** has been developed for each fellow (**Task 4.4**). The end-goal of this plan was to orient the training towards each fellow's career preference. The following points were covered:
- Long-term career objectives over 5 years, including the goals and the activity/training needed to attain these goals;
- Short-term objectives over 1-2 years, including the anticipated publications, anticipated participation in conferences/workshops/seminars; training in specific new areas and development of technical expertise; funding applications planned; communication activities; anticipated networking opportunities and other activities with professional relevance.
For more information regarding the Personal Career Development plan, including details for each fellow, refer to the document "Career Development Plan of ESRs" (D4.6 D32), submitted via continuous reporting on the EC Portal in February 2020.

The following **WP4 deliverables** were submitted:

Table III. Deliverables submitted for WP4.

Deliverable		Date of submission
D4.1 D18	Final Conference	31. Aug. 2023
D4.2 D24	Report on network school 1 (NS1)	16. Oct. 2019
D4.3 D25	Report on network school 2 (NS2)	09. May. 2022
D4.4 D26	Report on network school 3 (NS3)	02. Jul. 2021
D4.5 D27	Report on network school 4 (NS4)	09. May. 2022
D4.6 D32	Career development plan of ESRs	13. Feb. 2020
D4.7 D35	Secondment reports	31. Aug. 2023
D4.8 D37	Report on PhD courses	07. Set. 2020
D4.9 D42	Supervisory Board	09. Ago. 2019

The following **WP4 milestones** were achieved:

Table IV. Milestones achieved for WP4.

Milestone		Means of verification
MS7	Syn2Psy intermediate meeting	Syn2Psy intermediate meeting and project check was held online on May 3-4, 2021 with the participation of ESRs, PIs, Partners, one member of the EAB, the Project Officer and an external reviewer. The report was submitted as a deliverable (D6.4 D30) on July 2nd, 2021.
MS9	Personal career development plan	Written report concerning the ESRs personal career development plan was submitted as a deliverable (D4.6 D32) on February 13th, 2021.
MS13	Syn2Psy final meeting	The Final Conference was held at the EPFL in Lausanne on May 15-16, 2023 with the participation of ESRs, PIs, Partners and two invited keynote speakers. The report was submitted as a deliverable (D4.1 D18) on August 31st, 2023.

The following **WP4 milestones** was partially achieved:

Table V. Milestones partially achieved for WP4.

Milestone		Means of verification
MS12	PhD theses completed (13)	As of October 2023, the following ESRs have defended their theses: ESR10 Flavio Tomasi, ESR8 Elizabeth Brockman, ESR6 Daniel Hunter and ESR3 Giuseppe Cammarata. The remaining defences will be held from November 2023 to September 2024.

1.2.5 Work Package 5 – Dissemination & Communication and Exploitation

The objective of WP5 is to promote an efficient dissemination, communication and exploitation of the accomplishments of Syn2Psy, among the general public, the scientific community and other relevant stakeholders. The tasks within this work package include the creation of a public website to ensure project visibility, including contents for different audiences; the dissemination of Syn2Psy results through presentations, publications and reports; the organization of open seminars during the network schools to disseminate the outcomes of the project beyond the consortium; communication with relevant societal actors and multiple publics, including the production of articles for younger audiences and the creation of a theatre play on the activities of Syn2Psy.

The **public website** for Syn2Psy (**Task 5.1**), https://syn2psy.cnc.uc.pt/, was designed and implemented during the first months of the action and it is one of the main channels for dissemination of the information generated within the project. The *Home* area of the website gives an overview of the consortium, including information about the ESRs, the mission, the network, latest posts and news and a summary of past and future events. Furthermore, this section also has the purpose of highlighting the latest relevant activities of the consortium. Further details on each of the items, such as information about beneficiaries, partners, Principal Investigators and Early Stage Researchers, a description of the general objectives and work packages, as well as the specific individual projects and details on the network-wide events and secondments can be found on specific dedicated areas, through the *Menu*.
Relevant achievements of the consortium are shared through a *News* section and outreach materials produced within Syn2Psy are shared through a Blog Series, where the fellows write about a variety of topics in an informal manner, and through the SimpleSci video section, where videos about the individual Syn2Psy projects are published (described below).
The information available on the website was/is updated at regular, periodic intervals and aims at targeting the beneficiaries, partners and early stage researchers, as well as the wider scientific community, the general public and other stakeholders.
To increase visibility of Syn2Psy, profiles on social media were created for Syn2Psy on Twitter, Instagram, Youtube and Facebook platforms, where contents targeted at the general public, are shared.
Through the website, the implementation of outreach initiatives and with the presence on social media, Syn2Psy successfully reached a high number of people in the attempt to create awareness among the public about neuropsychiatric disorders and the need for research in this domain.
According to WP Statistics – Version 12.6.13, during the life-time of the project (until June 2023), Syn2Psy website had a total of 10 614 visitors and 34 141 visits.

Furthermore, Syn2Psy website also has a restricted area dedicated to internal communication. This restricted area was used as a virtual repository for relevant documentation, where beneficiaries and partners had access to documents regarding the networkwide events such as meetings, courses and internal Newsletters.



Figure 7. Print screen of the *Home* area of Syn2Psy website.

The **blog-post area** of the website had the purpose of allowing the ESRs to share their thoughts regarding science, their projects and/or the PhD experience in an informal and personal point of view. During the life-time of the project, the ESRs have actively participated in the creation of articles on various topics ranging from reflections regarding the current pandemic (loneliness, community, fake news), to scientific book reviews and creative writing.

All ESRs have written at least one blog-post (three ESRs contributed with two posts):

Table VI. Titles of blog-posts written by each ESR and uploaded to the blog area of the website.

ESR	Topic
ESR1 Orsolya Antal	An alternative approach to writing protocols
ESR2 Alessandro Chioino	A typical day in science
ESR3 Giuseppe Cammarata	1) Loneliness: the importance of social bonds; 2) The ethical emancipation of human neurodevelopmental studies
ESR5 Marcos Sintes	Journey logbook of a Ph.D. student
ESR6 Daniel Hunter	The Myth of Mirror Neurons: A Review of Hickok, 2014
ESR7 Elisa Corti	1) Email exchange with Ágata Silván; 2) Does free will exist?
ESR8 Elizabeth Brockman	A Note to Self: Reflecting on the Past and Present, Towards a Better Future
ESR9 Laura Upton	Animal Handling course and visit to the Musée Pasteur
ESR10 Flavio Tomasi	Back to the future
ESR11 Ágata Silván	Email exchange with Elisa Corti
ESR12 Manuela Rizzi	1) Diary of an early stage researcher; 2) The power of (science) communication
ESR13 Loredana Cumpana	One year of Swiss cheese and chocolate
ESR14 Vanesa Salazar	Chilling adventures of an early stage researcher

According to WP Statistics – Version 12.6.13, during the life-time of the project (until June 2023), the Blog Series had a total of 2 333 visits.

The **SimpleSci Series** is a collection of short-videos with the goal of describing, in a simple and attractive manner, each of the fourteen projects of this consortium. The target audience is the general public. The videos are created in a way that allows viewers with little to no scientific background to understand at least part of the contents, thus getting a general idea of what the main unanswered questions are, and the kind of research being done in the field of neuropsychiatric disorders. Furthermore, these videos also include a few elements for a general public with more scientific background or scientific interest.
Through the release of this video series we also aim to promote the consortium and the project's mission, to create awareness about neuropsychiatric disorders and the need for research in this domain, as well as to contribute to improvement of the population's scientific knowledge.

Six episodes have been released during the life-time of the project. They can be found on the Syn2Psy website (https://syn2psy.cnc.uc.pt/?page_id=4293) and on social media: Twitter - @Syn2Psy, Instagram - @msca_syn2psy, Facebook - @syn2psy and Youtube – syn 2psy:

Table VII. SimpleSci episodes released.

SimpleSci Episode	ESR
Episode 1	ESR12 Manuela Rizzi
Episode 2	ESR1 Orsolya Antal
Episode 3	ESR9 Laura Upton
Episode 4	ESR7 Elisa Corti
Episode 5	ESR2 Alessandro Chioino
Episode 6	ESR6 Flavio Tomasi
Episode 7	ESR13 Loredana Cumpana – soon to be released

According to WP Statistics – Version 12.6.13, during the life-time of Syn2Psy (until June 2023) SimpleSci Series had a total of 286 visits on the website, 910 views on Youtube and 553 views Instagram (total on all platforms 1749 views).

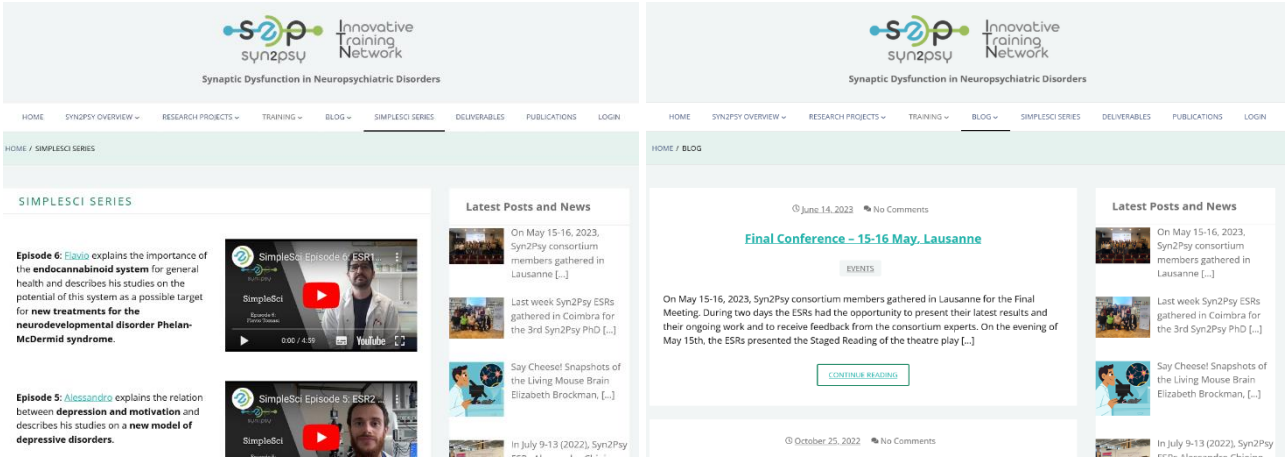


Figure 8. Print screens of the Blog and SimpleSci areas of the website.

Syn2Psy ESRs were encouraged to **discuss and present research results** and other Syn2Psy outcomes at major neuroscience events and to publish their research in main neuroscience scientific journals (**Task 5.2**). As mentioned on the "Note about COVID-19" of this report, the life-time of the project was severely impacted by COVID-19-pandemic and subsequent restrictions and confinement measures. This led to delays in the projects due to the closure of laboratories, with an extra waiting time for the animal models to be ready for the experiments, after the end of the lockdown.
As a consequence of the delay in the production of results, most of the fellows are now in the process of submitting manuscripts to scientific journals and thus, so far, several have not had the opportunity to publish in scientific journals.

Publications by the ESRs:

- **ESR2 Alessandro Chioino**, submitted as co-author a manuscript entitled "Mitofusin-2 in nucleus accumbens D2-MSNs regulates social dominance and neuronal function" to the journal *Molecular Psychiatry*. DOI: 10.1016/j.celrep.2023.112776.
- **ESR3 Giuseppe Cammarata**, published as co-author an article entitled "Generation and Characterization of Novel iPSC Lines from a Portuguese Family Bearing Heterozygous and Homozygous GRN Mutations" in the journal *Biomedicines*. DOI: 10.3390/biomedicines10081905

- **ESR5, Marcos Sintes**, posted as co-author on BioRxiv, a study on the potential effects of psilocybin for the therapy of autism spectrum disorder. Full posted article: https://www.biorxiv.org/content/10.1101/2020.09.09.289348v1.
- **ESR6, Daniel Hunter** published, as first author, a review under the title "Autoimmunity and NMDA receptor in brain disorders: Where do we stand?" in the Journal *Neurobiology of Disease*. DOI: 10.1016/j.nbd.2020.105161.
  Daniel also submitted as first author a manuscript entitled "Converging synaptic and network dysfunctions in distinct autoimmune encephalitis" to the journal *Brain*. Pending review.
- **ESR7 Elisa Corti**, published, as first author, a review under the title "The role of post-translational modifications in synaptic AMPA receptor activity" in the journal *Biochemical Society transactions*. DOI: 10.1042/BST20220827.
  Elisa also submitted as co-author a manuscript entitled "Amygdalar NT3-TrkC pathway underlies inter-individual differences in fear extinction and related synaptic plasticity" to the journal *Molecular Psychiatry*. Pending review.
- **ESR11 Ágata Silván**, submitted as first author a manuscript entitled "Gating small conductance calcium activated potassium channels in the thalamic reticular nucleus" to the journal *Synapse*. DOI: 10.1002/syn.22283.
- **ESR14 Vanesa Salazar**, submitted as co-author a manuscript entitled "Key roles of C2/GAP domains in SYNGAP1 related pathophysiology" to the journal Cell reports. Pending review.

Presentation of results in scientific events:

ESR1 Orsolya Antal

Conference	Date	Location	Poster/ Oral	Title of presentation
The Synaptic Dimension of Brain Disorders – "Current Trends in Biomedicine" workshop	4-6 Oct 2021	Baeza, Spain	Poster + Oral	Cognitive behavioural deficits in a knock-in mouse model with a human schizophrenia-associated mutation in the CACNG2 gene
XVII Meeting of the Portuguese Society for Neuroscience	1-3 Dec 2021	Coimbra, Portugal	Poster	Cognitive behavioural deficits in a knock-in mouse model with a human schizophrenia-associated mutation in the CACNG2 gene
8th European Synapse Meeting	20-21 Oct 2022	Coimbra, Portugal	Poster	Cognitive behavioural deficits in a knock-in mouse model with a human schizophrenia-associated mutation in the CACNG2 gene
ENCODS 2023 Meeting	1-2 May 2023	Faro, Portugal	Oral	Cognitive behavioural deficits in a knock-in mouse model with a human schizophrenia-associated mutation in the CACNG2 gene
FENS Regional Meeting	3-5 May 2023	Albufeira, Portugal	Poster	Cognitive behavioural deficits in a knock-in mouse model with a human schizophrenia-associated mutation in the CACNG2 gene

ESR2 Alessandro Chioino

Conference	Date	Location	Poster/ Oral	Title of presentation
49th Meeting of the European Brain and Behavior Society	4-7 Sep 2021	Lausanne, Switzerland	Poster	Impact of Mfn2 in the Nucleus Accumbens on motivated behavior and underlying neurobiological mechanisms
FENS regional meeting	25-27 Aug 2021	online	Poster	Impact of Mfn2 in the Nucleus Accumbens on motivated behavior and underlying neurobiological mechanisms
Meeting of Neuroleman Network school	7 May 2021	online	Poster	Impact of Mfn2 in the Nucleus Accumbens on motivated behavior and underlying neurobiological mechanisms
FENS Forum	9-13 Jul 2022	Paris, France	Poster	Impact of Mitofusin 2 on accumbal-related behaviors and underlying neurobiological mechanisms
8th European Synapse Meeting	20-21 Oct 2022	Coimbra, Portugal	Poster	Impact of Mitofusin 2 on accumbal-related behaviors and underlying neurobiological mechanisms

ESR3 Giuseppe Cammarata

Conference	Date	Location	Poster/ Oral	Title of presentation
XVII Meeting of the Portuguese Society for Neuroscience	1-3 Dec 2021	Coimbra, Portugal	Poster	Neurodevelopmental effects of valproic acid exposure in human brain organoids
FENS Forum	9-13 Jul 2022	Paris, France	Poster	Neurodevelopmental effects of valproic acid exposure in human brain organoids
8th European Synapse Meeting	20-21 Oct 2022	Coimbra, Portugal	Poster	Neurodevelopmental effects of valproic acid exposure in human brain organoids

ESR6 Daniel Hunter

Conference	Date	Location	Poster/ Oral	Title of presentation
FENS Forum	9-13 Jul 2022	Paris, France	Poster	Electrophysiological Characterisation of Hippocampal Networks in Anti-NMDA Receptor Encephalitis: From Synapse to Circuit

ESR7 Elisa Corti

Conference	Date	Location	Poster/ Oral	Title of presentation
XVII Meeting of the Portuguese Society for Neuroscience	1-3 Dec 2021	Coimbra, Portugal	Poster	Fragile X Mental Retardation Protein mediates BDNF-induced increase of synaptic NMDA receptors content
RNA Mechanisms and Brain Disease	20-23 Oct 2021	Rungstedgaard, Denmark	Poster	Fragile X Mental Retardation Protein mediates BDNF-induced increase of synaptic NMDA receptors content

FENS Forum	9-13 Jul 2022	Paris, France	Poster	Fragile-X Mental Retardation protein mediates BDNF-induced upregulation of synaptic NMDA receptors
8th European Synapse Meeting	20-21 Oct 2022	Coimbra, Portugal	Poster	Fragile-X Mental Retardation protein mediates BDNF-induced upregulation of synaptic NMDA receptors

ESR8 Elizabeth Brockman

Conference	Date	Location	Poster/ Oral	Title of presentation
FENS Forum	9-13 Jul 2022	Paris, France	Poster	Dissecting the contribution of astrocytes and upper layer neurons to human cortical circuit dynamics in Down Syndrome
8th European Synapse Meeting	20-21 Oct 2022	Coimbra, Portugal	Poster	Dissecting the contribution of astrocytes and upper layer neurons to human cortical circuit dynamics in Down Syndrome
British Neuroscience Association (BNA) 2023 Festival of Neuroscience	23-26 Apr 2023	Brighton, UK	Poster/ Oral	Illuminating the Mechanisms of Synaptic and Neural Network Dysfunction in Down Syndrome

ESR9 Laura Upton

Conference	Date	Location	Poster/ Oral	Title of presentation
Scientific days ED3C	8-9 Apr 2021	online	poster	Altered prefrontal cortex assembly in mice deficient in the autism-associated gene Glra2
FENS Forum	9-13 Jul 2022	Paris, France	Poster	No loss of glutamatergic neurons or interneurons in mice lacking the autism-associated gene Glra2 encoding the glycine receptor α2 subunit

ESR10 Flavio Tomasi

Conference	Date	Location	Poster/ Oral	Title of presentation
Scientific days ED3C	8-9 Apr 2021	online	poster	Assessing the endocannabinoid system as a therapeutic target in autism using Shank3-deficient mice
FENS Forum	9-13 Jul 2022	Paris, France	Poster	Assessing the endocannabinoid system as a therapeutic target in autism using Shank3-deficient mice
Second European Conference on Phelan-McDermid Syndrome	9-11 Jun 2023	Madrid, Spain	Oral + Poster	Inhibition of the cannabinoid CB1 receptor rescues deficits in a mouse model of Phelan-McDermid syndrome

ESR11 Ágata Silván

Conference	Date	Location	Poster/ Oral	Title of presentation
ENCODS	4-6 Jun 2021	online	Poster	Gating modulation of SK channels in the Thalamic reticular Nucleus
NeuroGrad Winter School	20 Jan 2022	online	Oral	Gating modulation of SK channels in the Thalamic reticular Nucleus

ESR12 Manuela Rizzi

Conference	Date	Location	Poster/ Oral	Title of presentation
6th Annual SIDB (Simons Initiative for the Developing Brain) Retreat	8-9 Sep 2022	Edinburgh, UK	Poster	Divergent translation profiles in CA1 pyramidal neurons from 2 mutant models of autism
8th European Synapse Meeting	20-21 Oct 2022	Coimbra, Portugal	Poster	Identifying convergent dysregulation of mRNA translation in multiple models of autism

ESR13 Loredana Cumpana

Conference	Date	Location	Poster/ Oral	Title of presentation
49th Meeting of the European Brain and Behavior Society	4-8 Sep 2021	Lausanne, Switzerland	Poster	The modulation of thalamic reticular nucleus neurons by corticotropin-releasing hormone
FENS Regional Meeting 2021	25-27 Aug 2021	online	Poster	The modulation of thalamic reticular nucleus neurons by corticotropin-releasing hormone
Neuroleman Network and Doctoral Schools Annual Meeting 2021	7 May 2021	online	Poster	The modulation of thalamic reticular nucleus neurons by corticotropin-releasing hormone
Annual Meeting of Swiss Society for Neuroscience 2022	29 Jan 2022	Fribourg, Switzerland	Poster	
FENS Forum	9-13 Jul 2022	Paris, France	Poster	The Modulation of Thalamic Reticular Nucleus Neurons by Corticotropin-Releasing Hormone

8th European Synapse Meeting	20-21 Oct 2022	Coimbra, Portugal	Poster	The Modulation of Thalamic Reticular Nucleus Neurons by Corticotropin-Releasing Hormone
Annual Meeting of Swiss Society for Neuroscience 2023	8-10 June 2023	Lugano, Switzerland	Poster	The Modulation of Thalamic Reticular Nucleus Neurons by Corticotropin-Releasing Hormone

ESR14 Vanesa Salazar

Conference	Date	Location	Poster/ Oral	Title of presentation
5th Annual SIDB (Simons Initiative for the Developing Brain) Retreat	30-31 Aug 2021	Edinburgh Hybrid	Poster	Reduced social interaction in two rat models of SYNGAP1 haploinsufficiency
Social Behavior Symposium - Alicante	7-9 Oct 2021	Online	Poster	Reduced social interaction in two rat models of SYNGAP1 haploinsufficiency
Gordon Research Conference: Novel Technologies to Advance Discovery of Disease Mechanisms and Therapeutics for Fragile X and Autism	15-20 May 2022	Lucca (Barga), Italy	Poster	Reduced social interaction in two rat models of SYNGAP1 haploinsufficiency
FENS Forum	9-13 Jul 2022	Paris, France	Poster	Reduced social interaction in two rat models of SYNGAP1 haploinsufficiency
6th Annual SIDB (Simons Initiative for the Developing Brain) Retreat	8-9 Sep 2022	Edinburgh, UK	Poster	Reduced social interaction in two rat models of SYNGAP1 haploinsufficiency

In order to **disseminate the outcomes of the project beyond the consortium** (**Task 5.3**) Network-School 1 was open to 6 external researchers from CNC and part of the Final Conference was open to 13-15 researchers from EPFL.

An important aim of Syn2Psy is to **increase awareness of the progress and benefits of brain research** (**Task 5.4**) by engagement with various audiences in European and global campaigns such as Brain Awareness Week and other public health initiatives:

Table VIII. ESR's participation in outreach activities.

ESR	Outreach activity
ESR1 Orsolya Antal	**European Researchers' Night (2021)** – Stand with materials and games to engage with public (children and adults) and promote knowledge of neuroscience.
ESR2 Alessandro Chioino	**EPFL Open days (2023)** – Informal conversations regarding scientific projects, including Syn2Psy project, with teenage visitors to EPFL.
ESR3 Giuseppe Cammarata	**Brain Awareness Week** (2021) – online event *AperiNeuro: fai la tua domanda!* (AperiNeuro: ask your question!), organized by *Biologi per la Scienza* (Biologists for Science), an Italian website dedicated to popularisation of science. **European Researchers' Night (2021)** – Stand with materials and games to engage with public (children and adults) and promote knowledge of neuroscience.
ESR7 Elisa Corti	**Brain Awareness Week** (2021) – online event *AperiNeuro: fai la tua domanda!* (AperiNeuro: ask your question!), organized by *Biologi per la Scienza* (Biologists for Science), an Italian website dedicated to popularisation of science.

	PubHD – event organized by PhD students, in Coimbra, to explain PhD projects to a non-scientific audience in a pub. **European Researchers' Night (2021)** – Stand with materials and games to engage with public (children and adults) and promote knowledge of neuroscience.
ESR10 Flavio Tomasi	Presentation and informal conversations with families of patients with Phelan-McDermid syndrome at the Second European **Conference on Phelan-McDermid Syndrome**.

For more information on the outreach activities, including description, target public, metrics and photographs, refer to deliverable "D5.2 Report on outreach and public engagement" submitted via continuous reporting on the EC Portal on August 31st, 2023.

With the goal of **writing communication articles shaped for younger audiences** (**Task 5.5**), Syn2Psy approached the journal Frontiers for Young Minds (FYM) with a description of Syn2Psy consortium. This led to the invitation to submit several abstracts for the FYM editors to judge the suitability of Syn2Psy topics for the Neuroscience section of this journal. After all abstracts were deemed suitable (abstracts on Annex 8), the ESRs, organised in seven groups, proceeded with the writing of the manuscripts, with the support and expertise of Syn2Psy's partner, PIN. One article has been published, one has been submitted and the others are in preparation.

Topics and authors are the following:

Table IX. Authors, titles of the articles and status of manuscripts for the journal Frontiers for Young Minds.

Groups	Title	Status
ESR2 Alessandro Chioino ESR13 Loredana Cumpana	Reaching for the top: the role of mitochondria.	Ongoing
ESR9 Laura Upton ESR10 Flavio Tomasi	Fantastic beasts and where to find them: how can we use animals to study neuropsychiatric disorders?	Ongoing
ESR7 Elisa Corti ESR12 Manuela Rizzi	The play-doh brain: shapeable neurons make you learn!	Submitted
ESR1 Orsolya Antal ESR11 Ágata Silván ESR4 Veronica Villeri	Schizophrenia - what causes hallucinations and distorted thinking?	Ongoing
ESR3 Giuseppe Cammarata ESR5 Marcos Sintes	How to build a mini human brain.	Ongoing
ESR6 Daniel Hunter ESR14 Vanesa Salazar	A brain composed by multilingual neurons: A story of coded messages and crosstalking.	Ongoing
ESR8 Elizabeth Brockman	Say Cheese! Snapshots of the Living Mouse Brain. Link to article.	Published

Under the guidance of Marionet, Syn2Psy fellows have written the **script and presented the play on the activities of Syn2Psy** (**Task 5.6**), under the title LAB 404 – A collective delirium about life in the Lab". The play was performed in the form of a Staged reading at the EPFL, Lausanne, as part of the Final Conference programme for Syn2Psy members and 11 external researchers. Link to complete performance here.
For more information regarding the theatre play, including full script and feedback from the fellows, refer to deliverable "D5.2 Report on outreach and public engagement" and "5.4 Theatre", both submitted via continuous reporting on the EC Portal on August 31st, 2023.

The following **WP5 deliverables** were submitted:

Table X. Deliverables submitted for WP5.

Deliverable		Date of submission
D5.1 D29	Syn2Psy public website	19. Nov. 2019
D5.2 D33	Report on outreach & public engagement	31. Aug. 2023
D5.3 D34	Public media dissemination	07. Out. 2019
D5.4 D38	Theatre	31. Aug. 2023
D5.5 D39	Report on the potential for results exploitation	

The following **WP5 milestones** were achieved:

Table XI. Milestones achieved for WP5.

Milestone		Means of verification
MS2	Syn2Psy internal website	Website available for the consortium members with updates on documentation, progress on ESR projects and other information.
MS6	Public website	Operational public website
MS10	Public media dissemination	Press releases were published in the beginning of the project.
MS11	Provisional patents established	ESR10 filed a patent application for the use of compound AEF0217 in Phelan McDermid Syndrome.
MS13	Syn2Psy final meeting	Final Conference was held at the EPFL in Lausanne, on May 15-16 2023.

1.2.6 Work Package 6 – Management

WP6 had the goal to warrant an efficient operational, administrative and financial management of Syn2Psy in order to meet with the European Commission rules. The tasks within this WP include overall project management and reporting; meeting objectives and deadlines; organizing project meetings and managing internal communication; the recruitment of the fellows according to the good practice procedures in the European Code of Conduct; project monitoring and quality control.

To ensure a seamless and efficient management of the project implementation, **a governance structure was implemented** (**Task 6.1**).
This governance structure is composed as follows (as described in the Consortium Agreement):

- **Supervisory Board (SB)** is the ultimate decision-making body of the consortium. The Supervisory Board was responsible for overseeing the quality of the network-wide training of ESRs and for ensuring that scientific/technological training is balanced with transferable skills training appropriate to the needs of each recruited researcher. The Supervisory Board also had the role to oversee the quality and quantity of supervision of the ESRs.
- **Research Coordination Committee** was responsible for oversight of the recruitment of ESRs and shall report and be held accountable by the Supervisory Board. This board was responsible for oversight of recruitment, for monitoring the supervision and co-supervision of all ESRs, and for approving secondments to Partner Organisations.
- **Training/Doctoral Studies Committee** had the role to provide advice to the Supervisory Board on all training and transfer of knowledge issues and to report to and be held accountable to the Supervisory Board. The Training Board was responsible for approving all training plans/reports and secondment reports, ensuring implementation of the training program.
- **D&C Committee** was responsible for managing the dissemination and outreach activities of the Project and for oversight of protection and exploitation of the research results.

- **Management Board** was composed of the Project Coordinator and the Project Manager. The Management Board had the responsibility to adopting main decisions taken by the Supervisory Board regarding financial issues, work plan, intellectual property rights and the progress of the network consortium, after decision of the Supervisory Board.
- **ESR Board** was composed of three ESRs. The ESR Board changed annually and represented the ESRs in all issues related to the work and contract conditions. The ESR Board provided feedback to the Supervisory Board. Feedback from each ESR was obtained through the annual progress monitoring meetings and the ESR Board.
- **External Advisory Board** consisted of two European and non-European top researchers in the research field of the Project, who were appointed to observe and advise on the strategic direction of Syn2Psy research training programme.
- A **project manager**, based at CNC, was recruited and was responsible for handling administrative, financial and legal management. The project manager provided direct assistance to the project coordinator, oversaw the ESRs recruitment process, the logistical preparation of the network-wide events, the production of technical and financial reports and managed the website as well as the outreach initiatives.

Five **project meetings** were held during the lifetime of the project (**Task 6.2**): Kickoff meeting, 1st and 2nd online PhD day, 3rd PhD day and Final Conference.

Syn2Psy **Kick-off meeting** took place on 26th and 27th of September 2019 at Exploratório in Coimbra with 41 participants, including Early Stage Researchers, Scientists-in-Charge representing each of the beneficiaries, and representatives from partner institutions. The purpose of this meeting was to introduce all team members of this consortium, allowing the start of the discussions regarding the activities planned within the scope pf Syn2sy.

The main topics covered by the discussions were the project structure, goals, timeline and events; finanancial aspects, management structures, reporting, open access policies and ethics; implementation strategy and plans for future work; Network Schools, secondments and Career Development plans; dissemination and communication activities, Syn2Psy presence on social media, participation on promoting awareness activities and the communication to different audiences through participation of PIN and Marionet;

The first official Supervisory Board meeting took place during this meeting, during which the members of the other governance bodies were assigned (described on task 6.1).
Throughout the two days of meetings, several informal and social activities took place, which allowed networking between the different actors of this consortium.
For more information regarding the Kick-off meeting, refer to the document "Report on the Kick-off meeting" (D6.1 D22), submitted via continuous reporting on the EC Portal in October 2019.



Figure 9. Group photography taken on September 26th, 2019 in Coimbra, during the Syn2Psy Kick-off meeting.

Due to the cancellation of all the Syn2Psy events planned for 2020 and early 2021, and given the uncertainty at that time regarding traveling in the months that followed, the consortium members agreed to participate remotely on the **1st PhD day** (October 5th 2020, Zoom) and **2nd PhD day (**October 23rd and 24th 2021, Zoom). The goal of these events was to promote scientific discussion and interaction between the consortium members and to give the opportunity to the ESRs to receive scientific feedback.
The Training and Doctoral Studies Committee (TDSC) had an active role in assessing the ESRs' progress in both events (based on the relevance of the scientific question, pertinence of experimental approach, project progress and training) and had the responsibility to prepare individual evaluation reports with feedback and recommendations, which were sent to each ESR. This was also the case for Syn2Psy External Advisors: Prof. Claudia Bagni from University of Lausanne, Switzerland and Prof. Gavin Rumbaugh from Scripps Research, Florida-USA, who had an active role in giving feedback and suggestions to the ESRs during the 2nd PhD day.



Figure 9. Photo taken during the 1st online PhD day.

The **3rd PhD day** was held in person in Coimbra on October 18-19, 2022 and had the participation of 23 members of the consortium. Similarly, to the 1st and the 2nd PhD days, this event had the purpose to give the opportunity to the ESRs to receive scientific feedback from the experts in the consortium. This event was further complemented with a session with Marionet and a Science communication course (see complete programme on Annex 7).

The **Final Conference** was held at the École Polytechnique Federal de Lausanne (EPFL), in Switzerland, on May 15-16, 2023, hosted by Prof. Carmen Sandi (principal investigator at IINS), Dr. Simone Astori (researcher at EPFL) and Barbara Goumaz (administrative assistant at EPFL). This event consisted of two days of presentations, including ESR's oral presentations and posters, keynote seminars, presentations from Syn2Psy PIs and Partners. The theatre play on the topic of neuropsychiatric disorders: "LAB 404 – A collective delirium about life in the Lab", written and presented by the ESRs under the guidance of Marionet, was presented in the form of a Staged Reading.
Furthermore, the keynote lectures from Nicola Allen, Salk Institute for Biological Studies (*Astrocyte-neuron interaction in health and disease*) and Gaia Novarino, Institute of Science and Technology Austria (*Illuminating human cortical circuit wiring and dysfunction in vivo*), were open to external researchers (13-15 researchers from the Brain Mind Institute at EPFL attended).
For more information on the final conference, including details of the programme, attendees and photographs of each presentation, refer to the document "Final Conference" (D4.1 D18), submitted via continuous reporting on the EC Portal on August 31st, 2023.



Figure 10. Syn2Psy Consortium members during the Final Conference at the EPFL.

For the same motives as described above for the online PhD days, a **remote meeting between the Project Officer (PO) and the ESRs**, originally planned for the Mid-term meeting, was held in October 2020. This was a confidential and private meeting between the PO and the ESRs only, with the goal of ensuring that administrative and supervision aspects of the project are appropriately implemented. The PIs were allowed to join the introductory part of this meeting, as well as the Project Coordinator and Project Manager, who were also requested to stay for a feedback session.
During the feedback session, the PO reported the fellows to be happy with the projects, the institutions and their work. The issues raised regarded secondments, allowances, teaching hours and the impact of the current pandemic on their projects.

During the life-time of the project, regular **internal communication** (**Task 6.2**) was achieved through electronic correspondence, dedicated mailing lists and virtual meetings. The internal area of the website was used to share Syn2Psy documents, including ESRs presentations/data and information on progress.
Moreover, two newsletters containing a compilation of activities carried out, were sent to all consortium members (newsletters 1 and 2 in Annex 9).

For the **recruitment of the fellows** (**Task 6.3**), ESR positions were advertised in EURAXESS, Era Careers, FENS Job Market, on the websites of the research centres where the action takes place, and through mailing lists from past and present collaborators and colleagues from the researchers. The candidates applied through the Consortium website (formerly www.syn2psy.eu, now www.syn2psy.cnc.pt) by stating their first second and third preferred project.
Applications included a Motivation Letter, Curriculum Vitae and contacts from 2 references. The eligibility criteria were described on the application section of the website as follows:
- MSc holder;
- Not a PhD-holder;
- Up to 4 years (full-time equivalent) of research experience;
- Up to 12 months in the last 36 leading to the recruitment, in the country hosting the ESR Position;
- Capacity to work in a multidisciplinary team, in an international environment and independently;
- Strong interest in research and science;
- Excellent English communication skills, orally as well as in scientific writing.

The Research Coordination Committee (RCC) prepared evaluation forms for the curricular analysis and the interviews and proposed the recruitment procedures to be implemented as follows:

1. Applications - deadline 15th May 2019;
2. Shortlisting (until 7th June 2019): Supervisors proposed a list of applicants for shortlisting. Each focused on the candidates with their project "first choice" but all supervisors were allowed to shortlist any of the applicants. An evaluation form was produced for each candidate (even for those not shortlisted). At least 3 candidates were shortlisted for each position.
3. Interview (local selection) (until 21st June 2019): Among the shortlisted candidates, each PI performed Skype interviews with those shortlisted candidates that marked their project as "first choice project". Each PI selected 1 candidate as their first choice. Subsequently (when necessary), shortlisted candidates (except PI's "first choice candidate") were interviewed by the PI of the "second choice project", etc. These interviews followed local institutional rules. A research paper was sent to the candidates to be interviewed, for discussion during the interview.
4. Confirmation (RCC and across consortium) (until 28th July 2019): The RCC checked the choices made for all positions. All PIs had the opportunity to confirm or oppose the choice done by each PI for their project.
5. Candidates were contacted (before 5th July 2019). In the case the candidate did not accept the position, the next candidate in the ranking was contacted.

For more details on the recruitment, refer to "Annual Progress Report 1" (D6.3 D28), submitted via continuous reporting on the EC Portal in February 2020.

After the resignation of ESR4 Diogo Soares in November 2020, ESR4 position was re-opened for applications. The recruitment procedures were carried out similarly to what described above for the recruitment of the 14 fellows. The position was opened on the CNRS website on November 23rd (2020). At first, until December 14th (2020) and then, due to the reduced number of suitable candidates who applied, the deadline was extended until January 31st (2021). It was further advertised through EURAXESS, Era Careers, FENS job market and mailing lists.
After a pre-screening of the applications, 7 candidates were interviewed. The interviews took place during the first week of February and the final candidate had a second interview on February 8th (2021). This selection process and the final candidate was supervised and approved by the RCC. The final candidate Veronica Villeri is a Syn2Psy fellow since May 2021.
Details of the position announcement can be found in the document "ESR4: Diogo Soares' resignation and plan for ESR4 project", submitted via formal notification on the EC Portal in January 2021.

A **quality control/project monitoring** (**Task 6.4**) process has been implemented in terms of revision of the project outputs, within which all deliverables are primarily revised by the coordinator, helped by the project manager.

The following **WP6 deliverables** were submitted:

Table XII. Deliverables submitted for WP6.

Deliverable		Date of submission
D6.1 D22	Report on the Kick-off meeting	16. Out. 2019
D6.2 D23	Internal website	19. Nov. 2019
D6.3 D28	Annual progress report 1	29. Feb. 2020
D6.4 D30	Report for the intermediate Syn2Psy meeting	02. Jul. 2021
D6.5 D31	Annual progress report 2	01. Mar. 2021
D6.6 D36	Annual progress report 3	28. Feb. 202
D6.7 D40	Final technical and financial project report	August 2023
D6.8 D41	Consortium Agreement	30. Oct. 2019

D6.9 D43	Data Management Plan	13. Dec. 2019
D6.10 D44	Potential input for Policy feedback	August 2023

The following **WP6 milestones** were achieved:

Table XII. Milestones achieved for WP6.

Milestone		Means of verification
MS1	Kick-off meeting	Kick-of meeting report with a summary of the meeting was produced.
MS2	Syn2Psy internal website	Website available for the consortium members with updates on documentation, progress on ESR projects and other information is available.
MS3	Accomplished ESR recruitment	All ESRs were recruited before M12.
MS4	Designing research/outreach plan	Full research plan for ESRs, including predicted Secondments have been established.
MS5	Annual progress report 1	Written report was produced.
MS7	Syn2Psy intermediate meeting	Written report was produced.
MS8	Project Check	Written report was produced.
MS9	Personal career development plan	Written report concerning the ESRs personal career development plan was produced.
MS13	Syn2Psy final meeting	Written report was produced.

1.2.7 Work Package 7 – Ethics requirements

WP7 focuses on the ethics requirements that Syn2Psy must apply, to ensure that the all possible domains of the research activities are in compliance with the fundamental ethical principles and legislation requirements.

Each beneficiary submitted to the coordinator authorisations for activities raising ethical issues. The documents produced for each requirement are described below.

H – Requirement No. 1
Ethics documentation regarding participation of humans in ESR3 project - *Production and testing of human derived neurons and brain organoids: advanced model probing in neurodevelopmental disorders* - supervised by João Peça at CNC, Coimbra – Portugal. Documents provided:

- Ethics self-assessment: criteria to identify research participants and informed consent procedures;
- Informed consent forms and information sheet;
- Project ethics approvals for the research with humans;
- Incidental findings;
- Statement by the Syn2Psy external ethics advisor.

HCT – Requirement No. 2
Ethics documentation regarding the use of human cells. Documents provided:

- Collaborator support letter for the use of human cells obtained within another project, including details on cells type and authorisation by primary owner of data for project ESR8 - *Illuminating the mechanisms of synaptic and neural network dysfunction in a new in vivo model of Down syndrome* - supervised by Vincenzo De Paola at ICL, London – UK.

- Documents regarding human cells obtained from a cell repository, for project ESR12 - *Identifying convergent dysregulation of mRNA translation in multiple models of autism* - supervised by Emily Osterweil at UEDIN, Edinburgh – UK.

POPD – Requirement No. 3
Data protection policy documentation for research participants of project ESR3 - *Production and testing of human derived neurons and brain organoids: advanced model probing in neurodevelopmental disorders* - supervised by João Peça at CNC, Coimbra – Portugal.
Data Protection Policy document approved by the Data Protection Officer at CNC includes a description of the technical and organisational measures that will be implemented to safeguard the rights and freedoms of the data subjects/research participants.

A – Requirement No. 4
Documentation regarding Project Specific Authorisations and/or Project Licence requests as well as Animal Facilities authorisations for each ESR's project; Personal Licences for animal handling for all ESRs except ESR3, ESR4 and ESR6 who have not performed animal experimentation.

EPQ – Requirement No. 5
Documents regarding facilities authorizations, security classification of laboratory, GMO authorizations (when applicable) and health and safety procedures conforming to relevant local/national guidelines/legislation are followed for Syn2Psy ESRs.

GEN – Requirement No. 6
Carla Barbosa has been appointed as Syn2Psy external ethics advisor and will monitor ethics issues associated with the project, especially the research involving children and persons unable to give informed consent. In this deliverable, submitted to the REA, we included:

- Syn2Psy external ethics advisor curriculum;
- Non-disclosure Agreement.

As stated on this deliverable, the present Technical Report includes an Ethics Report by Carla Barbosa (Annex 10).

The following **WP7 deliverables** were submitted:

Table XII. Deliverables submitted for WP7.

Deliverable		Date of submission
D7.1 D45	H - Requirement No. 1	29. Feb. 2020
D7.2 D46	HCT - Requirement No. 2	29. Feb. 2020
D7.3 D47	POPD - Requirement No. 3	29. Feb. 2020
D7.4 D48	A - Requirement No. 4	29. Feb. 2020
D7.5 D49	EPQ - Requirement No. 5	13. Feb. 2020
D7.6 D50	GEN - Requirement No. 6	13. Feb. 2020

There are no **milestones** in WP7.

2. Impact

The information described on section 2.1 of the DoA, regarding how the project will contribute to the expected impacts, is still relevant.

3. Update of the plan for exploitation and dissemination of results

The plan for exploitation and dissemination of results as described in the DoA does not need to be updated.

4. Update of the data management plan

The Data Management Plan (DMP) as updated on the Technical Report for the first reporting period was not further updated.

5. Follow-up of recommendations and comments from previous review(s)

(i) In October 2020, the ESRs had a (remote) private meeting with the REA Project Officer. Feedback comments provided by the Project Officer and information on how it was followed up:

- **ESRs expressed the need to have more clear information about the future secondments.**

  The ESRs, together with their supervisors made efforts to clearly establish their future secondments, despite the uncertainty of the current COVID-19 situation.

- **ESRs expressed the need to have more clarifications about living and mobility allowances from the host organizations;**

  The ESRs were advised to contact the National Contact Point, who should provide clear information regarding the net allowance, the mobility allowance and the taxation that each country applies.

- **Teaching hours can't be considered as part of the fellows' research project. The fellows should be focused 100% on the implementation of the action as per Grant agreement;**

  The fellows have worked work 100% exclusively for the implementation of the action.

- **Please provide the fellows with some fall-back plans in case of any deviations to the implementation of the action.**

  Each of the ESRs and PIs established a fall-back procedure to mitigate the consequences of the pandemic, in order not to jeopardise the implementation of the action. This included a slight modification of the research plan and changes in the planned secondments to a remote (or hybrid) mode and/or decrease in time, cancelation or adaptation of the objectives.

(ii) Syn2Psy intermediate meeting was held online on May 2 – 3, 2021. This meeting counted with the participation of ESRs, scientists-in-charge representing each beneficiary, representatives from each partner institution, the Project Officer (PO), the REA External Reviewer, appointed by the PO, and one member of the Syn2Psy External Advisory Board. Feedback comments provided by the Project Officer and information on how it was followed up:

- **The Project Officer started by acknowledging that the project encountered some issues and delays due to the COVID-19 pandemic and that Syn2Psy coped with this situation in a positive manner. Since some COVID-19-related restrictions were still ongoing at that time, the Project Officer advised Syn2Psy to have good communication among consortium members and to manage this situation by planning activities, trainings, secondments and future PhD defences in advance.**

Syn2Psy Supervisory Board effectively discussed and coordinated a strategy to implement the planned activities: Network Schools 2 and 4 and the course in Science Communication were deemed important to have in-person and were thus postponed for 2022 and 2023.
The 2nd PhD day (2021) was maintained in a remote mode and plans were made to have the 3rd PhD day in person in 2022. Secondments were adapted: non-academic secondments were transferred to a remote mode whenever the tasks to be performed were considered to be feasible remotely without a loss in content; modification in academic secondments was analysed on a case-by-case basis, which led to several changes to a hybrid mode, decrease in time and, in certain cases, cancelation.

6. Deviations from Annex 1 and Annex 2

During the life-time of the project, Syn2Psy submitted two Consortium Requested Amendments (AMD-813986-3 and AMD-813986-8) to the REA, which were accepted respectively on March 9th, 2021 and on August 11th, 2022. AMD-813986-3 amendment included modifications in four of the projects, changes to secondments' duration and/or host institution for several ESRs and modification of the dates for the network-wide events, while AMD-813986-8 consisted in the request for a 6 months extension of the project and the transfer of Veronica Villeri from CNRS to UEDIN (former ESR4, now ESR15).

Furthermore, several secondments were modified, cancelled or changed to a remote/hybrid mode. These modifications and respective justification were submitted via formal notification on the EC Portal:

- "Modification to Secondments due to COVID19-related delays", submitted on 14th March 2022;
- "Update secondment ESR4-ESR6-ESR7", submitted on 3rd March 2022;
- "Update secondments ESR2_ESR13", submitted on 22nd December 2022.

After the change of supervision of ESR5 Marcos Sintes from Prof. Thomas Knöpfel to Prof. Vincenzo De Paola, Marco's project was modified, and the secondments planned for the lab of Prof. Thomas Knöpfel were also replaced. These changes were submitted via formal notification on the EC Portal on 21 March 2022.

Apart from the changes included in the amendments and the documents submitted via formal notification, during the lifetime of Syn2Psy, other projects went through modifications either due to a change in supervision, due the consequences of the delays caused by COVID-19 pandemic or due to the natural progression of science and from new ideas being generated following the results of the previously proposed projects.

Importantly, all modifications described below are still part of the Syn2Psy topic and do not affect the ethical issues, work-packages and milestones. Furthermore, these changes were agreed between ESRs and Principal Investigators as being more suited to the project progression and career development plan of the students and do not affect the implementation of the action.

ESR4 – Diogo Soares/Veronica Villeri

Deliverable D2.1 - Identification of the link between synaptic nanoscale alterations and behavioural defects in animal and human models of Shank3-related ASD – corresponding to the presentation of scientific data in a peer-reviewed manuscript, in the context of ESR4 project, was eliminated.

This project was started by Diogo Soares, who resigned in November 2020. The project was continued by Veronica Villeri, who started her functions in May 2021. After Veronica's decision to stop working under the co-supervision of Eric Hosy and, due to this, not being able to continue this project (ESR4 project was conceived by Eric Hosy, and stems from previous data from his group), Syn2Psy Supervisory Board decided, with the accordance of Veronica Villeri, to transfer the fellow to another Syn2Psy laboratory. Veronica Villeri identified Emily Osterweil's group at UEDIN as being the one that that most fit to her scientific interests and training goals.
This led to the end of project ESR4 at IINS-CNRS and start of project ESR15 - Testing gene therapy for Fragile X Syndrome - at UEDIN. This modification was included in the amendment AMD-813986-8, approved in August 2022.
Veronica Villeri worked on ESR4 project at IINS-CNRS for 13.5 months and on ESR15 project at UEDIN for 9.5 months. Due to the short time of participation in each of the above-mentioned projects, it was estimated that

it would not be feasible to gather enough scientific data to present in a peer-reviewed manuscript. Thus, this deliverable was removed from the Grant Agreement in the amendment submitted in August 2022.

ESR5 – Marcos Sintes

The current version of ESR5 project included a secondment at EPFL, in the lab of Prof. Carmen Sandi. The goal of this secondment was to provide training in techniques that were not employed in Vincenzo de Paola's research group at Imperial College. The primary objective of this secondment was to gain skills in electrophysiology and RNAScope, and subsequently apply these techniques to generate useful data for Marco's project. These experiments were designed to be carried out on animal models that had been transplanted with human-derived cells. At that time, the production of these cells was done by a collaborator based in London. Unfortunately, this collaborator encountered difficulties in generating new cells, forcing Marcos and fellow group members to undertake the cell production process themselves, which required several months of optimization.

Consequently, as most of Marcos's experiments needed these cells, a significant setback occurred in the project timeline. As a result, Marcos was unable to generate the necessary animals within the planned timeframe for the secondment. Once the experiments were resumed, Marcos and his supervisor had to take the decision of cancelling the secondment due to having limited time left for the rest of experiments. Additionally, Marcos had the opportunity of continuing his project in Vincenzo De Paola's lab at Duke-NUS in Singapore (Prof. De Paola moved to Duke-NUS, while keeping some of his responsibilities at ICL, including the supervision of the ESRs Marcos Sintes and Elizabeth Brockman). This meant that alternative options for the experiments planned for the secondment became available with local collaborators, which also solved the logistic issues of sending animals or tissue samples overseas. In this institution Marcos has access to a new collaborative network including world-renowned experts in the field of iPSC-derived neuronal culture research, as well as in-house experts in electrophysiology and two-photon *in vivo* imaging. Thanks to these new resources Marcos will be able to expand the scope of the project, solve experimental issues, and produce more valuable data, which is proving to be a more beneficial option as compared to the previously planned secondment given the time limitations. This will allow Marcos to fulfil Syn2Psy objectives, including the publication of results and finishing the PhD studies.

ESR6 – Daniel Hunter

The objectives described in the Grant Agreement Annex I Part B are the following: A)Track NMDAR subtypes at the surface of pyramidal cells and interneurons in hippocampal brain slices using single nanoparticle (Quantum Dot) tracking; B) Apply super-resolution approaches (uPAINT and STORM) in order to provide the first high-density quantitative map of surface NMDAR subtypes in pyramidal cells and interneurons from brain slices; C) Study the physiological impact of the extracellular environment on the NMDAR dynamic, both in pyramidal cells and interneurons; D) Use rodent models of schizophrenia to determine the alteration of NMDAR surface distribution, dynamics and transmission in this disorder; and E) Manipulate the NMDAR surface dynamics in schizophrenia models in order to restore the proper transmission and prevent the behavioural deficits.

The initial primary objective for the project was to investigate the surface dynamics and organisation of NMDARs on hippocampal interneuron populations and principal cells (Objective A and C), and the subsequent organisation of NMDARs on these neuron populations (Objective B). Other objectives stem from this in such that we hoped to draw comparisons in our findings here to commonly used rodent models of schizophrenia and developing tools to rescue the deficits induced (Objectives D and E). However, we had difficulty in labelling hippocampal interneuron populations as initially planned, in both live cell cultures and fixed cell preparations of both intact and dissociated tissue. Since the primary focus of this work was to elucidate the impacts of NMDAR dysfunction on hippocampal interneurons, we focussed on hippocampal function by quantifying the impacts on both spontaneous excitatory and inhibitory postsynaptic currents onto hippocampal pyramidal cells. This approach allowed us to remain investigating the role of hippocampal interneuron dysfunction, as a consequence of NMDAR disorganisation on this cell population. Essentially, shifting our attention onto characterisation of excitatory and inhibitory signalling and synaptic organisation, and the dysregulation of these parameters after exposure to NMDAR-Abs maintains the core aims of the original proposal. Namely, to identify synaptic mechanisms that are dysfunctional in neuropsychiatric disorders. Our current and continuing work has already made strides towards this goal, by identifying a disinhibition in hippocampal networks by NMDAR-Abs, converging the etiology of disease with other psychiatric disorders such as schizophrenia. Finally, we hope our findings will still allow us to propose possible

routes of rescue in the employed in vitro models, and to develop and test possible drugs or other therapeutic interventions which may rescue the altered network imbalance.

ESR8 – Elizabeth Brockman

Initially, the secondment at IINS in the lab of Prof. Laurent Groc was planned for the 3rd year of Elizabeth's project, in the aftermath of COVID-19 pandemic. Since Elizabeth is Canadian, she would have needed a visa to work in another country for 4 months which, at that time, was not being issued due to COVID-19 restrictions still going on by then. When the issuing of visas was resumed, the lack of clarity regarding upcoming COVID-19 shutdowns meant that planning for a visa application was out of the question until the 4th year. Once COVID-19 travel restrictions were less of an issue, in Elizabeth's final year, it became clear that superresolution microscopy was no longer a useful avenue to explore in her project.

The plan for the secondment at IINS was to perform superresolution microscopy analysis of synaptic molecular alterations in neurons that harbour trisomy of chromosome 21 (Ts21). Unexpectedly, Elizabeth's experiments did not show differences between Ts21 neurons and controls. Since there was no difference found, the planned superresolution microscopy experiments at IINS was no longer of use for Elizabeth's project. Furthermore, traveling to another lab during the final year would have lost precious experimental time, as Elizabeth would have had to re-train in a new lab for use of animals there, a process that takes several months.

Additionally, COVID-19 lockdowns had prevented work for many months, meaning that the experiments at the home-lab required immediate and prioritized attention to be completed, for Elizabeth to be able to fulfil Syn2Psy objectives, such as gathering enough data to publish and defend the PhD thesis.

Overall, this cancelation is due to the experimental outcomes which negated the need for the experiments planned, due to the impact of COVID-19 restrictions in the project and the need to stay at the home-laboratory to perform important experiments and obtain results to fulfil the objectives of Syn2Psy.

ESR11 – Ágata Silván

Due to an extended period of sick leave, Ágata did not accomplish all the objectives for the secondment at UEDIN. This secondment had the objective of generating a relevant transgenic rat model to be used by Ágata at Lundbeck. Particularly, the main goal of the secondment was to use the CRISPR-Cas9 system to generate both knock-out (KO) and knock-in (KI) point mutation models for the cacna1i gene, which encodes for one of the most relevant ion channels pacemaking the activity in thalamic reticular neurons (CaV3.3).

Ágata did not perform the molecular characterization of the model at UEDIN. However, before the start of the sick leave, Ágata learnt about the designing process using CRISPR-Cas9. During this time, Ágata had meetings with Dr. Katie Paton and Dr. Peter Kind, where the plan on how to address the design of the models, exon of choice and guide RNAs was discussed. The approach taken for the KO model was a Cas9 double-strand break (DSB) induced within exon 13 to delete exon 13 of Cacna1i gene and create a frameshift in the transcript. For the design of the guide RNAs, the CRISPOR design tool (http://crispor.tefor.net/) was used to search within the exon sequence, or intron sequences flanking the exon of choice, to identify guide RNAs with predicted low off-target activity and high efficiency.

ESR12 – Manuela Rizzi

The initial methodology envisaged for answering the key question consisted in Ribosome Profiling applied to the exploration of disrupted mechanisms in rat models of *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ disease as well as human cellular models. COVID-19 pandemic and other technical difficulties encountered, paused this plan and redirected the objectives to the comparison between *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ mouse models instead by means of TRAP technology. ESR12 proceeded with the initial characterization of *Syngap*$^{+/-}$ dataset precedently pre-processed in the lab. DESeq analysis revealed misregulated targets which were compared with the ones previously published Fmr1$^{-/y}$ CA1 dataset leading to the observations described in WP3 Task 3.3.

Furthermore, delays caused by COVID-19 pandemic and the natural progression of the project forced Manuela to modify the secondment originally planned for the beginning of her project. Manuela's project was greatly impacted by the confinements and the restriction measures put in place at UEDIN, which led to the need to make adjustments in the project in order to prevent major delays.

Manuela's new planned secondment involved collaboration with Prof. Ana Luisa Carvalho to perform analysis of ribosome trafficking and with Prof. João Peça to explore human-derived neurons and brain organoids as models of neurodevelopmental disorders. Both secondments would have been carried out at CNC-Coimbra. However, prioritization of completion of the experiments and analysis of results pushed the starting period of the secondments to a point where it would longer be beneficial for Manuela. On the contrary, their accomplishment, which would have required Manuela to move abroad for 3 months in a critical step of her PhD (the final months are being dedicated to thesis writing and manuscript preparation), presented as potential disruption to meet the deadlines for her thesis submission, in accordance with her PhD program at the University of Edinburgh. Moreover, imminent move of her supervisor to Boston to start a new position, redirected the priority to maximization of the time left in Edinburgh to provide Manuela with support and guidance throughout the final steps of her PhD completion, which would not have been compatible with Manuela moving to Coimbra during this stage. The fellow and her supervisor, consider this choice to guarantee an optimal progression towards completion of the PhD at her home-institution, in Edinburgh and thus, request the cancelation of both secondments, with Prof. Ana Luisa Carvalho and Prof. João Peça at CNC.

In addition, although thought to have initial potential for Manuela's project, these secondments could have possibly ended up not presenting as a concrete addition to her final project and thesis. In fact, after exploration of Manuela's data the focus was directed towards mechanisms of function different from the initially hypothesized ones which would require other exploratory analysis for better comprehension of the data, far from the one envisaged for the secondments.

ESR13 – Loredana Cumpana

Due to the development of the CRH sensor, GRAB_CRH, Loredana and her supervisors added a new aim, allowing the investigation of the levels of CRH release at the TRN throughout vigilance states. This information was important for the project as it guided our decisions on how to approach the new task, regarding the optimal time to manipulate the CRH release in-vivo. In addition, towards the aims of the consortium, we have added behavioural characterization of anxiety for all sleep experiments, expanding the potential implications for the modulation of NREM sleep by CRH and psychiatric disorders.

ESR14 – Vanesa Salazar

One of the deviations between Vanesa's research and the original Grant agreement is that the fellow did not study Fragile X syndrome. Since Vanesa was doing research on two different models of SYNGAP1 haploinsufficiency instead of only one, in addition to the time shortage due to COVID-19, focusing only on SYNGAP1 haploinsufficiency was considered a good and realistic adaptation of the project to meet the network expectations. Given the unforeseen circumstances of the pandemic this change allowed to get a deeper understanding of the mechanisms of different genetic mutations of the SYNGAP1 gene.
The first task displayed in the Grant Agreement was changed since there was a shortage of time by the COVID pandemic and some results obtained by other members of the lab did not show successful results in that direction.
To date, the exact nature of social behaviour is not fully understood for SYNGAP1 haploinsufficiency models, nor addressed with robust paradigms. Given social impairments are a main trait in people with ASD and there is a need for robust paradigms in the pre-clinical field, understanding the role of SYNGAP1 in the expression of social behaviour was a solid alternative while still framed on pursuing the understanding behaviour and connectivity traits on these conditions as mentioned in the agreement. The tasks on the agreement aiming to determine critical periods and therapeutically interventions were re-designed given the obtained results on this project and the state-of-the-art at the time of the project execution. It was precisely, to keep the project on the cutting-edge knowledge of the field, that Vanesa tested the effects of a stimulating and naturalistic intervention using the Habitat, a naturalistic highly socially and environmentally enriched set during development.

ESR15 – Veronica Villeri

The ESR15 project was initially planned to investigate the possibility of gene-therapy in adult $Fmr1^{-/y}$ mice. P21 or P60 $Fmr1^{-/y}$ mice had to be subjected to the intracranial injection of an AAV9-Fmr1 gene therapy vector in inferior colliculus, a region involved in the audiogenic seizure typical of Fmr1 KO mice. Subsequently, mice would have been tested for Audiogenic Seizures in order to assess whether Fmr1 reinsertion in the adult could

rescue the phenotype and if eventually this could occur in both age conditions. Unfortunately, the injection of either the gene therapy vector or the empty vehicle in our Fmr1 KO mice induced an increase in general mice activity and in the predisposition to seizures, hindering a clear investigation of our hypothesis.
ESR15 project was thus adapted to investigate how defects at the cellular level may be related to behavioural phenotypes in model of neuropsychiatric disorders and study possible convergence at the cellular level. This project is in accordance with Syn2Psy's objectives and was designed to allow the ESR to develop skills in confocal imaging and image analysis and to be exposed to fruitful discussions about translational defects in autism spectrum disorders model during lab meetings.
Description of the adapted project/objectives/tasks: Fragile X and SYNGAP1 syndromes are among the most frequent forms of monogenic autism disorders. Previous studies from the Osterweil and Kind labs have shown that the SynGAP1$^{+/-}$ and Fmr1$^{-/y}$ share the same hippocampal pathophysiology, characterized by an increase in protein synthesis. Likewise, there is evidence that both models display deficiencies in the amygdala-mediated fear conditioning task. Current work in the lab is thus investigating whether they share common translational defects in the hippocampus and in the basolateral amygdala. To this last aim, mice have been subjected to fear conditioning and subsequently their amygdalae have been collected and used for TRAP sequencing. An important part of the project is to determine whether there is any difference in the amygdala cell activation between the two autism models and the respective wild-type.
C-Fos expression level after the shock exposure has been associated with the ability of mice to acquire tone-dependent freezing. The main task is to perform c-Fos and neural markers immunolabeling on fixed basolateral amygdala slides from conditioned/home cage and WT/ SynGAP1$^{+/-}$ or Fmr1$^{-/y}$ mice and carry out image analyses to investigate changes in neurons activation.

Annex 1 – Detailed description of the achievements for each project

ESR1 – Orsolya Antal

ESR1: overview of results towards the objective of the action

Project title – Cognitive disorders as defects in synaptic function: insight from human mutations in the CACNG2 gene encoding Stargazin

WP1 – New models of neuropsychiatric disorders

Early Stage Researcher 1 – Orsolya Antal

Supervisor – Ana Luísa Carvalho

Host Location – Centre for Neuroscience and Cell biology (CNC), University of Coimbra – Portugal

Encoded by the CACNG2 gene, stargazin is a postsynaptic protein that is crucial for several aspects of normal synaptic transmission: ranging from the functional modulation of AMPA-type glutamate receptors, it impacts the assembly and trafficking of the AMPAR complex and is important for synaptic plasticity[1,2]. Alterations in this gene have been associated with schizophrenia, with deficits in stargazin mRNA levels found in post-mortem studies[3,4]. Previous work from our group identified a missense variant (subsequently referred to as StgSN) in this gene in a patient with schizophrenia the in vitro profiling of which suggested that this variant leads to disruptions in the function of the protein and could possibly contribute to phenotypes of synaptic dysfunction that is characteristic of schizophrenia.

Here, we hypothesize that genetic susceptibility, as conferred by the StgSN stargazin variant generates alterations in synaptic function and neuronal morphology, leading to cognitive behaviour phenotypes characteristic of schizophrenia. To assess this, we are pairing the behavioural battery with morphological and functional characterisation of prefrontal cortex neurons, as well as assessing alterations in metabolism, to investigate how changes in neuronal morphology and activity in the PFC correlate with the observed cognitive behavioural phenotypes.

1. Behavioural assessment of StgSN animals

The first objective of the project focused on the characterisation of behavioural phenotypes presented by animals carrying the StgSN variant. In one thread of experiments, animals were exposed to a battery of tests aimed at assessing locomotion as well as some mood-related phenotypes through the open field, elevated plus maze and forced swimming tests. There were no gross changes in activity or any alterations in anxiety-like behaviours or alterations in coping strategies between genotypes in these tests.

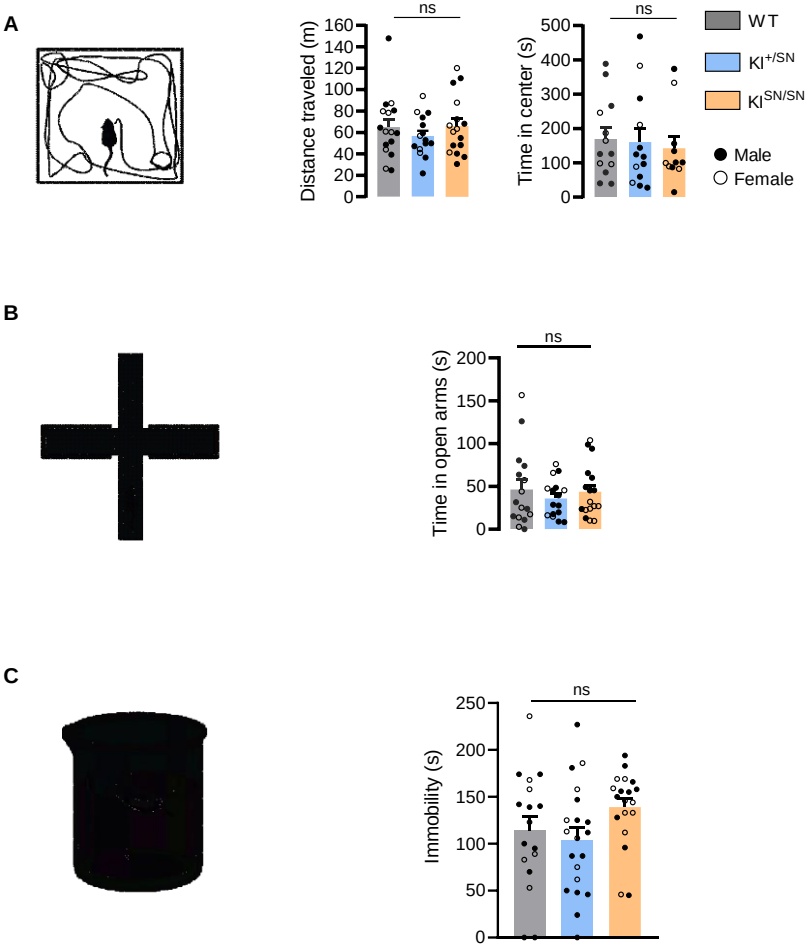


Figure 1. StgSN animals do not appear to present hyperactivity or changes in mood-related behaviours.
A) There were no alterations in the open field test either in locomotor activity (distance travelled) or time spent in the center of the arena (one-way Brown-Forsythe ANOVA, p=0.553, n≧16 for distance travelled, Kruskal-Wallis test, p=0.78, n≧16 for time in center). B) In the elevated plus maze test, there were no alterations in time spent in the open arms of the arena between genotypes (Kruskal-Wallis test, p>0.99, n≧16). C) There were also no significant changes between genotypes in time of immobility in the forced swim test (Kruskal-Wallis test, p=0.087, n≧17).

In parallel, animals were also assessed through a battery of cognitive tests aimed at assessing cognitive domains of spatial, spatial working and associative memory through the object displacement, T-maze and fear conditioning tests. Both heterozygous- and homozygous knock-in fail to alternate above chance level in the T-maze test and fail to interact with objects above chance level in the object displacement test compared to their wild-type counterparts. In the fear conditioning test, we have detected no changes in freezing behaviour between genotypes. However, when assessing fear conditioning, a higher proportion of hetero- and homozygous StgSN animals were successful at fear extinction (compared to wild-type counterparts).

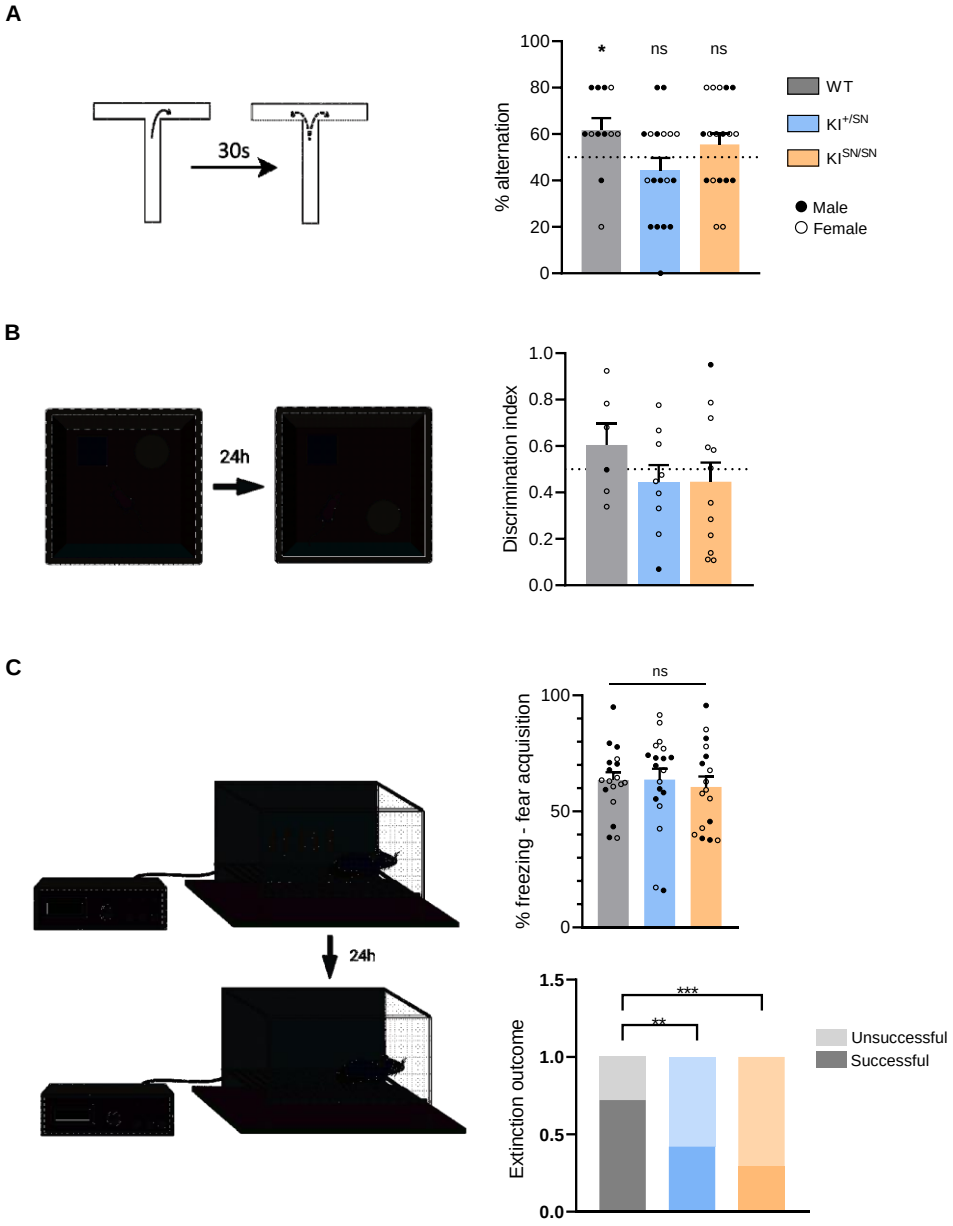


Figure 2. StgSN homozygous and heterozygous animals present alterations in some cognitive domains

A) Spontaneous alternation was assessed in the T-maze test, where wild-type animals alternated significantly above chance level (one-sample t-test, p=0.046), whereas heterozygous and homozygous animals did not (one-sample t-test, p=0.31 for heterozygous and p=0.26 for homozygous animals, n≧12 for all genotypes). **B)** In the object displacement task, preliminary results suggest a tendency for the heterozygous and the homozygous animals to fail to discriminate between the displaced and the original object. **C)** There were no changes between genotypes in the acquisition of fear memory in the fear conditioning test (Brown-Forsythe ANOVA, p=0.84). However, in the fear extinction phase, the proportion of animals that were successful at fear extinction presented a significant decrease compared to expected outcomes for both heterozygous and homozygous animals (binomial test, p=0.0079 for heterozygous and p=0.0003 for homozygous animals, n≧17 for all genotypes).

Overall, these results suggest cognitive deficits in spatial and associative memory as a consequence of the StgSN variant.

2. Neuronal and spine morphology in the prefrontal cortex

The prefrontal cortex is an area essential for many aspects of cognitive function and has been widely implicated as a site of dysfunction for neuropsychiatric disorders, including schizophrenia[5,6]. Impairments in PFC-related cognitive functions have been commonly observed in patients, while evidence from post-mortem studies from patients have shown changes in the expression of synaptic proteins[7,8], the dendritic morphology and spine density of PFC neurons[9], as well as differences in network activity[10]. Along with several other synaptic proteins, the expression of TARPs, including stargazin, has been shown to be altered in the PFC[11,12].

Given the essential function the PFC serves for many aspects of cognition, and its vulnerability to dysfunction due to various genetic or environmental factors in neuropsychiatric disorders, we have decided to focus subsequent analysis on the prefrontal cortex.

First, we aimed to assess phenotypes of neuronal morphology as well as dendritic spine density and morphology, focusing on layer 2/3 pyramidal neurons.

Sparse labelling of prefrontal neurons has been achieved by injecting animals with an adeno-associated virus (AAV) expressing GFP (AAV9.hSyn.eGFP.WPRE.bGH). GFP-expressing neurons residing in layers 2/3 in the PFC were then reconstructed in 3D and dendritic segments from both the apical and basal compartments of these neurons were randomly selected for the analysis of dendritic spine morphology.

Neuronal morphology was assessed, showing no alterations in dendritic complexity as assessed via Sholl analysis, and no changes in dendritic length either in basal or apical compartments, overall showing no gross changes in neuronal morphology between genotypes.

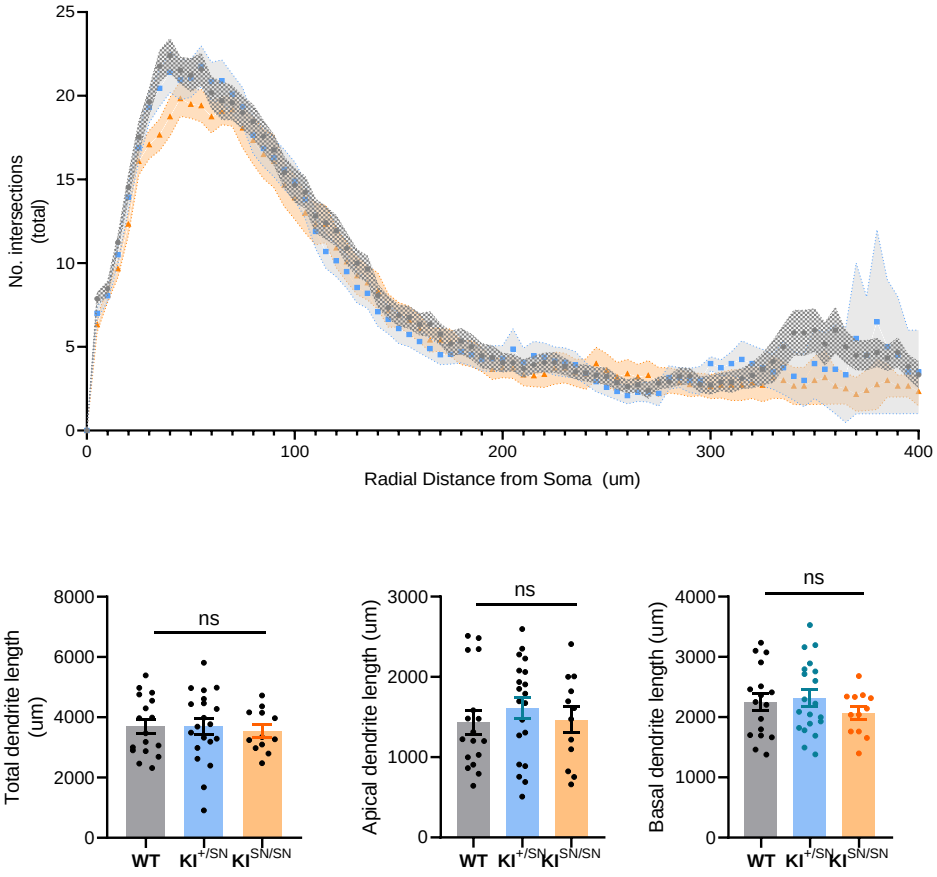


Figure 3. PFC layer 2/3 pyramidal neurons show no gross morphological alterations
Sholl analysis showed no changes in dendritic complexity between genotypes (Brown-Forsythe ANOVA computed for areas under the curve calculated for individual neurons, p=0.46, n≧12). There were also no changes in dendritic length, either total or considered for basal or apical compartments (Brown-Forsythe ANOVA, p=0.91 for total dendritic length, p=0.44 for basal and p=0.63 for apical dendritic length, n≧12).

To evaluate dendritic spine density and morphology, we selected dendritic segments from apical and basal compartments of neurons imaged for reconstruction and assessed spine density and spine morphology. To assess spine morphology, spines were manually categorised to five groups, as previously described[13].

There were no gross alterations in dendritic spine density either in apical or basal segments. Qualitative characterisation of spine morphology, however, revealed a change in spine subtypes, showing that more immature spine types (thin and filopodia-like spines) were enriched compared to mature spines (mushroom, branched and stubby-type spines) in heterozygous and homozygous knock-in animals, suggesting defects in synaptic function in these animals.

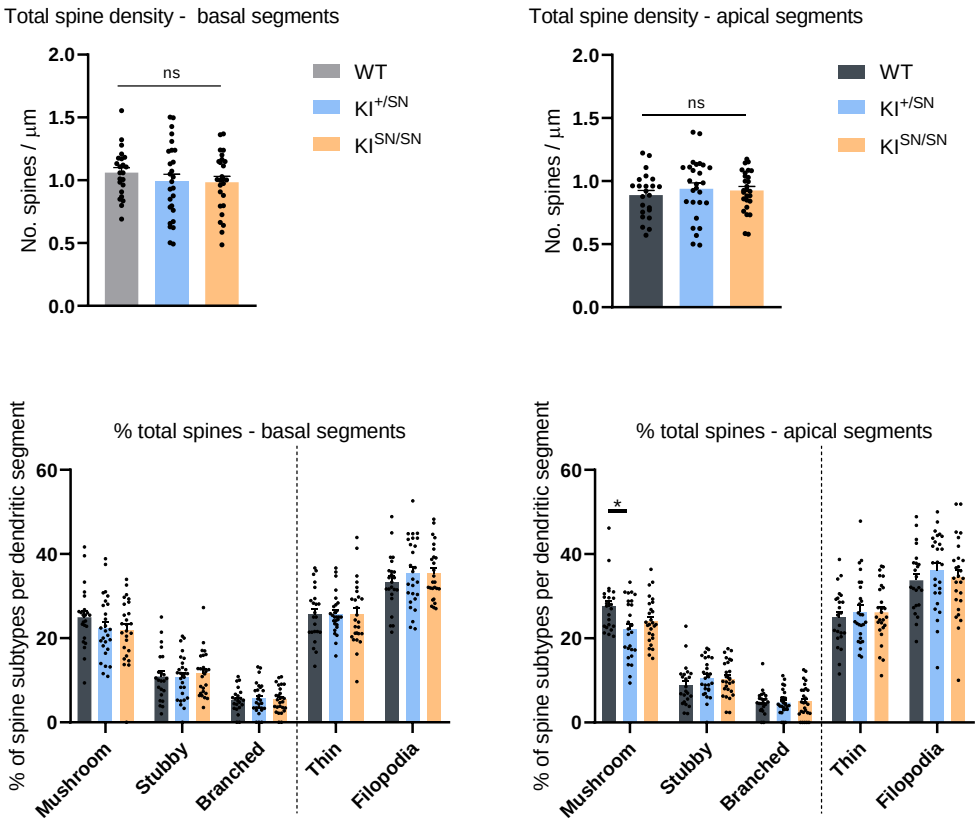


Figure 4. StgSN mice show subtle alterations of spine morphology in PFC layer 2/3 pyramidal neurons
Although no differences were detected in total spine density in dendritic segments from basal or apical compartments (points represent individual dendritic segments, Brown-Forsythe ANOVA, p=0.49 for basal segments and ordinary one-way ANOVA, p=0.65 for apical segments, n=5-9 segments from N=3 animals per genotype), heterozygous and homozygous StgSN animals present a tendency for decrease in the proportion of mushroom-type spines in apical and basal dendritic segments, which is significantly altered for mushroom-type spines in apical segments for heterozygous StgSN animals (two-way repeated measures ANOVA followed by Tukey's multiple comparisons test, adjusted p=0.011).

3. Assessment of mitochondrial structure and function

Mitochondria are crucially linked to many aspects of synaptic function – from being required for the maintenance of synaptic activity, involved in structural changes in response to activity, and play an overall crucial role for cellular function through their role as signal integration hubs[14] . Mitochondrial dysfunction is extensively linked to neuropsychiatric disorders. Even subclinical alterations to mitochondrial function can confer increased vulnerability to disease and contribute to diverse phenotypes seen in neuropsychiatric disorders [15,16]. Given the evidence linking synaptic and metabolic dysfunction[17], and considering evidence from our group for altered expression of mitochondrial proteins in animals carrying another, intellectual disability-associated variant of stargazin, we aim to explore changes to mitochondrial structure and activity in our model.

First, we aimed to assess mitochondrial function through measuring properties of mitochondrial respiration in the Seahorse assay, as previously described[18]. We analysed mitochondria isolated from the prefrontal cortex, with results so far showing a tendency for basal hyperactivity and increased H^+ leak in mitochondria isolated from homozygous knock-in animals (Fig 5. A). Concurrently, using the same assay for the functional assessment of elements of the electron transport chain (Fig 5. B), showing a similar increase in activity in homozygous animals. Finally, we have preliminarily assessed the capacity for calcium buffering and ROS production in these

mitochondria, with results suggesting an increase in ROS production, as well as an altered capacity for Ca uptake in mitochondria from the homozygous animals (Fig 5.C)

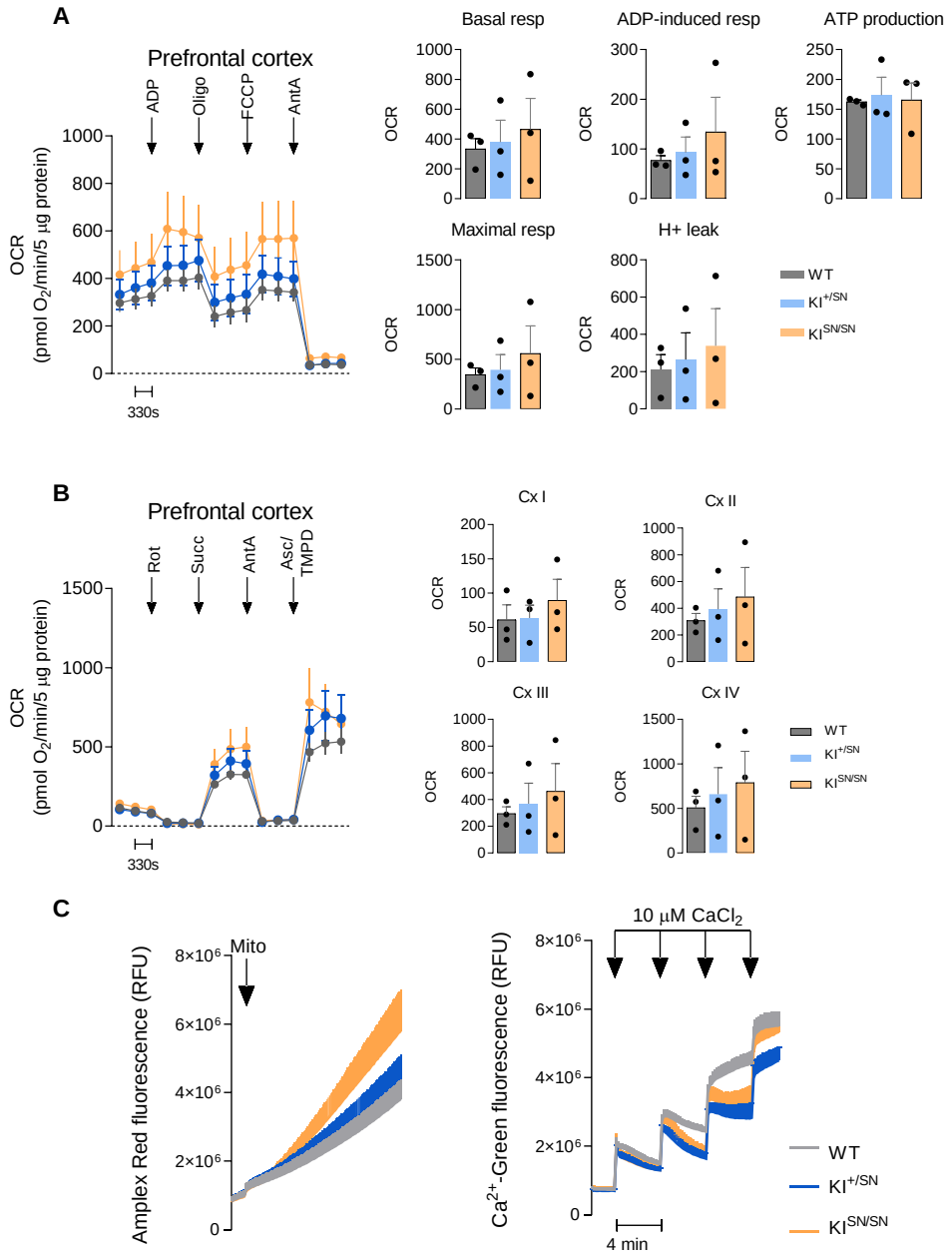


Figure 5. Preliminary results suggest altered mitochondrial activity in the prefrontal cortex

A) Assessment of mitochondrial respiration properties suggest a tendency for basal hyperactivity and increased H^+ leak in mitochondria isolated from homozygous StgSN animals, as well as a tendency for increased electron transport chain activity (B). C) ROS production (measured using H_2O_2 production and Amplex Red fluorescence as a proxy) appears to be increased in homozygous StgSN animals, while Ca-buffering capacity (as measured through Ca-uptake using Ca^{2+} Green fluorescence as a proxy) also appears to be altered in heterozygous and homozygous StgSN animals.

In parallel, we are evaluating mitochondrial morphology in StgSN animals, focusing on layer 2/3 in the infralimbic area of the PFC, to correlate changes in mitochondrial structure with functional alterations. Here, we visualised mitochondria using electron microscopy, followed by 3D reconstruction leveraging deep learning to quantify morphological parameters including the number, density and volume of mitochondria, and contacts with other organelles [19,20]. While further analysis of some of these morphological parameters is ongoing, results so far indicate that there are no gross overall changes, but a subtle shift to decreased mitochondria volume in mitochondria residing in dendritic compartments (Figure 6, A), whereas there are no such alterations in the volume of mitochondria in axonal compartments (Fig. 6, B). In addition to morphological properties, we have also assessed the properties of mitochondria-ER contacts (MERCs), showing a tendency for shortened contact sites in the heterozygous, but increased contact site length in the homozygous knock-in animals, suggesting potential alterations in some of the features of mitochondrial function mediated by MERCs.

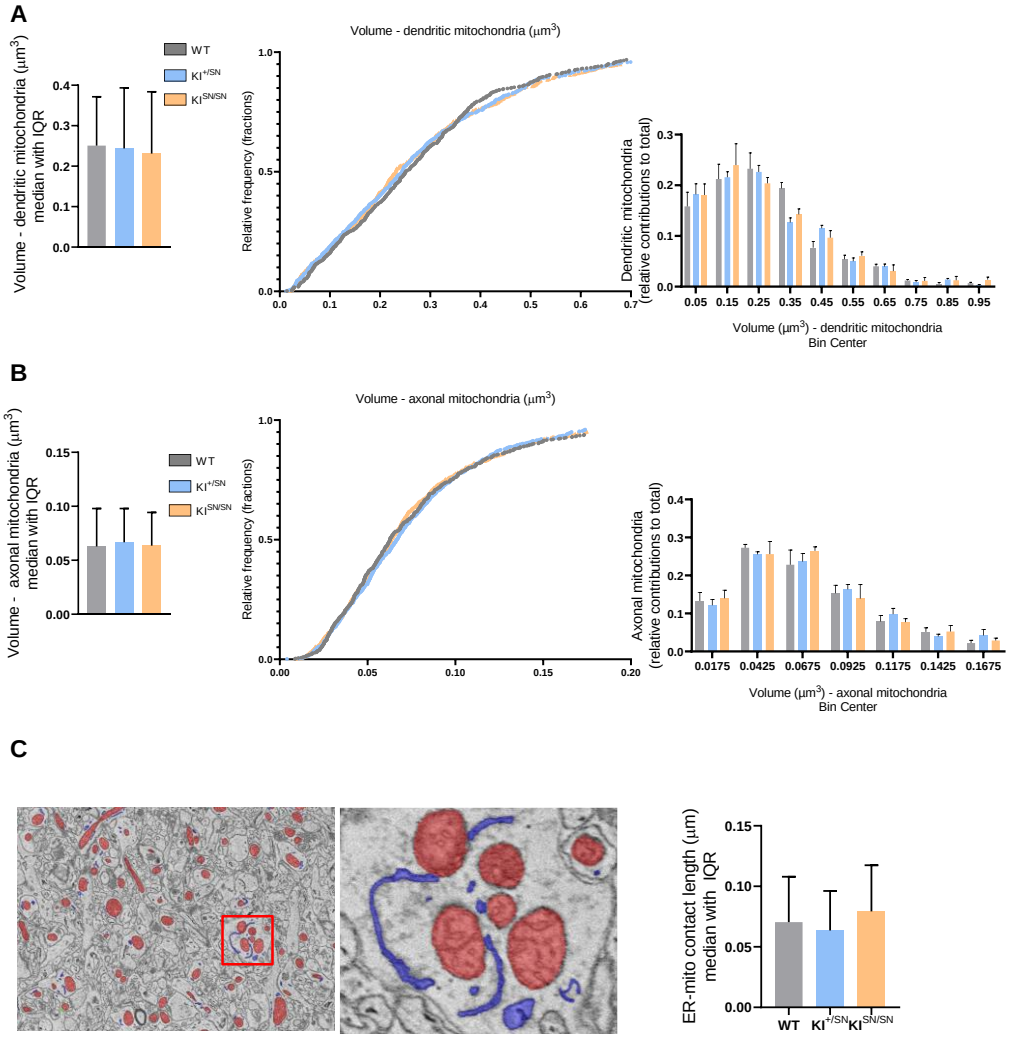


Figure 6. Preliminary results show that StgSN animals present subtle structural alterations and changes in mito-ER contacts
While mitochondrial volume does not appear to be changed overall either in dendritic (A) or axonal (B) compartments, in dendritic compartments (A) StgSN animals appear to have a higher proportion of small mitochondria, whereas the proportion of medium-sized and larger mitochondria show a tendency for a decrease. C) Preliminary assessment of mito-ER contact sites show a tendency for decrease in heterozygous, but an opposite tendency for increase in homozygous StgSN animals.

4. Secondment at CTI – training on the regulatory environment and design of clinical trials in neuropsychiatric disorders

In addition to the investigation of the neurobiological correlates of synaptic dysfunction and its involvement in the pathophysiology of schizophrenia, the project involved a secondment performed in collaboration with our partner CTI Clinical Trial & Consulting Services. This secondment provided an opportunity to leverage their expertise in clinical trials and the associated challenges with connecting the investigation of the neurobiology of disorders like schizophrenia to improved therapeutic options for patients, with the overall aim of the secondment achieved through designing a clinical trial proposal for a hypothetical drug aimed at improving cognitive performance in patients suffering from schizophrenia (as outlined below).

TRIAL SYNOPSIS

Trial Title:	A placebo-controlled, double-blind clinical trial to investigate the effects of a novel drug on cognitive functions in people with schizophrenia
Trial Rationale	Schizophrenia is one of the most common neuropsychiatric disorders, affecting around 1% of the population. While most often characterized by its positive and negative symptoms, disturbances in cognitive function are also common features of this disorder. Many currently available therapeutic options often fail to improve the cognitive impairments present in patients, making this an aspect of the disorder that presents a significant burden for patients' everyday life. Impairments circuit and synaptic function of the prefrontal cortex has been heavily implicated by recent research in contributing to the pathophysiological background of schizophrenia, with this area also crucial for many core cognitive functions. Restoring the normal functioning of circuitry within this area by improving diminished synaptic function therefore has promise in being able to improve cognitive performance in afflicted patients, improving the quality of life of patients suffering from cognitive impairments due to the disease. In this trial, a novel drug will be assessed for its effects on improving cognitive function in a population of patients diagnosed with schizophrenia but not currently suffering from an acute exacerbation of symptoms.
Primary Objective:	Evaluate the progression in cognitive functioning through assessing MCCB score changes (from baseline to MCCB score reassessed at 6 months)
Secondary Objective(s):	Evaluate progression in other schizophrenia-relevant symptoms (positive, negative or social) by assessing changes from baseline PANSS scores aimed at those domains at 6 months Evaluate potential changes in stress susceptibility by assessing changes to stress susceptibility scores compared to baseline at 6 months
Exploratory Objective(s), if any:	Evaluate neural correlates of changes in cognitive functioning by performing fMRI measurements of the prefrontal cortex at baseline and at 6 month

Trial Design:	The trial will be performed in randomized, double-blind groups. The trial drug or placebo will be administered on top of existing, stable treatment to remove potential deleterious effects as a consequence of discontinuing current treatment. The trial will be conducted in a single center in order to conform to concerns of feasibility (the recruitment of investigators and the homogeneity of patient population would be difficult to achieve with a multi-center trial at this step). The trial drug (or placebo) will be administered in a single dose, taken in a stable regimen (for example twice per week on the same days) over the course of the trial to establish potential effects of the trial drug at a stable concentration and remove potential bias due to changes in dosage.
Trial Population	Describe key inclusion and exclusion criteria. Inclusion: - Between 18-55 years of age - Diagnosis according to DSM-5, for at least 1 year - Capable of providing and have provided informed consent - Baseline MCCB score indicative of cognitive impairment Exclusion: - Acute exacerbation/episode of positive or negative symptoms in 3 months preceding start of trial - Change in medication in 3 months preceding start of trial, or use of recreational drugs that could confound trial data - Confounds to cognitive assays due to language concerns – native language should correspond to the language of the tests used for assessment during the trial - Confounding factors due to poor general health (severe cardiovascular disorders, conditions related to chronic pain, diagnosis with other neuropsychiatric disorders potentially impacting cognitive function)
Endpoints (strictly aligned with objectives hierarchy)	Primary endpoint: - Endpoint assessment (score change) in MCCB- after 6 months Secondary endpoints: - Score change related to positive or negative symptoms – assessed through the corresponding dimensions of PANSS- after 6 months - Score change in stress vulnerability scales - Change in social functioning- assessed through social dimension of PANSS - Safety – AEs during trial/discontinuation rate

OPTIONAL

Assessments/data to be collected	time point 1	time point 2	time point 3	time point 4	time point 5
	Baseline	1 months	3 months	6 months endpoint	
MCCB assay of cognitive performance	x			x	
General health check	X	X	X	X	
Assay of cognitive performance (different tests compared to baseline, but targeting same			X		
fMRI assessment of prefrontal cortex activity	X		X	X	
PANSS test for positive and negative symptoms and	X		X	X	
Vulnerability to stress using stress scales	X		X	X	

4. Conclusions

Previous work from our group has established that the StgSN variant of stargazin has altered functionality *in vitro*. Here, we have generated a knock-in mouse model to characterise the consequences of this variant *in vivo,* in assays ranging from assessing cognitive behavioural phenotypes and subsequently potential underlying neural correlates of such changes, to establish whether the StgSN variant generates disruptions in synaptic function, neuronal morphology and metabolic properties. Our results so far show deficits in StgSN animals in some cognitive domains, especially in spatial and associative memory, and subtle alterations in spine morphology in prefrontal pyramidal neurons. The assessment of mitochondrial activity and mitochondrial structure also appears to indicate hyperactivity in function and subtle alterations in mitochondrial structure, as well as altered properties of contacts between mitochondria and ER, a site that is crucial for various cellular and mitochondrial signalling processes. Taken together, evidence so far seems to indicate dysfunction in several properties related to normal synaptic function, downstream of alterations in cognitive performance in StgSN animals. However, several experiments to further assess synaptic function are ongoing. Synaptic function is being assessed through electrophysiology, targeting layer 2/3 pyramidal neurons to assess their intrinsic and functional properties, while further work focusing on assessing cellular and mitochondrial calcium signalling dynamics downstream of synaptic function could help shed light on the connection between altered mitochondrial signalling and synaptic dysfunction.

Overall, our results show that genetic susceptibility, as conferred by the StgSN stargazin variant generates some alterations in synaptic function and dendritic spine morphology, as well as producing cognitive behavioural phenotypes characteristic of schizophrenia. As ways of potential exploitation of the results of the project, we propose that the StgSN knock-in model has significant construct validity in modelling the impact of synaptic dysfunction in schizophrenia and provides a valuable tool for further investigation of the underlying pathophysiological mechanisms of features of the disorder as conferred by a genetic risk factor. We also propose that our model provides a platform to investigate how exposure to additional environmental factors may combine with preexisting genetic risk to exacerbate dysfunction and lead to disease-related phenotypes, leading to new insights on how disease states develop and revealing potential avenues for therapeutic intervention. Furthermore, in terms of generating new knowledge, investigating a variant of stargazin that impacts the normal functioning of the protein and its in vivo consequences on synaptic function also has the potential to provide a tool to further investigate the role of endogenous stargazin in regulating and maintaining normal glutamatergic transmission.

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ESR2: overview of results towards the objective of the action

Project title – Role of mitofusins in neurodevelopmental trajectories of psychopathology

WP1 – New models of neuropsychiatric disorders

Early Stage Researcher 2 – Alessandro Chioino

Supervisors – Carmen Sandi and Simone Astori

Host Location – Brain Mind Institute (BMI), École Polytechnique Fédérale de Lausanne – Switzerland

1. Evaluation of the activation of NAc circuits during an effort-related behavior:

To evaluate the impact of Mfn2-KD in D1-MSNs we initially employed a tamoxifen-inducible genetic mouse line: Mfn2-Tg(DrD1a-CreERT2)-R26-LSL-TdTom. The use of this mouse line ensures a homogenous downregulation of Mfn2 in D1-expressing cells (Mfn2-D1^{CreERT}).
Using this mouse line, we gained first indication that Mfn2 downregulation negatively impacts the engagement of the NAc during the exposure to an effortful task (forced swim test, FST), as indicated by cFos staining (Fig1A-C). Animals were sacrificed 90 minutes after the start of the test to target peak of cFos protein production.
To achieve region specific downregulation of Mfn2 in D1-MSNs, we injected Mfn2$^{lox/lox}$ mice with an AAV-Cre viral vector under the expression of a dynorphin promoter (DynCre) (Darvas and Palmiter, 2015), which was stereotactically delivered to the NAc (Fig2A).
The behavioral results show that Mfn2-deficient animals display a higher tendency to passive-coping strategy compared to control animals (Fig2A).
In line with the results obtained with Mfn2-D1^{CreERT} mice, D1-MSNs in NAc showed lower cFos protein expression after FST exposure (Fig2B). We performed cFos staining on multiple brain regions, which showed significantly lower activity in the infralimbic cortex (IL) and in the central amygdala (CeA) of Mfn2^{Dyn} mice. Worth noting, opposite to other brain areas, the ventral tegmental area (VTA) shows tendency to higher cFos proteins in Mfn2^{Dyn}. Network analysis performed separately on Mfn2^{Dyn} and Mfn2$^{+/+}$ animals (nodes based on cFos density, $p<0.1$; edges based on correlations, $r>0.5$; only regions meeting both requirements selected) showed that different brain circuits are activated during FST (Fig2C). Namely, WT subjects display higher activity in the anterior cingulate cortex (ACC) and motor cortex (MO). Compared to WT, Mfn2Dyn displayed lower functional connectivity between prelimbic, infralimbic and anterior insula cortexes (PL, IL, aIC), Thalamus and bed nucleus of stria terminalis (BNST).
Together, our findings suggest that Mfn2-KD in D1-MSNs induces a significant reduction in NAc engagement during the exertion of an effortful behavioral task, accompanied by a shift in the functional connectivity between the brain areas recruited.

2. Analyses of electrophysiological and morphological alterations in NAc MSNs:

We performed patch-clamp recordings in NAc shell MSNs in acute slices from mice with either virally mediated (Mfn2^{Dyn}) or conditional (Mfn2-D1^{ERT}) Mfn2 downregulation to assess the role of Mfn2 in D1-MSN excitability.
In Mfn2-D1^{ERT} mice, we examined the synaptic inputs by recording miniature excitatory synaptic

currents (mEPSCs) in D1-MSNs. Cumulative distribution analyses of inter-event interval and peak amplitude showed that Mfn2-D1^{ERT} MSNs receive less excitatory events with comparable amplitude (Fig 3A).
In Mfn2^{Dyn} mice, we evaluate E/I balance by recording spontaneous excitatory and inhibitory synaptic currents (sEPSCs, sIPSCs) from the same MSNs (Fig 3C). In D1-MSNs, we observed an increase in the peak amplitude of the excitatory events and in the inter-event interval, while the inhibitory currents displayed smaller peak amplitude and shorter interval between events. Overall, the E/I balance showed decreased excitation in D1-MSNs from Mfn2-D1^{Dyn} mice (Fig 3B).

Morphological analysis was carried out in cells recorded in electrophysiological experiments. Cells were infused with biocytin via the patch pipette. D1-MSNs with Mfn2 downregulation have reduced dendritic complexity, as indicated by Sholl analysis of dendritic arborization, higher spine density but less functional spines, as highlighted by the reduction in the percentage of mushroom spines (Fig3C).
To infer whether the observed changes induced by Mfn2 are specific to NAc and not a general MSNs feature, we used Mfn2-D1^{ERT} mice and recorded miniature excitatory currents (mEPSC) in D1-MSNs from either NAc or the dorsal striatum. Interestingly, whereas in NAc we confirmed that Mfn2 loss reduced excitatory inputs, now difference was found in the dorsal striatum (Fig 3D). Morphological analyses also indicated no difference in the dendritic complexity in D1-MSNs in WT vs. Mfn2-D1^{ERT} mice.
This suggests that NAc neurons may be particularly vulnerable to mitochondrial deregulations. To probe for alterations in mitochondrial structure, we infused Mfn2-D1^{Dyn} and WT animals with a mix of Dyn^{Cre} and mitoDsRED viruses to label mitochondria in D1-MSNs, and performed qualitative evaluation via confocal microscopy on the organelle morphology in fixed brain slices. The results indicate an increase in mitochondria circularity following Mfn2 downregulation (Fig 4A-C).
Due to the low resolution of this approach, we implemented our study with light-correlative electron microscopy (CL-EM) technique, which allowed us to identify mitochondria in D1-MSNs with super resolution (Fig 4D). The CL-EM results, in line with the mitoDsRed data, confirmed reduction in mitochondria complexity following Mfn2-KD (complexity index calculated as Vincent et al., 2019). Similar to the complexity, mitochondrial volume was decreased. The direction of the changes may reflect the fragmentation of mitochondria due to the lack of the fusional protein, which in the literature is reported to lead to mitochondria swelling in fibroblast (Filadi et al. ,2015; Luo et al., 2021).
Correlation between volume and complexity index per each mitochondrion averaged per dendrite, showed clear segregation between WT and Mfn2^{Dyn} conditions (Fig 4E).
Spine analysis from the same samples indicated no differences in total spine density, but a clear reduction in the percentage of mushroom spines and smaller head diameter of mature spines upon Mfn2 downregulation.

3. Identification of the molecular determinants of morphological and synaptic alterations in NAc MSNs:

To further deep in the molecular underpinnings of Mfn2 actions in D1-MSNs, we performed RNA-sequencing of D1-MSNs from ribosome-associated mRNA from both using theMfn2$^{lox/lox}$ and Mfn2$^{+/+}$ genotypes (Fig5A).
More than 400 genes were differently expressed between the two genotypes and survived the correction (adjusted p value<0.1, Fig5B). GOterms on these significantly changing genes mostly belong to synaptic physiology, axonal guidance, neurites growth and cellular response (Fig5C).
Interestingly, no clear difference in mitochondrial-related genes (Park7, Pink1, Trap1 among others) were detected between genotypes.
The sequencing results, together with the morphological and electrophysiological data, seem to point to a major impact of Mfn2 on neuronal remodeling and synapse physiology.
Worth noting, different genes belonging to cilia remodeling and cytoarchitecture are differently expressed (Crocc, Shh among others). Because of the cilia function as sensory element for the cells,

the role on striatum signaling and the growing evidences of their functional role on animals' behavior (Jovasevic et al., 2021; Stubbs et al., 2022; Sheu et al., 2022) we plan to manipulate candidate genes to revert the neuronal dysfunction induced by Mfn2-KD in D1-MSNs.

4. Investigation of the impact of accumbal Mfn2 deficiency on glutamate receptors dynamics:

Evidence from the literature highlights the essential role of dendritic mitochondria to support synapses, that synaptic activity modulates the motility and fusion/fission balance of mitochondria and their distribution on dendrites (Li et al., 2004).

Based on that and on the electrophysiological results we obtained, we hypothesized that differences in glutamate receptor dynamics might contribute to behavioral differences observed upon Mfn2 downregulation in D1-MSNs.

To that aim, primary mixed striatal cultures were generated from WT pups (P0-P1) and a CRISPR/dCas9 packaged in Lentivirus system adopted to achieve downregulation of Mfn2 in the population of interest. Lentivirus expressing LacZ was used as control.

Due to the absence of a reliable Mfn2 antibody, Mitotracker was used to stain for mitochondria morphology in cell cultures upon viral infection. Confocal images showed significant differences in mitochondria morphology between control and dCas9-infected cells, consistent with previous results (data not shown).

Cultures were infected at DIV 3 and transfection of Homer1C-DsRed (to identify spines) and CMV-GluN1-Sept (to identify the subunit of interest) performed at DIV 10. Single particle tracking (SPT) of the GluN1 subunit of the NMDARs was performed two days after transfection. On the experimental day, rabbit anti-GluN1 and quantum dots (QDs) anti-rabbit incubations were performed. This system allows the tracking of the GluN1 subunit on the cell surface via imaging of the highly photostable QDs. Pilot experiment showed a clear difference on QDs behavior between LacZ and dCas9-treated MSNs, with a strong reduction in receptor mobility, as indicated by lower mean square displacement (MSD) and lower diffusion coefficient (data not shown).

However, due to the pilot nature of the study and the below scale value for MSD and diffusion coefficient (as compared to reported in literature) for dCas9 condition, further work is needed to corroborate whether Mfn2-KD in MSNs leads to changes in NMDARs dynamic.

In order to address functional impact of Mfn2 loss on mitochondria, we used the same primary neuronal cultures and checked the calcium buffering capacity of the organelles in WT and Mfn2-KD conditions via mitoGCaMP6 probe. Interestingly, upon depolarizing stimulus such as KCl, a significantly lower calcium accumulation is reported in Mfn2 deprived mitochondria in D1-MSNs (data not shown).

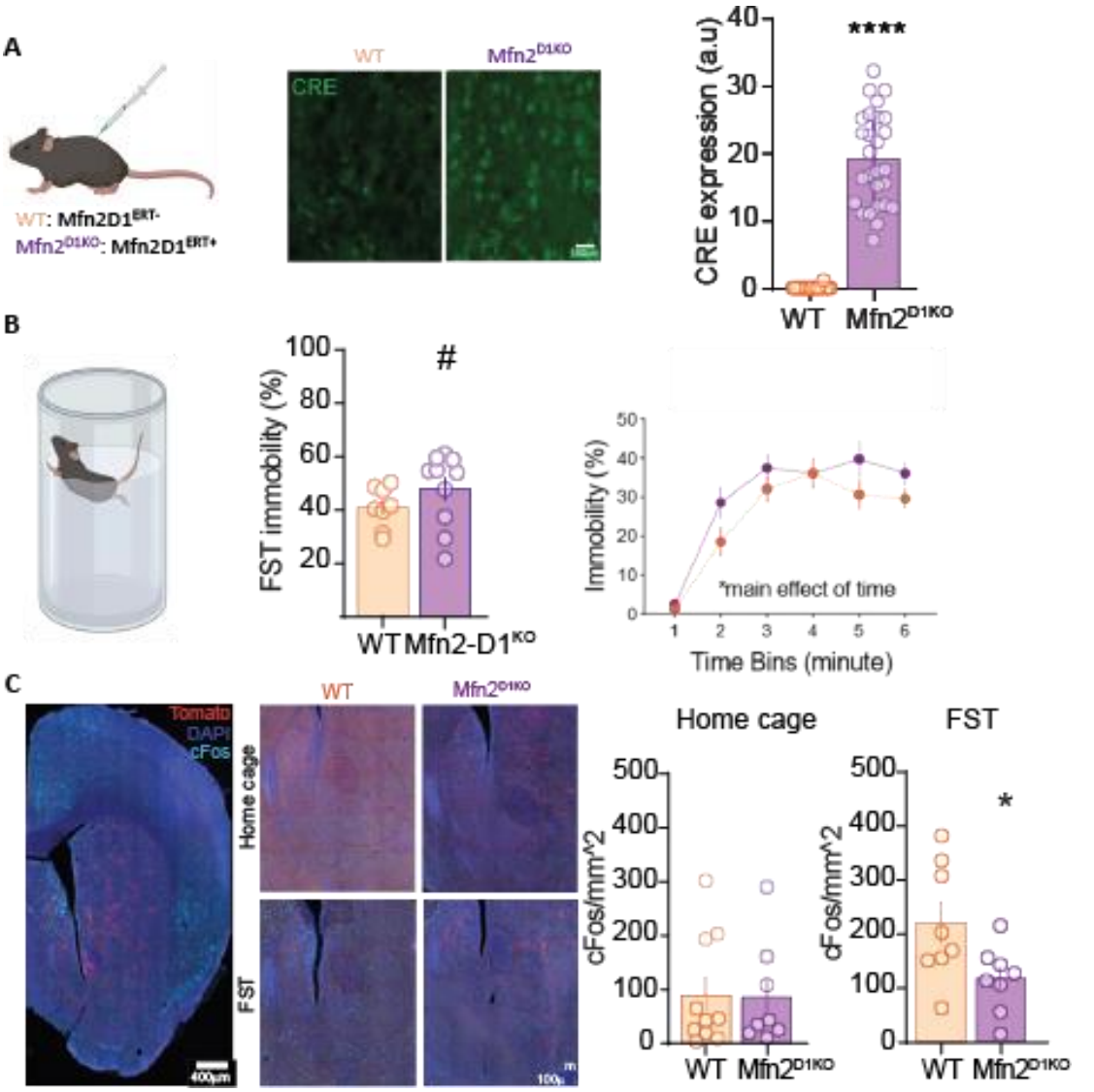


Figure 1. **A**. Tamoxifen-inducible Mfn2-KD in D1-expressing neurons. Animals were intraperitoneally injected for 5 consecutive days. Cre expression (green) was quantified in the NAc at least 4 weeks after the end of the injections. Mann-Whitney test, $p<0.0001$. **B**. Coping behavior was assessed with a Forced Swim Test (FST). $Mfn2^{D1KO}$ mice show tendency to passive coping behavior (Mann-Whitney, $p=0.059$). **C**. Left, example of immunofluorescence for cFos (cyan), DAPI (blue) and tomato, marking D1- MSNs (red). Right, high magnification images with example of cFos activation after home cage of FST exposure in WT and $Mfn2^{D1KO}$ mice. cFos quantification shows significant less expression during effortful task in $Mfn2^{D1KO}$ mice, Mann-Whitney test $p<0.05$. No difference is observed during a control home cage condition.

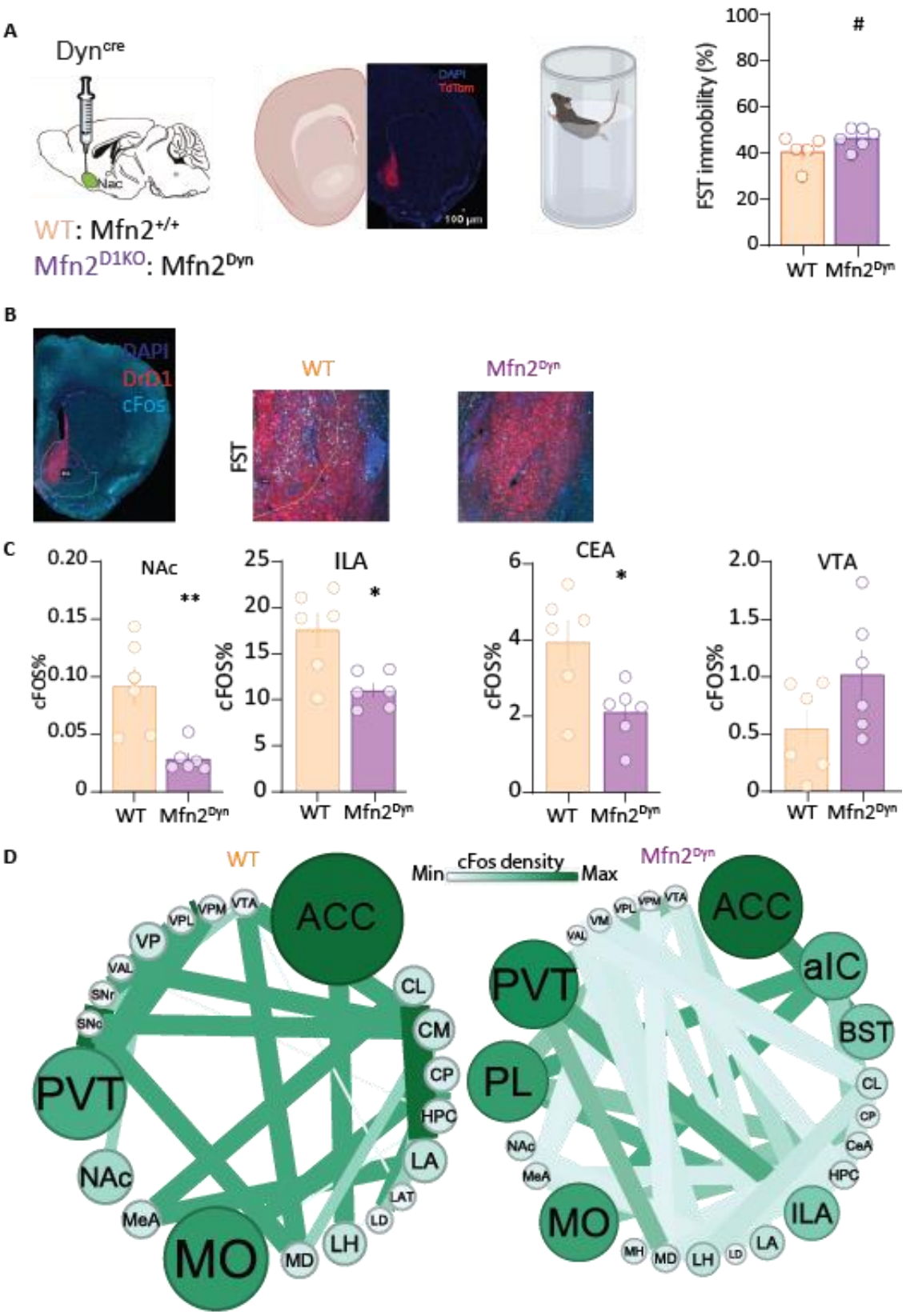


Figure 2. A. Viral-mediated downregulation of Mfn2 in D1-MSNs in NAc and representative image of viral expression. 4 weeks after injection, animals were tested for their coping behavior during a FST. Mfn2Dyn mice show tendency to passive coping behavior (p=0.059). B. cFos expression was quantified upon FST exposure. Left, representative images for DAPI (blue), D1-MSNs (red) and cFos (cyan). Mfn2Dyn mice show reduced cFos expression in NAc (p=0.008). C. Furthermore, ILA and CeA show significant reduction (p=0.02; 0.04), and VTA a tendency towards increase. D. Left, network analysis from WT group. Right, network analysis from Mfn2Dyn group. Brain regions selected for p<0.08 and r>0.5.

3. Mfn2 downregulation reduces synaptic excitatory inputs in D1-MSNs and increases intrinsic excitability

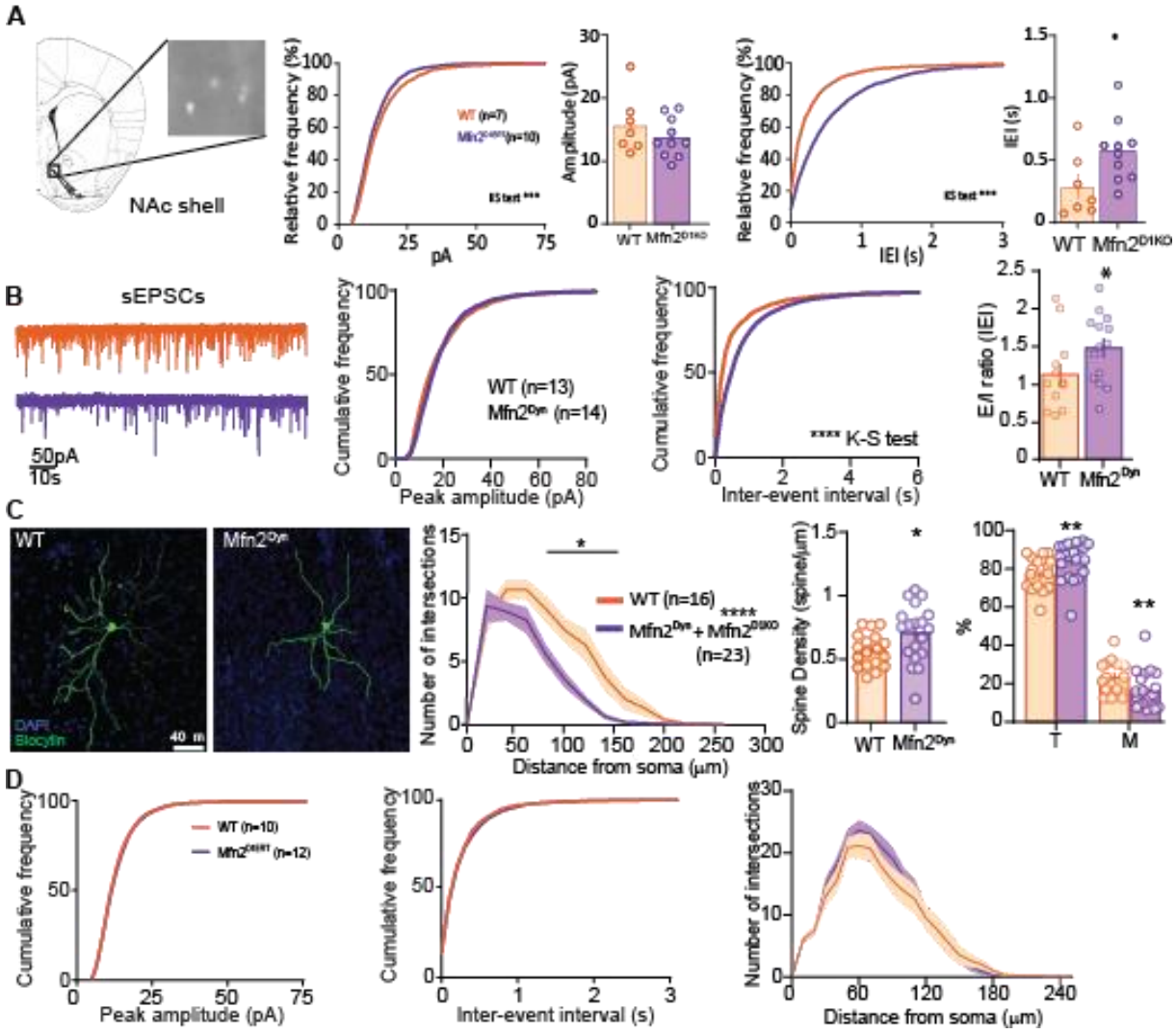


Figure 3 **A.** Image of NAc shell portion with a fluorescent reporter in D1-MSNs**. B.** Example of spontaneous excitatory postsynaptic currents (sEPSCs). Viral-mediated downregulation of Mfn2 in D1-MSNs (Mfn2Dyn) increases the inter-event interval of sEPSCs, with no changes in peak amplitude (KS test, $p<0.0001$). **C**. Overall, the Mfn2-KD leads to an E/I imbalance, as evidenced by a change in the ratio of the inter-event interval of excitation and inhibitory currents (Mann-Whitney test, $p<0.01$). D. Mfn2-KD in D1-MNSs from dorsal striatum does not change peak amplitude, frequency of events and dendritic complexity as compared to WT, $p>0.05$.

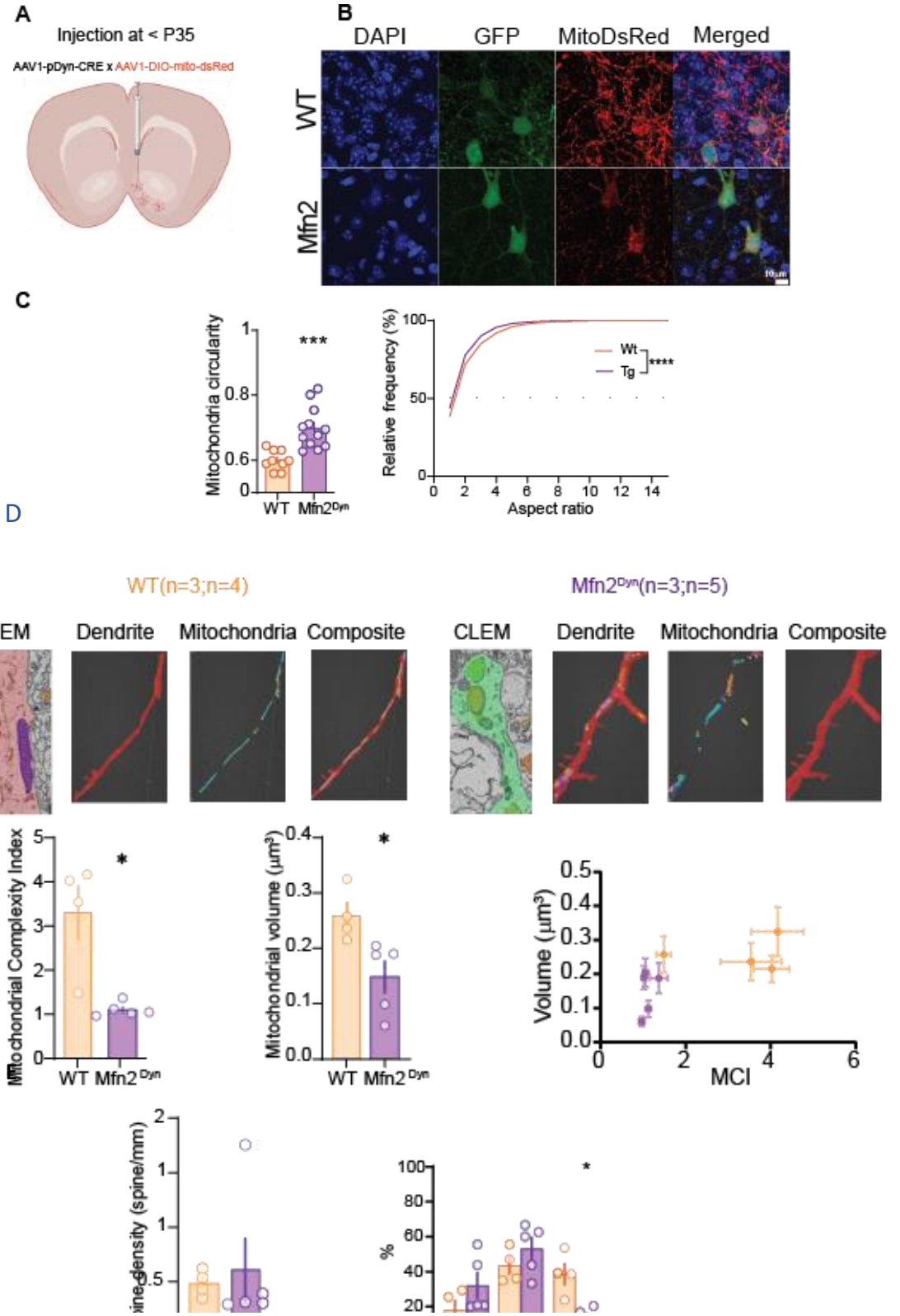


Figure 4 Quantification of mitochondrial morphology in NAc D1-MSNs upon Mfn2-KD. **A**. Injection scheme in NAc. **B**. Representative confocal images of D1-MSNs co-labeled with eGFP (green – dendrite and soma) and mito-dsRed (red – mitochondria) in WT and Mfn2^{Dyn} animals.

C. Left panel, D1-MSNs display increased mitochondria circularity concomitant to Mfn2-KD in comparison to WT D1-MSNs (WT: n=9; Mfn2^{Dyn}: n=12). Right panel, mitochondria aspect ratio relative frequency per mitochondrion shows enhanced smaller mitochondria in Mfn2-KD D1- MSNs. **D**. Correlative-light electron microscopy experiment in D1-MSNs displays reduced mitochondria complexity and volume upon Mnf2KD, $p<0.05$. **E**. Spine density does not change between genotype, while fewer mushroom spines are detected upon Mfn2 deletion, $p<0.05$.

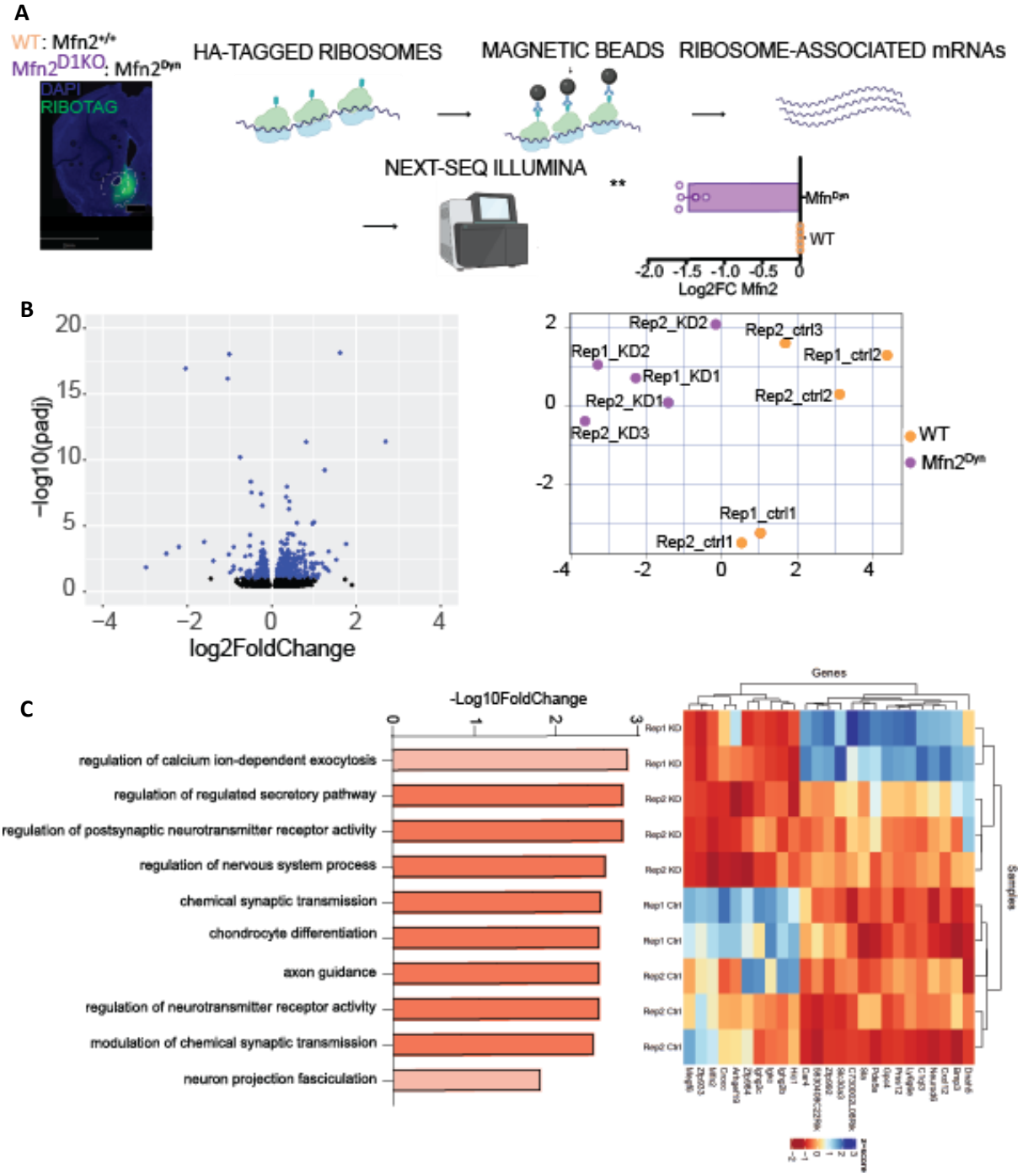


Figure 5 **A**. Scheme of injection and isolation of translationally-active mRNA from D1-MSNs in NAc. Bar plot shows abolished levels of Mfn2 in Mfn2^{Dyn} group. **B**. Volcano plot shows >400 genes differentially expressed surviving multiple correction, PCA analysis reveals that WT and Mfn2^{Dyn} group segregate separately. **C**. GOterms analysis for Biological process shows that most of the DEGs belong to synapse- and morphology-related genes. Heatmap from the top and down most DEGs, including Crocc.

Potential of these results for exploitation

The project will contribute to the dissection of nucleus accumbens function and dysfunction in the context of psychiatric conditions. We report here for the first time the causal role of mitofusin 2 deficits

in a specific subpopulation of NAc during learned helplessness. We provide novel information on the downstream morphological and molecular adaptations happening into the ventral striatum following reduced mitofusin 2 expression levels. Finally, we provide new insights on specific brain-regions adaptation and response to such change such as the prefrontal cortex, the amygdala and the thalamus: all areas related to stress and depressive-like conditions.

Our findings can deepen our understandings of the mechanisms underlying motivational disorders and pave the way for the development of novel treatment strategies to depression.

ESR3: overview of results towards the objective of the action

Project title – Production and testing of human-derived neurons and brain organoids: advanced model probing in neurodevelopmental disorders

WP1 – New models of neuropsychiatric disorders

Early Stage Researcher 3 – Giuseppe Cammarata

Supervisors – João Peça and Catarina Seabra

Host Location – Centre for Neuroscience and Cell biology (CNC), University of Coimbra – Portugal

Neurodevelopmental effects of VPA on unguided neural organoids

Introduction

Valproic acid (VPA) is a short-chain fatty acid naturally synthesized by the *Valeriana officinalis* plant (Chateauvieux et al. 2010). This compound is well known for its broad anticonvulsant action and mood stabilization effect which are linked to the direct effect of VPA on GABA neurotransmitter production and degradation and on the inhibition of voltage-gated channels (Romoli et al., 2018). Therefore, VPA has been considered an effective drug in regulating synaptic potentials and treating epileptic seizures and manic bipolar disorder (Peraudeau et al., 2018, Cerrizuela, Vega-Lopez & Aybar, 2020). The use of VPA by epileptic women during pregnancy has been the main concern due to the fact that the children exposed to this drug presented "fetal valproate spectrum disorder" which manifests in an increased rate of major congenital anomalies (MCAs) mostly observed at the cardiac and laryngeal level (Ornoy, 2009, Clayton-Smith, Jill, et al., 2019). Apart from the manifestation of MCA features, children with "fetal valproate spectrum disorder" are characterized by an increased risk to develop neurodevelopmental disorders such as autism spectrum disorder (ASD), intellectual disability and speech impairment (Ornoy, 2009, Clayton-Smith, Jill, et al., 2019). Considering the strong impact of VPA exposure on the cognitive functions and neurodevelopment observed by retrospective and prospective studies, the drug has been heavily restricted by the European Medicines Agency (Agency., E. M. Assessment report, 2014, Tomson et al. 2015a, Tomson et al. 2015c, Tomson et al., 2016).

However, the research interest towards the mechanisms affected by VPA that lead to ASD manifesting in the children still remains. ASD is a mental disorder that is usually diagnosed solely through a behavioural assessment to determine the mental health of patients, that feature makes the research study on this topic with animal models significantly challenging. However, several tools for behavioural neurosciences are available and aspects of ASD-like behaviours, such as repetitive stereotypes and social communication, can be now investigated in rodents (Crawley, 2004, Roullet and Crawley, 2011, Wohr et al., 2011).

In utero administration of VPA has been widely used in several studies to assess the impact of this exposure on the development, social behaviour and stereotypic behaviours of rodents (Kataoka et al., 2013, Markram et al., 2008, Roullet et al., 2010, Schneider and Przewłocki, 2005). Delay in development (Schneider and Przewłocki, 2005), the transgenerational inheritance of the ASD-like features (Choi et al., 2016), and excitation and inhibition imbalance are the most prominent discoveries on the effects of VPA (Iijima et al., 2016).

The ASD *in vivo* model with the administration of VPA is an important tool to analyse this neurodevelopmental disorder both at the behavioural and molecular level. However, this model

has shown some limitations; 1) the administration protocol given to rodents does not mimic the human foetal exposure to VPA; 2) human and rodent neurodevelopment have critical differences; 3) VPA animal model cannot always address all the molecular pathways, structural and cellular alterations of the human ASD example. Therefore, the possibility to perform experiments on suitable VPA human models, such as human brain organoids (Quadrato, G., et al., 2017, Lancaster et al., 2017, Kelava et al., 2016, Pasca et al., 2015), would provide critical information on the mechanisms linked to the onset of neurodevelopmental disorders, like ASD.

Effects of environmental factors on neurodevelopment in 3D cultures

To overcome the aforementioned limitations, new ways of culturing hiPSCs have been developed like three-dimensional (3D) cultures. These cultures are formed by aggregates of self-organizing hiPSCs that differentiate in a free-floating environment. By now, the most known methods of 3D cultures are mainly two: the regionalized neural organoids, developed by Paşca et colleagues, which replicate specific regions of the human brain and have the advantage to be assembled into assembloids in order to study the interaction between different systems (A. M. Paşca et al., 2015; S. P. Paşca, 2019); and unguided neural organoids, developed by Lancaster et colleagues, which instead resemble the development of the cerebrum without guidance toward a particular region (Lancaster & Knoblich, 2014; Paşca et al., 2022).

These techniques have been extensively employed in studying the aetiology of various neurodevelopmental disorders. Notably, unguided neural organoids have been utilized to investigate microcephaly (Lancaster et al., 2013) as well as the impact of Zika virus infection on human brain development (Qian et al., 2016). Furthermore, 3D culture models have provided insights into the effects of environmental exposures on brain development. For instance, alcohol exposure has been modelled using unguided neural organoids (Zhu et al., 2017), cadmium exposure (Yin et al., 2018), prenatal nicotine exposure (Wang et al., 2018), and the effects of a hypoxic environment on regionalized organoids have also been investigated (A. M. Paşca et al., 2019). Nefiracetam and PHA 543613 have been used to provide insights on the effects of methyl-CpG binding protein 2 gene (MECP2) modulation in Rett syndrome (RTT) (Amir et al., 1999; Mellios et al., 2018).

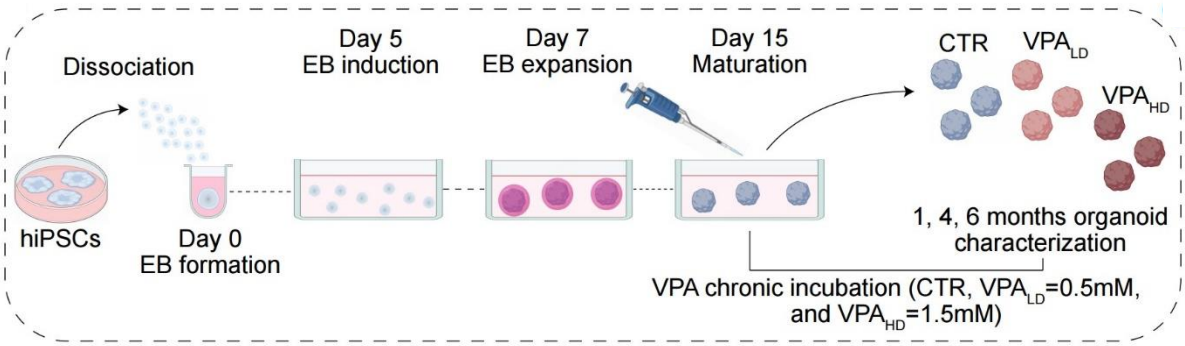
When considering the use of 3D cultures in investigating the impact of VPA on neurodevelopment, there are a limited number of studies available. A recent study found that VPA exposure at the early stage (day 18) of cortical organoids derived from one male hiPSC line impairs neurodevelopment (Cui et al., 2020). In a different study, forebrain organoids (hFOs) were utilized to investigate the impact of valproic acid VPA on ASD risk in humans. The hiPSCs derived from healthy individuals were used to generate hFOs, which were then exposed to VPA at a concentration of 1 mM for 3 days, mimicking clinically relevant conditions. By employing proteomics, genomics, and electrophysiology analyses, researchers identified several genes, including AMK4, CLCN4, DPP10, GABRB3, KCNB1, PRKCB, SCN1A, and SLC24A2, that were affected by VPA exposure. Notably, these findings showed significant overlap with dysregulated pathways observed in the brains or organoids derived from ASD patients (Mariani et al., 2015; Meng et al., 2022). Single-cell RNA sequencing analysis revealed that VPA exposure altered gene expression in specific cell types within the hFOs, such as the choroid plexus, excitatory neurons, immature neurons, and medial ganglionic eminence cells. Additionally, microelectrode array experiments demonstrated that VPA exposure disrupted synaptic transmission in the hFOs (Meng et al., 2022).

Experimental design of ESR-3 project

While previous studies have investigated the effects of VPA during the initial stages of unguided neural organoids organoid development, there remains a considerable gap in our understanding of the long-term consequences of chronic VPA exposure on human brain development. Recognizing this limitation, our study aims to bridge this gap by administering the drug chronically, starting from day 15 and continuing until 6 months of development, encompassing crucial early and later stages of organoid maturation.

Unguided neural organoids were kept exposed to VPA at low and high concentrations from day 15 of the differentiation protocol, respectively 0.5 mM (VPA_{LD}) and 1.5 mM (VPA_{HD}). The former

being the concentration of VPA found in the blood of women who took the drug for treatment, while the latter being a higher to highlight possible features found in the low dose.



Objectives

Task 1. Characterization of stem cell-derived unguided neural organoids: Characterization of unguided neural organoids was performed in my project at 2 main levels: molecular level, with immunohistochemistry (IHC) and bulk RNA-sequencing, and at the functional level, with calcium imaging and electrophysiology. Taking together all these methods, we observed that our control condition (CTR) of unguided neural organoids generate neural progenitor cells (NPCs) that were organizing into 3D structures called ventricular-like zone (VZ) **(Figure 1a)**.

When comparing the NPCs and neuronal populations in different conditions at different timepoints, we observed that even though the NPCs population was similar at early stages of development, VPA_{LD} showed lower presence compared to CTR at later stages of development. Regarding NEUN presence, instead, VPA_{LD} showed a higher presence of NEUN (double stained with MAP2) compared to CTR.

From these structures, neurons differentiate into different subpopulations: early born and later born neurons. These neuronal subpopulations usually localize outside the VZ and form respectively the deeper and superficial layer **(Figure 1c)**.

At later stages, we also observed the appearance of glial cells very important in neuronal and synaptic maturations, astrocytes and oligodendrocytes. Even though we did not witness myelination we are able to record network activity, using calcium imaging, and action potentials, using electrophysiology, in mature organoids.

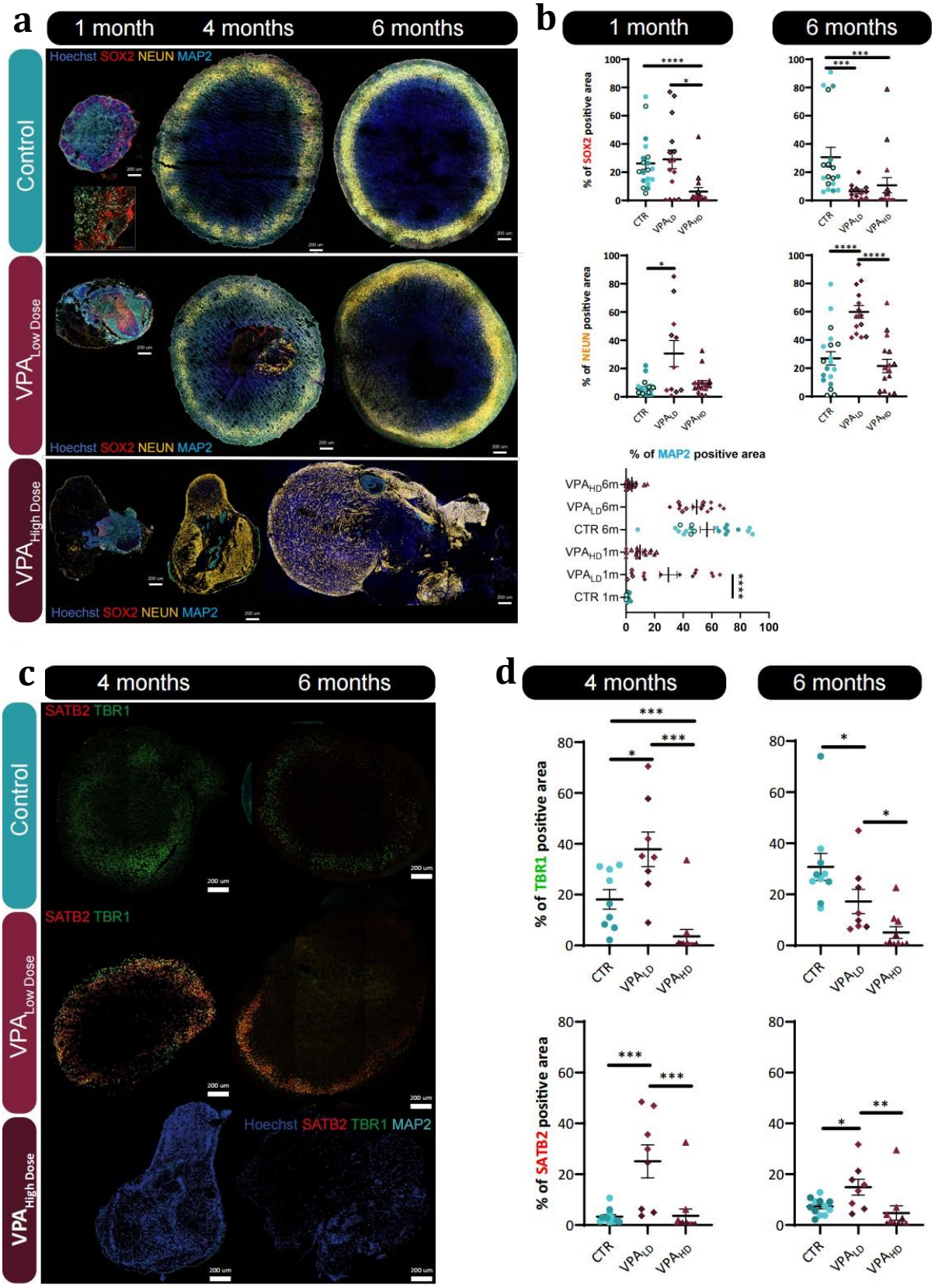
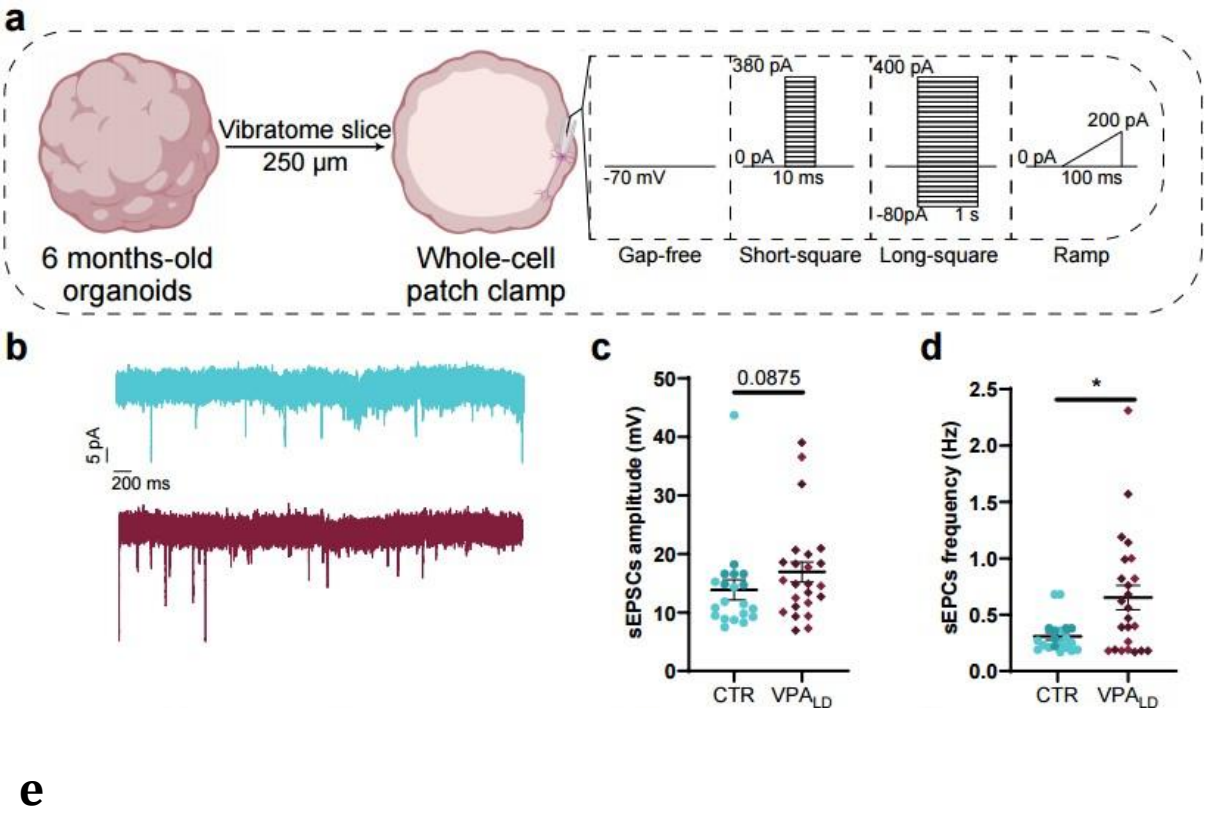


Figure 1. Human neural organoids cell distribution in CTR and VPA-treated conditions. a) Representative pictures of entire slices of human neural organoids from all conditions at 1, 4 and 6 months of development. Panel of antibodies used are SOX2 for neural progenitors cells (NPCs) and NEUN together with MAP2 for neurons. **b)** Quantification of the area occupied by SOX2, NEUN and MAP2 at 1 and 6 months (N=18-19/6/3 for CTR and N=11-15/5-6/3 for VPA_{LD}, slices/organoids/batch). **c)** Representative pictures of entire slices of human neural organoids from all conditions at 1, 4 and 6 months of development. Panel of antibodies used are TBR1 for

early-born neurons and SATB2 for later-born neurons. **d)** Quantification of the area occupied by TBR1 and SATB2 at 4 and 6 months (9-12/4/2 for CTR and 8/4/2 for VPA_{LD}, slices/organoids/batch). Also here, MAP2 was used to confirm the staining. All scale bars in the representative pictures are 200 µm. Graphs with analysis Mann-Whitney test illustrates $p<0.05$ = *, $p<0.01$ = **, $p<0.001$ = ***.

Task 2. Probe neurophysiological and network activity properties in hiPSC-derived brain organoids compared to VPA-treated organoids: The network activity was analysed by performing two experiments: electrophysiology gap free experiment at 6 months **(Figure 2a)**, and calcium imaging at 4 and 6 months **(Figure 2e)**. The former showed a significant increase in the frequency of action potentials and a tendency to have increased amplitude of action potentials in VPA_{LD} compared to CTR when looking in spontaneous post synaptic currents (sEPSCs) **(Figure 1b-d)**. The calcium imaging experiments have also shown increased frequency in VPA_{LD} at both 4 and 6 months **(Figure 2g)**.
These results, taken together, suggest that the VPA_{LD} unguided neural organoids generated networks that were hyperactive **(Figure 2d and g)**.



e

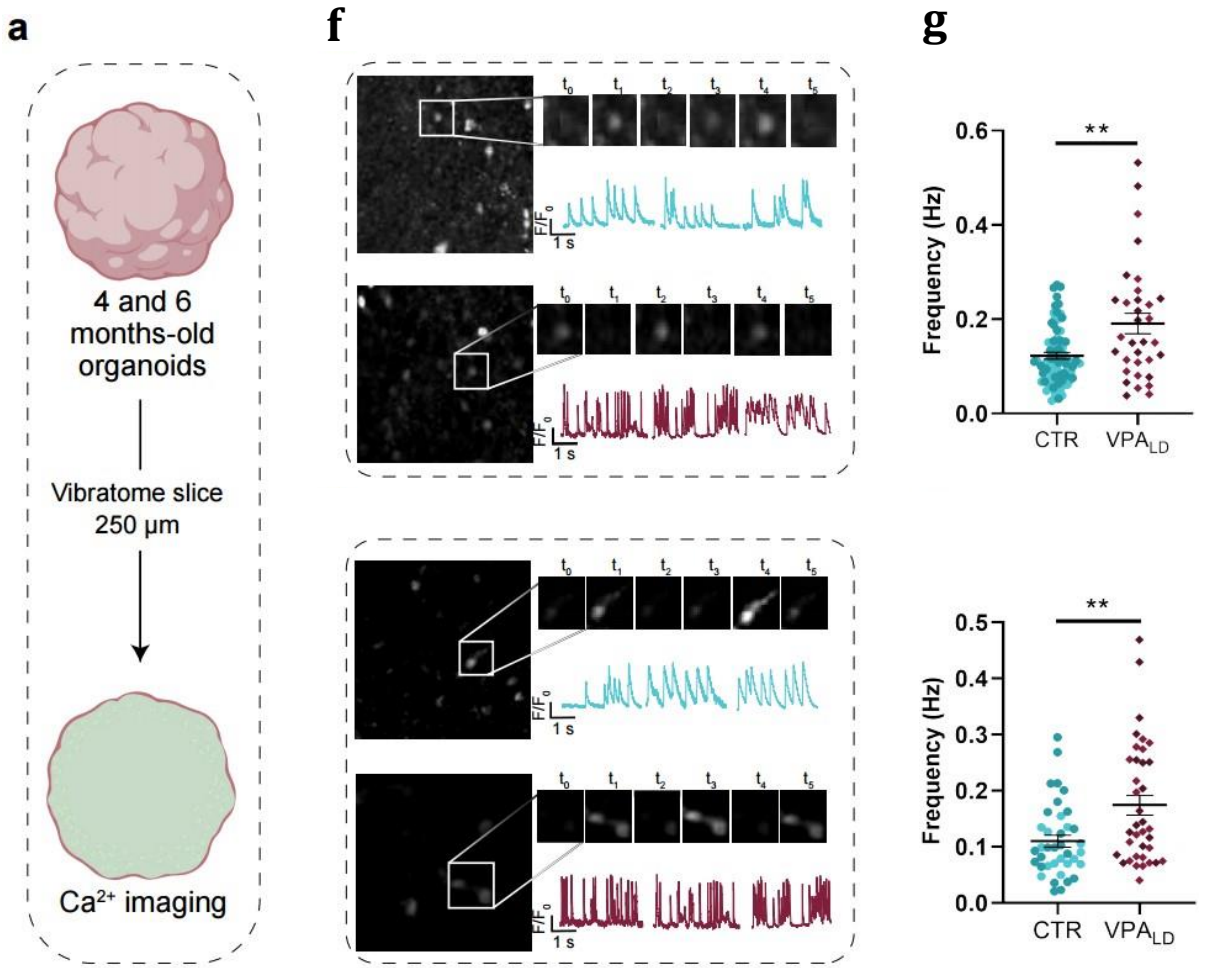
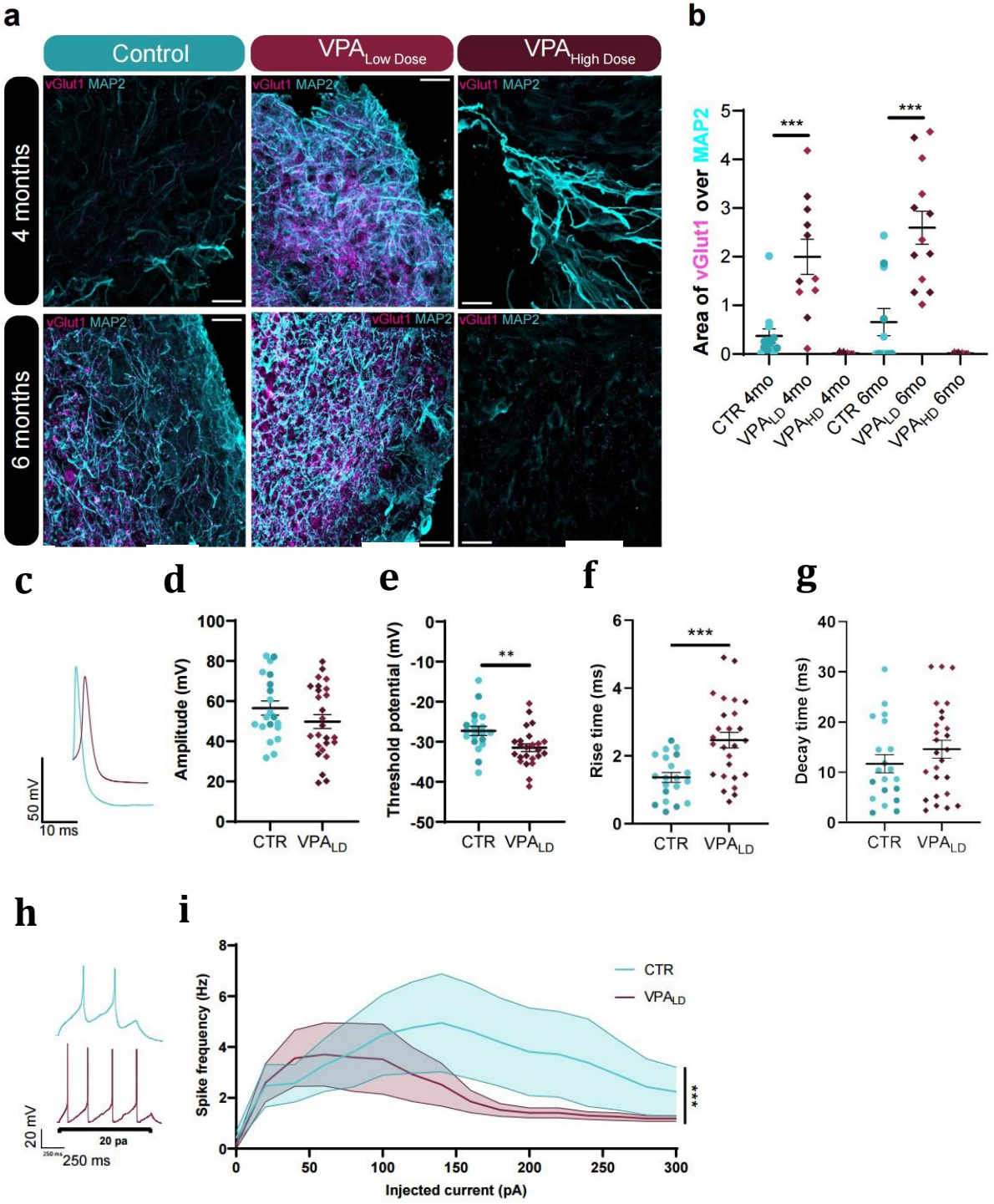


Figure 2. Network activity of VPA_{LD} shows hyperactivity compared to CTR in electrophysiology and calcium imaging experiments. a) Schematics of electrophysiology experiments: *Gap free* experiment aims in assessing the spontaneous post synaptic activity in a neuron, *short-square* aims to assess the properties of an action potential, *long-square* aims to assess the parameters of bursts of action potentials, and the *ramp* aims to assess the amount of current needed to elicit an action potential (rehobase). **b)** Representative traces of CTR and VPALD of spontaneous post synaptic excitatory currents. **c-d)** The amplitude of sPSECs tended to be higher in VPA_{LD} compared to CTR (p=0.088) while the frequency was significantly higher in VPA_{LD} compared to CTR (N=20/6/2 for CTR and for 24/6/2 for VPA_{LD}, cells/organoids/batches). **e)** Schematic of calcium imaging experiments at 4 and 6 months. **f)** representative pictures and representative traces of 3 different ROIs of CTR and VPA_{LD} at 4 and 6 months**. g)** Average frequency of each video is represented as a point on the graphs which show a significant higher frequency in CTR and VPA_{LD} both at 4 and 6 months (for 4 months N=74/6/2 for CTR and N=33/6/2 for VPA_{LD}, for 6 months N=38/6/2 for CTR and 37/6/2 for VPA_{LD}, average per region/organoids/batches). Graphs with analysis Mann-Whitney test illustrates p<0.05 = *, p<0.01 = **, p<0.001 = ***.

Task 3. Develop neuronal cultures from hiPSCs to determine Electrophysiological and synaptic properties in VPA-treated neurons: Unguided neural organoids reach synaptic maturation around 5 and 6 months of age. Using IHC experiments on synaptic vesicles, we observed that there is a significant difference in the presence of vGLUT1, glutamatergic synaptic marker, presence between VPA_{LD} and CTR was significantly different **(Figure 3a-b)**. More precisely, vGLUT1 had a higher presence in VPA_{LD} compared to CTR at all time-points. The higher presence of vGLUT1 in VPA_{LD} may suggest that VPA chronic exposure may induce excitatory/inhibitory imbalance (E/I imbalance) which is one the hallmarks of ASD. Regarding the physiology of the single neurons exposed to VPA, we also observed that they were easily excitable with a lower rheobase, current needed to induce an action potential, and threshold

potential compared to CTR **(Figure 3e and m)**. These experiments were performed prevalently at 6 months when the maturation of neurons reached the peak.



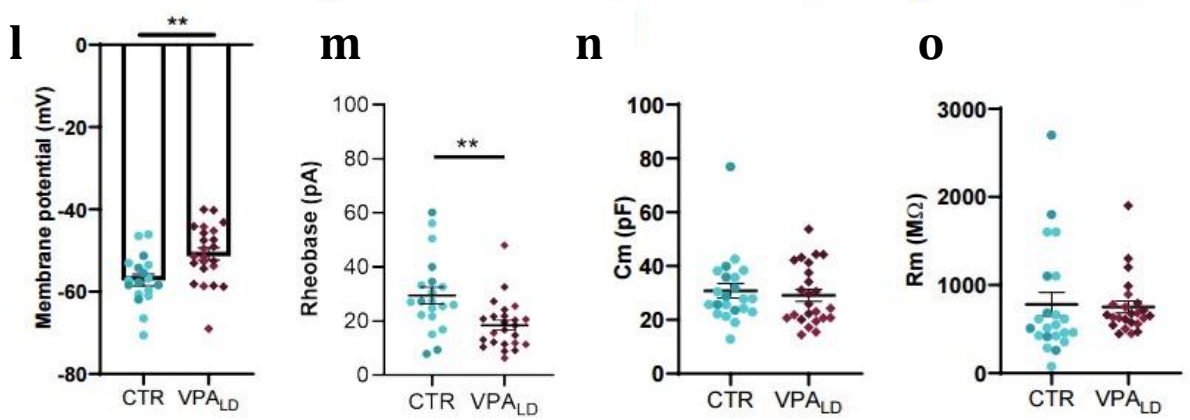
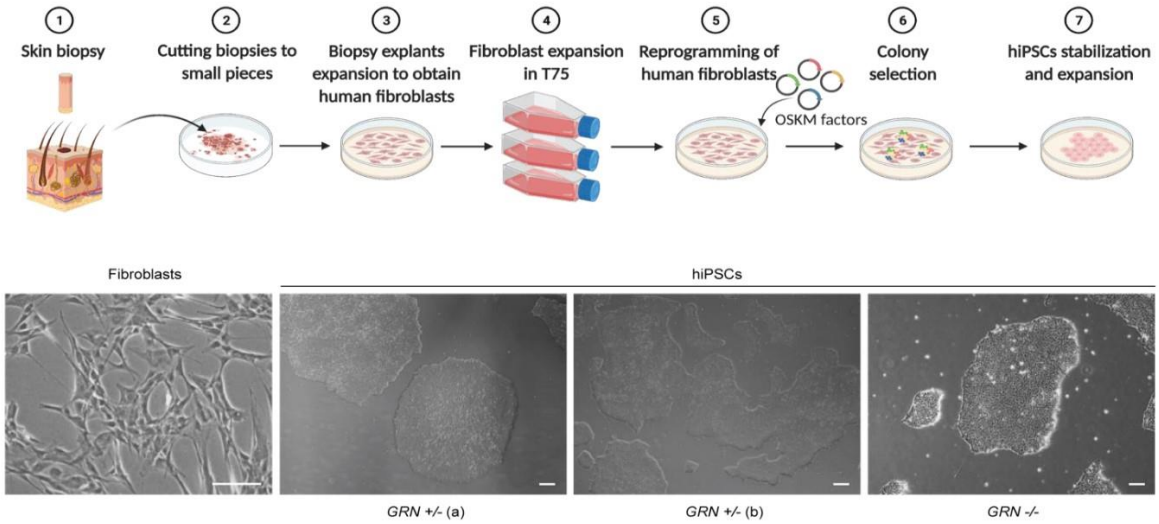


Figure 3. VPA chronically exposed neural organoids generate neurons that are hyperexcitable at low doses. a) Representative pictures of organoid slice regions at 4 and 6 months from CTR, VPALD and VPAHD. Slices were stained with MAP2 (dendritic marker) and vGLUT1 (excitatory synaptic marker)**. b)** Quantification of the area of overlap between MAP2 and vGLUT1. On the graph, represented as points, the average of 4 regions selected randomly within the slice of the organoid (N=11-13/4/2 for CTR, N=11-13/4/2 for VPA_{LD} and N=9/4/2 for VPA_{HD} at all time-points, average of 4 random regions/organoids/batches)**. c)** Representative traces of action potentials of both CTR and VPA_{LD}. **d-g)** the threshold potential is shown to be significantly lower in VPA_{LD} compared to CTR (N=20/6/2 for CTR and N=24/6/2 for VPA_{LD}, cells/organoids/batches), and rise time is shown to be significantly different in VPA_{LD} compared to CTR (N=20/6/2 for CTR and N=26/6/2 for VPA_{LD}). **h-i)** Representative traces of bursts of action potentials of both CTR and VPALD on step 20pA of current injection**.** Upon current injection, VPA_{LD} showed a higher frequency only around 20pA. At later steps, VPA_{LD} frequency drops showing a lower frequency compared to CTR (N=21/6/2 for CTR and N=27/6/2 for VPA_{LD}, cells/organoids/batches). **l-o)** Membrane potential and rheobase were shown to be respectively higher and lower in VPALD compared to CTR (N=20/6/2 for CTR and N=24/6/2 for VPA_{LD}, cells/organoids/batches).

Task 4. Proof-of-concept applications of brain organoids as a biomedical platform to test drugs: Being able to replicate complex structures and molecular signature of human brain development, unguided neural organoids have several advantages in *in vitro* studies of neurodevelopment. In particular, in our project we exploited the capability 3D cultures to be kept for a long period of time. Together with their use for drug testing, we were able to establish a method that aids us in the exploration of the long-term effects of chronic exposure to VPA which provides valuable insights into the impact of this drug over an extended period. This knowledge can be leveraged in drug development and regulatory processes to assess the safety and efficacy of VPA and potentially other similar drugs. Furthermore, establishing this method may help us to explore the effects of similar drugs in the future.

Task 5. Enlarge the sample biobank from patients and from healthy controls: In regard of this task, throughout of my PhD I helped the lab in collecting samples together with Catarina Seabra and Frederico Duque. NCBL1C5 was a line from healthy control that was reprogrammed into human induced pluripotent stem cells to use for my VPA project. We used this line in order to repeat some of the experiments that were already done on another control line established in the laboratory (31f-r1). Other lines were reprogrammed also by me, but they were used in another project already published (Oliveira et al., 2022). These lines were 2 hiPSC lines from GRN+/- and one from GRN-/-. The aim of this project was to generate and characterize new hiPSC lines that will be used in the future to study the effects of GRN mutations. GRN is a gene encoding for granulin and mutations in this gene accounts for 5 to 10% of all frontotemporal lobar degeneration (FTLD) and neuronal ceroid lipofuscinosis (NCL). After the reprogramming and characterization we also experimented their capability to generate 3D cultures, such as embryoid bodies, in order to use these lines in the future as tools to elucidate the pathophysiological mechanisms associated with the GRN mutations in the context of FTLD (Oliveira et al., 2022). *Below*

the procedure used to reprogramme skin biopsies as well as dental stem cells and representative pictures of different colonies of hiPSCs.



Potential of these results for exploitation

While we have already mentioned the advantages of human unguided neural organoids in drug testing, when looking to the results of our project we can draw some points that could be exploited in the future. For example, in performing chronic exposure to VPA for 6 months we explored the effects of VPA in long-term exposure. Surely, one of the next steps may be to also explore the short-term effects of the drug. Additionally, further experiments may be done on the physiological effects of VPA on the inhibitory system developed by the unguided neural organoids. The implications of the project's results for ASD research are particularly noteworthy. By establishing a platform that recapitulates the hallmarks of ASD using human in vitro models like unguided neural organoids, researchers can gain a deeper understanding also on other similar drugs and their effects. This knowledge can be utilized to advance diagnostic methods, identify potential biomarkers, and develop novel therapeutic strategies for ASD and other neurodevelopmental disorders. Moreover, the project's focus on identifying the mechanisms impacted by VPA, which are also associated with ASD phenotypes, opens up avenues for the development of targeted drugs. By designing drugs that act on these specific mechanisms, it may be possible to develop more effective treatments for ASD and related neurodevelopmental disorders.

ESR4 – Diogo Soares

ESR4: overview of results towards the objective of the action

Project title – Linking autism related mutations in Shank3 over synaptic, network and behaviour defects

WP2 – Testing the Excitation/Inhibition imbalance hypothesis in neuropsychiatric disorders

Early Stage Researcher 4 – Diogo Soares (resigned October 2020)

Supervisors – Daniel Choquet and Eric Hosy

Host Location – Interdisciplinary Institute for Neuroscience (IINS) – CNRS, Bordeaux University – France

Alterations in glutamatergic synaptic transmission are a prominent feature of numerous forms of ASD. In particular, numerous mutations in synaptic protein genes have been identified as risk factors for ASD with almost half of ASD being now recognized as synaptic diseases. The majority of these synaptic protein mutations concerns proteins that molecularly organize the synapse such as trans-synaptic proteins or scaffolding proteins. Among these, SHANK3 has been highlighted as responsible for more than 1% of ASD cases. Despite numerous studies on Shank3 mutant mice, surprisingly little is known yet on the mechanistic links between the molecular impact of Shank3 mutations on synapse organization and the effect of such dis-organization on circuit and behavioural phenotypes. This originates in large part from our lack of knowledge of Shank3 precise role in organizing synapses and controlling their function. The aim of our project is to unravel the multiscale consequences of Shank3 mutations on synaptic physiology. More precisely, we plan to determine both the nanoscale co-organization of the three main types of post-synaptic glutamate receptors (mGluR, NMDAR and AMPAR), and their co-activation in a shank3 mutant model.

Three models have been initially retained, the shank3 shRNA expression (mir KD) which should reduce of around 50% the endogenous shank3 expression (based on previous publications), the shank3 ko mouse (available at the Jacksons' lab) and the implementation of pluripotent human stem cells that we will derive in excitatory neurons. We started experiments with the shRNA expression, which was already available at lab, and order during this time both hIPS and ko mice. The first characterization of the effect of the shank3 shRNA expression on shank3 organization (Figure 1) reveals a 40% decrease of the amount of shank3 per spine and a 30% decrease of the spine number. These results encouraged us to continue to use this model to determine the effect of shank3 decrease on the glutamate receptor activation and organization.

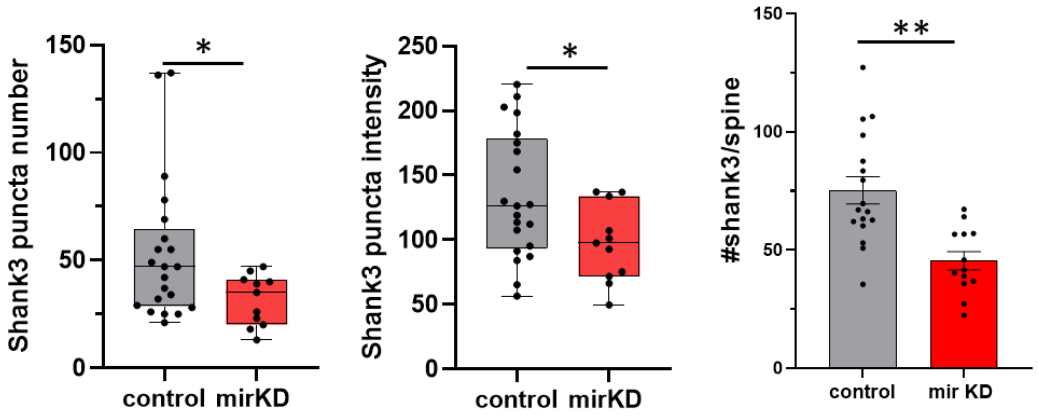


Figure 1. Effect of the shank3 shRNA expression on both spine density (right panel) and the intensity (middle panel) or the amount (right panel) of shank3.

We then performed a characterization of the neuronal phenotype following Shank3 shRNA expression by determining spines morphology with confocal imaging experiments. The type of spine (mushroom or stubby) has been determined as the morphological characteristics (size, width, etc), but no significant differences has been observed as illustrated in figure 2.

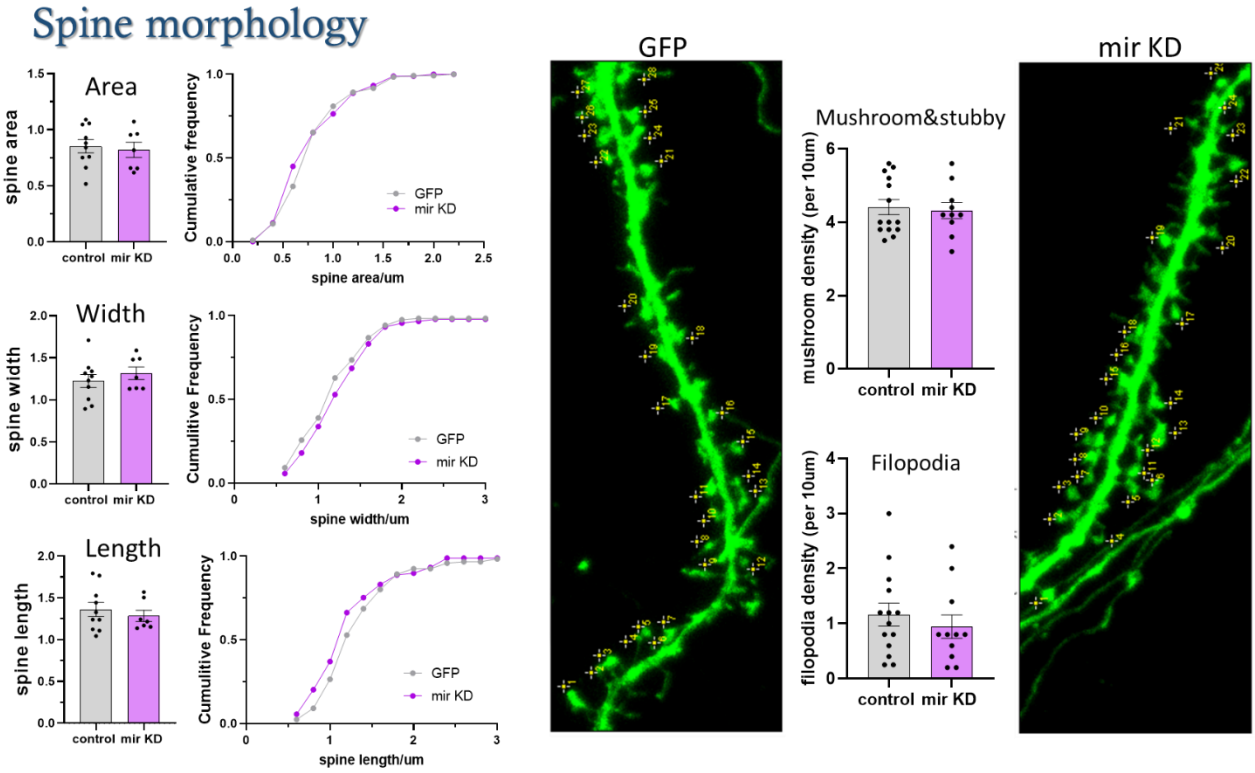


Figure 2: Determination of the spine morphology characteristic in control condition and when the shank3 shRNA is expressed. No significant differences are observed.

Then we estimated the effect of the 40% reduction of Shank3 expression on fast synaptic transmission response. We know this response is based on the activation of AMPA type glutamate receptors. Proper activation necessitates the nanoscale organization of a post-synaptic cluster of AMPA receptors under the pre-synaptic glutamate release site. This parameter is thought to be dependent of SHANK3 which is one of the main organizers of the PSD which structured the AMPAR clusters. We performed electrophysiology experiments to determine the amplitude of single synapse activation (called miniature EPSC). As shown figure 3, shank3 shRNA expression reduced of around 25% the amplitude of AMPAR currents without affecting the frequency or the kinetics of the currents.

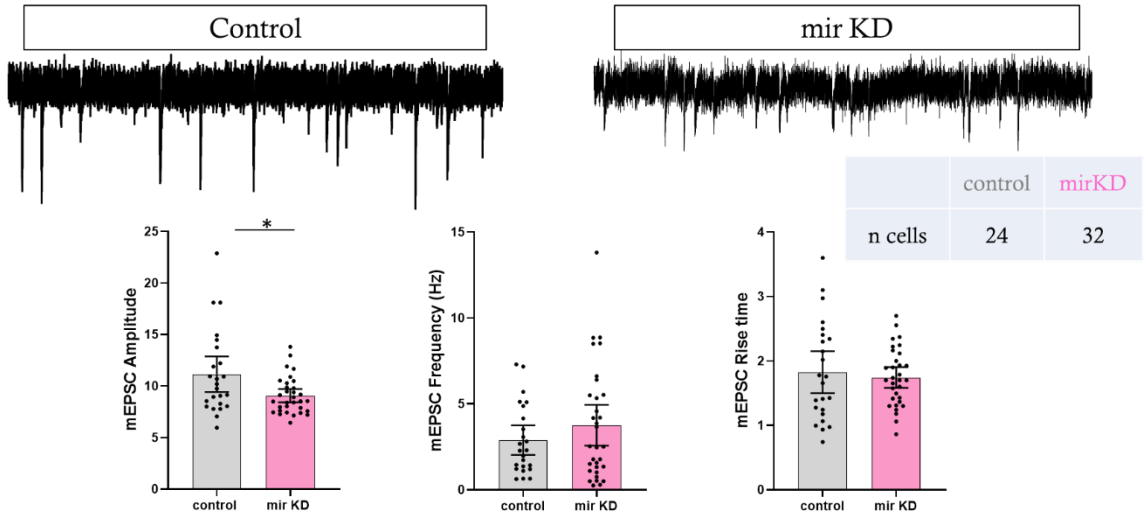


Figure 3: Shank3 shRNA expression decreases of 25% the amplitude of AMPAR dependent fast synaptic transmission currents (left panel) without affecting the frequency (middle panel) nor the rise time (right panel).

To explain this decrease of glutamatergic synaptic transmission we performed super-resolution imaging techniques on the three main type of glutamate receptors localized at the post-synapse (the mGluR, the NMDAR and the AMPAR). D-STORM experiments have been done (Figure 4). Preliminary results revealed surprisingly an increase of AMPAR amount per synapses and per nanoclusters which is at the opposite of what expected based on electrophysiology experiments. In parallel, we did not observe modification of NMDA receptor number and organization. Interestingly, we observed an almost 50% decrease of the mGluR5 density both at dendrite and on spines, this can reveal a lack of neuronal maturation, this hypothesis has to be explored.

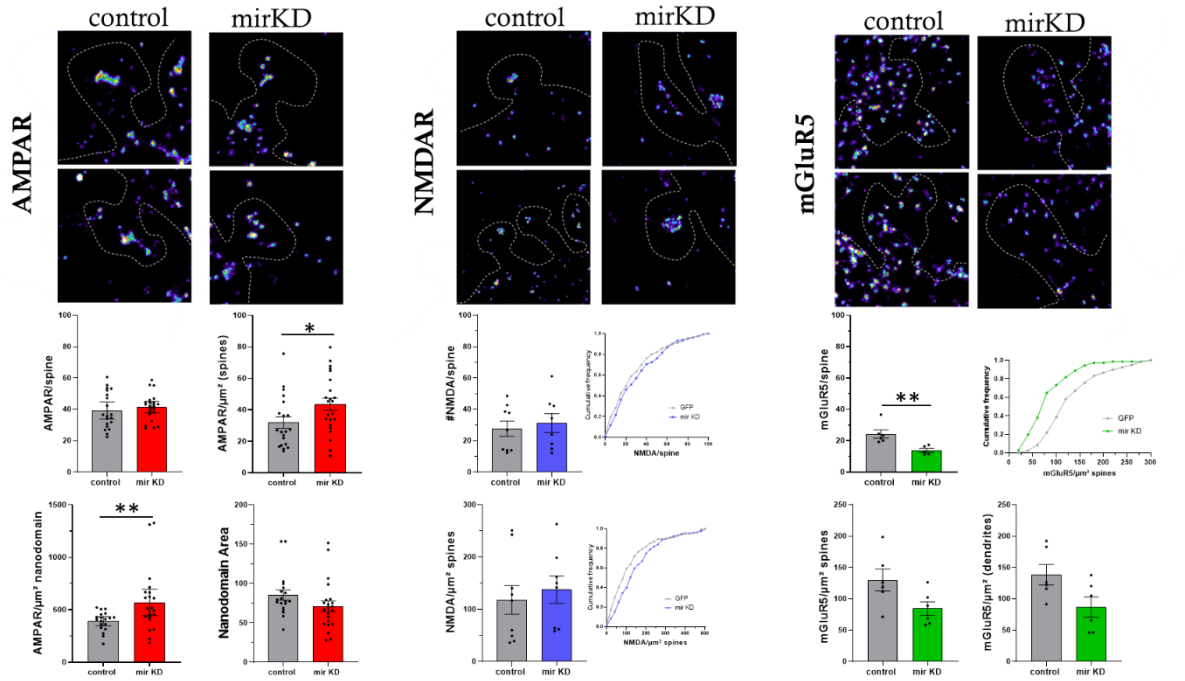


Figure 4: Single color d-STORM super-resolution experiments allow to decipher nanoscale organization and to determine the amount of both AMPAR (left panel), NMDAR (middle panel) and mGluR5 (right panel) at control and with shank3 shRNA expression. AMPAR amount per spine and inside nanodomain is increased, NMDAR are not affected while mGluR5 amount is highly decreased.

These preliminary results need to be reproduced, and then multiple color to determine the pre-post synaptic alignment between pre-synaptic release site and AMPAR cluster has to be done to try to understand the discrepancy between electrophysiology and imaging experiments. In parallel, we will determine the AMPAR/ NMDAR/ and mGluR5 co-organization and co-activation to estimate how pathological synapses (expressing shRNA) behave following glutamate release.

Finally, we questioned the accuracy of the shRNA model, while the human patients present a 50% decrease of shank3, only a weak phenotype on mice is observed in the heterozygote. This probably mean that the 40% of SHANK3 decrease triggers by shRNA expression is not enough to recapitulate the neuronal phenotype able to reproduce the pathology.

We are implementing both pluripotent human stem cell of patients and the ko mouse for shank3 to determine how such model affects the co-organization / activation of the various glutamate receptors.

ESR5 – Marcos Sintes

ESR5: overview of results towards the objective of the action

Project title – Human cortical circuit wiring in ASD and the role of psychedelics

WP2 – Testing the Excitation/Inhibition imbalance hypothesis in neuropsychiatric disorders

Early Stage Researcher 5 – Marcos Sintes

Supervisor –Vincenzo De Paola

Host Location – Institute of Clinical Sciences, Imperial College London – United Kingdom

The aim of this project is to study human cortical circuit wiring in autism spectrum disorder (ASD) and assess the therapeutic potential of psychedelic drugs. ASD models have been shown to present deficits in the establishment of normal cortical connectivity, but the mechanisms that produce abnormal circuit wiring are not well understood. Psychedelic drugs act as modulators of neuronal function through the activation of 5HT2A and other serotonergic receptors, which, we hypothesise, may induce a remodelling of the neuronal connections and restore normal cortical circuit activity. To achieve this, the following two objectives were defined:

- In vitro characterisation of cell identity and the effects of psychedelic drugs on human cortical neuron 5HT2AR expression and neuronal plasticity.
- In vivo analysis of human synaptic and neural network activity dynamics in ASD and the effects of psychedelic drugs.

The experimental approach was designed as illustrated in figure 1. Neuronal progenitors derived from human iPSCs are generated and either plated for in vitro experiments or transplanted into the somatosensory cortex of mice to form cortical grafts. In vitro, molecular, and functional approaches are used to characterise the expression of 5HT2A receptors and the acute and subacute effects of drug treatment on neuronal activity and plasticity. In vivo, two-photon imaging is used to study the differences of structural neuronal connectivity between patient and donor cell lines before and after treatment.

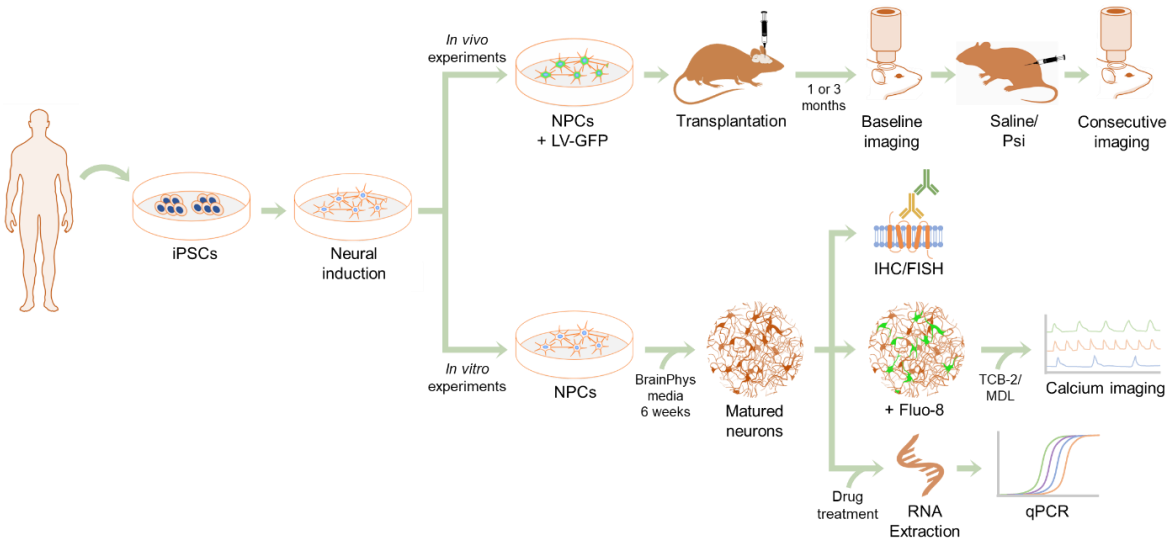


Figure 1: Overview of the experimental design.

We first matured iPSC-derived neuronal progenitors, from control donors, in vitro over a period of eight weeks and characterised the maturation of the cells by immunocytochemistry. Over several weeks cells organised in distinct and tightly packed three-dimensional neuronal colonies that communicated with other in the well by axonal bundles (figure 2A). At the eight week timepoint the cultures became unstable, and their overall health appeared compromised. Six weeks post-final plating (wpp) was chosen for the experiments as the longer duration the cells could be feasibly maintained. At this stage, a small percentage of cells still presented the progenitor marker PAX6 and the proliferation marker KI67, while most of the cells were expressed the mature neuron marker MAP2 (figure 2B). Out of the neuronal population, a majority of the cells were positive for the deep layer markers TBR1 and CTIP2, and only a small percentage of cells presented the upper layer marker SATB2. Matured neurons expressed both the presynaptic marker Synaptophysin and the postsynaptic marker PSD-95, both involved in the formation and maintenance of synapses. Expression of 5HT2A receptors was confirmed both by immunohistochemistry and in situ fluorescence hybridisation (figure 2C). The relative expression levels were quantified by qPCR at three, six and eight weeks of maturation and compared to adult human brain samples (figure 4A). There was a five-fold increase in 5HT2AR expression between three and six weeks, consistent with the maturation of the neurons. At eight weeks, 5HT2A expression decreased, possibly due to the reduced fitness of the cells. At six weeks, the expression level was around half of the average expression of the adult human brain samples. Serotonin receptor expression varies on different regions of the cortex, so we considered the result for the six-week timepoint to be reasonably high. Overall, these results show that the six-week maturation timepoint leads to the generation of predominantly mature neuron populations that express 5HT2AR and other synaptic proteins, making this maturation protocol promising for later experiments.

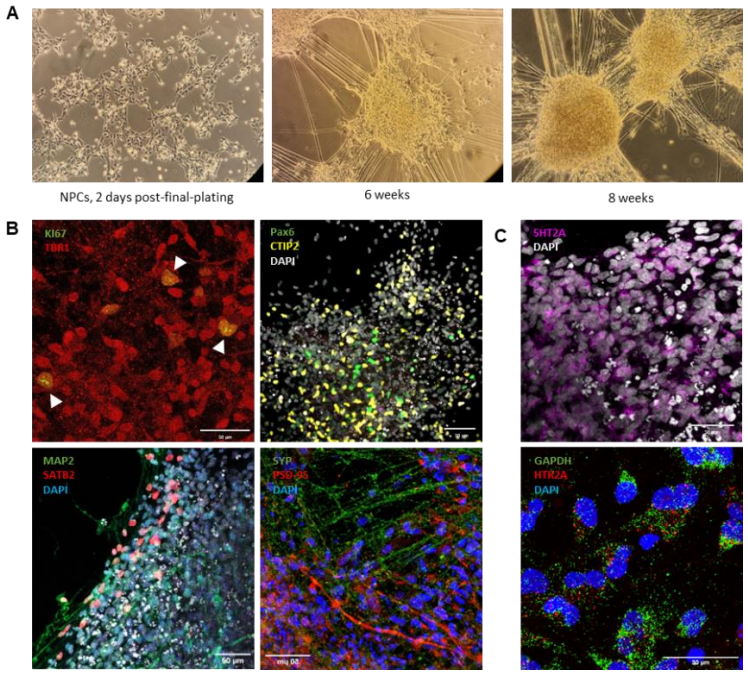


Figure 2: Characterisation of cell maturation and 5HT2A expression in vitro.

A) Representative examples of human iPSC-derived neurons at different maturation stages in BrainPhys media after final plating.
B) ICC detection of cortical maturation markers in neuronal cultures at 6 weeks of maturation. Ki67 for proliferation (indicated by arrows), Pax6 for NPCs, TBR1 and CTIP2 for deep layer neurons, SATB2 for upper layer neurons, MAP2 for mature excitatory neurons, Synaptophysin (SYP) for presynaptic terminal, and PSD-95 for postsynaptic terminal.
C) Top panel shows the ICC detection of 5HT2A protein at 6 weeks of maturation on top, while the bottom panel depicts HCR-FISH detection of *HTR2A* and *GAPDH* gene expression.

The expression of the serotonin receptors alone is not sufficient to assume the amount of receptor protein levels in the cell at any given time will be large enough to be physiologically relevant. Furthermore, 5HT2A receptors are G-coupled protein receptors (GPCRs) that require the action of a complex intracellular signalling pathway, after an agonist binds to them, in order to induce changes in the cell activity and the expression of genes associated with neuronal plasticity. To test whether this serotonergic signalling system is present in the neuronal cultures, a functional approach was used. Cells were treated with a calcium indicator dye that emits fluorescent light with a variable intensity positively correlated with the intracellular calcium levels, which was utilised as a proxy to study neuronal activity (figure 3). Live-cell confocal imaging was used to record the cellular calcium signals before and after treatment with the psychedelic and 5HT2AR selective agonist TCB-2, and the selective antagonist MDL100907 (MDL). Cells treated with TCB-2 at 10µM and 40 µM presented an immediate and significant increase in the rate of calcium events when compared to the cells treated with just media as a control (figure 3D), while MDL treatment abolished the activity increase. This increase in firing activity is consistent with the canonical effects of serotonin upon activation of the 5HT2A receptors, which evidences the presence of a biologically relevant receptor concentration in and a functional intracellular signalling cascade. Cells

treated with the psychedelic drug psilocybin did not show an effect when compared to the control, which could be due to different factors. Firstly, in vivo, psilocybin must be converted to its active form psilocin to exert its psychotropic effects, but it is not clear whether this is due psilocybin not being able to activate the receptors or if it is due to its inability to cross the blood brain barrier. Secondly, psilocybin could be primarily activating a different intracellular signalling cascade that does not lead to calcium release.

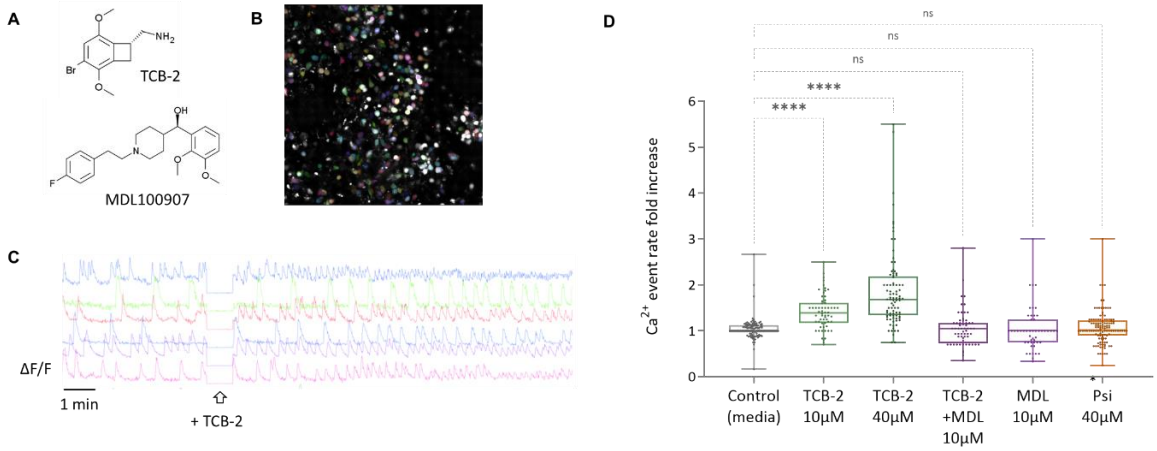


Figure 3: Functional study of 5HT2A expression in vitro.

A) Molecular structure of the selective 5HT2AR agonist TCB-2, and the selective antagonist MDL100907 (MDL).
B) Representative example of the automatic ROI detection by the software Suite2P. Each ROI corresponds to a single cell.
C) Examples of calcium activity traces detected from cells treated with 40µM TCB-2, immediately before and after the treatment.
D) The Ca^{2+} event rate was calculated for each ROI as the number of distinct calcium events per minute of recording. The plot shows a comparison between the fold increase in Ca^{2+} event rate from basal after treatment with just media (Control) and with drugs dissolved in media to the appropriate final concentration. Nested one-way ANOVA and multiple comparisons with Bonferroni correction (**** $p<0.0001$).

Cells were treated with media alone, TCB-2 at 10µM/40 µM or psilocybin for 1.5 hours before the RNA was collected (figure 4B), and C-FOS expression was quantified by qPCR. The expression of this gene is associated with an early response to an increase in cellular activity. Both TCB-2 at 10µM and psilocybin did not induce a noticeable early C-FOS expression change, while TCB-2 40 µM resulted in a 5-fold increase. To study the subacute effects of the drug treatment (figure 4C), cells were then treated with media, TCB-2, MDL or ketamine for 12 hours. After an additional period of 12 hours post-treatment, RNA was collected and the expression of 5HT2A and different genes associated with neuronal plasticity was measured by qPCR. In this case, TCB-2 10µM produced an increase of all plasticity markers, while the higher concentration did not present a noticeable change in expression when compared to the control.

This may be due to regulatory mechanisms that could get triggered to protect the cell from excitotoxicity induced by the initial higher activation. MDL prevented the increase in marker expression. Ketamine, which has been shown in the literature to increase neuronal plasticity, did not have a prominent effect. This could be due to its target NMDA receptors not being expressed at a high level at this stage of cell maturation.

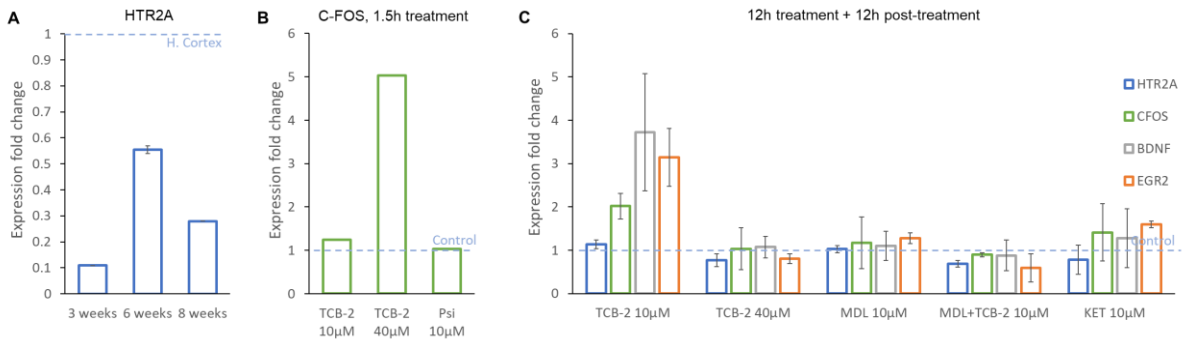


Figure 4: Quantification of HTR2A expression and transcriptional response to psychedelics in vitro.

A) qPCR quantification of *HTR2A* gene at different neuronal maturation stages, using post-mortem human cortex mRNA as reference and normalised to *GAPDH* expression.
B) qPCR quantification of *FOS* gene after 1.5h of treatment with TCB-2 and psilocybin, using untreated control as reference and normalised to *GAPDH* expression.
C) qPCR quantification of different genes, used as markers of neuronal activity and plasticity. Samples were collected after 12h of treatment with TCB-2, Ketamine (KET) and MDL100907 (MDL) plus 12h of post-treatment with normal media. Untreated control is used as reference, and the expression levels are normalised to *GAPDH* expression.

We next performed craniotomies on mice and transplanted neuronal progenitor cells derived from control iPSCs expressing GFP into the somatosensory cortex by stereotaxic injections. A cranial window was implanted for long-term in vivo imaging. The resulting cortical grafts matured for either one or three months before a preliminary psychedelic treatment experiment was conducted (figure 5A). Using two-photon microscopy on anaesthetised mice, the axonal-projection terminals from GFP-expressing human neurons were located and imaged at two timepoints separated by a 12-hour interval. After the second imaging session, mice were treated with psilocybin, and, one day later, two post-treatment imaging sessions 12 hours apart were performed. For the analysis of the results, a reference point was defined for each axon at both timepoints either pre- or post-treatment and the distance between that reference point and the end of the axon were measured. The distance measured at the first timepoint was subtracted to the one measured at the second timepoint to obtain the total growth distance, which was then divided by the elapsed time between imaging sessions to obtain the axonal growth rate (figure 5C). This first preliminary in vivo experiment was done using only three animals per graft maturation timepoint, and all animals received the psilocybin treatment, so its scope is limited. Some of the grafts presented a visible increase in axonal growth rate after the treatment when compared to basal, but there was a large degree

of variability between individual grafts (figure 5D). Increasing the number of animals and including a control treated with saline will increase the power of this experimental approach in ongoing and future experiments.

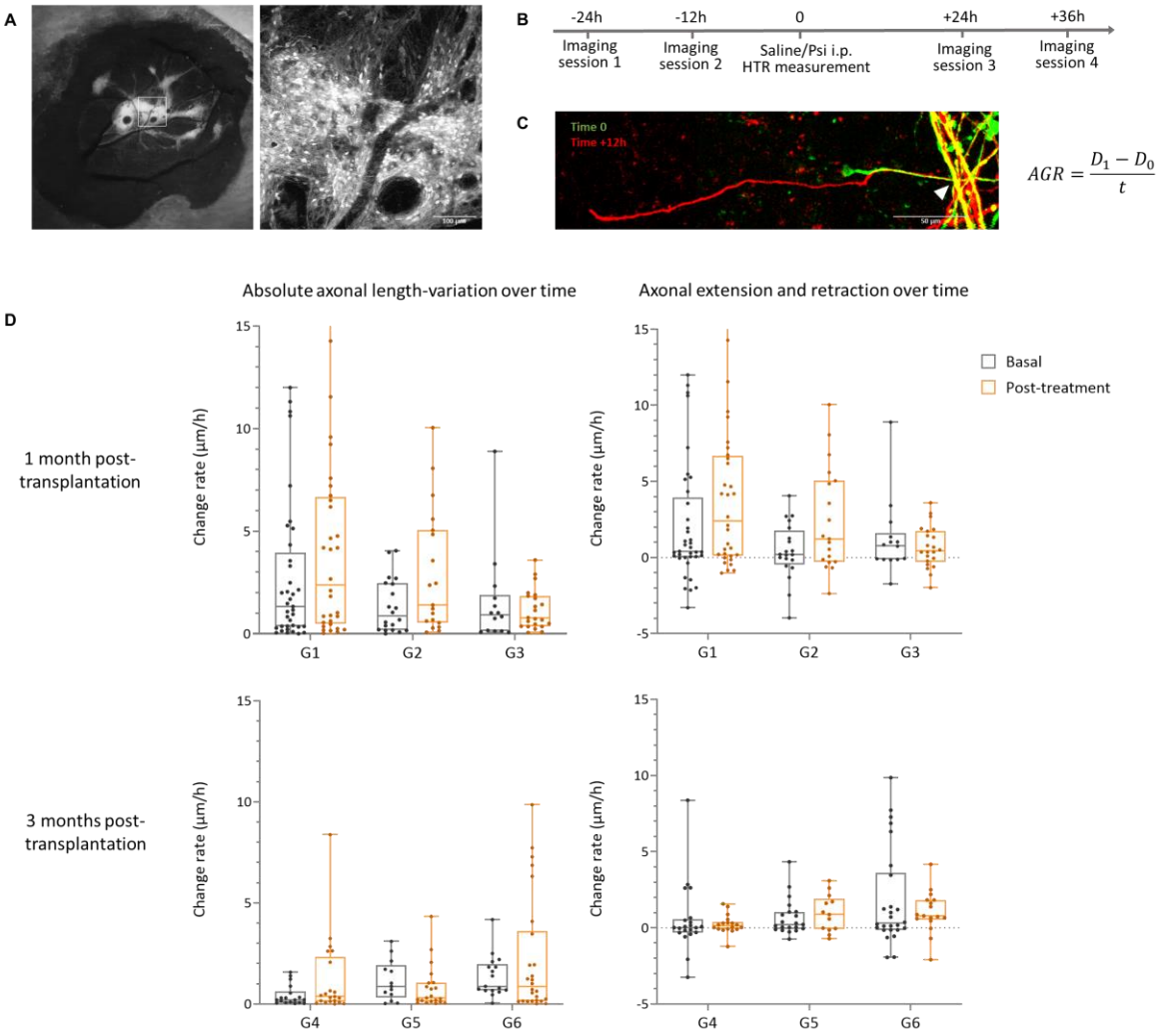


Figure 5: Effects of psilocybin on axonal growth in human iPSC-derived neurons in vivo.

A) Example of a GFP-expressing cortical graft. Left image shows an overview of the whole cortical window. Right image shows a magnified view of the graft area.

B) Experiment design timeline, including two imaging sessions before treatment to establish basal axonal growth rate, and two imaging sessions after treatment with psilocybin (Psi) to evaluate changes following treatment.

C) Example of axonal extension, showing an overlay of two different timepoints for the same ROI. The formula depicts how the growth rate for each axon (AGR) was calculated. The distances from a reference point to the axon terminal at two timepoints (D_0 and D_1) were subtracted and divided by the elapsed time between imaging sessions (t).

D) Axonal growth rate measurements for individual grafts before and after treatment. The grey and orange colours respectively represent the measurements before and after treatment. Each graft is labelled with a unique identifier (G1 to G6). The top row displays the results for the three grafts at 1 month post transplantation (mpt), while the bottom row presents the results for the three grafts at 3 mpt. The left graphs illustrate the absolute axonal length-variation rate, which considers both extension and retraction as positive values, providing an overview of the tendency of the axons to change over time. The graphs on the right present the actual growth rate for each axon, with axonal extension treated as positive and axonal retraction treated as negative.

Overall, these results provide, to our knowledge, the first insights on the effects of psychedelics on neuronal plasticity in human iPSC-derived neurons. These models provide unique perspective into the effects of psychedelic drugs on human cortical circuits that is not attainable in the clinical, animal and animal-derived cell studies published to date. We are currently using these experimental approaches on both control and ASD patient-derived neurons, which could provide the first data on the potentially therapeutic mechanisms of psychedelic drugs for the treatment of neurodevelopmental disorders.

ESR6: overview of results towards the objective of the action

Project title – Physiopathological regulation of single NMDA receptor dynamics in psychosis

WP2 – Testing the Excitation/Inhibition imbalance hypothesis in neuropsychiatric disorders

Early Stage Researcher 6 – Daniel Hunter

Supervisor – Laurent Groc

Host Location – Interdisciplinary Institute for Neuroscience (IINS) – CNRS, Bordeaux University – France

NMDA receptor encephalitis and GABAA receptor encephalitis are two autoimmune disorders that share similar clinical symptoms despite different underlying mechanisms. In NMDA receptor encephalitis, autoantibodies cause a reduction in the expression and localization of NMDA receptors, leading to a decrease in excitatory function and altered network connectivity. On the other hand, GABAA receptor encephalitis involves the direct antagonism of GABAA receptors by autoantibodies, resulting in a decrease in inhibitory function. Despite these distinct mechanisms, both diseases exhibit common symptoms such as seizures, cognitive dysfunction, and autonomic instability. However, NMDA receptor encephalitis is also associated with neuropsychiatric symptoms resembling schizophrenia, which are rarely observed in GABAA receptor encephalitis cases. The mechanisms underlying the neuropsychiatric symptoms in NMDA receptor encephalitis are not fully understood. It is hypothesized that the autoantibodies may have distinct impacts on inhibitory interneuron populations in the hippocampus, which play a crucial role in regulating overall circuit behaviour. As such, interneuron dysfunction may contribute to the psychotic symptoms observed in NMDA receptor encephalitis. Furthermore, interneurons in the hippocampus are more reliant on NMDA receptors for excitatory synaptic transmission compared to pyramidal cells. This higher reliance, along with the presence of specific NMDA receptor subunits, makes interneurons more susceptible to the effects of NMDA receptor autoantibodies. The disruption of excitatory drive onto inhibitory networks can lead to local disinhibition and widespread epileptiform activity. Overall, while NMDA receptor encephalitis and GABAA receptor encephalitis have different underlying mechanisms, their clinical manifestations show considerable overlap. Understanding the shared and distinct mechanisms of these diseases is crucial for developing targeted therapeutic interventions.

The primary objective of this thesis is to understand the functional mechanisms behind hippocampal pathophysiology caused by NMDA and GABAA receptor autoantibodies. The study aims to investigate synaptic, cellular, and network deficits induced by autoantibody actions in hippocampal cell preparations. The first aim involves examining the synaptic phenotype by analysing excitatory and inhibitory synaptic activity using electrophysiology and immunohistochemistry. The second aim focuses on the cellular phenotype by studying the intrinsic excitability of CA1 hippocampal neurons and their response to NMDA and GABAA receptor autoantibodies. The third aim explores the network phenotype by assessing the overall network

activity and synchronicity of cell activations in hippocampal circuits exposed to autoantibodies. The ultimate objective is to gain a comprehensive understanding of the impact of NMDA and GABAA receptor autoantibodies on hippocampal function, shedding light on the mechanisms underlying autoimmune encephalitis and its associated symptoms.

Early studies on the molecular pathogenicity of patient autoantibodies were limited by the availability of samples, such as serum and cerebrospinal fluid (CSF). Patient samples contain a combination of different autoantibodies with varying targets, making it challenging to determine the precise pathogenicity of specific autoantibodies. However, advances in recombinant monoclonal antibody production have allowed researchers to generate patient-replicated monoclonal autoantibodies in unlimited quantities. Using these monoclonal antibodies avoids the presence of unknown factors in patient samples and allows for precise control of the concentration of pathogenic immunoglobulins. We have employed two pathogenic autoantibodies in our investigations: one targeting the NMDA receptor and the other targeting the GABAA receptor. These monoclonal antibodies were derived from patient samples and extensively characterized in previous studies. The anti-NMDA receptor monoclonal antibody binds to the GluN1 subunit of the receptor, affecting its clustering at postsynaptic compartments and reducing NMDA receptor-mediated synaptic currents. The anti-GABAA receptor monoclonal antibody binds to α1-containing GABAA receptors and induces a reduction in channel function and synaptic displacement of the receptors. The use of these monoclonal autoantibodies in research allows for the replication of the disease state and provides a controlled environment for studying the pathogenic mechanisms of autoimmune encephalitis. While monoclonal antibodies may not fully replicate the complexity of polyclonal disease states, they offer greater replicability and precise characterization of the effects of autoantibodies on specific receptor targets. Ultimately, monoclonal autoantibodies serve as valuable tools for studying autoimmune encephalitis. They provide a controlled and reproducible system for investigating the pathogenic effects of autoantibodies on neuronal proteins, shedding light on disease mechanisms and potential therapeutic approaches.

In Chapter II we aimed to evaluate the quality and suitability of the human-derived anti-NMDA receptor monoclonal autoantibody, #003-102, for investigating antibody-mediated pathology in autoimmune encephalitis. We performed immunocytochemical and histochemical analyses to determine its reactivity to the neuronal surface, specificity for the target, and its impact on astrocytes and microglia in organotypic hippocampal cultures. The results showed that the antibody bound to hippocampal neurons, correlated with NMDA receptor expression, and met pre-set criteria for maintained binding and specificity. Further, we found that it did not cause immune activation of astrocytic and microglial cells in the organotypic culture system. However, we acknowledge that the possibility of cytokine release by autoantibodies without altering cell morphology and immune marker expression cannot be ruled out, suggesting the need for further investigation. Based on these findings, we determined that a concentration of 0.5 µg/ml and a 24-hour exposure time of the antibody were appropriate for the planned experimental work in in vitro hippocampal preparations.

Given the array of homeostatic systems in place, each dependent on effective cross-talk between excitatory and inhibitory synaptic signalling, the mechanistic underpinnings of autoimmune epileptogenesis may reside in pathological co-regulation of excitatory and inhibitory neurotransmission. We would expect NMDAR-Abs and GABAaR-Abs to elicit downstream perturbations across both neurotransmission systems, inhibitory and excitatory. However, the characterisation of this cross-synaptic disruption remains to be fully investigated. In Chapter III we hypothesise that antibody-mediated interference of NMDA- or GABAA receptor organisation and function will metastasise into global dysregulation of ionotropic synaptic signalling, impacting

glutamatergic and GABAergic neurotransmission. Investigations into the co-regulation of glutamatergic and GABAergic systems, in the context of autoimmune encephalitis, may provide novel avenues for therapeutic development and intervention for these disease states. Moreover, identification of EI co-regulation in these diseases may further expand our knowledge regarding the interaction between excitatory and inhibitory systems in the maintenance of hippocampal homeostasis on a more fundamental level. In this Chapter we aimed to investigate the synaptic effects of NMDA and GABAA receptor autoantibodies on excitatory and inhibitory neurotransmission. This demonstrated that both types of autoantibodies led to a reduction in AMPA receptor-mediated currents, indicating a decrease in excitatory synaptic transmission. We also observed a moderate decrease in the localization of AMPA receptors at postsynaptic sites. Interestingly, the reduction in current magnitude was greater than the depletion of synaptic AMPA receptors, suggesting possible alterations in the nanoscale organization of these receptors. Additionally, both types of autoantibodies caused a substantial reduction in GABAA receptor-mediated currents, indicating a decrease in inhibitory synaptic transmission. This reduction was expected in the case of GABAA receptor autoantibodies but was also observed with NMDA receptor autoantibodies. The findings suggest that autoantibodies targeting single neuronal antigens can impact synaptic function through multiple mechanisms, affecting both excitatory and inhibitory synaptic activity.

Additionally, we further investigated the balance between spontaneous excitatory and inhibitory synaptic inputs, finding that the balance was shifted towards hyperexcitation despite the overall reduction in both excitation and inhibition. Moreover, we exposed CA1 principal cells to autoantibodies and recorded excitatory and inhibitory synaptic currents in the absence of action potential-driven activity. In these experiments we found that NMDA receptor autoantibodies reduced AMPA receptor-mediated excitatory inputs, while GABAA receptor autoantibodies did not substantially alter miniature excitatory postsynaptic current (mEPSC) amplitudes. However, we observed a reduction in the frequency of miniature inhibitory postsynaptic current (mIPSC) events, after exposure to either pathogenic autoantibody samples. This data was used to model the synaptic multiplicity, as a measure of the degree of connectivity between principal cells, and interneurons-to-principal cells. This data suggested that neither type of autoantibody altered the density of excitatory synapses, but both caused a downregulation of inhibitory synapses on the CA1 pyramidal neurons. To investigate the molecular mechanisms underlying synaptic destabilization, we then examined the role of excitatory and inhibitory synaptic scaffolding proteins, homer-1c and gephyrin, respectively. We found no significant impact on the excitatory synaptic scaffolds; however, incubation with autoantibodies in the absence of astrocytes prevented the reduction of synaptically localized AMPA receptors, suggesting that astrocytes may play a role in the pathogenic alterations induced by autoantibodies. Additionally, we also observed a decrease in the density of inhibitory synaptic scaffolding protein, gephyrin, which was mediated by hyperphosphorylation of a specific serine residue, S270. Interestingly, similar levels of phosphorylation and degradation of inhibitory synaptic scaffolds were observed with both GABAA and NMDA receptor autoantibodies, indicating a common substrate for pathogenicity in autoimmune encephalitis, regardless of the specific target antigens.

While we observed significant perturbations to both excitatory and inhibitory synaptic phenotypes, after incubation with NMDA- and GABAA receptor autoantibodies, we hypothesised that such autoantibodies may impact the functional properties of extrasynaptic ion channels involved in neuronal excitability. While reductions in tonic GABAergic inhibition might be expected in conditions of reduced inhibitory synaptic activity, evidence suggests that loss of synaptic inhibition during status epilepticus either does not alter – or alternatively, can increase – tonic GABAergic conductance. Moreover, a large body of literature has demonstrated that GABAA receptors located at the axon initial segment can influence intrinsic neuronal

function and action potential generation. Regarding NMDA receptor autoantibodies, they may reduce tonic excitatory currents, particularly in inhibitory neuronal populations. The presence of magnesium-insensitive GluN2D-containing NMDA receptors on inhibitory interneurons contributes to their reliance on tonically active channels. This disinhibitory mechanism has been implicated in psychotomimetic effects and rapid antidepressant effects. The text speculates that NMDA receptor autoantibodies may decrease tonic excitatory currents, potentially leading to both seizure and psychosis phenotypes. Together, providing a solid basis for the possibility that action of NMDA- and GABAA receptor autoantibodies, targeting non-synaptic receptor populations, may strongly modulate the intrinsic excitability of hippocampal neurons. To investigate these ideas, in Chapter IV, we performed whole-cell current clamp and cell-attached voltage-clamp recordings on CA1 pyramidal neurons and inhibitory interneurons to measure action potential output, intrinsic excitability, and spontaneous activity without intracellular manipulation. Our results showed that GABAA receptor autoantibodies increased the excitability of pyramidal neurons, leading to increased action potential output, depolarization of resting membrane potential, decreased rheobase and spike latency, and increased input resistance. In contrast, NMDA receptor autoantibodies did not have any significant effect on these parameters. The loss of tonic GABAergic inhibition on principal cells was observed, which may promote cellular hyperactivity and depolarization of the resting potential. Further, GABAA receptor autoantibodies, uniquely, were found to alter action potential waveforms, reducing amplitude and prolonging duration, possibly by affecting the function of voltage-gated sodium and potassium channels. Conversely, NMDA receptor autoantibodies selectively reduced GABAergic neuronal excitability, leading to a negative shift of input-output plots, hyperpolarization of resting potential, and increased rheobase. The mechanism by which these autoantibodies exert their effects is not fully understood, but strongly suggests the modulation of extrasynaptic GABAA and NMDA receptors. Cell-attached voltage-clamp experiments further demonstrated that inhibitory cellular outputs were silenced after exposure to NMDA receptor autoantibodies, while excitatory neuron activity increased after exposure to either autoantibody. Taken together, these findings suggest that the combined effects of synaptic input alterations and changes in cellular excitability contribute to pyramidal cell hyperexcitation in hippocampal circuits.

Given our prior results, and the knowledge that seizure phenotypes are highly prevalent in both NMDA- and GABAA receptor encephalitis, we hypothesised that autoantibody actions would converge to induce hyperactivity in hippocampal networks. To address this hypothesis in Chapter V, we conducted calcium imaging experiments on cultured hippocampal slices exposed to NMDA and GABAA receptor autoantibodies, aiming to examine their impact on network activity. Given the substantial role of the CA1 hippocampal subfield in memory processing and cognitive dysfunction, as well as its association with epilepsy, we focussed primarily on dysfunction within this subfield. We also note that the use of multi-electrode array (MEA) recording techniques to further study network activity has led to some disparity in previous reports, specifically regarding the impact of NMDA receptor autoantibodies on dissociated network activity. We suggest that both autoantibodies are likely to induce hyperexcitability at the network level, with NMDA receptor autoantibodies possibly affecting synchronicity through their specific impact on GABAergic interneurons. Our experiments using calcium imaging revealed that both autoantibodies lead to hyperactivity in CA1 pyramidal neurons, indicating a hyperactive network state. As such, we expect that this antibody-mediated hyperactivation is likely due to altered synaptic inputs favouring hyperexcitation and selective impacts on cellular excitability. We further propose that destabilization and hyperactivation of hippocampal networks could be a key factor in seizure generation in patients with autoimmune encephalitis. The hippocampus is widely associated with various cognitive functions, and hippocampal hyperactivation may result in deficits in cognitive processing. As such, we considered the importance of fine coordination in the activity of CA1 output cells for healthy cognitive

processes. We uncovered a selective alteration by NMDA receptor autoantibodies, leading to a hypersynchronous network state. With this data, it is tempting to speculate that dysfunction in inhibitory interneurons and reduced feedback and lateral inhibition could be linked to the development of hypersynchronous hippocampal discharges. We further suggest that future work may aim to characterise the impact of autoantibodies on specific subpopulations of GABAergic interneurons. Additionally, we also performed multi-electrode array (MEA) recordings on dissociated hippocampal cultures and found that NMDA receptor autoantibodies increased network activity, while GABAA receptor autoantibodies did not have a noticeable effect. The lack of consistency between the results of calcium imaging and MEA recordings may be attributed to high degree of variability – inherent in MEA datasets – or may reflect hippocampal circuit architecture-dependent impacts of autoantibodies. Overall, we emphasize the role of NMDA and GABAA receptor autoantibodies in altering hippocampal network activity and highlight the potential implications for seizures, cognitive dysfunction, and synaptic plasticity.

ESR7 – Elisa Corti

ESR7: overview of results towards the objective of the action

Project title – FMRP regulation of synapse dynamics

WP2 – Testing the Excitation/Inhibition imbalance hypothesis in neuropsychiatric disorders

Early Stage Researcher 7 – Elisa Corti

Supervisors – Carlos Duarte, Ramiro Almeida, Paulo Pinheiro

Host Location – Centre for Neuroscience and Cell biology (CNC), University of Coimbra – Portugal

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability and it is characterised by a high incidence of autism. FXS is caused by transcriptional silencing of the *Fmr1* gene and consequent loss of the expression of the Fragile X messenger ribonucleoprotein (FMRP, previously known as fragile X mental retardation protein. Martin and Bell, 1943; Berger et al., 1985). FMRP is an RNA-binding protein (Ashley et al., 1993) that is involved in the trafficking of a wide array of mRNAs that encode for proteins essential for a proper synaptic formation and physiology (Brown et al., 2001, Pasciuto and Bagni, 2014). Moreover, FMRP exerts translational control over these mRNAs (Osterweil et al., 2010) in the distal compartments of the neurons (namely the dendrites and the axons), a relevant mechanism for synaptic plasticity.
The main hallmarks of FXS are an aberrant dendritic spine morphology and density, deficits in Hebbian forms of long-term depression (LTD) and long-term potentiation (LTP), altered neuronal excitability, and delayed synaptic maturation (reviewed in Bagni and Zukin, 2019).
Due to the broad heterogeneity of FMRP target mRNAs, it is difficult to understand the contribution of FMRP in maintaining a proper balance of the physiology of the synapse. In this project we are investigating the role of FMRP in (i) the number and formation of presynaptic sites and (ii) NMDA receptors physiology, with eventual implications in synaptic plasticity.

(i) Over the last decades, extensive efforts have been made to understand the role that FMRP has in the more distal compartments of neurons. However, the majority of research was focused in the postsynaptic compartment, with little resources allocated to the study of the role of FMRP in axons and presynaptic terminals. Hence, in the first part of this project we focused on studying how FMRP can shape the development of presynaptic terminals in the axonal shaft.

For this, we prepared pseudo explants from WT and *Fmr1* KO hippocampal neurons. This is a particular neuron seeding technique that allows separating the somato-dendritic compartment from the axonal compartment on a glass coverslip. With this tool we could study a variety of presynaptic proteins over time by immunocytochemistry, such as bassoon (bsn), synapsin (syn), vesicular glutamate transporter 1 (vGluT1) and synaptic vesicle protein 2 (SV2). Since the staining for these proteins was represented by discrete clusters, we quantified the number and the integrated signal density of such clusters. We choose different timepoints, namely days-in-vitro (DIV) 7, DIV 9, DIV 12 and DIV 15, that span from early synaptogenesis to mature synapses.

The results are summarised in table 1. We also describe in detail the results of the two timepoints where we observed significant changes: DIV 7 and DIV 12. In DIV 7 neurons we did not observe any differences in the number of bsn (Fig. 1B), syn (Fig. 1D), vGluT1 (Fig. 1F) and SV2 (Fig. 2B) clusters between WT and *Fmr1* KO neurons. We found a significant reduction of bsn intensity (Fig. 1C) in *Fmr1* KO neurons, while the intensity of the other markers was not impaired by the absence of FMRP (Fig. 1E, Fig. 1G, Fig. 2C for syn, vGluT1 and SV2 respectively).

Interestingly, in slightly older neurons, namely at DIV 12, we observed a marked reduction of bsn (Fig. 3C), syn (Fig. 3E) and vGluT1 (Fig. 3G) in axons lacking FMRP, while SV2 levels were comparable across genotypes (Fig. 4C). The number of clusters of all these markers was unaffected by the KO of *Fmr1* (Fig. 3B, Fig. 3D, fig 3F, fig 4B for bsn, syn, vGluT1 and SV2 respectively).

Overall, the lack of FMRP impairs the accumulation of presynaptic proteins in the axonal shaft, in a differential fashion, depending on the protein of interest and the stage of development.

Considering that FMRP is regarded to as a suppressor of protein synthesis (Osterweil et al., 2010) and that factors necessary for protein synthesis, including ribosomes and mRNAs are localised in the axon (evidences reviewed in Perrone-Capano et al, 2021), we evaluated whether the axons of *Fmr1* KO neurons show impaired local protein synthesis. To do so, we labelled the phosphorylated form of the 4E-Binding Protein 1 (p4E-BP1) on Thr37/46. When 4E-BP1 is not phosphorylated, it is known to interact with the eukaryotic initiation factor 4E (eIF4E), preventing the initiation of cap-dependent protein synthesis. On the other hand, the phosphorylation of 4E-BP1 prevents such interaction, allowing the binding of eIF4E to the cap found in the 5' region of many eukaryotic mRNAs, followed by translation initiation (Gingras et al., 2001). Interestingly, we found that the number (Fig. 5B) and the integrated density (Fig. 5C) of p4E-BP1 clusters was reduced in axons of *Fmr1* KO neurons at DIV 7. On the other hand, at DIV 12, *Fmr1* KO axons presented an increase in the integrated density of p4E-BP1 clusters (Fig. 6C), while the number of clusters was not affected by the lack of FMRP (Fig. 6B). We can conclude that regulation of local protein synthesis is unbalanced in axons lacking FMRP, and this seems to be dependent on the developmental stage of the neurons.

Synaptogenesis is known to rely on axonal protein synthesis (Batista et al, 2017), and we want to understand whether this process is impaired in neurons lacking FMRP. Therefore, we are currently investigating if FMRP affects cue-induced synaptogenesis, by stimulating WT and *Fmr1* KO neurons with two synaptogenic factors: Fgf22 and PDL-coated beads. In the future, we plan to complement the results obtained by performing functional experiments, such as multielectrode array (MEA) recording and calcium imaging, to gather a wider perspective on the functional consequences of presynaptic defects caused by the lack of FMRP.

(ii) Long term potentiation (LTP) is a long-lasting form of synaptic plasticity, characterised by a persistent, activity-dependent strengthening of synaptic connections (Douglas, Goddard, 1975), that underlies memory formation and learning (Bliss and Collingridge, 1993). *Fmr1* KO mice exhibit deficits in learning (Ding et al., 2014), and this is correlated with deficits in LTP in CA3-CA1 synapses within the hippocampal circuit (Lauterborn et al., 2007). In this second part of the project we aim to understand how lack of FMRP causes such deficits in LTP and what is the role of FMRP in the regulation of NMDA receptor dynamics in a context of synaptic plasticity.
It is known that some forms of LTP rely on the activation of the Tropomyosin receptor kinase B (TrkB) (Kang et al., 1997), and that blockade of the GluN2B subunit of NMDA receptors (NMDAR) abolishes the facilitatory effect that brain-derived neurotrophic factor (BDNF) has on LTP (unpublished data). To assess whether alterations in TrkB activation and in the synaptic accumulation of GluN2B-containing NMDAR mediate the LTP deficits observed in *Fmr1* KO neurons, we are currently recording field

excitatory postsynaptic potentials (fEPSPs) in acute WT and *Fmr1* KO hippocampal slices. We are preincubating the slices with TrkB-FC, a scavenger of BDNF, and with ifenprodil, to pharmacologically block GluN2B-containing NMDAR. We are inducing LTP by stimulating Schaffer collaterals with a theta burst protocol and recording in the apical dendrites of CA1 pyramidal neurons (Fig. 7A). From the preliminary results we already obtained, both TrkB-FC and ifenprodil are effective in dampening LTP in WT slices, but not in *Fmr1* KO slices, indicating that TrkB signalling and the activity of GluN2B-containing NMDAR are already impaired in *Fmr1* KO slices. This is true especially considering the early phase of LTP, immediately after induction (Fig. 7C), but further data are needed to draw meaningful conclusions.
The initial phase of LTP is mainly driven by NMDAR (Grosshans et al, 2001) and the deficits seen in *Fmr1* KO slices may be due to differences in the synaptic content of these receptors. To tackle this question, we isolated synaptoneurosomes (a subcellular fraction consisting of a presynaptic and a postsynaptic terminal attached together) from WT and *Fmr1* KO hippocampi. We allowed them to adhere on glass coverslips and labelled the surface fraction of GluN2A (Fig. 8A) and GluN2B (Fig. 8E)-containing NMDAR by briefly incubating synaptoneurosomes with an antibody that recognizes the extracellular N-terminus of each subunit in non-permeabilizing conditions. We subsequently also immunostained for PSD95 and vGluT1. For each NMDAR subunit we quantified the percentage of positive synaptoneurosomes, the area and the integrated density of the clusters. We observed no differences across genotypes in the percentage of GluN2A^{+} synaptoneurosomes (Fig. 8B); however, the area (Fig. 8C) and the integrated density (Fig. 8D) were significantly increased in synaptoneurosomes isolated from FMRP-lacking hippocampi. In contrast, no differences were observed regarding the surface staining of GluN2B (Fig. 8F, 8G, 8H).

To dissect the role of FMRP in regulating NMDAR physiology, we prepared primary rat hippocampal neurons and transfected the cells with a short hairpin (shFMRP_A) to knock down (KD) the expression of *Fmr1*, or with the correspondent short hairpin control (shCTRL_A). Three days later we activated TrkB by treating neurons with BDNF for 30 min, in order to mimic what happens in the early phase of LTP, and labelled the surface GluN2B-containing NMDAR together with the synaptic markers PSD95 and vGluT1, as described above for synaptoneurosomes (Fig. 9A). We quantified the number and intensity of GluN2B clusters that colocalise with the aforementioned synaptic markers. BDNF treatment upregulated the intensity of GluN2B puncta in the synaptic compartment, but such effect was abrogated upon KD of FMRP (Fig. 9C), while the total number of GluN2B clusters was unaffected by BDNF (Fig. 9B). Since PSD95 is a target of FMRP (Zalfa et al., 2007), and is a main scaffold protein at the postsynaptic density to which NMDAR are anchored, we quantified the number and integrated density of PSD clusters. We observed that BDNF treatment upregulated the number of PSD95 clusters in neurons transfected with the shCTRL_A, but this did not happen in neurons with lower levels of FMRP (Fig. 9D). Moreover, BDNF treatment also upregulated the integrated density of PSD clusters too. Interestingly, *Fmr1* KD alone increased the integrated density of PSD clusters, but this phenotype was reverted upon stimulation with BDNF (Fig. 9E).

Since the lateral diffusion of NMDAR in the membrane is a powerful mechanism to finely tune synaptic receptor content (Tovar and Westbrook, 2002), we examined whether FMRP influences the lateral movements of GluN2B-containing NMDAR by means of single particle tracking with quantum dots (QD). We co-transfected rat hippocampal neurons with a short hairpin to knock down *Fmr1* expression and a plasmid encoding for an intrabody conjugated to a green fluorophore, that binds to endogenous PSD95. This way, postsynaptic densities are visible and we can discern the synaptic GluN2B-containing NMDAR from the extrasynaptic ones. We treated neurons with BDNF for 30 min and tracked the movement of surface GluN2B-containing NMDAR, quantifying the mean square displacement (MSD) as a proxy to measure the area in which the receptors move, and the diffusion coefficient to measure the velocity of such receptors. We observed that the MSD of extrasynaptic receptors was not affected neither by the treatment with BDNF nor by the KD of *Fmr1*, but the combination of the two factors significantly decreased their MSD (Fig. 10A). The same trend was observed considering the diffusion

coefficient: the combination between *Fmr1* KD and BDNF treatment significantly decreased the velocity of extrasynaptic GluN2B-containing NMDAR compared to all the other experimental conditions (Fig. 10B).

A protein that has a pivotal role in the BDNF-induced upregulation of GluN2B-containing NMDAR at the synapse is the proline-rich tyrosine kinase 2 (Pyk2). It is known that BDNF induces synaptic accumulation and *de novo* protein synthesis of Pyk2, and these steps are required for the neurotrophin-induced synaptic accumulation of GluN2B-containing NMDAR (Afonso et al., 2019). Since Pyk2 mRNA is a target of FMRP as well (Darnell et al., 2011), we wondered whether the lack of FMRP would impair the BDNF-induced Pyk2 accumulation. For this purpose, we transfected rat hippocampal neurons with a shRNA to KD *Fmr1* expression (shFMRP_B) or the respective shRNA control (shCTRL_B). After a 30-min BDNF treatment, we immunolabelled Pyk2 and analysed its abundance by measuring the integrated signal density. As expected, BDNF treatment increased Pyk2 abundancy in the dendrites. Moreover, this effect was abrogated in neurons lacking FMRP (Fig. 11B).

A similar set of experiments was performed analysing GluN2A-containing NMDAR upon a 10-min treatment of cultured hippocampal neurons with BDNF (results not shown). Briefly, we found that BDNF stimulation upregulates the number and intensity of GluN2A puncta in the synaptic compartment, and such effect was abrogated upon KD of FMRP. Moreover, we observed that PSD95 levels increased, even after a short treatment with BDNF, and this effect was abrogated following *Fmr1* KD. We also tracked the lateral diffusion of GluN2A-containing NMDAR and found that their MSD was decreased upon *Fmr1* KD and that BDNF increased the MSD by a mechanism independent of FMRP. Finally, we observed that a 10-min treatment with BDNF increased Pyk2 levels in the dendrites of cultured hippocampal neurons, and this effect was abrogated upon *Fmr1* KD.

Taken together, our data show a role for FMRP in BDNF-induced upregulation of synaptic GluN2A- and Glun2B- containing NMDAR at glutamatergic synapses, probably exerted through Pyk2 accumulation in the dendrites and PSD95 translational control.

In the near future, we will complete the fEPSP recordings to conclude about the mechanisms underlying the alterations in LTP of CA1 synapses in *Fmr1* KO mice. Furthermore, we will investigate whether de novo Pyk2 protein synthesis induced by BDNF is impaired in neurons transfected with the shRNA to reduce FMRP levels.

TABLES AND FIGURES:

Table 1: Summary of the characterization of presynaptic proteins accumulation in the axonal shaft throughout development. Data related to the integrated density of the clusters are reported. Results are expressed as *Fmr1* KO versus WT.

Marker	DIV7	DIV9	DIV12	DIV15
Bassoon	↓	=	↓	=
Synapsin	=	↓	↓↓	=
SV2	=	↓	=	=
vGluT1	=	=	↓	=

The symbol "=" means that there are no significant differences across genotypes. The symbols "↓" and "↓↓" mean that KO neurons exhibit less protein compared to WT neurons.

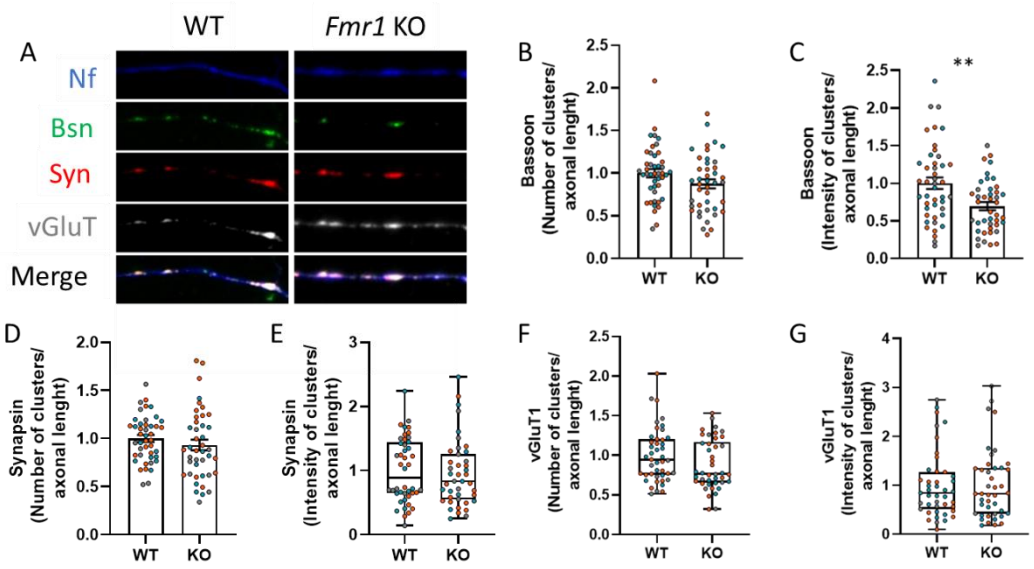


Figure 1: Representative images of DIV 7 neurons immunolabelled for neurofilament (Nf), Bassoon (Bsn) synapsin (Syn) and vGluT (A). Quantification of the number (B) and integrated density (C) of bassoon clusters. Quantification of the number (D) and integrated density (E) of synapsin clusters. Quantification of the number (F) and integrated density (G) of vGluT1 clusters. Results are normalised to the WT and are expressed as mean ± SEM if data are normally distributed, or using a box and whiskers plot (the box indicating the median, the 25th and 75th percentile, the whiskers indicating the maximum and minimum values) if data are not normally distributed. An unpaired t-test has been used if data are normally distributed, a Mann-Whitney test if data are not normally distributed. ** $p < 0.01$

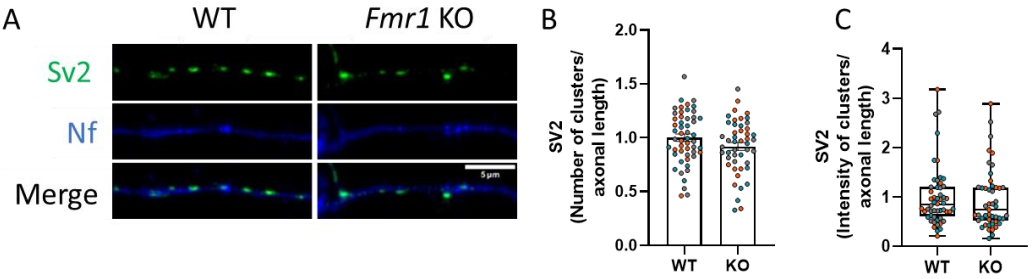


Figure 2: Representative images of DIV7 neurons immunolabeled for synaptic vesicle protein 2 (Sv2) and neurofilament (Nf) (A). Quantification of the number (B) and integrated density (C) of SV2 clusters. Results are normalised on WT and are expressed as mean ± SEM if data are normally distributed, or using a box and whiskers plot (the box indicating the median, the 25th and 75th percentile, the whiskers indicating the maximum and minimum values) if data are not normally distributed. An unpaired t-test is used if data are normally distributed, a Mann-Whitney test if data are not normally distributed.

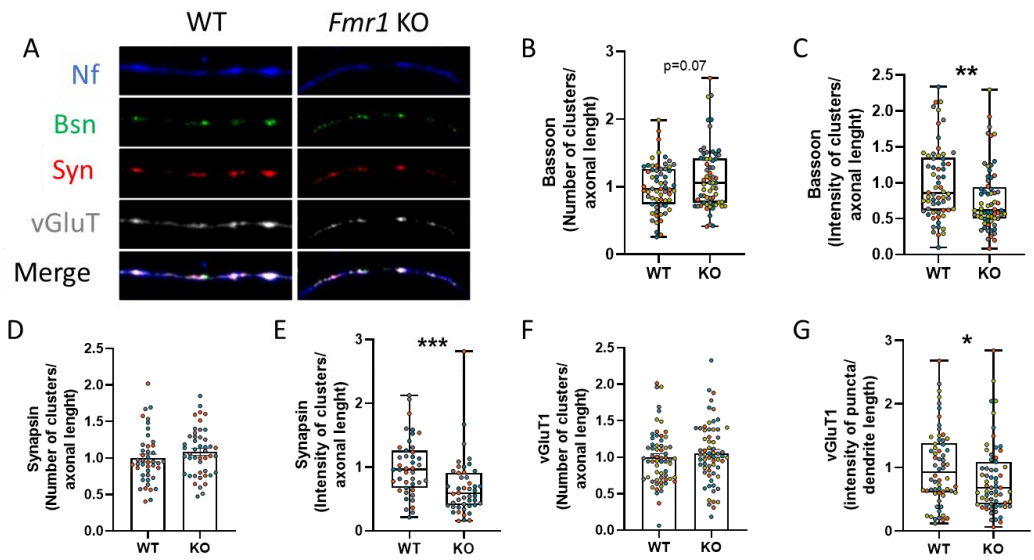


Figure 3: Representative images of DIV 12 neurons immunolabelled for neurofilament (Nf), Bassoon (Bsn) synapsin (Syn) and vGluT (A). Quantification of the number (B) and integrated density (C) of bassoon clusters. Quantification of the number (D) and integrated density (E) of synapsin clusters. Quantification of the number (F) and integrated density (G) of vGluT1 clusters. Results are normalised on WT and are expressed as mean ± SEM if data were normally distributed, or using a box and whiskers plot (the box indicating the median, the 25th and 75th percentile, the whiskers indicating the maximum and minimum values). An unpaired t-test is used if data are normally distributed, a Mann-Whitney test if data are not normally distributed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

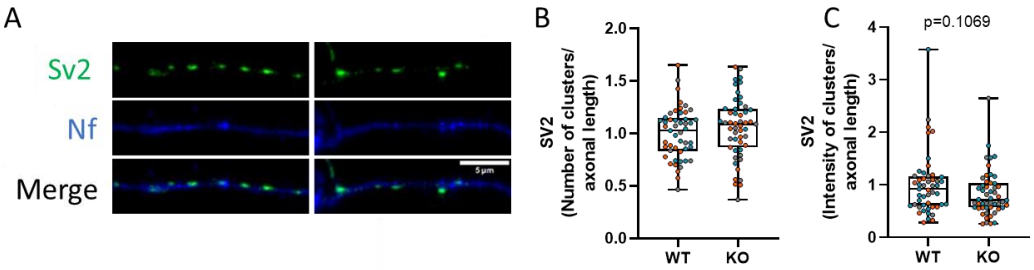


Figure 4: Representative images of DIV12 neurons immunolabeled for synaptic vesicle protein 2 (Sv2) and neurofilament (Nf) (A). Quantification of the number (B) and integrated density (C) of SV2 clusters. Results are normalised on WT and are expressed using a box and whiskers plot (the box indicating the median, the 25th and 75th percentile, the whiskers indicating the maximum and minimum values). Data are analysed using a Mann-Whitney test.

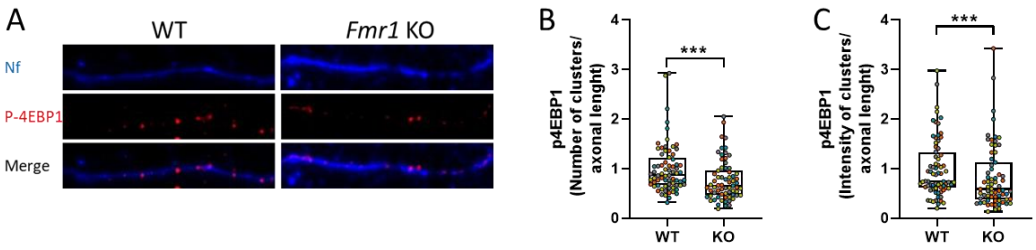


Figure 5: Representative images of DIV7 neurons immunolabeled for neurofilament (Nf) and P-4EBP1 (A). Quantification of the number (B) and integrated density (C) of p4E-BP1 clusters. Results are normalised on WT

and are expressed using a box and whiskers plot (the box indicating the median, the 25th and 75th percentile, the whiskers indicating the maximum and minimum values). Data are analysed using a Mann-Whitney test. *** p value < 0.001.

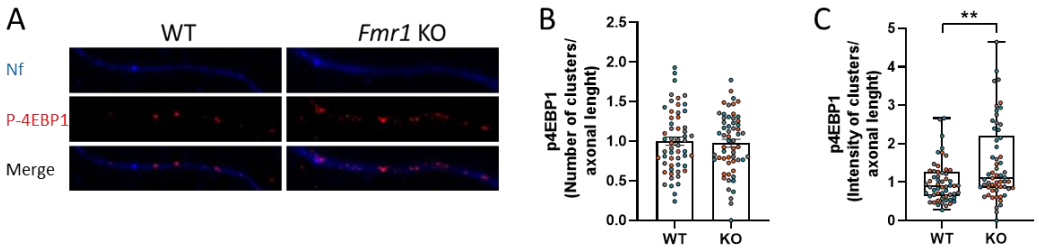


Figure 6: Representative images of DIV12 neurons immunolabeled for neurofilament (Nf) and P-4EBP1 (A). Quantification of the number (B) and integrated density (C) of p4E-BP1 clusters. Results are normalised for WT and are expressed as mean ± SEM if data are normally distributed, or using a box and whiskers plot (the box indicating the median, the 25th and 75th percentile, the whiskers indicating the maximum and minimum values) if data are not normally distributed. An unpaired t-test is used if data are normally distributed, a Mann-Whitney test if data are not normally distributed. ** p < 0.01

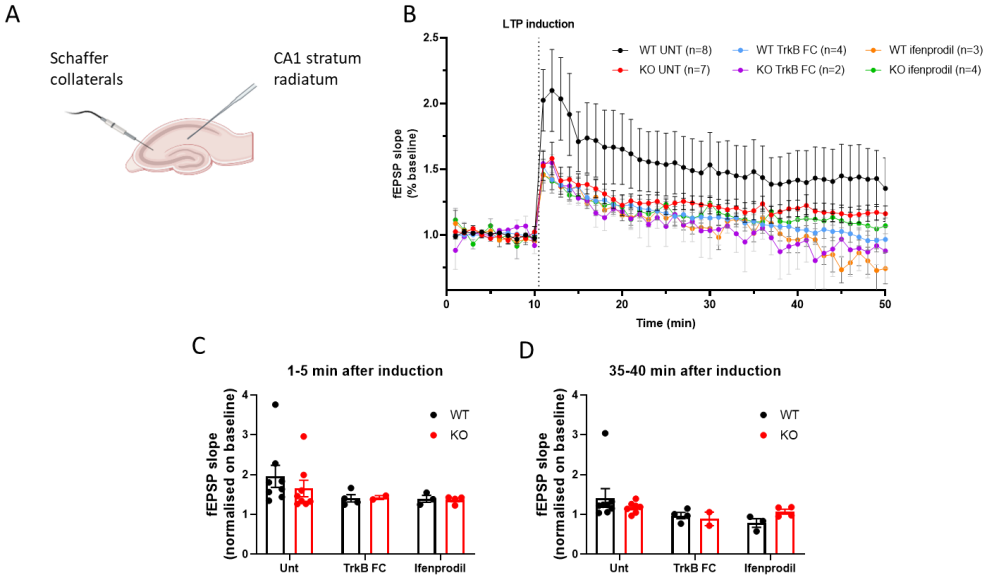


Figure 7: Representation of the placement of the stimulating electrode and the recording pipette in the hippocampus (A). Mean slope of field excitatory postsynaptic potential recorded for 40 minutes after LTP induction, expressed as percentage of the baseline (B). Average of the first (C) and last (D) 5 minutes of recording, expressed as mean ± SEM.

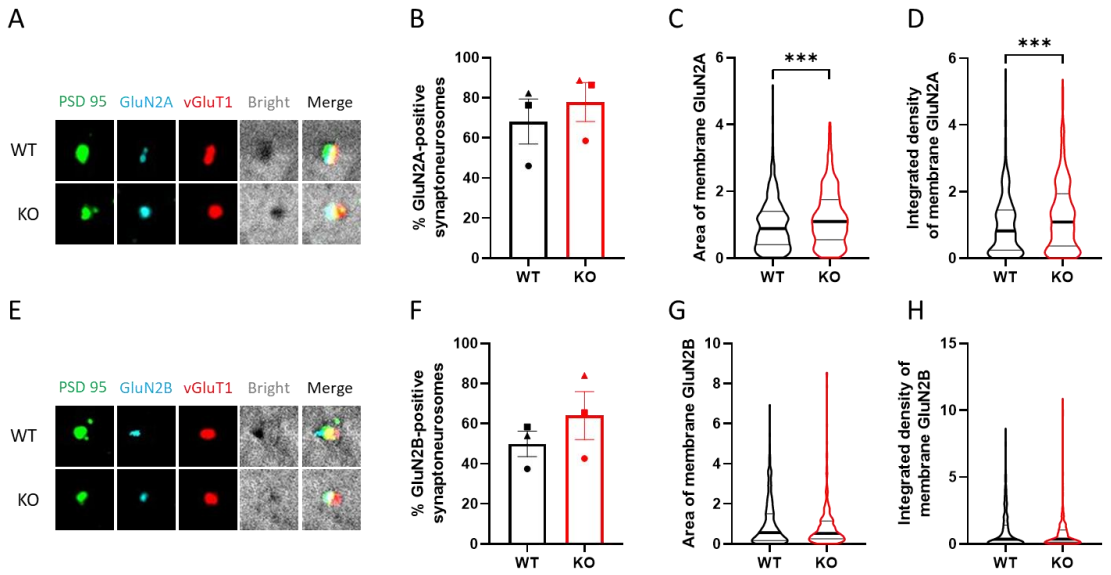


Figure 8: Representative images of synaptoneurosomes, immunolabelled for PSD95, vGluT1 and GluN2A (A). Quantification of the percentage of GluN2A^{+} synaptoneurosomes (B), of the area of GluN2A clusters (C) and of the integrated density of GluN2A clusters (D). Representative images of synaptoneurosomes, immunolabelled for PSD95, vGluT1 and GluN2B (E). Quantification of the percentage of GluN2B^{+} synaptoneurosomes (F), of the area of GluN2B clusters (G) and of the integrated density of GluN2B clusters (H). In panels B and F, the raw values are represented as mean ± SEM. In panels C, D, G and H data are normalised to the WT condition and represented by truncated violin plots, in which the 25th percentile, the median and the 75th percentile are highlighted. Data are analysed using a Mann-Whitney test. *** $p < 0.001$.

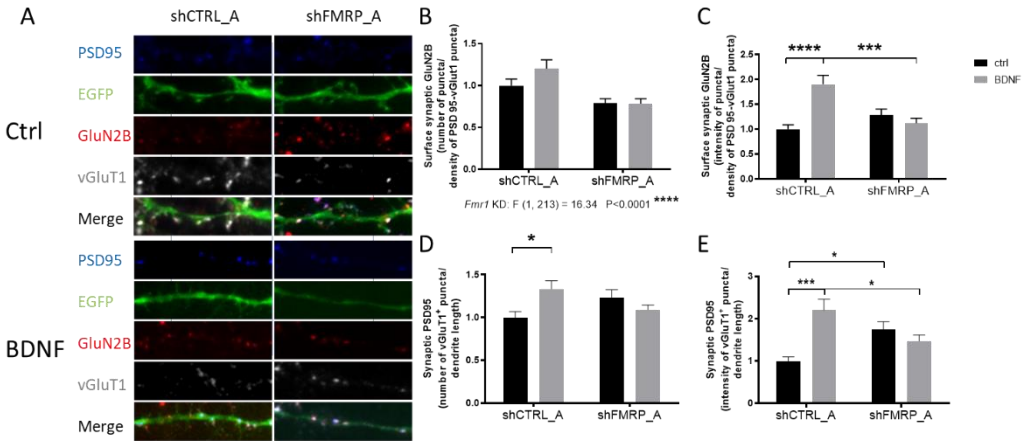


Figure 9: Representative images of rat hippocampal neurons transfected with a shCTRL_A and a shFMRP_A to lower the expression of *Fmr1*. Neurons were then treated with BDNF and the surface GluN2B-containing NMDA receptors were labelled by mean of a brief incubation with a primary antibody that recognizes an extracellular epitope. Synaptic markers PSD95 and vGluT1 were stained with immunocitochemistry (A). Quantification of the number (B) and integrated density (C) of synaptic GluN2B-containing NMDA receptor clusters. Quantification of the number (B) and integrated density (C) of PSD95 clusters. Data are normalised to the control condition (neurons transfected with shCTRL_A that did not receive BDNF) and expressed as mean ± SEM. Data were analysed using a two-way ANOVA with Tukey correction for multiple comparisons. * $p < 0.05$, *** $p < 0.001$, ****$p < 0.0001$

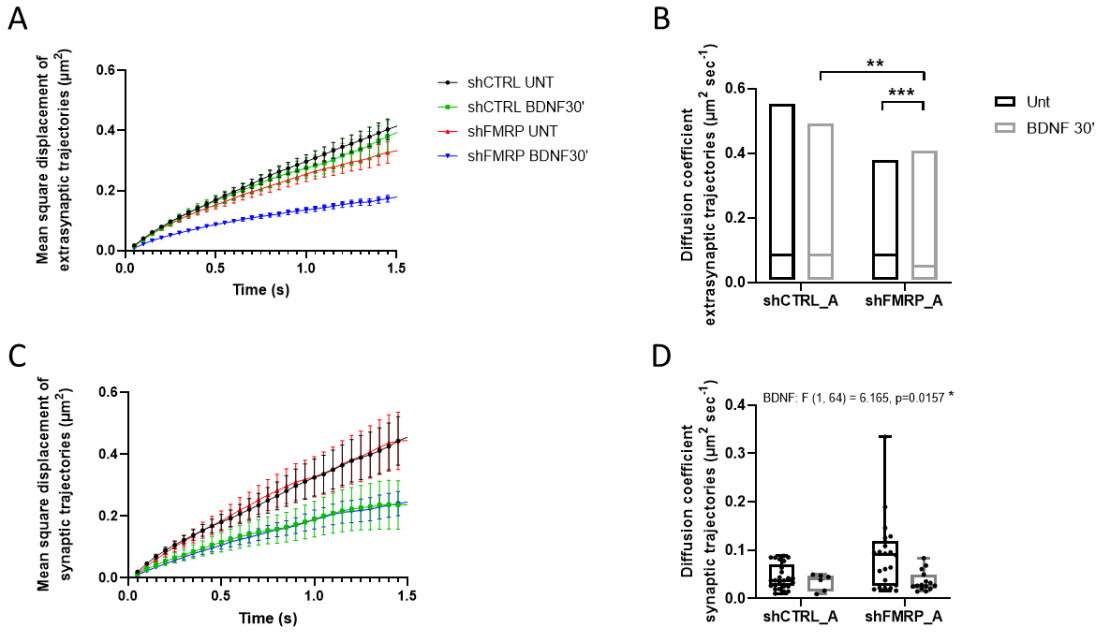


Figure 10: Quantification of the mean square displacement of extrasynaptic GluN2B-containing NMDA receptors (A). Quantification of the diffusion coefficient of extrasynaptic GluN2B-containing NMDA receptors (B). Quantification of the mean square displacement of synaptic GluN2B-containing NMDA receptors (C). Quantification of the diffusion coefficient of extrasynaptic GluN2B-containing NMDA receptors (D). Raw data are plotted and they are expressed as mean ± SEM in panels A and C, with floating bars are used for data in panels B and D, showing the minimum and maximum, as well as the mean. Here data were analysed using a two-way ANOVA with Tukey correction for multiple comparisons. ** $p < 0.01$, *** $p < 0.001$

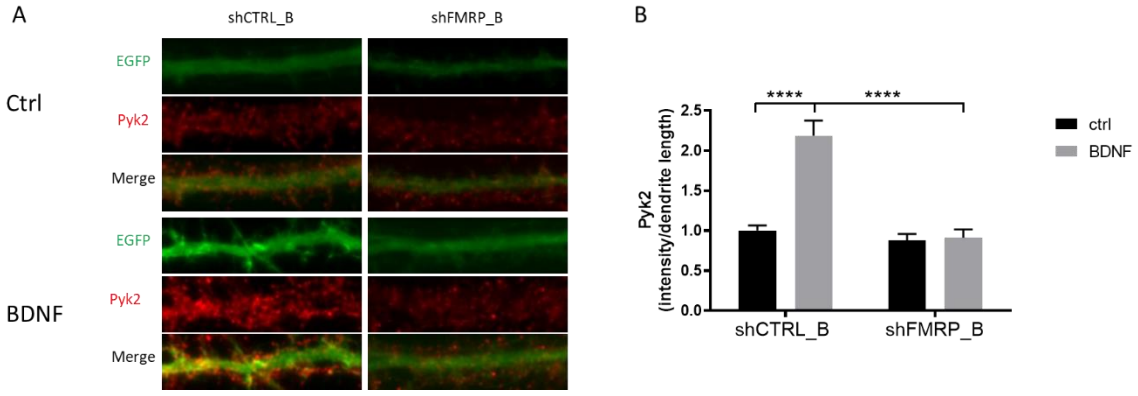


Figure 10: Representative images of rat hippocampal neurons transfected with a shCTRL_B and a shFMRP_B to lower the expression of *Fmr1* (A). Quantification of the integrated density of Pyk2 staining in dendrites (B). Data are normalised on the control condition (namely neurons transfected with shCTRL_B that did not receive BDNF) and expressed as mean ± SEM. Data were analysed using a two-way ANOVA with Tukey correction for multiple comparisons. ****$p < 0.0001$

Potential of results for exploitation

In our project we described a wide range of synaptic proteins that are dysregulated upon *Fmr1* knock out or knock down. Our findings will boost the research to understand how the phenotypes that are caused primarily by the absence of FMRP can be rescued, ultimately to individuate new therapeutic targets.

ESR8 – Elizabeth Brockman

ESR8: overview of results towards the objective of the action

Project title – Illuminating the mechanisms of synaptic and neural network dysfunction in a new *in vivo* model of Down's syndrome

WP2 – Testing the Excitation/Inhibition imbalance hypothesis in neuropsychiatric disorders

Early Stage Researcher 8 – Elizabeth Brockman

Supervisor – Vincenzo De Paola

Host Location – Institute of Clinical Sciences, Imperial College London – United Kingdom

Down's syndrome (DS) is a genetic disorder caused by trisomy of chromosome (Chr) 21 (Ts21). It is the most frequent cause of genetic intellectual disability, but the underlying synaptic and neural network mechanisms remain poorly understood. Most studies looking into the mechanisms of DS have been performed using mouse models. However, these models can only partially recapitulate the chromosomal abnormality present in DS patients. First, rodents do not have a Chr 21 and the > 200 genes contained in the human Chr21 are distributed into 3 sections of the mouse genome. Therefore, different transgenic mouse models are partially trisomic for a distinct set of genes present in the human Chr 21. In addition, there is evidence that also other chromosomes contribute to the disease. Because of these limitations, human induced pluripotent stem cells (iPSCs) hold great promise for modelling neurodevelopmental brain disorders such as DS. Our group has recently developed a radically new paradigm to model the synaptic and functional network phenotypes of DS, by transplanting human iPSC-derived neurons from healthy and DS donors in the adult mouse brain (in collaboration with Dr. Rick Livesey, Cambridge University). This approach combined with longitudinal structural and functional multiphoton imaging allowed the study human neuron synaptic and calcium dynamics in vivo for the first time and revealed that synapses are denser and more stable on Ts21 neurons and have a defect in the ability to build functional neural networks in vivo (Real *et al*., 2018; Science).

This project's main aim was to 1) dissect of the mechanisms involved in the increased synaptic stability found in previous grafted Down Syndrome (DS) induced pluripotent stem cells (iPSCs) that had been differentiated into neuronal progenitors, injected into an immunodeficient mouse brain, and subsequently monitored with longitudinal multiphoton imaging over several months (Task 8.1 in the GA); and 2) monitor these same grafts for calcium activity fluctuations to determine the mechanisms behind previously found deficits in oscillatory calcium activity in DS grafts (Task 8.2 in the GA).

To study the synaptic stability of Ts21 axons and the oscillatory calcium activity of Ts21 somas, I first utilized a new differentiation protocol to develop novel grafts. The aim of this was to eliminate a confounding variable from the previous graft type: that the amount of SATB2+ upper layer cortical neurons and the amount of hGFAP+ astrocytes were both significantly different in Ts21 grafts compared to control grafts. These differences in cell type distribution made it difficult to compare the Ts21 and control grafts accurately. Additionally, I had hoped that the use of a novel differentiation protocol would create grafts that also contained inhibitory interneurons (which were absent from the Real et al., 2018 grafts), making the grafts more realistic developmental models. I therefore switched from the Shi et al., 2012 neuronal differentiation protocol that was used in the past, to a novel protocol developed by our collaborators in Dean Nizetic's lab at Queen Mary University of London. As depicted in Figure 1, the use of this novel protocol did normalize the number of all cell types across the genotypes. I was not successful in producing grafts with inhibitory interneurons, but the normalization of SATB2+ and hGFAP+ cell levels across genotypes was a success that eliminated a confounding factor present in our group's previous work.

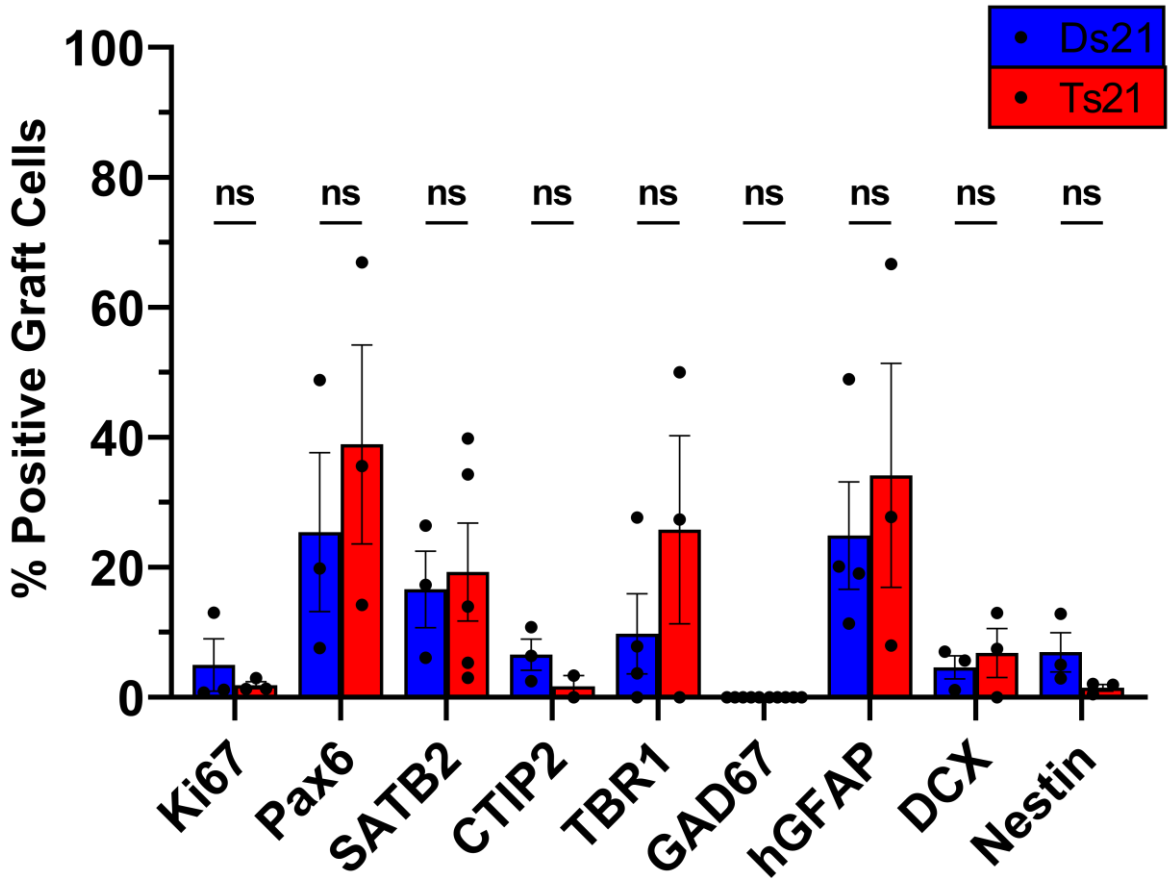


Figure 1. Graft characterization graph depicting the percentage of a graft that is composed of cells positive for specific stainings. These data indicate that there is no significant difference in any of the amounts of cell types across genotypes. Furthermore, both genotypes contain neuronal progenitors and stem cells (Ki67+, Pax6+, DCX+, Nestin+), astrocytes (hGFAP+), deep layer cortical excitatory neurons (CTIP2+, TBR1+), upper layer

excitatory cortical neurons (SATB2+), and are completely absent of inhibitory interneurons (GAD67+). Each point represents the average for one animal.

Once the new grafts were developed, I performed longitudinal multiphoton imaging of the Ts21 and isogenic control graft cells to test whether the novel graft type also presented with an increase in synaptic structural stability and a decrease in oscillatory calcium activity. To measure synaptic structural stability, axons containing en-passant boutons (EPBs) were imaged three times over the course of five days at both 3 months post-transplantation (mpt) and 5mpt. The same axons were followed and imaged at all three imaging sessions and then were comparatively analysed to determine the number of EPBs that were gained over time, lost over time, or were stable across multiple imaging sessions. Figure 2 depicts an example of a Ts21 axon imaged over three sessions with arrows marking examples of gained, lost, and stable EPBs.

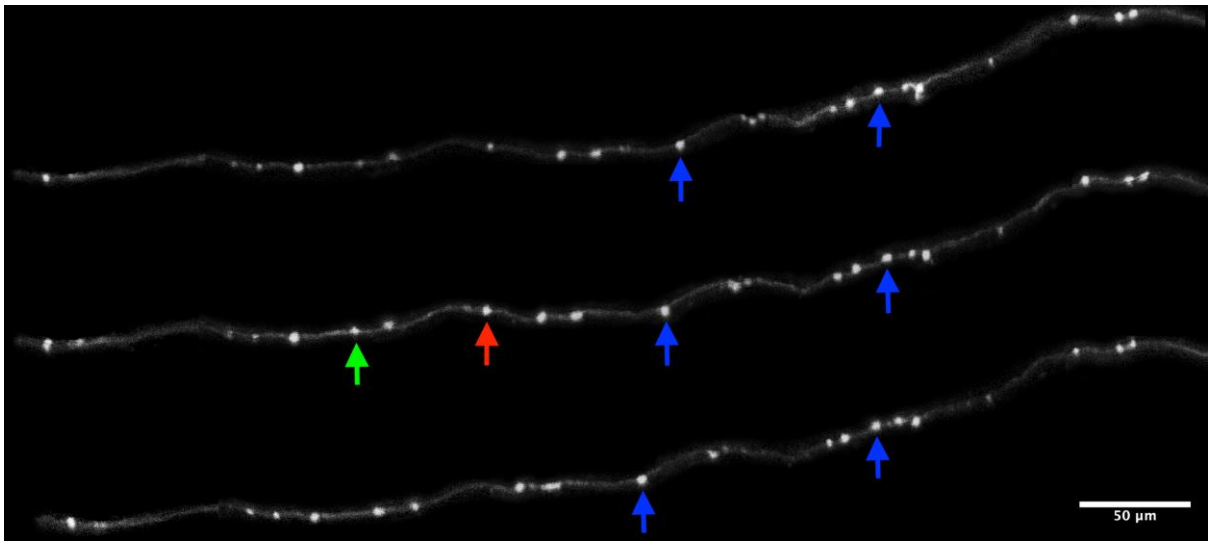
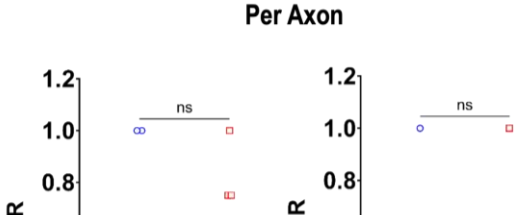


Figure 2. Example of a Ts21 axon with en-passant boutons imaged three times over a week (Top: Day 0, Middle: Day 2, Bottom: Day 4). The green arrow indicates an example of a gained EPB, which was not present at a high enough intensity to be classified as a bouton until Day 2. The red arrow indicates and example of a lost EPB, as the EPB indicated is not present in the following image. The blue arrows indicate two examples of stable EPBs.

These data were used to calculate the turnover rate (TOR) and survival rate (Fr) of the axonal EPBs of each genotype at 3mpt and 5mpt. TOR indicates the amount of gains and losses over time, while survival rate is specifically related to the number of EPBs present in the first session that remain over the subsequent two imaging sessions. Surprisingly, analysis indicated no significant difference in EPB TOR (see Figure 3) or EPB Fr (see Figure 4) between Ts21 and isogenic control grafts at 3mpt or 5mpt. These were analyzed both by animal and by axon. I therefore concluded that in the current grafts, the presence of a third copy of chromosome 21 is not sufficient to create a significant change to the synaptic stability phenotype of grafts in early development.



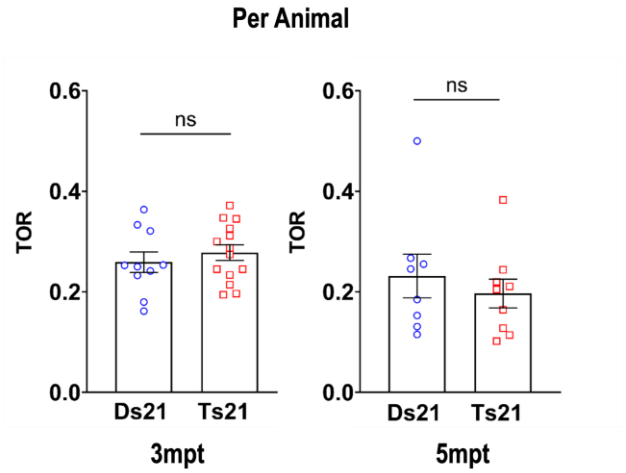


Figure 3. Turnover rate (TOR) of axonal en-passant boutons analyzed per animal (left) and per axon (right) in both genotypes (blue = Ds21 controls; red = Ts21) at 3mpt and 5mpt. No significant difference was found between the groups at any timepoint.

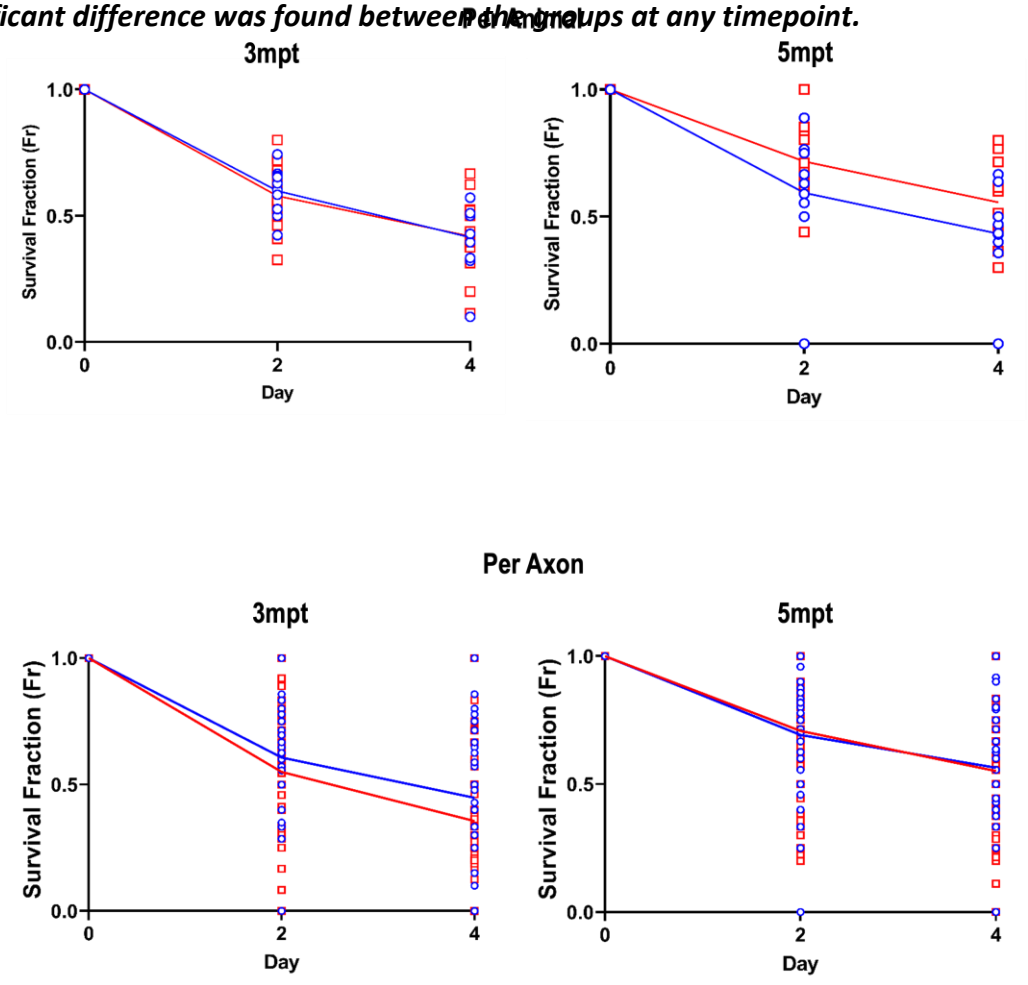


Figure 4. Survival rate (Fr) of axonal EPBs at 3mpt and 5mpt in both Ds21 control grafts (blue) and Ts21 grafts (red) quantified per animal (top) and per axon (bottom). No significant difference was found between the genotypes at any timepoint.

These synaptic stability findings represent Task 8.1 and revealed a surprising and unexpected lack of significant difference between the genotypes that was not present in previous grafts with a different differentiation protocol. Despite this outcome not being expected, I have developed a model within which the presence of Ts21 does not affect the stability of synapses, and which provides additional information on the mechanisms behind DS phenotypes.

To confirm whether this model has indeed normalized the effect of Ts21, I also evaluated spontaneous and oscillatory calcium activity (fulfilling the outcomes of Task 8.2). Longitudinal multiphoton imaging was used to measure GCaMP6+ calcium fluctuations in graft cell neuronal somas at 1mpt, 3mpt, 5mpt, and in a pilot study group at 7-8mpt. Calcium activity was determined as a measure of the change in fluorescent intensity in relation to background intensity (ΔF/F0). The threshold change in fluorescence was optimized and set to 30ΔF/F0. The subsequent activity data was analyzed using a MATLAB script provided by our collaborator Dr. Samuel Barnes (ICL) to achieve output data of calcium activity integral (ΔF/F0), frequency (Hz) and amplitude (ΔF/F0). Figure 5 outlines the spontaneous calcium activity outcomes from each of these outputs, as well as the percentage of active somas, with

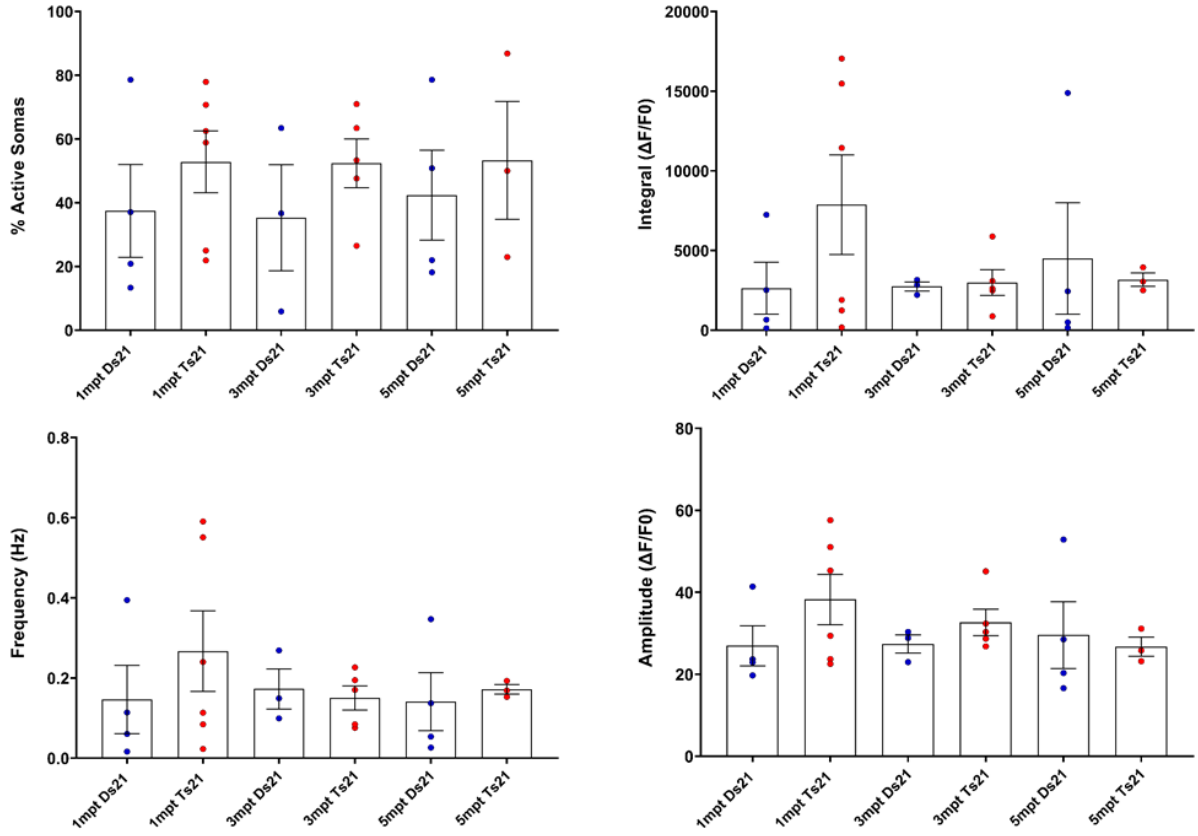


Figure 5. Spontaneous calcium activity outcomes at 1mpt, 3mpt, and 5mpt in Ts21 and isogenic control (Ds21) grafts based on the change in fluorescent intensity post-thresholding (30ΔF/F0 threshold). All data was statistically analyzed, and no significant differences were found between any genotypes at any of the timepoints. Top left: The percentage of active somas per animal at each timepoint. Top right: Integral outputs (ΔF/F0) by animal. Bottom left: Frequency (Hz) outputs by animal. Bottom right: Amplitude (ΔF/F0) outputs by animal.

A similar lack of significant difference was found in the pilot test group at 7-8mpt but the n-value is not high enough to provide statistical analyses.

These data indicate that the presence of Ts21 is also not sufficient to alter spontaneous somatic calcium activity levels at early developmental stages in these grafts. This is expected, given the findings from the synaptic stability dataset in Task 8.1. For the second half of Task 8.2, I wanted to determine whether this normalization of Ts21 effect also applied to oscillatory network calcium activity. All ROIs containing only 1 active soma or no active somas were removed from the dataset, and the remaining ROIs were analyzed to determine the amount of oscillatory network calcium activity. Figure 6 depicts the results of oscillatory network activity outcomes at 1mpt, 3mpt, 5mpt, and 7-8mpt in Ds21 controls (blue) and Ts21 grafts (red) over many thresholds. Comparisons of the curve of the average percentage of coactivity in an ROI compared at each threshold indicated no significant differences between the genotypes. Furthermore, the level of coactivity is generally low at the threshold of 30 used for other calcium activity experiments (10% or less; see Figure 6).

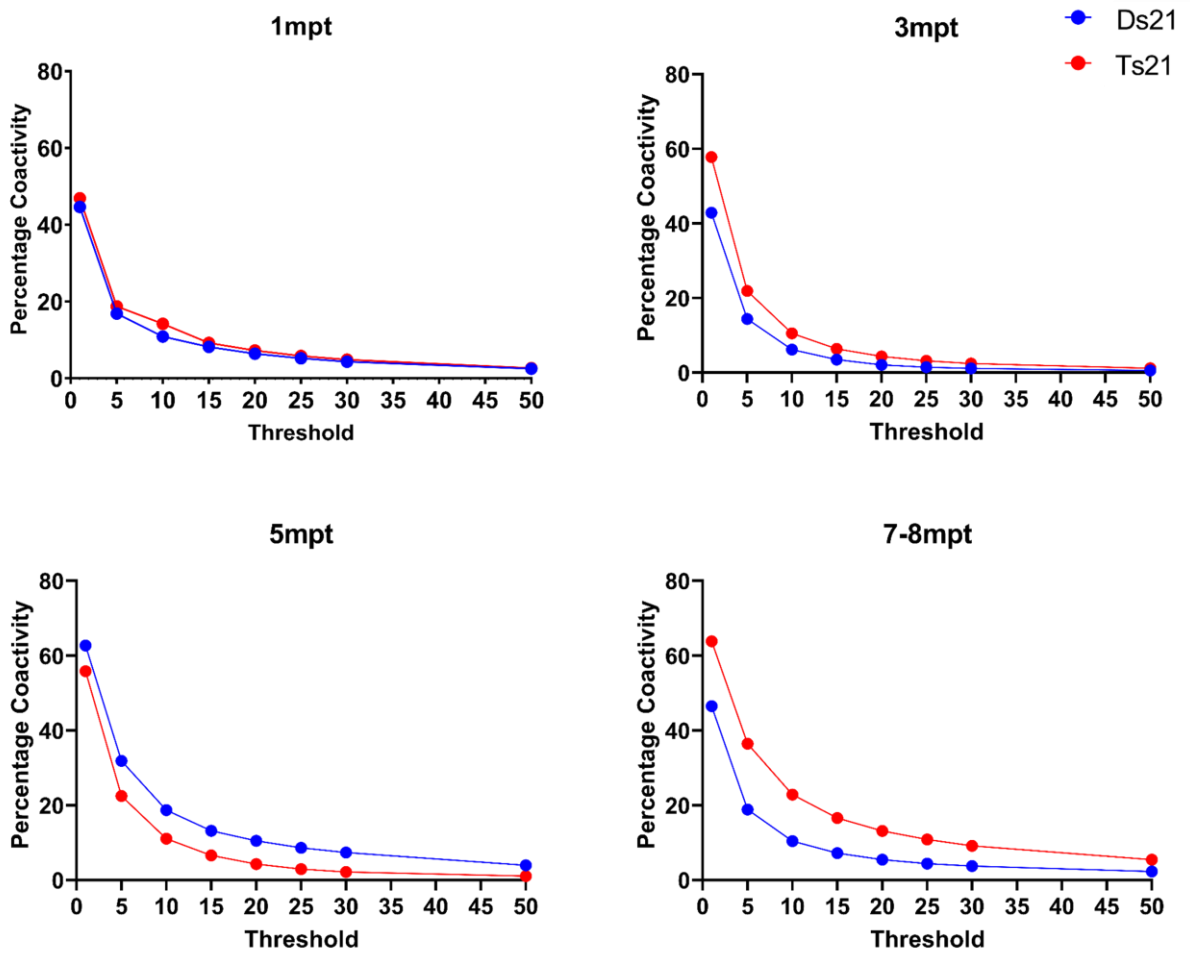


Figure 6. The percentage of coactive somas in an ROI averaged by animal at 1mpt, 3mpt, 5mpt, and 7-8mpt over a range of thresholds as a measure of oscillatory network activity. Thresholds are measured as the change in fluorescent intensity (ΔF/F0). Red points = Ts21; Blue points = Ds21 control.

Overall, these data demonstrate that in the current grafts where the levels of all cell types are equivalent across genotypes, the presence of Ts21 is not sufficient to alter the stability of synaptic structures or the spontaneous or oscillatory network calcium activity of the graft cells. This is not what we expected, as it does not follow the same trends as previous publications from our group (Real *et al*., 2018). However, the grafts here are very different in cell type distribution than the previous publication. As such, we can infer mechanisms of Ts21 early development by comparing the two graft types and evaluating the effect of cell types that were particularly altered in their distribution in current grafts: in particular, SATB2+ upper layer cortical excitatory neurons and hGFAP+ astrocytes. To evaluate the role of these cell types, I performed correlational analyses. The amount of these cell types was correlated to synaptic stability and spontaneous calcium activity outputs from the same animal. The figure below (see Figure 7) depicts the results from these correlations, where the major outputs used were EPB TOR and calcium integral. Importantly, the only significant correlation found was between the number of hGFAP+ astrocytes per cubic micrometer and the EPB TOR of the same animal. SATB2+ correlations with EPB TOR outputs and calcium integral outputs were not significant, nor was the correlation between hGFAP+ number per cubic micrometer and calcium integral.

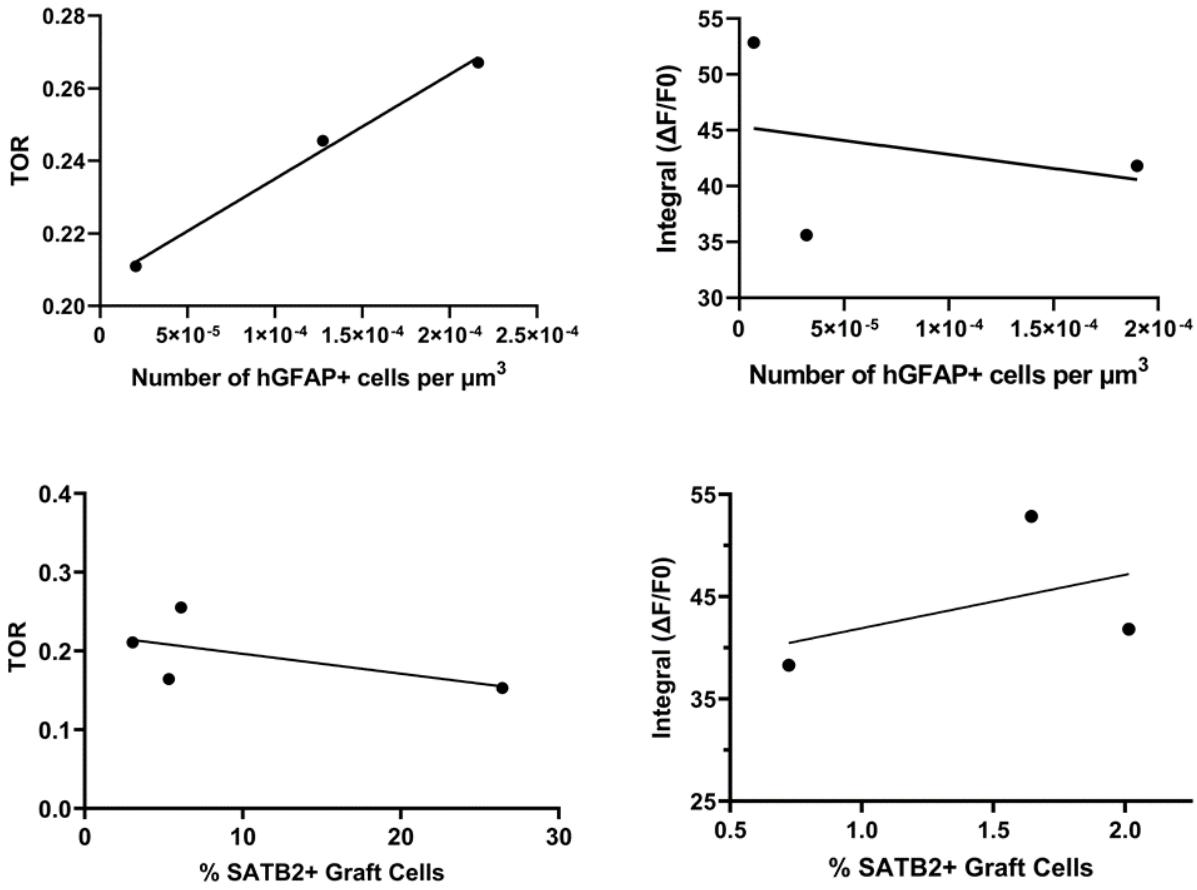


Figure 7. Correlational analyses between cell type distribution and major experimental outcomes. Top Left: Correlation between EPB TOR and the number of hGFAP+ astrocytes per cubic micrometer. This is a significant (p<0.05) correlation. Top Right: Correlation between calcium integral (spontaneous) and the number of hGFAP+ astrocytes per cubic micrometer.

This correlation is not significant. Bottom Left: Correlation between EPB TOR and the percentage of graft cells that are positive for SATB2. This is not a statistically significant correlation. Bottom Right: Correlation between calcium integral (spontaneous) and the percentage of graft cells that are SATB2+. This correlation is not significant.

Overall, my experimental achievements have successfully addressed the main Tasks outlined in the ESR8 Actions (Task 8.1 and Task 8.2). The findings are that the presence of Ts21 in current grafts is not sufficient to alter the stability of synaptic structures or the spontaneous and network oscillatory activity of graft cells. This finding was not expected, but nonetheless provides interesting information. The correlative analyses performed between major outcomes and the number of astrocytes per cubic micrometer indicates that GFAP+ graft astrocytes may be a main mechanistic contributor to the lack of phenotypic change in Ts21 grafts. Future work by our lab will focus on narrowing down these mechanisms using transcriptomic and genomic experiments on current grafts and on grafts developed using the same protocols as the Real *et al.,* 2018 paper.

Potential of these results for exploitation

The results from this project (ESR8) provide a model and foundational knowledge upon which further DS research can be applied. This applies to research on DS neuron structure, neurodevelopment, calcium activity, and even pharmacological treatments. Future research could utilize this model for the study of the role of astrocytes in DS, as they are prevalent in our grafts. Furthermore, this body of work provides a secondary dataset in which the novel model from Real et al., 2018 was successfully used to study neurological development and activity. Future work on this model will allow for the expansion of the knowledge base on DS as well as a wide range of other neurological and neurodevelopmental disorders.

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ESR9 – Laura Upton

ESR9: overview of results towards the objective of the action

Project title – Role of glycinergic signalling in autism spectrum disorder

WP2 – Testing the Excitation/Inhibition imbalance hypothesis in neuropsychiatric disorders

Early Stage Researcher 9 – Laura Upton

Supervisor – Catalina Betancur

Host Location – Neuroscience Paris-Seine (INSERM, CNRS), Institut de Biologie Paris-Seine, Sorbonne University, Paris – France

Pathogenic variants in the *GLRA2* gene, which encodes the glycine receptor alpha-2 subunit, have been implicated as a novel cause of autism spectrum disorder (ASD) and intellectual disability[1]. Our group previously showed that *Glra2*-deficient male (*Glra2*$^{Y/-}$) mice present with deficits in object recognition and altered synaptic plasticity in the prefrontal cortex (PFC)[1]. In this project, I investigated the cellular and functional properties underlying these behavioural and synaptic defects.

Glra2$^{-/Y}$ mice show many of the hallmarks of neurodevelopmental brain dysfunction observed in other rodent models of ASD. In the PFC of adult mice, we observed decreased inhibitory synapses, increased spine density and dendritic complexity of pyramidal neurons, increased cortical somatostatin interneurons, and in P14-P21 mice, an increased frequency of miniature excitatory post-synaptic currents in layer V pyramidal neurons. The electrophysiology experiments were performed during my secondment at the University of Edinburgh, where I had the opportunity of learning to patch. Overall, these findings point to excitatory hyperconnectivity in the PFC and suggest an imbalance of excitatory and inhibitory transmission in *Glra2*$^{-/Y}$ mice.

In addition, we assessed c-Fos expression as a marker of neuronal activation following novel object recognition, and found that the rostral infralimbic PFC was hypoactivated in *Glra2*$^{-/Y}$ mice following this task. To further assess the altered PFC activity in *Glra2*$^{-/Y}$ mice during cognition, we are currently recording calcium transients from infralimbic glutamatergic neurons during this task using fiber photometry.

Finally, as part of a collaboration during my secondment at the University of Edinburgh, we assessed the possibility of sub-threshold seizure activity and sleep dysfunction in *Glra2*$^{-/Y}$ mice, since both epilepsy and sleep disorders are common features in ASD and rodent models of autism. To do this, 24-48 h multi-electrode EEG recordings were obtained from *Glra2*$^{-/Y}$ mice and wild-type littermates, and the analysis of this experiment is ongoing.

a. Overview of progress

Altered assembly and functionality in the PFC of *Glra2*$^{-/Y}$ mice results in altered excitatory-inhibitory balance

Synapses, spines and dendrites

Alterations in the density of excitatory and/or inhibitory synapses are a frequent finding in mouse models of ASD, and are an indicator of excitatory-inhibitory imbalance. To ascertain whether the absence of *Glra2* alters the density of excitatory or inhibitory synapses, we quantified the colocalisation of excitatory and inhibitory pre- and post-synaptic markers in the PFC of *Glra2*$^{-/Y}$ and wild-type (WT) mice via immunofluorescence, and observed a 27% reduction in the density of functional inhibitory synapses in knockout (KO) animals (Fig. 1), with no effect on the colocalisation of excitatory synaptic markers.

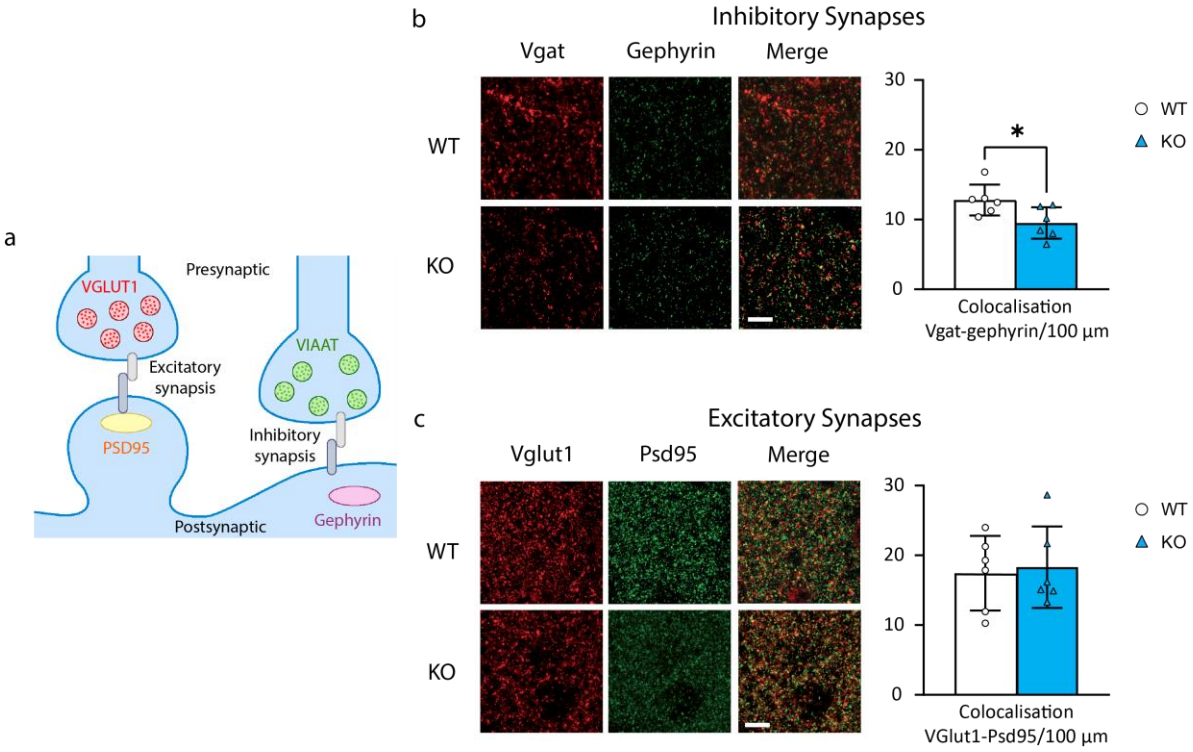


Figure 1. *Glra2*$^{-/Y}$ mice have an imbalance of functional excitatory and inhibitory synapses in the PFC. (a) Schematic representation of the colocalisation of pre- and post-synaptic marker proteins at excitatory and inhibitory synapses. (b, c) Representative images at 63x magnification of the colocalisation of inhibitory and excitatory markers. Post-synaptic markers Vgat and Vglut1 are shown in red, whilst pre-synaptic markers gephyrin and Psd95 are represented in green. Scale bar = 3 µm. Quantification of the colocalisation of inhibitory and excitatory synaptic markers. Data represent mean ± s.e.m. (n = 5 KO, 5 WT); Mann-Whitney test, *p<0.05.

Alterations in the density and morphology of dendritic spines and arborization are also commonly observed in genetic mouse models of autism and are a marker of dysfunctional brain development. In order to assess the impact of a loss of *Glra2* on neuronal morphology, prefrontal pyramidal neurons were filled with biocytin via patch-clamp and stained with fluorophore-coupled streptavidin for

morphological analysis. The density of dendritic spines in layer II/III and V pyramidal neurons of the PFC was increased in *Glra2*$^{-/Y}$ mice (Fig. 2c). Dendritic spines are often classified by their morphology into immature 'stubby' spines and mature 'thin' or 'mushroom' shaped spines. Although a significant increase in the density of mature spines was observed in *Glra2*$^{-/Y}$ mice, this can likely be attributed to the fact that mature spines represented the vast majority of total spines (84-88%), which were increased in KO animals (data not shown). In addition, the dendritic length was increased in apical neurons in *Glra2*$^{-/Y}$ mice and there was an increase in the dendritic complexity of pyramidal neurons, with an increase in number of total and apical branch points (Fig. 2). Overall, these findings suggest that pyramidal neurons are hyperconnected in the PFC of *Glra2*-deficient mice, as has been previously described in other mouse models of ASD.

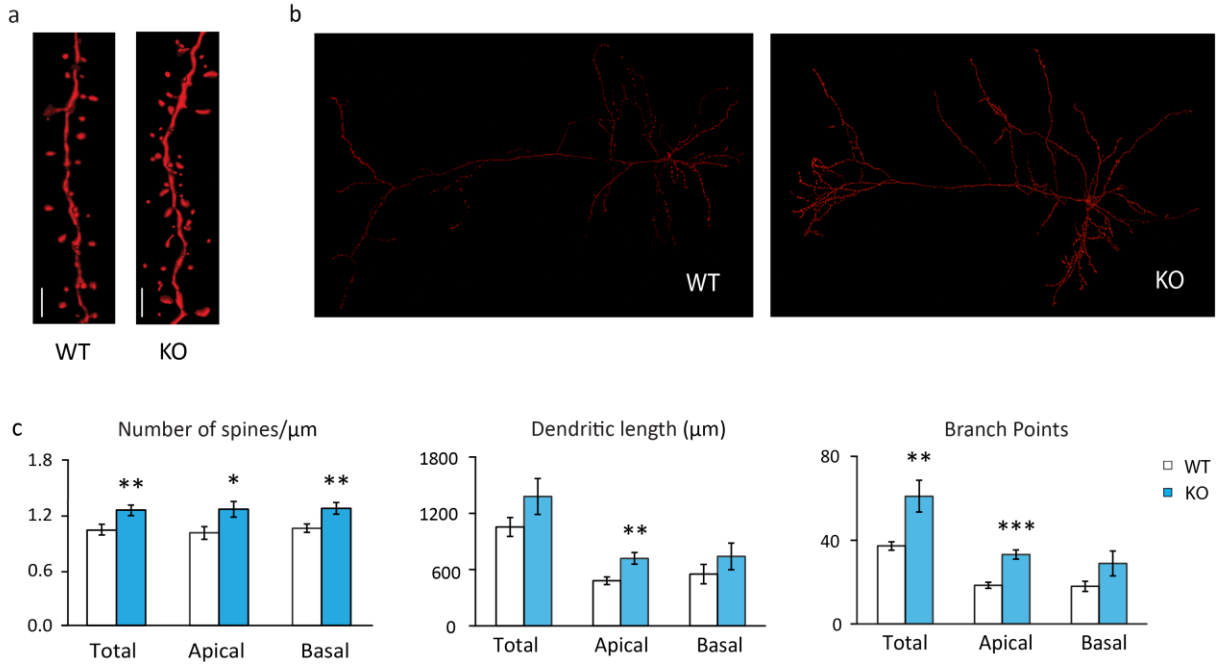


Figure 2. Prefrontal pyramidal neurons are hyperconnected in *Glra2*$^{-/Y}$ mice. Representative images of (a) deconvoluted dendritic spines (scale bar = 2 µm) and (b) dendritic arborisation of pyramidal neurons. (c) Number of spines, dendritic length and number of branch points of WT and *Glra2*$^{-/Y}$ prefrontal pyramidal neurons. Data represent mean ± s.e.m. (n= 11-13 neurons from 6 WT mice, 8-10 neurons from 5 KO mice); Mann-Whitney test, *p<0.05, **p<0.01, ***p<0.001.

Neuronal quantification

The absence of *Glra2* during development has been shown to impact interneuron migration, and another group previously reported a severe loss of cortical glutamatergic neurons and interneurons in P0-P14 *Glra2*-deficient mice, as well as a marked microcephaly[2-4]. By contrast, our colony of *Glra2*$^{-/Y}$ mice possessing the same mutation but bred on a different C57BL/6J genetic background had normal brain weight and cortical thickness (data not shown), and the relatively mild behavioral findings that we observe seemed incompatible with a severe neuronal loss. To rule out neuronal loss in our *Glra2*$^{-/Y}$ mice, we quantified glutamatergic neurons and GABAergic interneurons in the PFC using fluorescent *in situ* hybridisation (FISH). No global loss of glutamatergic neurons or interneurons was observed; however, quantification of sub-populations of interneurons using cell-type specific markers revealed a 36% increase in somatostatin mRNA-expressing neurons in the PFC of adult *Glra2*$^{-/Y}$ mice (Fig. 3). In order to confirm our findings, and to rule out the possibility that apparent neuronal loss in *Glra2*$^{-/Y}$ detected via

immunofluorescence by the other group[3,4] could be due to reduced protein expression in these neurons, we also quantified neuronal populations in the prefrontal and somatosensory cortices of adult (Fig. 3) and P14 (data not shown) mice using immunofluorescence, and obtained similar results.

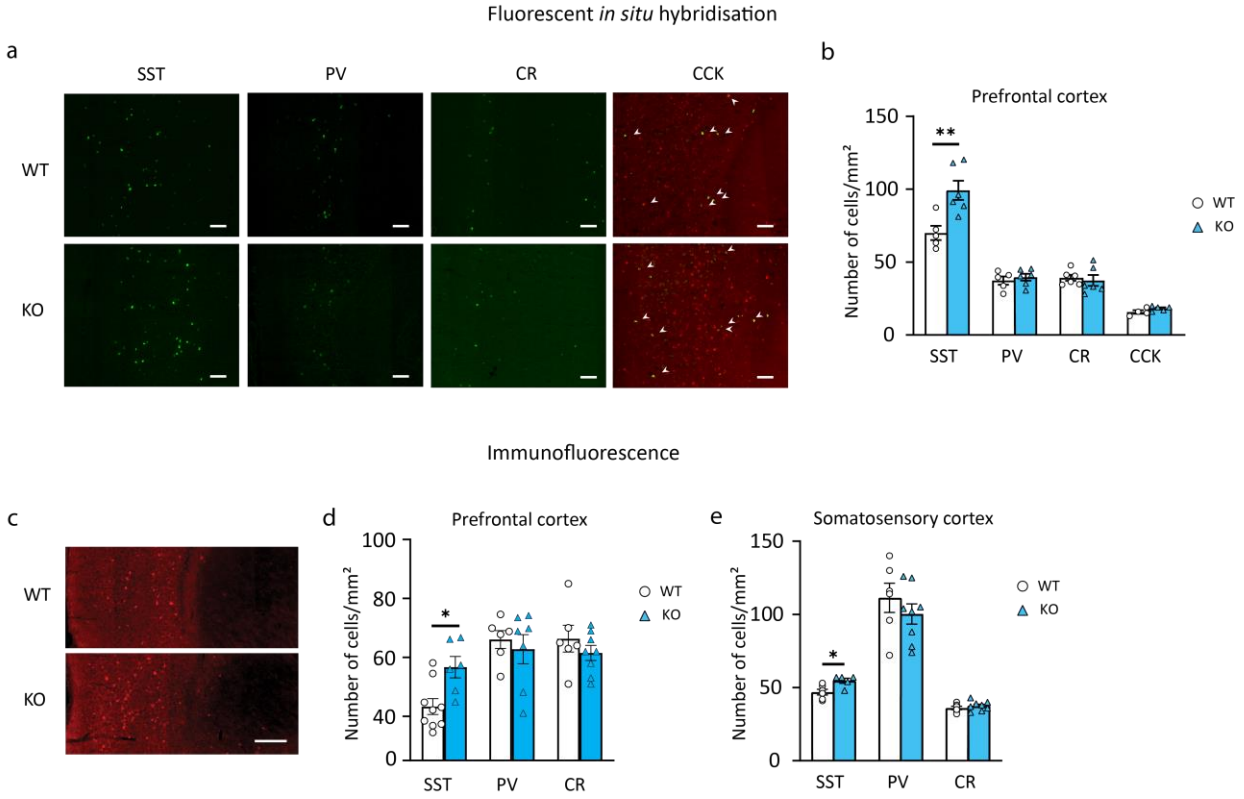


Figure 3. *Glra2*$^{-/Y}$ mice have increased cortical somatostatin interneurons with no change in glutamatergic or other interneuron populations. Representative 10x images from the PFC (a) and quantification (b) of somatostatin (SST), parvalbumin (PV), calretinin (CR) and cholecystokinin (CCK) interneurons via FISH in the PFC of WT and KO mice. Representative 10x images of somatostatin-positive cells in the PFC (c) and quantification of SST, PV and CR interneurons via immunofluorescence in the prefrontal (d) and somatosensory (e) cortices. Scale bar = 100 µm. Data represent mean ± s.e.m. (n = 5-9 WT, 5-6 KO); Mann-Whitney test, *p<0.05, **p<0.01.

Electrophysiology

In order to assess the impact of these alterations in synapses, pyramidal dendritic morphology and interneuron sub-populations in the PFC of *Glra2*$^{-/Y}$ mice, we studied the synaptic and firing properties of P14-P21 *Glra2*$^{-/Y}$ prefrontal pyramidal neurons in layer V using patch-clamp electrophysiology during my secondment in Peter Kind's lab at the University of Edinburgh. To assess the synaptic properties of neurons, miniature excitatory and inhibitory post-synaptic currents (mEPSCs and mIPSCs) were recorded from pyramidal neurons at -70 mV and 0 mV, respectively, in the presence of tetrodotoxin. The frequency of mEPSCs was significantly increased in *Glra2*$^{-/Y}$ layer V pyramidal neurons with no effect on amplitude or inter-event interval, indicating an increase in glutamatergic transmission at synapses onto this neuronal population (Fig. 4). Although these findings were obtained in P14-P21 neurons and cannot therefore be directly correlated to the adult morphological data, this finding is consistent with the increased spine density observed in adult prefrontal pyramidal neurons in this region. By contrast, no difference was observed in the frequency, amplitude or inter-event interval of mIPSCs (data not shown), providing further evidence of an alteration in the excitatory-inhibitory balance in the PFC of *Glra2*-

deficient mice, and suggesting a shift towards excitation. In addition to synaptic properties, current-clamp recordings were also acquired to assess firing properties and the current-voltage relationship of *Glra2*$^{-/Y}$ pyramidal neurons, and the analysis of these experiments is still ongoing.

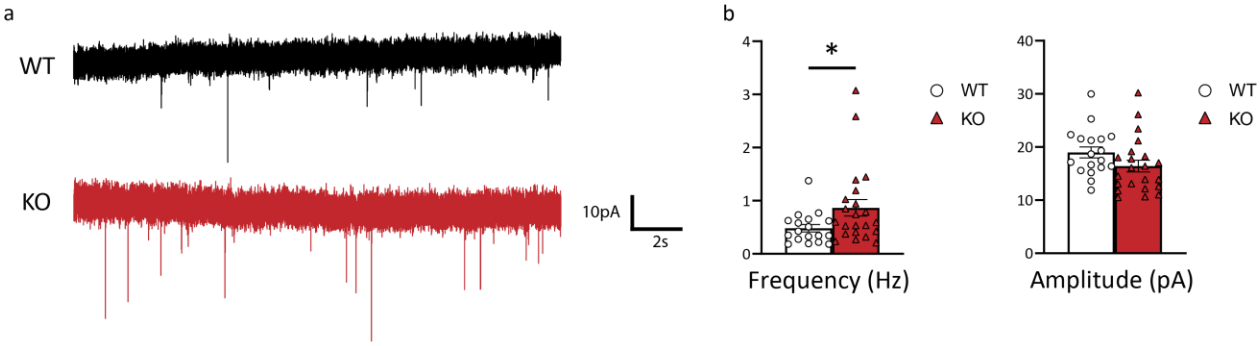


Figure 4. Excitatory synaptic events are increased in layer V pyramidal neurons of *Glra2*$^{-/Y}$ mice. (a) Representative trace of mEPSCs detected during the first 20 seconds of -70mV voltage-clamp recordings from WT and KO layer V pyramidal neurons. (b) The frequency of mEPSCs is increased in layer V pyramidal neurons in P14-P21 *Glra2*$^{-/Y}$ mice with no effect on the amplitude of events. Data represent mean ± s.e.m. (n= 18 neurons from 8 WT mice, 22 neurons from 9 KO mice); mixed-effects model, *p<0.05.

Cortical regions associated with cognitive deficit

Since *Glra2*$^{-/Y}$ mice show a deficit in novel object recognition, we hypothesized that neuronal activation during the task might be altered in one or more brain regions associated with this behavioural task. In order to assess which brain regions were affected, we quantified c-Fos activation, an immediate early gene commonly used as a marker of neuronal activation, following either basal experimental conditions (no exposure to a novel object during the test phase) or novel object recognition (training and object recognition test). Brains were collected from *Glra2*$^{-/Y}$ mice and WT littermates 1 h following the behavioural task, and slices from brain regions that have previously been found to be functionally important in novel object recognition, including the PFC, perirhinal and entorhinal cortices, and hippocampus, were stained for c-Fos analysis. Quantification of c-Fos-positive neurons in the rostral infralimbic PFC revealed a hypoactivation of neurons in *Glra2*$^{-/Y}$ mice following novel object recognition, which was absent from the basal group (Fig. 5). By contrast, quantifications of c-Fos-positive cells in the rostral and caudal prelimbic PFC, as well as the perirhinal and entorhinal cortices, did not reveal any differences in neuronal activation following the task, suggesting regional-specificity of the neuronal dysfunction observed during this task. Quantification of c-Fos activation in the hippocampus is ongoing. Overall, these findings suggest that neuronal activation is functionally altered in the rostral infralimbic PFC, resulting in a deficit in performance in the task.

To further assess the source of hypoactivation in the rostral infralimbic PFC, we performed c-Fos colocalisation immunofluorescence experiments on a subset of PFC slices with protein markers of different sub-populations of neurons: Satb2 as a marker of glutamatergic neurons, and somatostatin and parvalbumin as markers of the two major interneuron populations found in the PFC. No difference in the proportion or number of c-Fos-positive somatostatin interneurons was observed in the PFC of *Glra2*$^{-/Y}$ mice (Fig. 5), suggesting that the overall hypoactivation observed is not driven by somatostatin interneuron hypoactivation. However, somatostatin interneuron dysfunction could still play a role in the

functional hypoactivation observed in this region. Quantification of activated Satb2 and parvalbumin neurons following novel object recognition is ongoing.

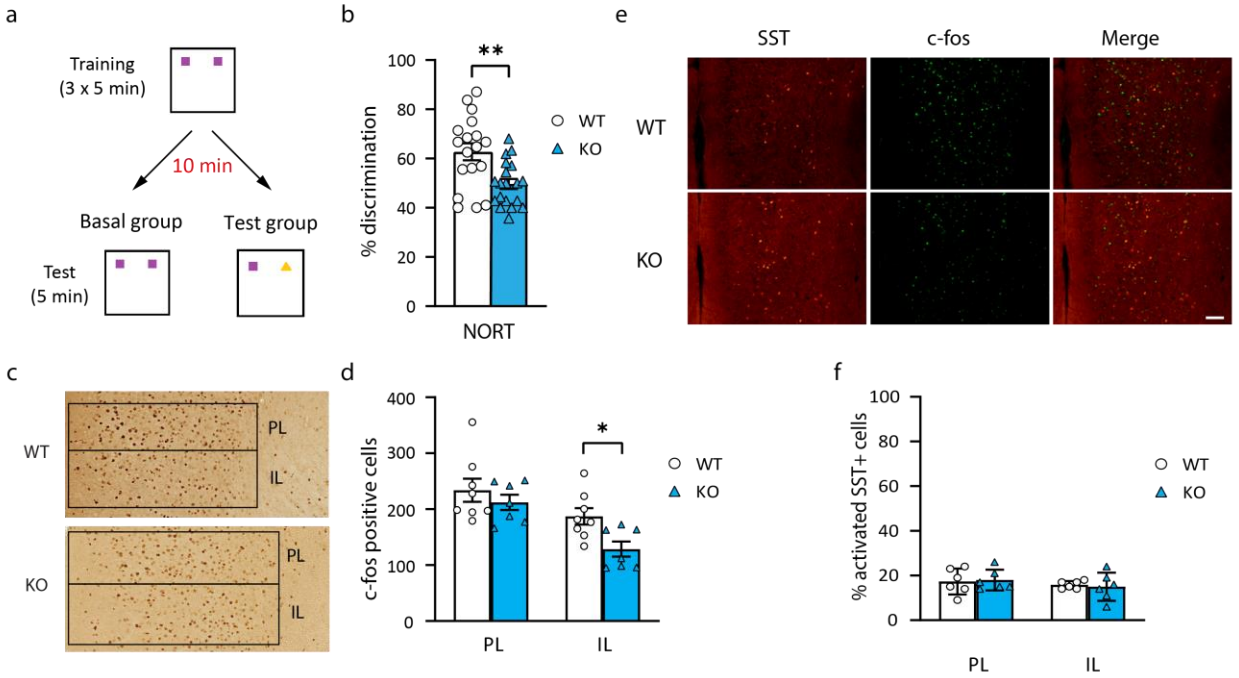


Figure 5. The deficit in novel object recognition observed in *Glra2*$^{-/Y}$ mice is associated with a hypoactivation of the rostral infralimbic PFC. (a) Schematic outline of the experimental conditions of mice in the basal and novel object recognition test groups prior to c-Fos analysis. (b) *Glra2*$^{-/Y}$ mice are deficient in novel object recognition (n = 18 WT, 18 KO). (c) Representative images at 10x magnification of c-Fos expression in the rostral prelimbic (PL) and infralimbic (IL) PFC following novel object recognition. (d) Mean density of c-Fos-positive cells in the rostral PFC of *Glra2*$^{-/Y}$ and WT littermates (n = 8 WT, 7 KO). (e) Representative images at 10x magnification of somatostatin and c-Fos colocalisation in the PFC following novel object recognition. (f) Mean percentage of activated somatostatin-positive neurons in the rostral PFC of *Glra2*$^{-/Y}$ and WT mice (n = 6). Data represent mean ± s.e.m., Mann-Whitney test, *p<0.05, **p<0.01. NORT, novel object recognition test; SST, somatostatin.

Analysis of network activity and connectivity of the PFC *in vivo*

Fiber photometry during novel object recognition and social novelty

C-fos analysis of neuronal activation following novel object recognition revealed a hypoactivation of the rostral infralimbic region of the PFC of *Glra2*$^{-/Y}$ mice. In order to assess how the pattern of activation in this region is altered in *Glra2*$^{-/Y}$ mice during novel object recognition, we are recording aggregate calcium transients from rostral infralimbic glutamatergic neurons via fiber photometry. To do this, we first injected an adeno-associated virus (AAV) containing a calcium sensor (GCaMP6f) expressed under a glutamatergic neuronal promoter (CaMKII) into the rostral infralimbic PFC of *Glra2*$^{-/Y}$ mice and WT littermates, and placed a fiber optic implant at the same location. The next phase of this experiment, the recording phase, is currently underway. We are recording infralimbic PFC glutamatergic activity of *Glra2*$^{-/Y}$ mice and WT littermates during novel object recognition, as well as during a social novelty task in which *Glra2*$^{-/Y}$ mice show no deficits, and during which we therefore do not predict to observe altered functional activity.

EEG and coherence of PFC with other brain regions

Both epilepsy and sleep disorders are common features in individuals with ASD, and seizures and sleep dysfunction have been described in several rodent models of neurodevelopmental disorders. As part of my secondment at the University of Edinburgh, I collaborated with the laboratory of Alfredo Gonzalez-Sulser to assess possible sub-threshold seizure activity and sleep dysfunction in *Glra2*$^{-/Y}$ mice. To assess these, 24-48 h multi-electrode EEG recordings were obtained from *Glra2*$^{-/Y}$ mice and WT littermates, and the analysis of this experiment is ongoing.

Potential of results for exploitation

In humans, loss of *GLRA2* causes a recently described X-linked neurodevelopmental disorder in males that includes ASD and intellectual disability. The findings described in this report, observed in a mouse model deficient for *Glra2*, provide insights into the neurobiological impact of the loss of this gene. The characterization of the underlying brain dysfunction caused by the loss of *Glra2* in this mouse model can contribute to a better understanding of the cellular and circuit mechanisms underlying ASD and intellectual disability in affected individuals and could point toward future therapeutic strategies. In addition, our studies uncover insights into the biological roles of *GLRA2* in the brain.

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ESR10 – Flavio Tomasi

ESR10: overview of results towards the objective of the action

Project title – The endocannabinoid system as a therapeutic target in *Shank3*-deficient mice, a model of autism spectrum disorder

WP3 – Modulators of neuropsychiatric disorders

Early Stage Researcher 10 – Flavio Tomasi

Supervisor – Catalina Betancur

Host Location – Neuroscience Paris-Seine (INSERM, CNRS), Institut de Biologie Paris-Seine, Sorbonne University, Paris – France

SHANK3 is one of the most frequently mutated genes in autism spectrum disorder (ASD). It encodes a major scaffold protein of the postsynaptic density and the loss of one copy of the gene results in a rare genetic disorder called Phelan-McDermid syndrome (PMS). PMS is a neurodevelopmental disorder characterized by intellectual disability, speech deficits, ASD, hypotonia and motor deficits, for which there are no specific therapies.

Shank3-deficient mice are a well-studied model of PMS and ASD, characterized by deficits resembling the manifestations observed in affected individuals and an imbalance between excitatory and inhibitory neurotransmission. This alteration has been observed in many animal models of ASD and other neurodevelopmental disorders and is thought to underlie the behavioural manifestations of ASD. Thus, correcting the excitatory/inhibitory imbalance could be a possible therapeutic target in many genetic forms of ASD and other neurodevelopmental disorders. The endocannabinoid system, and more specifically, the activity of the CB1 receptor, can modulate the excitatory/inhibitory balance in the brain and the inhibition of this receptor has already shown promise as a therapeutic target in two mouse models of neurodevelopmental disorders that share features with PMS, fragile X syndrome and Down syndrome.

The objective of the project was to assess the endocannabinoid system as a therapeutic target in autism and PMS, using *Shank3*-deficient mice as a model. Specifically, the two main aims were:

1. To assess the effects of the inhibition of the CB1 receptor on the phenotype of *Shank3*-deficient mice;
2. To evaluate the consequences of *Shank3* deficiency on the endocannabinoid system.

We used a *Shank3*-deficient mouse model with a deletion affecting exons 4-22, an optimized model of PMS and autism, characterized by full disruption of the Shank3 protein and behavioural deficits resembling patient features. First, we studied the effects of repeated administration of the CB1 receptor antagonist rimonabant (1 mg/kg, intraperitoneal) for seven days in *Shank3* wild-type (WT), heterozygous and KO male and female mice. Males and females were analysed separately and the results pooled together in the figures because there was no significant Sex x Treatment interaction in any of the studies performed. Rimonabant rescued deficits in novel object recognition in heterozygous and KO mice (Figure 1a), and improved motor coordination in the rotarod in KO mice

of both sexes (heterozygous mice did not show any deficits in motor coordination) (Figure 1b). In contrast, no improvements were observed in the hypolocomotion (Figure 1c) and anxiety-like behaviour (Figure 1d) in KO mice.

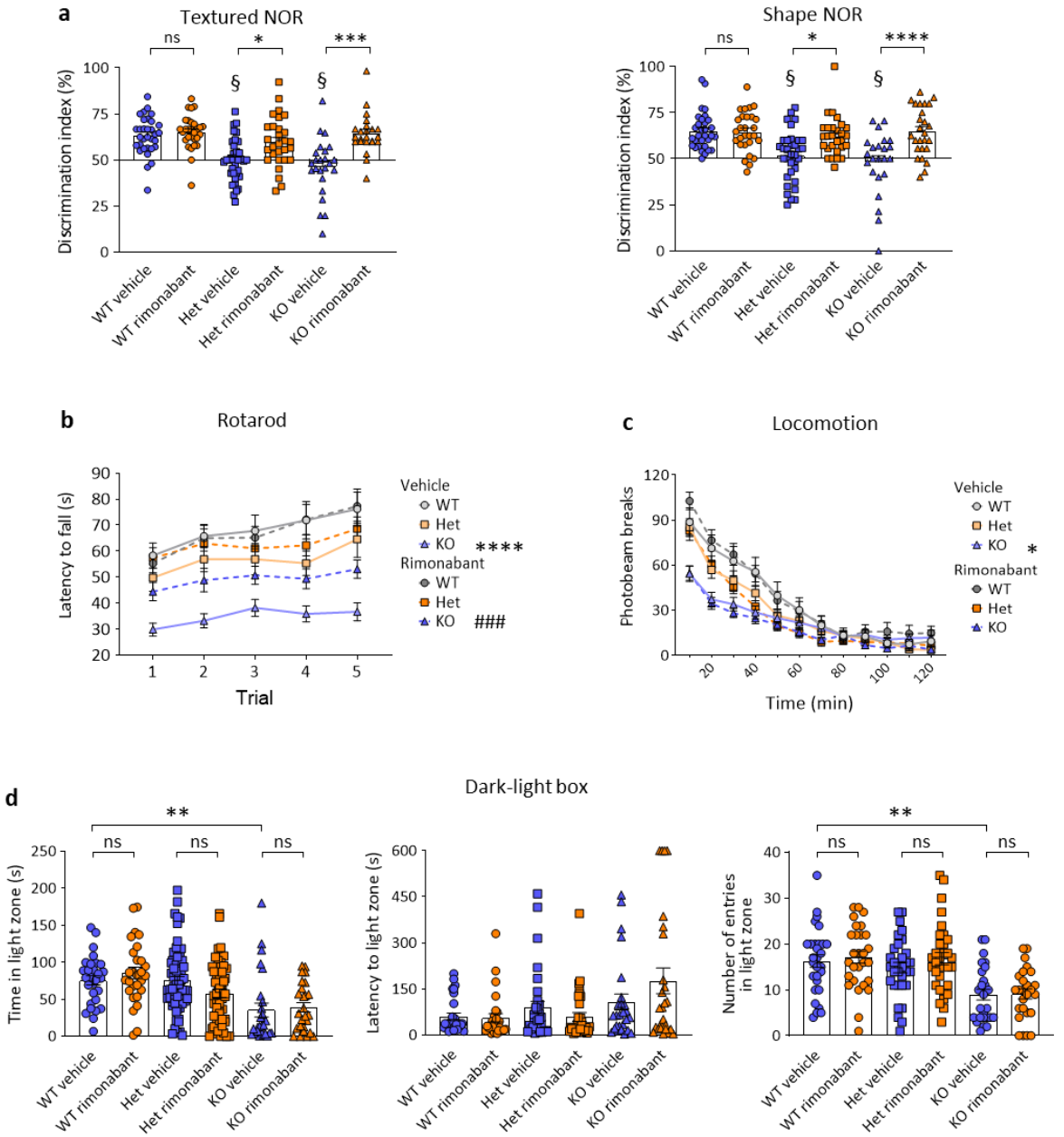


Figure 1. Behavioural effects of rimonabant in *Shank3*-deficient mice. Seven days of treatment with rimonabant rescued object recognition impairments in heterozygous and KO mice (a), and improved coordination deficits in KO animals (b). The hypolocomotion (c) and the increased anxiety-like behaviour (d) observed in KO mice were not rescued by rimonabant.

One of the objectives of this study was to identify a possible pharmacological treatment for individuals with PMS and eventually other forms of ASD. Rimonabant cannot be used in patients because of serious neuropsychiatric side effects. However, the positive effects of rimonabant on the behavioural deficits of *Shank*3-deficient mice suggested the possible efficacy of a novel compound developed for clinical use by the company Aelis Farma. AEF0217 is a signalling-specific inhibitor of the CB1 receptor that blocks a subset of the pathways of CB1 receptors without modifying the effects of the receptor on adenylate cyclase. AEF0217 did not show any toxicity or side effects in

previous preclinical studies and could thus be a candidate for the pharmacological treatment of PMS and more generally ASD. Furthermore, this compound has already been tested in a phase 1 clinical trial in healthy volunteers, confirming its safety profile and the possibility to be used in patients.

After promising results obtained in a first study involving WT and KO male mice, AEF0217 was evaluated in a dose-response study following oral administration through drinking water for at least seven days and up to four weeks. Five different doses of AEF0217 (0.006, 0.02, 0.06, 0.2 and 0.6 µg/ml) were tested in KO male and female mice, with WT mice receiving vehicle used as controls. In this study, AEF0217 normalized object recognition (Figure 2a) and improved motor coordination (Figure 2b) in KO animals, both males and females, confirming the promising results obtained with rimonabant. In contrast, the compound did not improve the hypolocomotion in KO mice (Figure 2c). No significant alterations due to the genotype were observed in anxiety-like behaviour in the dark-light box. KO mice showed a reduced number of entries in the light zone of the box, likely due to the hypolocomotion, and AEF0217 did not rescue this phenotype (Figure 2d).

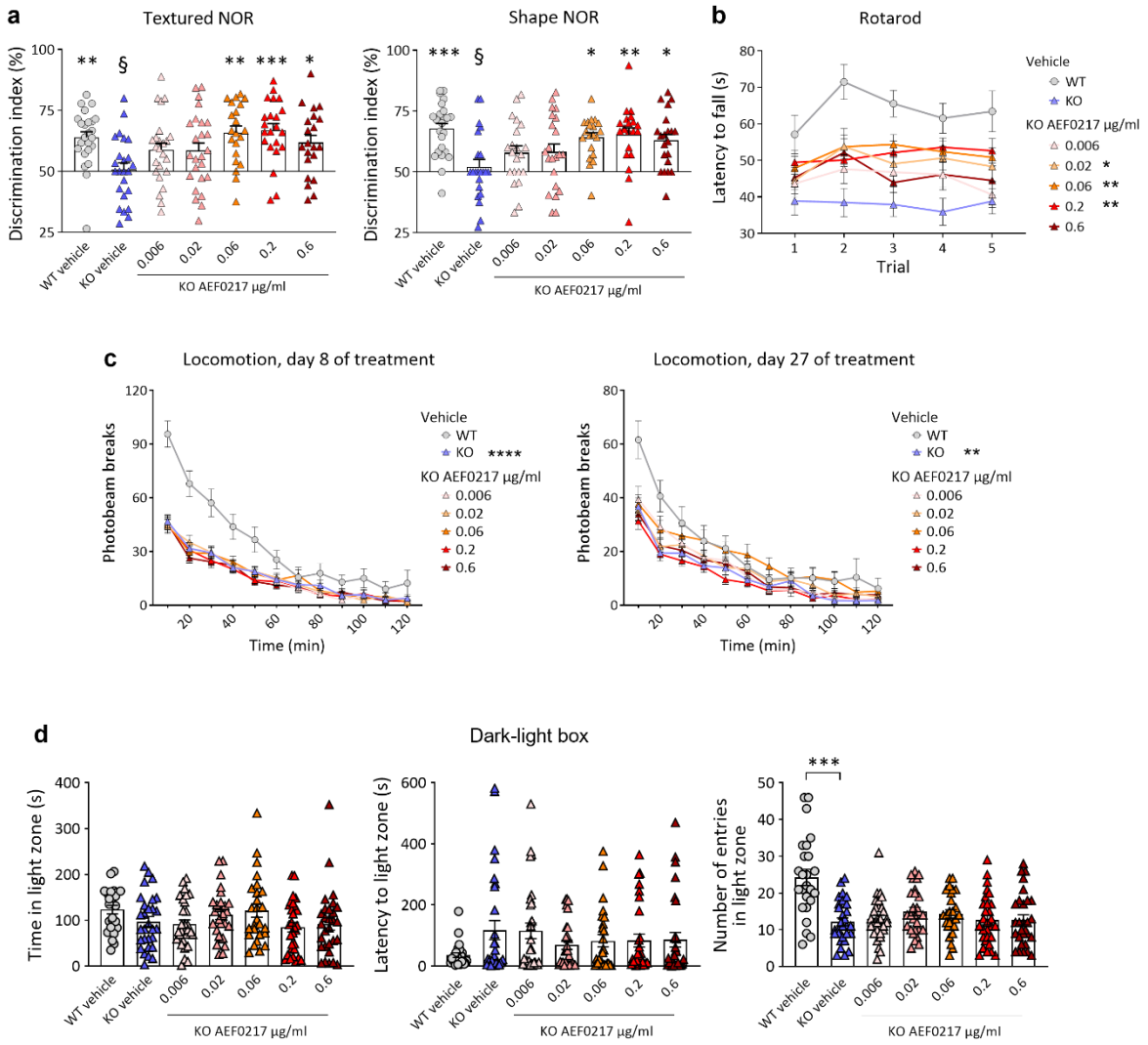


Figure 2. Behavioural effects of AEF0217 in a dose-response study in *Shank3* KO mice. After at least seven days of treatment with the three highest doses of AEF0217, 0.06, 0.2 and 0.6 µg/ml, administered in drinking water, KO mice showed a rescue in the object recognition deficits (a). Three doses of AEF0217 (0.02, 0.06 and 0.2 µg/ml) also improved coordination deficits in KO animals (b). The hypolocomotion (c) observed in KO mice was not rescued by AEF0217 after 8 or 27 days of treatment. KO mice did not show increased anxiety-like

behaviour in the dark-light box, but entered the light zone less frequently than WT animals. This alteration was not rescued by AEF0217.

The most effective dose of AEF0217, 0.06 µg/ml, was used in a final experiment that included, in addition to KO animals, heterozygous mice, to explore the effects of the compound on the model that more closely resembles the human genetic defect leading to PMS (the loss of only one copy of *SHANK3*), and WT mice, to assess possible side effects. In this study, AEF0217 confirmed its ability to reverse the impairment in object recognition (in KO and heterozygous mice) (Figure 3a) and to improve motor coordination (in KO mice; no deficits were observed in heterozygous mice) (Figure 3b). Moreover, AEF0217 was also shown to be effective in improving marble burying deficits in KO mice (Figure 3c). We did not observe any alterations in social or repetitive behaviours in either heterozygous or KO mice, thus we did not have the possibility to assess the effects of AEF0217 in these phenotypes (Figure 3d).

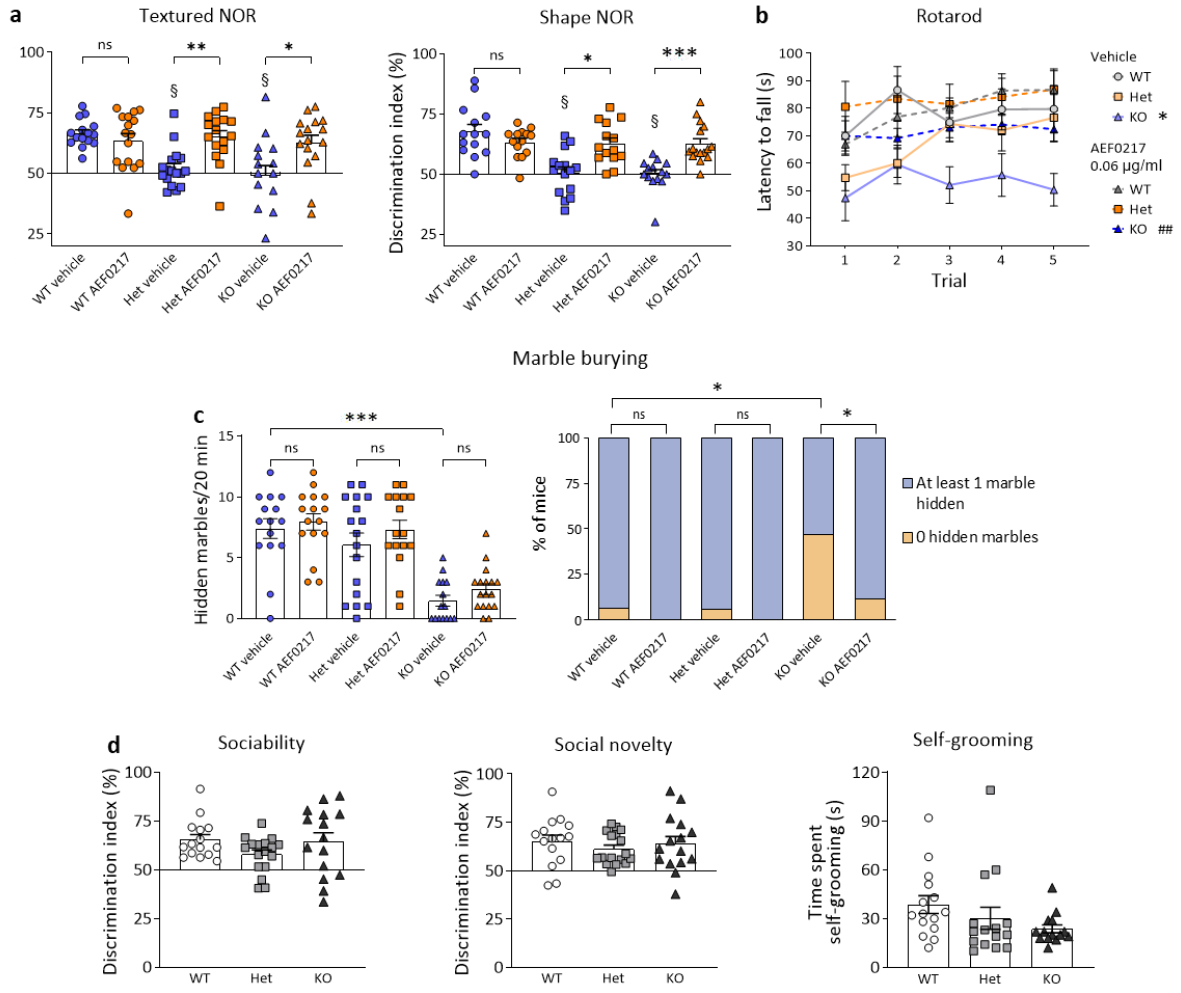


Figure 3. Behavioural effects of AEF0217 0.06 µg/ml in mice of the three genotypes. After at least seven days of treatment with AEF0217 0.06 µg/ml, administered in drinking water, heterozygous and KO mice showed a rescue of object recognition deficits (a). AEF0217 also improved coordination deficits in KO animals (b). In the marble burying test, AEF0217 did not improve the number of marbles hidden by KO animals but the percentage of animals that did not hide any marbles (approximately 50% in KO mice) was significantly reduced by the treatment (c). No genotype effects were observed in social and repetitive behaviours (d).

We subsequently tested the effects of the administration of AEF0217 (0.06 µg/ml) for one week on the electrophysiological alterations in medial prefrontal cortex pyramidal neurons of KO mice (layers 2/3 and 5). Interestingly, these neurons showed a marked hyperexcitability in response to

depolarizing steps of current that was rescued by AEF0217 (Figure 4a). This effect was associated with a reduced action potential decay time in neurons from KO mice treated with vehicle, which was also rescued by the compound (Figure 4b). These findings suggest a possible biological mechanism involving voltage-gated potassium channels located in the axon initial segment of pyramidal neurons, which could explain the cortical hyperexcitability. Cortical hyperexcitability has been observed in many rodent models of autism and other neurodevelopmental disorders. The effect of AEF0217 in *Shank3* KO mice suggests that this compound could also be effective in other rodent models characterized by a similar phenotype, potentially expanding its therapeutic potential. We also observed alterations in other intrinsic properties of prefrontal pyramidal neurons, such as the capacitance, the resting membrane potential, and the rheobase, which were all normalized by the treatment (Figure 4c). Finally, we measured the spontaneous excitatory and inhibitory activity of these neurons and we did not find any alterations due to the genotype in excitatory or inhibitory postsynaptic currents (Figure 4d). However, AEF0217 had different effects in neurons of WT and KO mice, inducing an increase in the frequency of excitatory events only in WT neurons and a reduction in the amplitude of excitatory and inhibitory events only in KO neurons. These findings indicate that the electrophysiological effects of AEF0217 are different depending on the genotype, suggesting a possible alteration of the endocannabinoid system in KO animals.

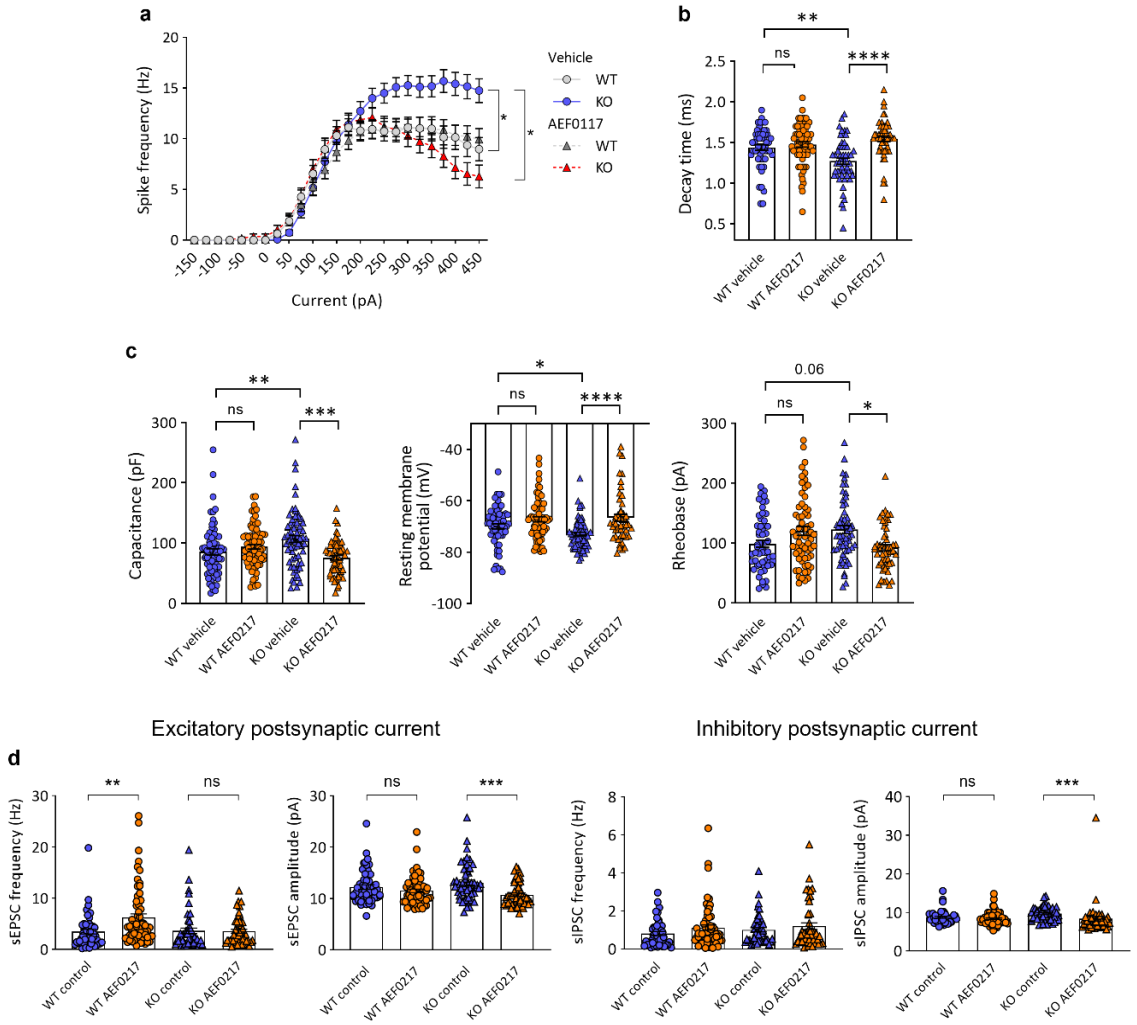


Figure 4. Electrophysiological effects of AEF0217 0.06 µg/ml on prefrontal pyramidal neurons in KO mice. Pyramidal neurons of the medial prefrontal cortex of KO mice showed an increased spike frequency in response to depolarizing steps of current compared to WT neurons, which was normalized after seven-eight days of treatment with AEF0217 0.06 µg/ml, administered in drinking water (a). The kinetics of the action

potentials showed that KO neurons presented a decreased decay time of action potentials that was rescued by the treatment (b). AEF0217 also reversed other alterations in intrinsic properties in KO neurons (c): the capacitance that was increased compared to WT neurons; the resting membrane potential that was hyperpolarized; and the rheobase that was increased in KO neurons. No genotype effects were observed in spontaneous excitatory or inhibitory postsynaptic currents (d). AEF0217 specifically increased the frequency of spontaneous excitatory postsynaptic currents only in WT neurons and decreased the amplitude of both spontaneous excitatory and inhibitory postsynaptic currents only in KO neurons.

In addition, we investigated the effects of AEF0217 on the molecular composition of synapses in the prefrontal cortex and hippocampus of *Shank3*-deficient mice. Specifically, we assessed the levels of Homer1 and mGluR5, a scaffold protein and a receptor frequently altered in *Shank3*-deficient mice. We observed a reduction in Homer1 but not in mGluR5 in the postsynaptic density of the prefrontal cortex in KO mice (Figure 5a,b). However, AEF0217 did not rescue this alteration. Moreover, we did not observe any alterations in Homer1 or mGluR5 in the hippocampus of *Shank3* KO mice (Figure 5c,d).

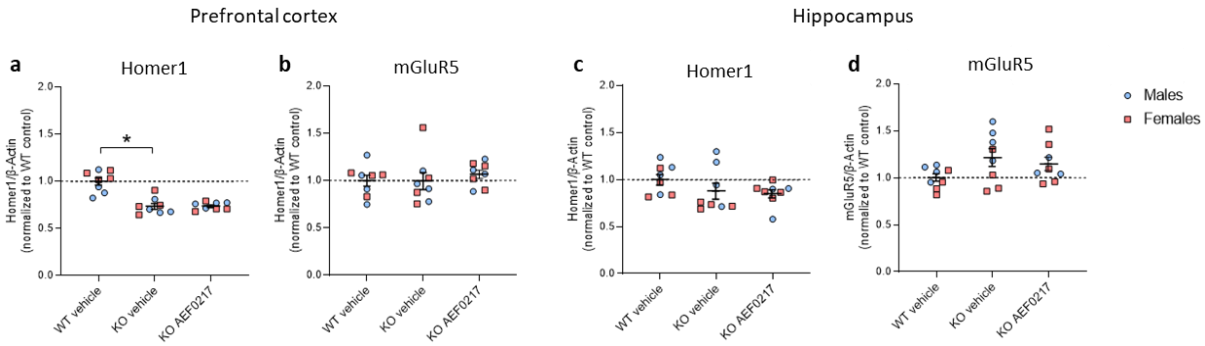


Figure 5. AEF0217 did not modify the levels of Homer1 and mGluR5 in the hippocampus and prefrontal cortex of *Shank3* KO mice. In the postsynaptic density of the prefrontal cortex, KO mice treated with vehicle showed a reduction in Homer1 (a) but not mGluR5 (b). AEF0217 did not rescue the deficit in Homer1 and did not alter the levels of mGluR5.In the postsynaptic density of the hippocampus, KO mice treated with vehicle or AEF0217 did not show any alteration of Homer1 (c) or mGluR5 (d).

In order to assess the effects of the loss of *Shank3* on the endocannabinoid system, we examined the levels of various endocannabinoids in brain and plasma and of CB1 receptors in various brain regions in untreated *Shank3* WT and KO mice but we did not observe any significant alterations (data not shown). Even if the levels of the endocannabinoids and CB1 receptor are not altered we cannot exclude functional alterations. For this reason we are studying the consequences of *Shank3* deficiency on the activity of the CB1 receptor using an electrophysiological approach.

To better explore the effects of AEF0217 in *Shank3*-deficient mice we are currently analysing the effects of the compound on: 1) dendritic arborization and spine density and morphology of prefrontal pyramidal neurons, and 2) synaptic plasticity, focusing on LTP in the hippocampus. In addition we used RNA-seq to explore the downstream modifications induced by AEF0217 in the prefrontal cortex of WT and KO mice. The results of this experiment only showed a significant reduction of Shank3 levels in KO mice. However, other transcripts non-significantly altered will be further examined for their possible biological interest.

Potential of these results for exploitation

Our work represents the first attempt to specifically modulate CB1 receptor activity in a *Shank3*-deficient mouse model, thus proposing a new pharmacological target for PMS. Importantly, given the characteristics of AEF0217 in terms of therapeutic profile, our results suggest that this compound is a

potential pharmacological agent for the treatment of PMS and more generally of ASD. The results allowed us to file a patent application for the use of AEF0217 in PMS and to start planning a clinical trial in PMS patients. Our results will be published in an article with Aelis Farma describing the development of AEF0217, including *in vitro* and *in vivo* preclinical studies and a phase 1 clinical trial in healthy volunteers. The manuscript is in preparation and will be submitted soon.

ESR11 – Ágata Silván

ESR11: overview of results towards the objective of the action

Project title – Investigating the excitation/inhibition (E/I) imbalance hypothesis of neuropsychiatric disorders in the cortico-thalamic system with electrophysiological methods

WP3 – Modulators of neuropsychiatric disorders

Early Stage Researcher 11 – Ágata Silván

Supervisors – Niels Plath and Kjartan Herrik

Host Location – H. Lundbeck A/S, Copenhagen – Denmark

The TRN is comprised of a thin sheet of GABAergic cells that receive synaptic inputs from cortex, thalamus, and subcortical regions, and provide local inhibition to the thalamus (Clemente-Perez et al., 2017; Crabtree, 2018; Pinault, 2004). Several genes linked to neurodevelopmental and neuropsychiatric disorders are highly expressed in the TRN (Chaudry et al., 2015; Iossifov et al., 2012; Ripke et al., 2014; Pinto et al., 2010). This, together with its importance in sensory gating, focused attention, cognitive flexibility, and sleep, points out the TRN as a particularly relevant area of study (Krol et al., 2018), as well as a potential target for drug discovery (Pratt & Morris, 2015).

SK channels play a crucial role in regulating neuronal excitability by mediating the medium afterhyperpolarization (AHP) following a single or multiple action potentials (APs). Three SK channel subunits (SK1, SK2 and SK3) have been cloned so far (Köhler et al., 1996) and their expression is widely distributed in the nervous system (for detailed reviews, see Stocker and Pedrazani, 2000; Rimini et al, 2000). In the brain, while SK channels are predominantly expressed in neurons, intermediate conductance K^+ channels (IK) are mainly located in microglia and endothelial cells (Pedrazani and Stocker, 2008; Kshatri et al., 2018). TRN neurons express mainly SK2 channels, and SK1 channels to a lesser extent. Importantly, neuronal activity in the TRN is paced by the coordinated action of low threshold Ca^{2+} channels (Ca_V3) and SK channels. $Ca_V3.3$ channels mediate burst firing, and Ca^{2+} ions entering through these channels stimulate SK2 channels, which are responsible for the AHP (Astori et al., 2011; Fernandez & Lüthi, 2020). The AHP allows TRN cells to be ready to fire again and therefore ensures that the burst can occur repeatedly over time. This is crucial for the generation of thalamocortical oscillations during sleep and wakefulness. Reduced SK2 channel function in the TRN has been described in models of attentional disorder and the epileptic encephalopathy called Dravet

syndrome (Ritter-Makinson et al., 2019). On the contrary, SK2 channel-overexpressing mice showed increased bursting activity in TRN neurons, prolonged thalamic oscillations and less fragmented sleep compared with their WT littermates (Wimmer et al., 2012). However, less is known regarding how pharmacological modulation of SK channels affect TRN activity. During my PhD project, we sought to address the impact of pharmacological modulators of SK channels on AP firing and intrinsic properties of TRN neurons. Our pharmacological tools were two SK positive allosteric modulators (PAMs) and one negative allosteric modulator (NAM): CyPPA, NS309 and NS8593 respectively (figure 1). Whole cell patch clamp recordings in current clamp configuration were performed in horizontal rat brain slices containing TRN. NS309 and NS8593 elicited opposite effects on TRN firing patterns and intrinsic properties. By acting on the burst AHP, negative modulation of SK channels by NS8593 resulted in increased AP firing, increased burst duration and decreased intervals between bursts. Conversely, both CyPPA and NS309 increased the afterburst AHP, prolonging the interburst interval, which additionally resulted in reduced AP firing in case of NS309. Alterations in SK channel activity would be expected to alter functioning of thalamocortical circuits. When taken together, the results suggest that SK channel allosteric modulators are useful tools for modulating TRN activity. Targeting SK channels could be promising in treating disorders involving thalamic reticular dysfunction such as psychiatric and neurodevelopmental disorders.

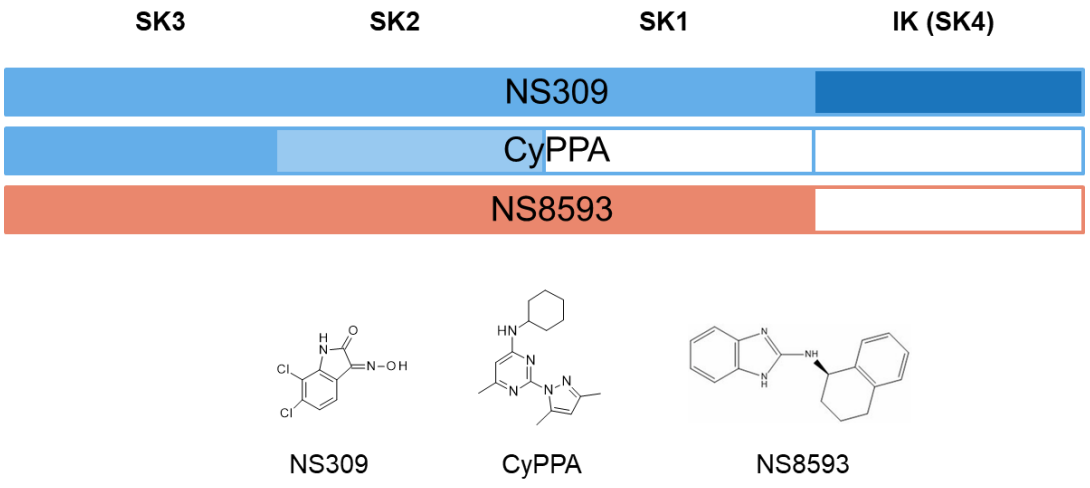


Figure 1. SK modulators. Modified from Hougaard et al., 2007; Strøbaek et al., 2006; Strøbaek et al., 2004. Positive allosteric modulators of SK channels are shown in blue (NS309, CyPPA) and negative modulators are shown in red (NS8593). The color code reflects the potency of the target; the darker the color the more potent on the target.

TRN neurons typically exhibit two distinct firing patterns (tonic or burst firing) depending on their resting membrane potential (RMP). Burst firing is characterized by low-threshold T-type Ca^{2+} spikes elicited at hyperpolarized membrane potentials, followed by high-frequency Na^{+} spike trains. When depolarized, TRN neurons fire regular Na^{+} spikes in a tonic firing pattern. TRN can be visually identified as a dense layer of cells located between the internal capsule (ic) and the thalamus, and cells typically display an elongated soma (figure 2A). In response to hyperpolarizing current step injections, TRN neurons exhibited a characteristic rebound burst (figure 2B) followed by an afterburst AHP. Under the current experimental conditions, TRN cells showed an average RMP of -73.3 ± 5 mV, input resistance (Rin) of 202.9 ± 10 MΩ and AP threshold of -43 ± 1 mV (n=31; N=11). Although recordings were obtained in P15-21 animals, firing profiles agree with what is shown in the literature for rodent TRN neurons (Lee et al., 2007; Clemente-Perez et al., 2017).

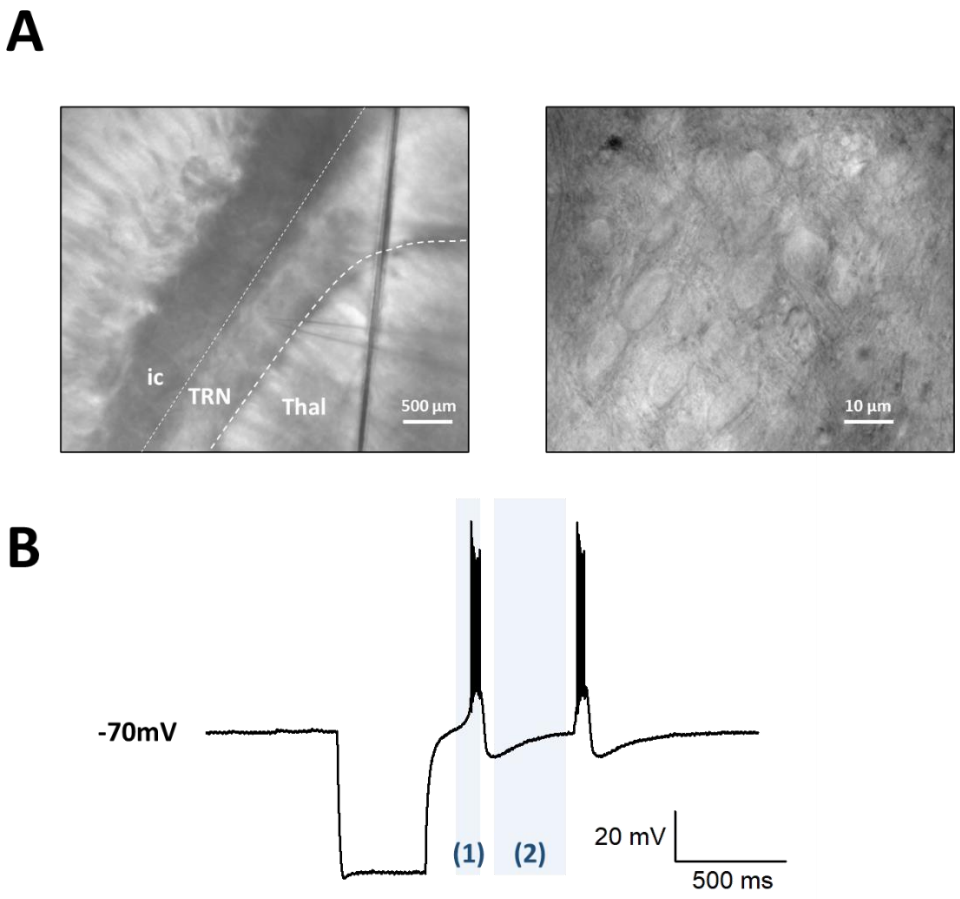


Figure 2. TRN neurons exhibited rebound bursts in response to a hyperpolarizing current. A) Differential contrast microscopy images depicting TRN. Left: TRN was visually identified as a dense layer of cells located in between the internal capsule (ic) and the thalamus (Thal). Right: under higher magnification, TRN cells display an elongated/oval shape. B) Typical rebound burst firing of a TRN neuron (1) followed by the afterburst AHP (2).

Number of APs were studied as a function of increasing current injection (from -200 to 200 pA; step size 25 pA) before (baseline) and 10 minutes after each compound was administered to the bath. No rebound bursts were observed in response to hyperpolarizing current steps at the RMP of the cells. Therefore, only depolarizing current steps (from 0 to 200 pA) are shown (figure 3). Results obtained showed that opposite effects were exerted on TRN firing by NS309 and NS8593 (figure 3B); while NS8593 (1µM) significantly increased the number of APs compared to baseline ($F [1, 18] = 12.29$; $p=0.003$), NS309 (3µM) reduced it ($F [1, 22] = 20.57$; $p=0.0002$). No significant effect was observed when applying CyPPA (10 µM), which acts less potently on SK2 channels ($F [1, 10] = 0.26$; $p=0.62$). Similar results were obtained when observing the effect of SK modulators across time (figure 3C). In this situation, APs were activated by injection of a squared current pulse (150 pA, 1s) repeated every 5 min. None of the compounds showed a washout effect when switching back to regular ACSF in the bath for more than 5 minutes (figure 3C). NS8593-treated cells displayed a significant increased number of APs compared to DMSO time-matched control cells at 10, 15 and 20 minutes after bath application of the compound ($F [1, 84] = 45.20$ $p<0.0001$), and the opposite effect was observed when applying NS309, which reduced significantly the number of APs across time ($F [1, 14] = 13.96$; $p=0.002$). No significant effects were observed when CyPPA was bath applied to the slice ($F [1, 8] = 0.31$; $p=0.59$) compared to DMSO.

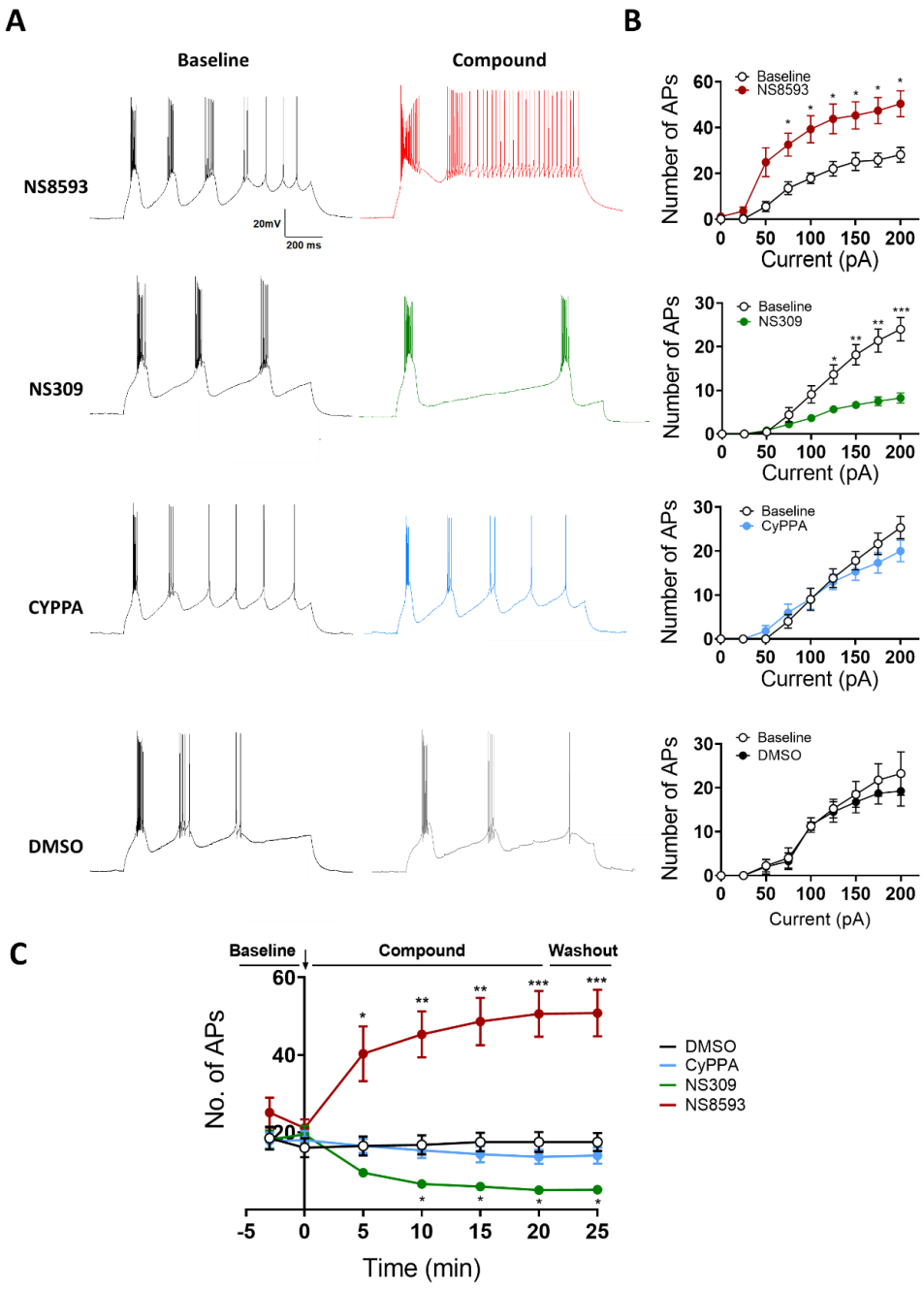


Figure 3. AP firing in TRN neurons are sensitive to SK channel modulators. A) Representative traces of AP firing in TRN neurons activated by a depolarizing current injection (150 pA, 1s) before (baseline) and following 10 minutes of compound application. B) Number of APs as a function of increasing current injection (from -200 to 200 pA; step size 25 pA) before (baseline) and 10 minutes after each compound is administered (black baseline; red NS8593 1µM; green NS309 3µM; blue CyPPA 10µM; gray DMSO CTRL). For statistical analyses, two-way repeated measures ANOVA followed by Holm-Šídák post-hoc test was performed (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). C) Number of APs as a function of time. APs were elicited by injection of a squared current pulse (150 pA, 1s) repeated every 5 min. Data are shown as mean ± SEM (DMSO n=4 N=3, black; NS8593 1µM n=9 N=3,

red; NS309 3µM n=12 N=3, green; CyPPA 10µM n=6 N=3, blue). For statistical analyses, mixed-effects analyses followed by Holm-Šídák post-hoc test was performed (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Afterburst AHP regulates neuronal excitability and ensures that the burst firing in TRN neurons can occur repeatedly. To gain insight into how SK modulators affect intrinsic excitability and burst properties in TRN neurons, we assessed the effect of SK modulators on the amplitude of AHP, burst duration, intraburst firing frequency and time between bursts (interburst interval) elicited by a depolarizing current injection (1 s, 150 pA), before (baseline) and 10 minutes after compound administration (figure 4). Afterburst AHP was defined as the hyperpolarizing phase after the first burst, and it was measured as the difference in voltage compared to the AP threshold. In line with the results showed previously, when applying NS8593 to the slice TRN cells exhibited a clear reduction in the AHP ($t(7) = 8.8$, $p < 0.0001$) and a shorter interbust interval ($t(6) = 4.3$, $p = 0.005$). Conversely, both SK PAMs increased the afterburst AHP (CyPPA $t(5) = 4.99$, $p = 0.004$; NS309 $t(11) = 2.8$, $p = 0.02$) and the time between bursts (CyPPA $t(5) = 3.35$, $p = 0.02$; NS309 $t(9) = 9.6$, $p < 0.0001$). Additionally, NS8953-treated cells showed a longer burst duration ($t(7) = 6.4$, $p = 0.0004$) and lower intraburst burst firing frequency ($t(7) = 2.98$, $p=0.02$). Even though the overall intraburst frequency was reduced in presence of NS8593, the traces show an increase in the frequency during the first spikes and a reduced spike frequency by the end of the burst (figure 4A). Applying DMSO to the bath did not show a significant effect on any of the parameters studied.

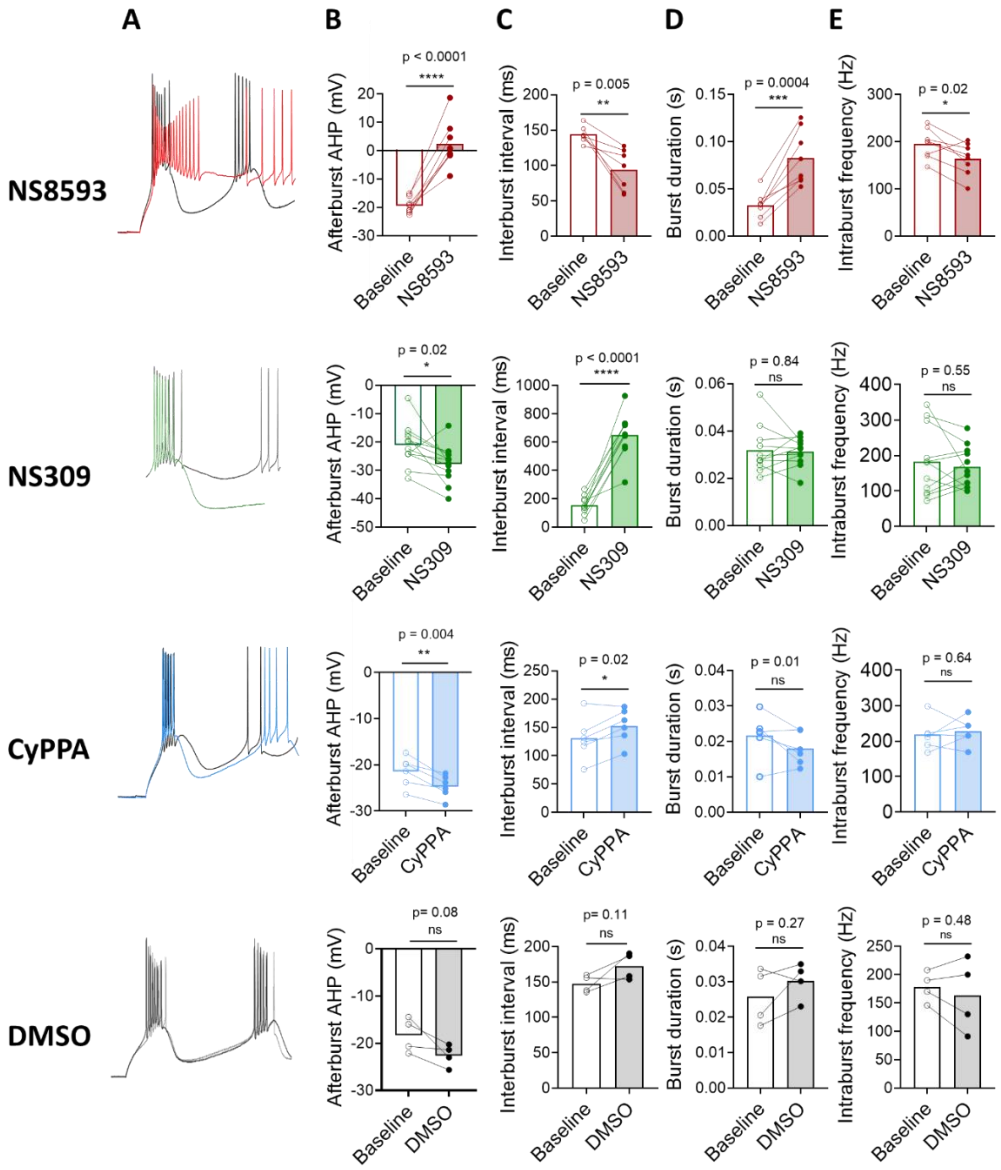


Figure 4. SK channel modulators affect the AHP and the interburst interval in TRN neurons. A) Magnification of representative traces highlighting the afterburst AHP elicited by a depolarizing current injection (1 s, 150 pA), before (Baseline) and 10 minutes after compound administration. Afterburst AHP, interburst interval, burst duration and intraburst spike frequency values before and after compound application are depicted in graphs (B), (C), (D) and (E) respectively. Data are shown as mean ± SEM (DMSO n=4 N=3, black; NS8593 1µM n=9 N=3, red; NS309 3µM n=12 N=3, green; CyPPA 10µM n=6 N=3, blue). For statistical analyses, paired student T- test were performed for each condition (baseline vs compound). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

When taken together, results show that SK channel modulators affect firing pattern and frequency in TRN neurons. By reducing the AHP as a consequence of SK negative modulation, NS8593 increases excitability on TRN cells, prolongs the burst duration, and shortens the refractory period between bursts, leading to a higher firing frequency. On the other hand, positive modulation of SK channels increases the AHP in TRN neurons, which reduces intrinsic excitability and increases the time between bursts. This effect is particularly remarkable for NS309, that showed a significant reduction in firing frequency. Thus, targeting SK channels could be promising in treating disorders involving TRN dysfunction. Based on previous literature, this might be particularly important in the context of neurodevelopmental and neuropsychiatric disorders. For example, reduced TRN function which includes marked deficits in TRN-generated sleep spindles has been noted in schizophrenia which supports genetic findings that a spontaneous mutation leading to suppression of SK channel currents is present in human schizophrenia patients (Miller et al, 2001). Accordingly, pharmacological agents which enhance SK function offer hope of normalization of TRN firing and restoration of more normal attentional processes and sleep spindle behavior, which has been suggested to be a novel treatment target for improving cognitive symptoms of schizophrenia (Manoach et al., 2016). Future studies using SK modulators in animal models will be key in finding the potential of these compounds as therapeutical tools.

The potential of the results for explotiation

The results obtained in the present study point out to develop compounds targeting SK channels, particularly SK2, as a potential therapeutical strategy in the context of neuropsychiatric and neurodevelopmental disorders.

ESR12 – Manuela Rizzi

ESR12: overview of results towards the objective of the action

Project title – Identifying convergent dysregulation of mRNA translation in multiple models of autism

WP3 – Modulators of neuropsychiatric disorders

Early Stage Researcher 12 – Manuela Rizzi

Supervisor – Emily Osterweil

Host Location – Centre for Discovery Brain Sciences (CDBS), University of Edinburgh – Scotland

Following TRAP methodology used to isolate the CA1 specific-ribosome-bound population from *Fmr1*$^{-/y}$ and WT mice described in (Thomson et al. 2017), previous members of the lab similarly collected CA1 TRAP and Total fractions from *Syngap* heterozygous (*Syngap*$^{+/-}$) and WT mice, specifically from 4 littermate pairs (Fig 1 A). Differential expression analysis using DESeq2 revealed 69 upregulated and 76 downregulated transcripts composing the population of mistranslated mRNAs in *Syngap*$^{+/-}$ CA1 TRAP fraction (Figure 1 B). Gene ontology analysis of the upregulated and downregulated fractions from *Syngap*$^{+/-}$ CA1 TRAP did not yield any significant enriched pathway. This could be ascribed to the modest nature of these changes. To this end, a different interrogation on the dataset was performed by means of Gene Set Enrichment Analysis, enabling the focus on more global changes interesting the dataset. Indeed, exploration of top differentially regulated categories in *Syngap*$^{+/-}$ TRAP dataset revealed upregulation of terms interesting regulation of transcription (ATP dependent activity acting on DNA, Catalytic activity acting on DNA, Helicase activity, Transcriptional regulation of gene expression, Chromosome organization) as well as DNA repair and DNA replication (Recombinational repair, Double strand break repair). (Figure 1 C). Ribonucleoprotein assembly (Ribonucleoprotein complex biogenesis, ncRNA processing, Exoribonuclease activity) ribogenesis, RNA catabolism, ubiquitin like protein ligase activity categories were also upregulated. In contrast, to the upregulated fraction the differentially expressed downregulated categories represented a lower proportion and included terms referring to Glycoprotein metabolism and G-protein coupled receptor activity.

After initial characterization of the *Syngap*$^{+/-}$ dataset, we proceeded to explore the hypothesis of convergence between *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ datasets. Indeed, exploration of the overlapping population from *Fmr1*$^{-/y}$ and *Syngap*$^{+/-}$ CA1 datasets revealed significant overlap of 25 transcripts in the TRAP fraction (p=0.03205504) (Figure 1 D). Furthermore, the population of misregulated transcripts was not informative by itself. The minimal magnitude of the overlap introduced the hypothesis of different regulation mechanisms characterizing the two mutants. Following up with overlap analysis at the level of differentially expressed categories in *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ TRAP datasets, 26 categories were found significantly converging (p=8.227324e-07) and interestingly out of these 4 were similarly upregulated and 22 were divergently regulated (Figure 1 E). When looking at these categories, terms implicated in the regulation of transcription, chromatin remodeling, histone modification, mitochondrial transport, glycoprotein synthesis and monoamine transport were oppositely regulated in the two TRAP fractions. However, ncRNA processing, ribosome biogenesis, ribonucleoprotein complex biogenesis and rRNA metabolic process categories were upregulated in both fractions (Figure 1 E). Although these results suggest different rather than convergent regulation interesting the two disorders, the overlap found at the level of exaggerated ribogenesis was further explored since its implication in the mechanism of mGluR-LTD revealed by dissection of the translation fraction associated to LTD induction in WT and *Fmr1*$^{-/y}$ mice (Seo et al. 2022). Interestingly, the overtranslated population induced by LTD and occluded in *Fmr1*$^{-/y}$ mice consisted of ribosomal proteins at

the expense of the downregulated synaptic transcripts. This led to hypothesize that excessive ribogenesis rather than a general increase in protein synthesis production would determine a shift in the translatome with consequential phenotypes in synaptic plasticity. Thus, convergent exaggerated ribogenesis in *Syngap*$^{+/-}$ TRAP would suggest similar mechanisms underlying exaggerated mGluR-LTD as the ones showed in *Fmr1*$^{-/y}$. To this end we explored whether the same shift in the translation of short vs long transcripts was observed for *Syngap*$^{+/-}$ as well. Information about length for most abundant transcripts expressed in CA1 specific neurons was retrieved and populations were binned accordingly to define a population of short (<1kb) and long (>2kb) genes that served to interrogate *Syngap*$^{+/-}$ dataset. Oppositely to what observed in *Fmr1*$^{-/y}$ this shift was in the opposite direction for *Syngap*$^{+/-}$ translated transcripts, with short genes being downregulated and long genes being upregulated (Figure 2 A). It was however of interest to dissect the nature of these populations in relation to LTD targets. Long genes >2k differentially downregulated (FDR<0.1) after LTD induction overlapped to a certain extent with population of transcripts translated in *Syngap*$^{+/-}$ TRAP and *Fmr1*$^{-/y}$ TRAP (Figure 2 D). Gene ontology analysis on these specific overlapping fractions revealed significantly enriched categories spanning from cell adhesion, axon development, postsynaptic density organization, calcium ion transmembrane transport (Figure 2 E). The same analysis was performed for the significantly upregulated transcripts including short genes <1kb induced by LTD and revealed enrichment of ribosome and translation related categories that was shared between the two sets (58 genes) (Figure 2 H,I,J,K). These observations would strongly sustain the hypothesis that the mGluR-LTD mechanism is shared at a certain extent between the two models and more specifically reproducing the shift observed in translation of specific population of short and long genes. However, they would also introduce ulterior mechanisms of regulation in *Syngap*$^{+/-}$ that were not explored before such as the observed increase in categories related to processes of transcription and DNA repair, which could represent a novel point of focus to disentangle molecular mechanisms and pathways in physiological and disease state.

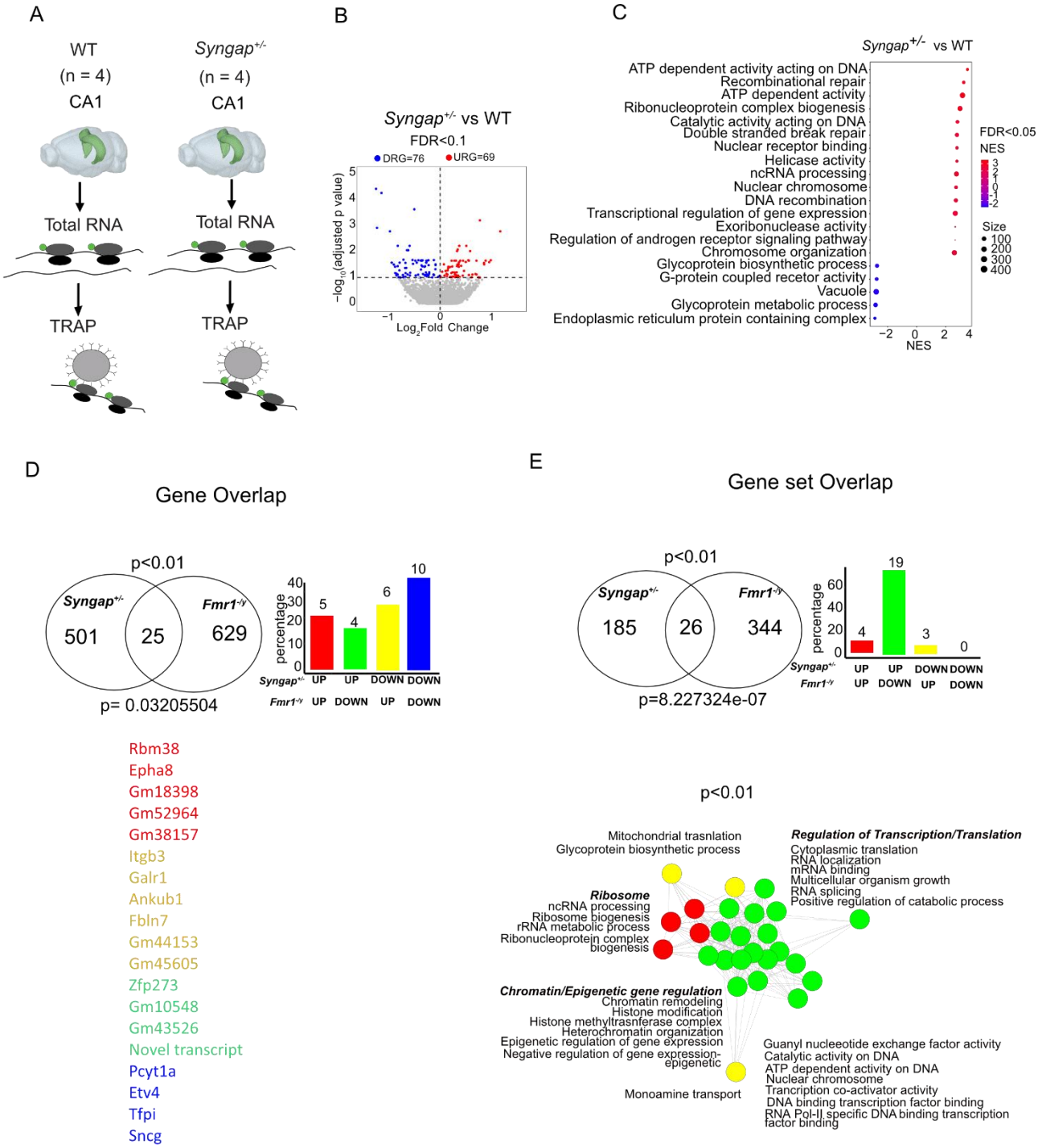
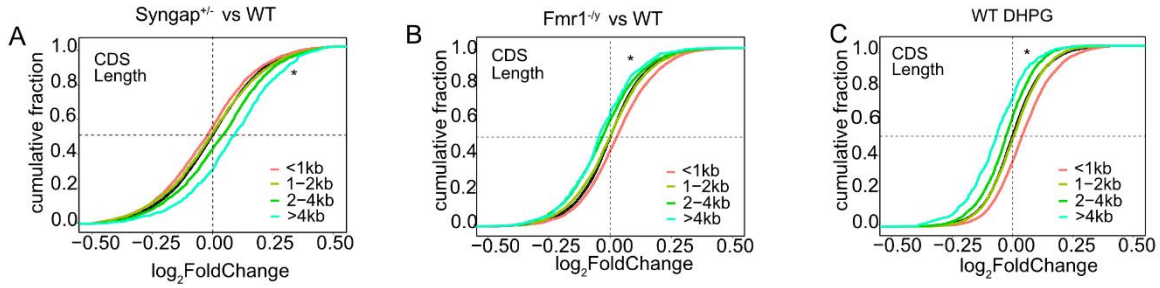
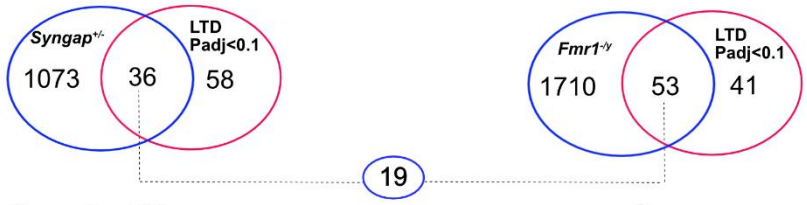
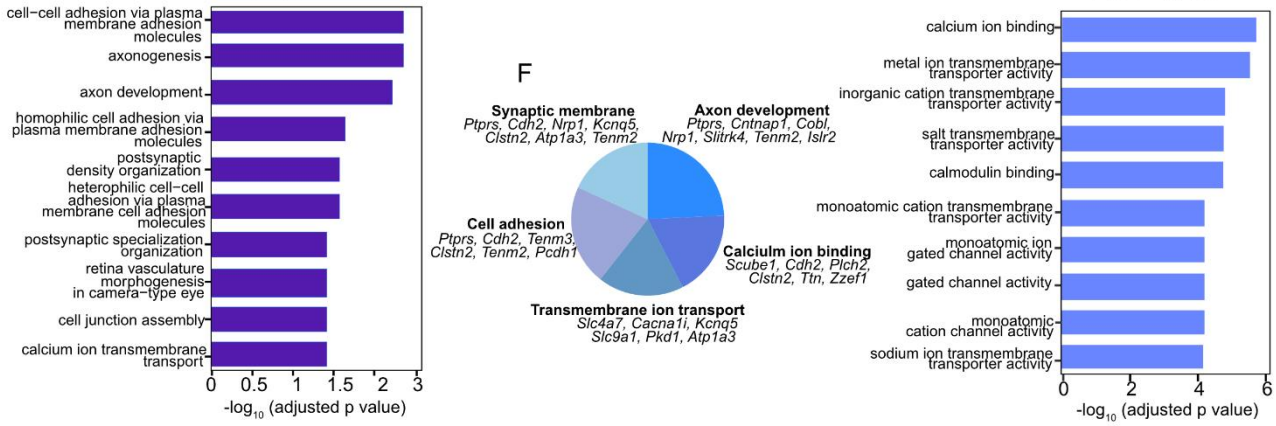

A
WT
(n = 4)
CA1
Syngap+/-
(n = 4)
CA1
Total RNA
Total RNA
TRAP
TRAP
B
Syngap+/- vs WT
FDR<0.1
DRG=76
URG=69
−log10(adjusted p value)
Log2Fold Change
C
Syngap+/- vs WT
ATP dependent activity acting on DNA
Recombinational repair
ATP dependent activity
Ribonucleoprotein complex biogenesis
Catalytic activity acting on DNA
Double stranded break repair
Nuclear receptor binding
Helicase activity
ncRNA processing
Nuclear chromosome
DNA recombination
Transcriptional regulation of gene expression
Exoribonuclease activity
Regulation of androgen receptor signaling pathway
Chromosome organization
Glycoprotein biosynthetic process
G-protein coupled recetor activity
Vacuole
Glycoprotein metabolic process
Endoplasmic reticulum protein containing complex
FDR<0.05
NES
Size
NES
D
Gene Overlap
p<0.01
Syngap+/-
Fmr1-/y
501
25
629
p= 0.03205504
percentage
5
4
6
10
Syngap+/- UP UP DOWN DOWN
Fmr1-/y UP DOWN UP DOWN
E
Gene set Overlap
p<0.01
Syngap+/-
Fmr1-/y
185
26
344
p=8.227324e-07
percentage
4
19
3
0
Syngap+/- UP UP DOWN DOWN
Fmr1-/y UP DOWN UP DOWN
p<0.01
Mitochondrial trasnlation
Glycoprotein biosynthetic process
Ribosome
ncRNA processing
Ribosome biogenesis
rRNA metabolic process
Ribonucleoprotein complex biogenesis
Chromatin/Epigenetic gene regulation
Chromatin remodeling
Histone modification
Histone methyltrasnferase complex
Heterochromatin organization
Epigenetic regulation of gene expression
Negative regulation of gene expression-epigenetic
Monoamine transport
Regulation of Transcription/Translation
Cytoplasmic translation
RNA localization
mRNA binding
Multicellular organism growth
RNA splicing
Positive regulation of catabolic process
Guanyl nucleeotide exchange factor activity
Catalytic activity on DNA
ATP dependent activity on DNA
Nuclear chromosome
Trancription co-activator activity
DNA binding transcription factor binding
RNA Pol-II specific DNA binding transcription factor binding
Rbm38
Epha8
Gm18398
Gm52964
Gm38157
Itgb3
Galr1
Ankub1
Fbln7
Gm44153
Gm45605
Zfp273
Gm10548
Gm43526
Novel transcript
Pcyt1a
Etv4
Tfpi
Sncg
Poc1a
Gm42930
Fam174c
Csgalnact1
Clrn1
Tmem232

Figure1: Characterization of *Syngap*$^{+/-}$ CA1 TRAP dataset and overlap analysis with *Fmr1*$^{-/y}$ CA1 TRAP dataset. A) Schematic of the experimental strategy. CA1 region was dissected from WT and mutant littermates (N=4 *Syngap*$^{+/-}$ /WT). Collected immunoprecipitated ribosome-bound fractions (IP) and Total homogenates (Input) were sequenced and analysed. **B)** Differential expression analysis of *Syngap*$^{+/-}$ TRAP dataset shows upregulation of 69 genes and downregulation of 76 genes. Significant population was determined as presenting a False Discovery Rate (FDR) <0.1. **C)** GSEA analysis was performed on *Syngap*$^{+/-}$ TRAP dataset and plotted are the top 10 significantly upregulated and downregulated categories (FDR<0.05). Amongst the downregulated categories only 5 passed the threshold of significance and are thus shown so extra 5 are shown from the upregulated fraction. **D)** Overlap analysis in differentially expressed transcripts from TRAP fractions of *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ (Pvalue<0.01) reveals 25 common transcripts (hypergeometric test, p= 0.03205504). Out of these 25 transcripts 5 are commonly upregulated, 10 are commonly downregulated and 10 are divergently regulated. **E)** Overlap analysis in TRAP fraction from *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ differentially expressed categories (Pvalue<0.01) reveals 26 common categories (hypergeometric test, p= 8.227324e-07). Out of these 26 transcripts 4 are commonly upregulated, 0 are commonly downregulated and 22 are divergently reg

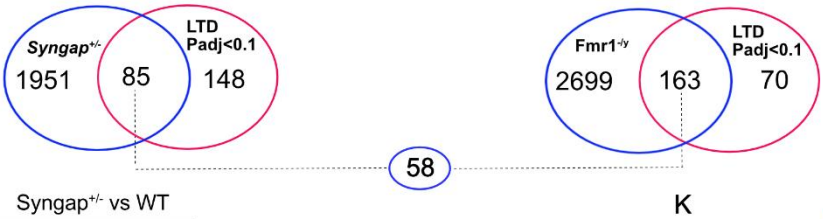


D 94/109 Downregulated Long genes (>2KB) in WT DHPG , FDR<0.1





H 233/272 Upregulated Short genes (<1KB) in WT DHPG , FDR<0.1



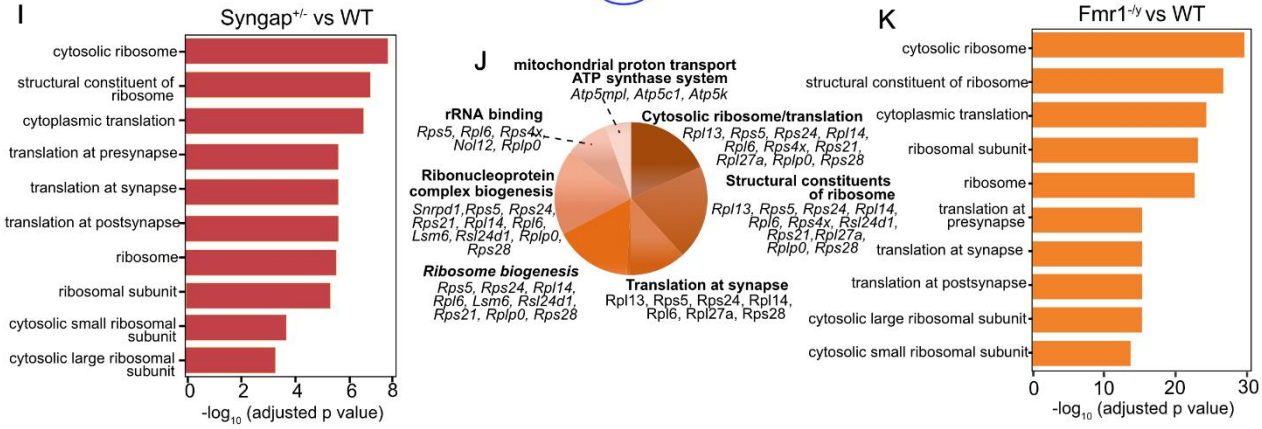


Figure 2. Dissection of upregulated short and downregulated long transcripts from differentially expressed genes in WT DHPG dataset. Overlap with *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ TRAP datasets reveals convergent pathways. Information about length for most abundant transcripts expressed in CA1 specific neurons was retrieved and populations were binned accordingly to define a population of short (<1kb) and long (>2kb) genes that served to interrogate *Syngap*$^{+/-}$ dataset **(A)**, *Fmr1*$^{-/y}$ dataset **(B)** and WT DHPG dataset **(C)**. **A)** Relative to *Syngap*$^{+/-}$ vs WT IP population longer transcripts are found upregulated and shorter ones downregulated; **B)** The opposite is observed in comparison to *Fmr1*$^{-/y}$ vs WT IP population where longer genes are downregulated and shorter genes are upregulated; **C)** and in comparison to WT treated with DHPG vs Vehicle. **D)** A population of 94 transcript differentially expressed in WT DHPG dataset (FDR<0.1) represents downregulated long genes. Comparison of this population with the total populations of downregulated long genes found in *Syngap*$^{+/-}$ (1073) dataset reveals 36 commonly downregulated long genes(left) and out of the total population found in *Fmr1*$^{-/y}$ (1710) 53 commonly downregulated long genes. In addition, within these overlaps *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ datasets share 19 of these downregulated long genes. **E)** Gene Ontology analysis of these transcripts reveals enrichment of synaptic-related categories (top 10 showed) in *Syngap*$^{+/-}$ dataset as well as in **G)** *Fmr1*$^{-/y}$ dataset (top 10) that also overlap in the common fraction **F)** here displayed the top 5 significantly enriched categories with relative genes: axon development, synaptic membrane, cell adhesion, calcium ion binding, transmembrane ion transport. **H)** A population of 233 transcript differentially expressed in WT DHPG dataset (FDR<0.1) represents upregulated short genes. Comparison of this population with the total populations of upregulated short genes found in *Syngap*$^{+/-}$ (1951) dataset reveals 85 commonly upregulated short genes (left) and out of the total population found in *Fmr1*$^{-/y}$ (2699) 163 commonly downregulated long genes. In addition, within these overlaps *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ datasets share 58 of these upregulated short genes. **E)** Gene Ontology analysis of these transcripts reveals enrichment of metabolic related categories (top 10 showed) in *Syngap*$^{+/-}$ dataset as well as in **G)** *Fmr1*$^{-/y}$ dataset (top 10) that also overlap in the common fraction **F)** here displayed the top 6 significantly enriched categories with relative genes: mitochondrial proton transport ATP synthase system, rRNA binding, ribonucleoprotein complex biogenesis, ribosome biogenesis, translation at synapse, structural constituent of ribosome, translation at synapse,cytosolic ribosome/translation.

After investigation of CA1 TRAP, we further proceeded interrogating the basolateral amygdala (BLA) region in these two models of disease. The observed phenotypes affecting this brain regions led to hypothesize that dysregulation of protein synthesis could also play a role here. To this end we characterized the BLA specific translational profiles for the two models of disease, asking whether in line with our hypothesis they would present with misregulated transcripts. These results would also allow comparison of regulation in BLA vs the one described for CA1.

At first basal changes in BLA were inspected. In *Fmr1*$^{-/y}$ BLA TRAP 16 transcripts were upregulated and 49 downregulated (Figure 3 C). Gene ontology analysis of the significantly changed fraction reveals enrichment of GTPase activity and GTPase complex terms in the upregulated fraction, while downregulation of external encapsulating structure, extracellular matrix, external side of plasma membrane, side of membrane and apical part of cell categories (Figure 3 E). Differential expression analysis in *Syngap*$^{+/-}$ BLA TRAP revealed 26 genes upregulated and 11 genes downregulated (Figure 3 D). Gene ontology analysis of the significantly changed fraction reveals enrichment of receptor binding, GTP-dependent protein binding terms in the upregulated fraction, while downregulation of proton-transporting two-sector ATPase complex and cell-cell adhesion categories among others (Figure 3 F). However, due to the minor changes observed in both datasets no clear cluster of genes interesting a particular category was observed. Gene set Enrichment analysis was then used to elucidate on global changes in these datasets. 10 top upregulated categories in *Fmr1*$^{-/y}$ BLA TRAP spanned across synaptic transport and endocytosis while translation and ribosomes represented the top downregulated categories (Figure 3G). The latter regulation is opposite to what observed for CA1, where translation processes were upregulated. *Syngap*$^{+/-}$ dataset revealed upregulation of RNA processing categories and downregulation of synaptic ones (Figure 3H). For synaptic categories negative regulation opposed the positive observed in the *Fmr1*$^{-/y}$ dataset. Overall, these first observations delineate a minor fraction of mistranslated transcripts, leading to question whether abnormal protein synthesis is an actual phenotype for the two models of disease in this brain region. Moreover, a different profile emerges for the

BLA region in relation to CA1. Overlap analysis at the gene level between the two populations of mistranslated transcripts revealed 14 common mRNAs (Figure 4A). Overlap at the level of gene set categories instead, revealed a larger number of shared categories (92 terms) (Figure 4 B) that did not show a particular bias towards regulation since a similar number of categories were either convergent or divergent. This analysis further corroborated the idea of different misregulation interesting BLA and CA1. Indeed, downregulation of ribosomal categories in BLA was found not associated with a shift in the translation of synaptic terms towards upregulation, since these were as well found downregulated. This was further elucidated when comparing specific population of targets representing synaptic-related terms such as mRNAs that bind to FMRP proteins as well as autism risk genes collected in the SFARI (Simons Foundation Autism Research Initiative) database and interrogating long vs short transcripts in the population. If similarly to what observed in CA1, long transcripts were downregulated in *Fmr1*$^{-/y}$ BLA dataset they were not changed in *Syngap*$^{+/-}$ BLA dataset (Figure 4 C), contrary to their upregulation in CA1. Moreover, looking at the population of ribosomal proteins which was found upregulated and not changed respectively in *Fmr1*$^{-/y}$ and *Syngap*$^{+/-}$ CA1 datasets, in BLA this was found downregulated in both models (Figure 4 D).

Together this data suggests that BLA possibly presents a minimal basal dysregulation in protein synthesis, which would be interesting to further validate. However, different mechanisms of regulation seem to interest the two models despite convergent regulation of processes such as translation and DNA transcription. Interestingly the downregulation in ribosome related terms is opposite to what observed in CA1, introducing the hypothesis of a different mechanistic dysfunction interesting the BLA.

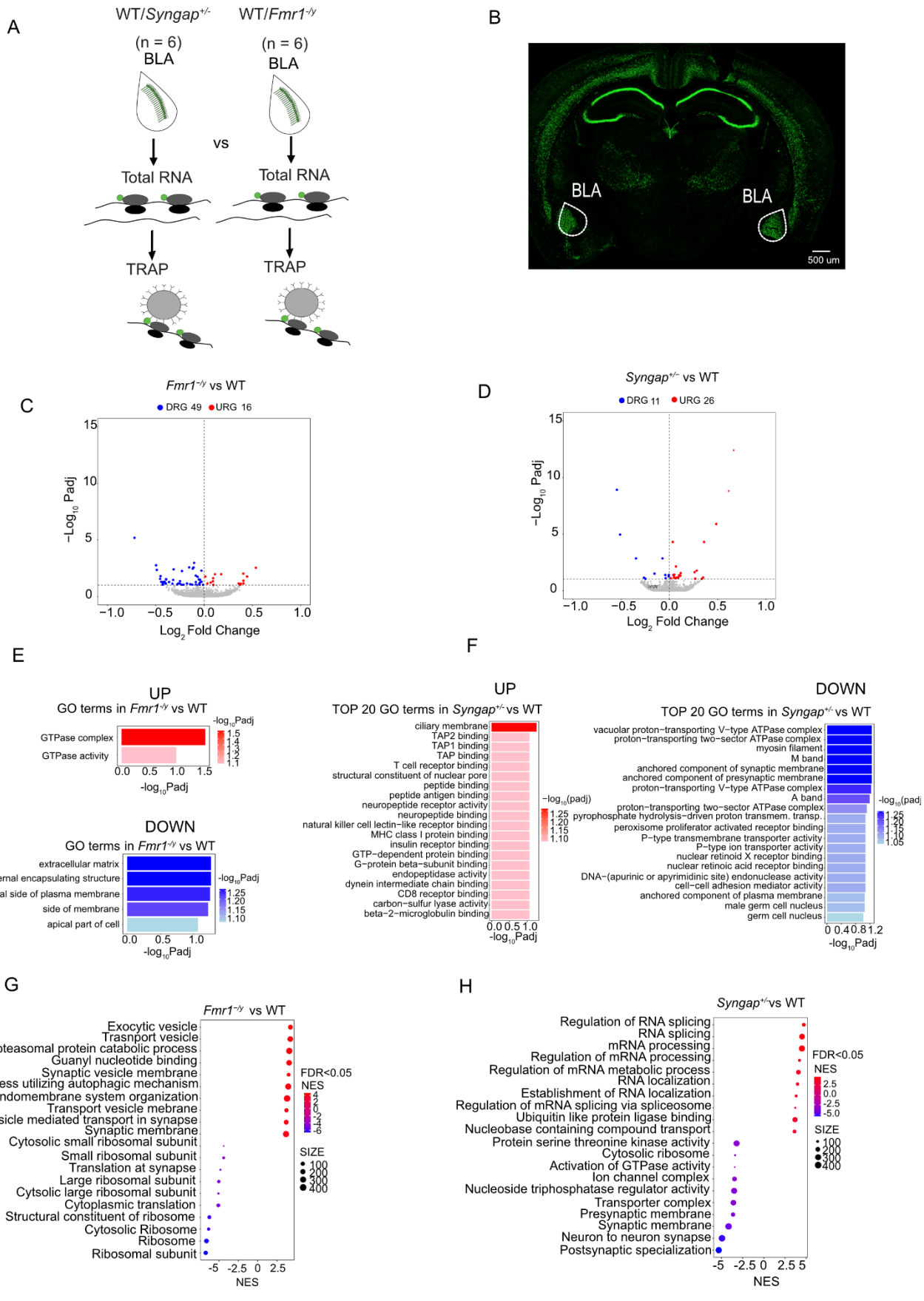
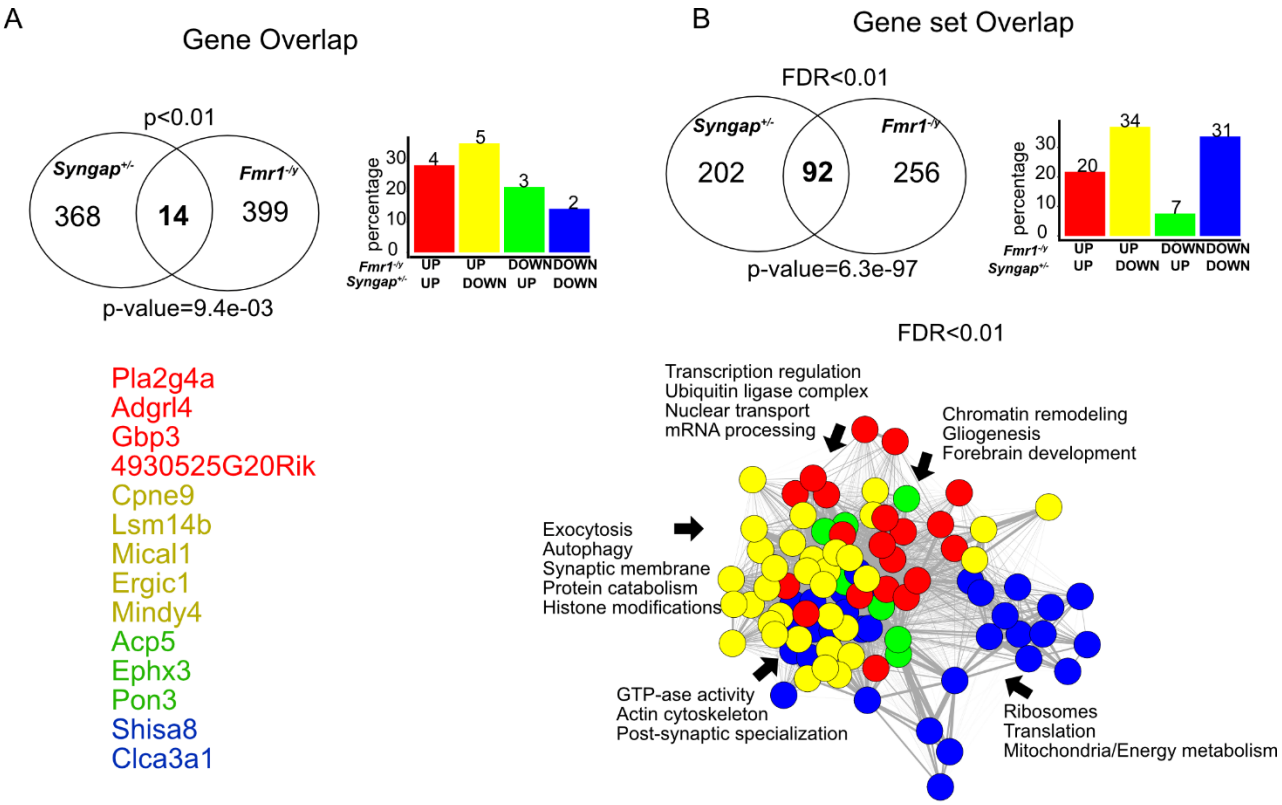
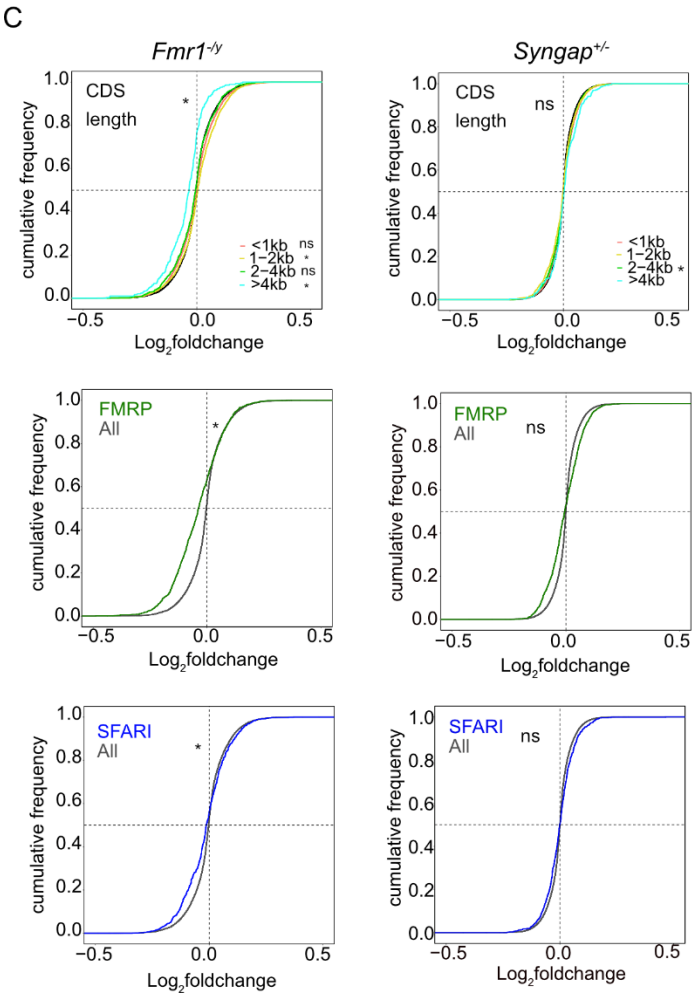

A
WT/Syngap+/-
WT/Fmr1-/y
(n = 6)
BLA
vs
Total RNA
TRAP
B
BLA
BLA
500 um
C
Fmr1-/y vs WT
DRG 49
URG 16
−Log10 Padj
Log2 Fold Change
D
Syngap+/- vs WT
DRG 11
URG 26
E
UP
GO terms in Fmr1-/y vs WT
GTPase complex
GTPase activity
-log10Padj
DOWN
extracellular matrix
external encapsulating structure
external side of plasma membrane
side of membrane
apical part of cell
F
UP
TOP 20 GO terms in Syngap+/- vs WT
ciliary membrane
TAP2 binding
TAP1 binding
TAP binding
T cell receptor binding
structural constituent of nuclear pore
peptide binding
peptide antigen binding
neuropeptide receptor activity
neuropeptide binding
natural killer cell lectin−like receptor binding
MHC class I protein binding
insulin receptor binding
GTP−dependent protein binding
G−protein beta−subunit binding
endopeptidase activity
dynein intermediate chain binding
CD8 receptor binding
carbon−sulfur lyase activity
beta−2−microglobulin binding
−log10(padj)
DOWN
TOP 20 GO terms in Syngap+/- vs WT
vacuolar proton−transporting V−type ATPase complex
proton−transporting two−sector ATPase complex
myosin filament
M band
anchored component of synaptic membrane
anchored component of presynaptic membrane
proton−transporting V−type ATPase complex
A band
proton−transporting two−sector ATPase complex
pyrophosphate hydrolysis−driven proton transmem. transp.
peroxisome proliferator activated receptor binding
P−type transmembrane transporter activity
P−type ion transporter activity
nuclear retinoid X receptor binding
nuclear retinoic acid receptor binding
DNA−(apurinic or apyrimidinic site) endonuclease activity
cell−cell adhesion mediator activity
anchored component of plasma membrane
male germ cell nucleus
germ cell nucleus
-log10(padj
G
Fmr1-/y vs WT
Exocytic vesicle
Trasnport vesicle
Proteasomal protein catabolic process
Guanyl nucleotide binding
Synaptic vesicle membrane
Process utilizing autophagic mechanism
Endomembrane system organization
Transport vesicle mebrane
Vesicle mediated transport in synapse
Synaptic membrane
Cytosolic small ribosomal subunit
Small ribosomal subunit
Translation at synapse
Large ribosomal subunit
Cytsolic large ribosomal subunit
Cytoplasmic translation
Structural constituent of ribosome
Cytosolic Ribosome
Ribosome
Ribosomal subunit
FDR<0.05
NES
SIZE
NES
H
Syngap+/- vs WT
Regulation of RNA splicing
RNA splicing
mRNA processing
Regulation of mRNA processing
Regulation of mRNA metabolic process
RNA localization
Establishment of RNA localization
Regulation of mRNA splicing via spliceosome
Ubiquitin like protein ligase binding
Nucleobase containing compound transport
Protein serine threonine kinase activity
Cytosolic ribosome
Activation of GTPase activity
Ion channel complex
Nucleoside triphosphatase regulator activity
Transporter complex
Presynaptic membrane
Synaptic membrane
Neuron to neuron synapse
Postsynaptic specialization
FDR<0.05
NES
SIZE
NES

Figure 3. Characterization of BLA translation profiles in *Fmr1*$^{-/y}$ and *Syngap*$^{+/-}$ models at basal state. A) Schematic of the experimental strategy. BLA region was dissected from WT and mutant littermates (N=6 *Syngap*$^{+/-}$/WT; N=6 *Fmr1*$^{-/y}$/WT). Collected immunoprecipitated ribosome-bound fractions (IP) and Total homogenates (Input) were sequenced and analysed. **B)** Representative immunohistochemistry of the validated RiboTag mouse, targeting ribosome-bound population specifically from BLA brain region. **C)** Differential expression analysis of *Fmr1*$^{-/y}$ TRAP dataset shows upregulation of 16 genes and downregulation of 49 genes. Significant population was determined as presenting a False Discovery Rate (FDR) <0.1. **D)** Differential expression analysis of *Syngap*$^{+/-}$ TRAP dataset shows upregulation of 26 genes and downregulation of 11 genes. Significant population was determined as presenting a False Discovery Rate (FDR) <0.1. **E)** GO enrichment of differentially expressed population in *Fmr1*$^{-/y}$ TRAP reveals significantly upregulated and downregulated categories. **F)** GO enrichment of differentially expressed population in *Syngap*$^{+/-}$ TRAP reveals significantly upregulated and downregulated categories. **G)** GSEA analysis was performed on *Fmr1*$^{-/y}$ *Syngap*$^{+/-}$ TRAP dataset and plotted are the top 10 significantly upregulated and downregulated categories (FDR<0.05). **H)** GSEA analysis was performed on *Syngap*$^{+/-}$ TRAP dataset and plotted are the top 10 significantly upregulated and downregulated categories (FDR<0.05).


A
Gene Overlap
p<0.01
Syngap+/-
Fmr1-/y
368
14
399
p-value=9.4e-03
percentage
4
5
3
2
Fmr1-/y UP DOWN
Syngap+/- UP DOWN
B
Gene set Overlap
FDR<0.01
Syngap+/-
Fmr1-/y
202
92
256
p-value=6.3e-97
20
34
7
31
Pla2g4a
Adgrl4
Gbp3
4930525G20Rik
Cpne9
Lsm14b
Mical1
Ergic1
Mindy4
Acp5
Ephx3
Pon3
Shisa8
Clca3a1
FDR<0.01
Transcription regulation
Ubiquitin ligase complex
Nuclear transport
mRNA processing
Chromatin remodeling
Gliogenesis
Forebrain development
Exocytosis
Autophagy
Synaptic membrane
Protein catabolism
Histone modifications
GTP-ase activity
Actin cytoskeleton
Post-synaptic specialization
Ribosomes
Translation
Mitochondria/Energy metabolism


C
Fmr1-/y
Syngap+/-
CDS
length
FMRP
All
SFARI
All
cumulative frequency
Log2foldchange
<1kb ns
1–2kb *
2–4kb ns
>4kb *
ns
*

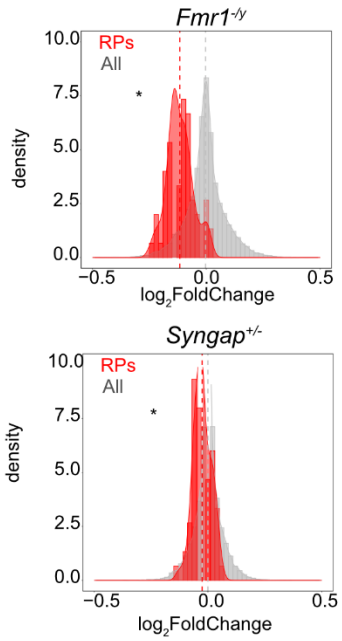

D
Fmr1-/y
Syngap+/-
RPs
All
density
log2FoldChange
*

Figure 4. Overlap analysis at gene and gene set level and exploration of distribution of synaptic vs metabolic transcripts in BLA. A) Overlap analysis in differentially expressed transcripts from TRAP fractions of *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ (Pvalue<0.01) reveals 14 common transcripts (hypergeometric test, p= 9.4e-03). Out of these 14 transcripts 4 are commonly upregulated, 2 are commonly downregulated and 8 are divergently regulated. **B)** Overlap analysis in TRAP fraction from *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ differentially expressed categories (FDR<0.01) reveals 92 common categories (hypergeometric test, p= 6.3e-97). Out of these 92 transcripts, 20 are commonly upregulated, 31 are commonly downregulated and 41 are divergently regulated. Overlapping upregulated categories comprise processes related to transcription and ubiquitination while translation is commonly downregulated. Processes interesting development, exocytosis and apoptosis are divergently regulated. **C)** Interrogation of SFARI and FMRP targets distribution in *Syngap*$^{+/-}$ reveals no significant changes (FMRP: z = -1.3849, p-value = 0.1661; SFARI=z = 0.092245, p-value = 0.9265). SFARI and FMRP targets are significantly downregulated in *Fmr1*$^{-/y}$ population (FMRP: z = -9.2399, p-value < 2.2e-16; SFARI=z = -2.0501, p-value = 0.04036). Information about length for most abundant transcripts expressed in BLA specific neurons was retrieved and populations were binned accordingly to define a population of short (<1kb) and long (>2kb) genes that served to interrogate *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ datasets. Relative to *Syngap*$^{+/-}$ population no shift is present in translation of long vs short transcripts (Ks <1kb vs >4kb, D = 0.070097, p-value = 0.1212). However binned population >2kb is significantly downregulated (<1kb: z = 0.15061, p-value = 0.8803; 1kb-2kb: z = -1.1921, p-value = 0.2332; >2kb: z = -2.6633, p-value = 0.007738; >4kb: z = 1.8993, p-value = 0.05752). Relative to *Fmr1*$^{-/y}$ population, shift is present in translation of long vs short transcripts, however it is mostly driven from the downregulated long transcript, since short ones (<1kb) are not changed (Ks <1kb vs >4kb, D = 0.22744, p-value = 1.366e-12). However binned population >2kb is significantly upregulated and >4kb significantly downregulated (<1kb: z = -0.62823, p-value = 0.5299; 1kb-2kb: z = 4.3275, p-value = 1.508e-05; >2kb: z = -1.5063, p-value = 0.132; >4kb: z = -8.0979, p-value = 5.59e-16). **D)** Ribosomal population is downregulated in both *Syngap*$^{+/-}$ (z= -5.6608, p-value =1.506e-08) and *Fmr1*$^{-/y}$ (z= -17.37, p-value<2.2e-16) datasets.

In order to dissect the specific changes that would infer insights on the amygdala functionality, BLA neuronal population in the two models of diseases was interrogated in response to the behavioural paradigm of Fear Conditioning, that relies on the activity of this specific brain region. More in detail, mice were habituated to the contextual chamber and conditioned during a single training session consisting of administration of 3 shocks of 0.6 mA for 2 sec with an inter trial period of 70 sec. 1 hour after mice were sacrificed to collect the actively translating ribosome bound population (Figure 5). We hypothesized that looking at this fraction of translated mRNAs at this specific time point corresponding to acquisition of memory, could be key to detect possible mechanisms dysregulated at early stages of memory formation that could relate to deficit in learning and memories displayed by these mice. In addition, here as well we hypothesized the regulation interesting the two models of disease would converge as for the exaggerated mGluR-LTD observed in hippocampus. Impaired Long-Term Potentiation (LTP) is observed in the amygdala of these mice, correlating with the deficient learning abilities. Fear Conditioning is believed to induce LTP-like changes, thus exploring the translated fraction activated following the paradigm could reveal insights in the regulation of this mechanism.

Starting from characterization of *Fmr1*$^{-/y}$ dataset we interrogated the fraction translated in WT state following conditioning as well as the fraction translated in *Fmr1*$^{-/y}$ mice after conditioning and the baseline activation in the *Fmr1*$^{-/y}$ not exposed to conditioning (home cage condition). We could appreciate that at basal state the *Fmr1*$^{-/y}$ mice presented with a significative mistranslated fraction consisting of 93 upregulated and 110 downregulated mRNAs. (Figure 6 A) GO analysis of these fractions revealed upregulation of transport activity including calcium channel activity and G-protein coupled receptor signalling and downregulation of processes interesting neuronal development (Figure 6 B), indicating aberrant protein synthesis in BLA at later developmental stage since these mice were tested at adult age (day 100). Interestingly, when looking at the gene sets categories differentially regulated at global level with GSEA analysis, ribosome and translation processes were upregulated while categories related to synaptic activity, transport and development were downregulated (Figure 7 A). These global changes would reproduce the ones observed in the juvenile

hippocampal translatome. A similar profile was observed in WT state upon conditioning (Figure 6 B, 7 B). However, at the gene level in WT the activation state was confirmed by the significant upregulation of Immediate Early Genes with consequent enrichment in categories related to miRNA transcription, calcium ion channel activity, long term memory as well as skeletal muscle differentiation and increase of vascular activity which are the cellular correlates of the freezing response displayed by the mice in response to the shock. In contrast the downregulated fraction was enriched for presynaptic activity and neurotransmission. This translational profile resembled in addition the one observed in CA1 during LTD induction in WT mice. This would possibly suggest that Fear Conditioning induces a molecular profile that mimics LTD-induced changes but whether this is a proxy for memory and learning is not clear since we did not test the consolidation of memory in these mice. Moreover, these changes were mimicked in BLA of *Fmr1*$^{-/y}$ mice at basal state (home cage condition) (Figure 6 C, 7C) which could indicate that these mice present basally with an hyperactive state. Interestingly, the changes were occluded in the *Fmr1*$^{-/y}$ mice under activity. When examining the response of the BLA upon conditioning in *Fmr1*$^{-/y}$ mice, the induced profile resulted opposite to the one observed in WT state. At the gene level, the only similarity between WT and *Fmr1*$^{-/y}$ activation was found in the upregulation of immediate early genes that similarly elicit upregulation of categories related to freezing response and transcription (Figure 6 I). Interestingly the downregulated fraction is composed of categories that span across synaptic transmission, long term potentiation, cellular response to dopamine that suggest an impaired LTP for these mice, possibly a consequence of their basal hyperactive state on which further activation through Fear Conditioning induces decrease of LTP. Moreover, gene set analysis of *Fmr1*$^{-/y}$ activation resulted in upregulation of terms related to neuron development and downregulation of ribosome/ translation related categories, a profile that was completely divergent from what observed in WT (Figure 7 C). Cfos imaging of the BLA specific population validated the activation of both WT and *Fmr1*$^{-/y}$ mice upon conditioning. Moreover, it also revealed a significant decrease in the level of cFos activation in *Fmr1*$^{-/y}$ compared to WT (Figure 8). These preliminary observations open to different hypothetical mechanisms which would require further validation. Interestingly it may seem that in *Fmr1*$^{-/y}$ BLA, induction of LTP would be dependent on protein synthesis. Upon conditioning, LTP like response is occluded in *Fmr1*$^{-/y}$ as displayed by the downregulation in categories related to long term potentiation. This can be the result of their basal alteration, that depicts an hyperactive state. A further stimulus such as conditioning, that induces new protein synthesis, is causing a shift in regulation towards the opposite direction, indicating that LTP like induction is dependent on protein synthesis as already demonstrated in the literature. This is different from independence of mGluR-LTD of de novo protein synthesis showed in CA1 for *Fmr1*$^{-/y}$ mice, where the system appears saturated and occluded regardless of new protein synthesis. This would however require further validation, interrogating for example the role of ribogenesis in the modulation of the activity in BLA.

Potential of results for exploitation

We then interrogated Syngap+/- dataset to explore the overlap hypothesis. However, characterization of the molecular profile in the WT after conditioning (Figure 9), prevented us from further pursuing this question. Conditioning caused an opposite response to the one observed in the WT littermates from Fmr1-/y mice. Overlap would thus be misleading since putative differences displayed by the mutants could be confounded by the different response observed in the WT. Indeed, strain specifications of Syngap+/- mice, differing from the Fmr1-/y mice, could be a possible impacting factor for this difference since it has been observed in literature that diverse strains could determine heterogeneity in response to behavioural paradigms(Nguyen et al. 2000). Although it would be still interesting to analyze and characterize the Syngap+/- dataset independently, ulterior validations are prioritized for the Fmr1-/y dataset having collected multiple evidence in the lab about the mechanistic insights impacting this model. Thus, retrieving further information about regulation of BLA region, which is still less explored compared to other brain regions, by means of validation of the results observed using TRAP technology, represent a great source for valuable insights in the molecular mechanisms regulating the system in health and disease state. Overall, exploration of protein synthesis proved to be a potential target to retrieve key and relevant information for the design of new therapeutic strategies that can more precisely address subtle aspects of misregulation observed in disease state. In addition, these datasets represent a great resource that will be of public availability and allow scientists to build a far clearer picture of the molecular characterization interesting these complex disorders. They can be looked at from different perspectives depending on the focus of the analysis and this would allow to retrieve various pieces of information. Furthermore, the exploration at the level of multiple brain regions is an added point towards characterization of circuitry disfunctions. This would ultimately lead to generation of new experimental and scientific questions which pursue is targeted at the understanding of how the system works and how its manipulation could impact on healthy and disease state. The sets of results generated would potentially be the basis for new research projects to be carried out by future members of the lab.

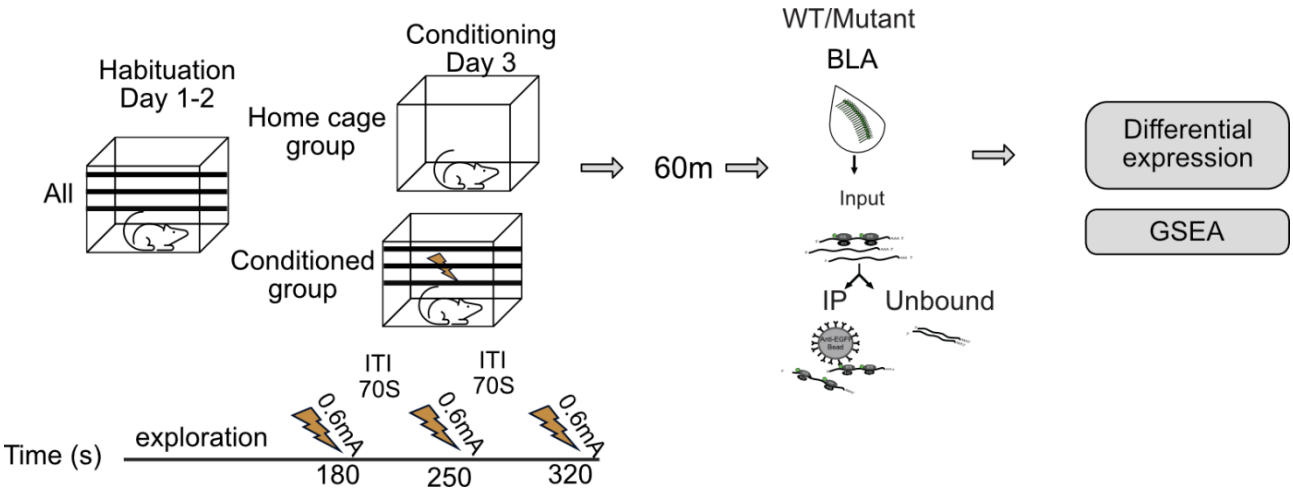


Figure 5. Schematic of strategy for TRAP coupled to Fear Conditioning paradigm. Mice were habituated for 2 days to the contextual chamber and on the 3rd day the conditioned group underwent a training session of 3 shocks of 0.6 mA of the duration of 2 sec each 70sec, instead the home cage group was left in the cage. 1 hour after the conditioning mice were sacrificed to retrieve the BLA specific ribosome bound fraction by means of TRAP in order to interrogate the differentially expressed transcripts in response to activity.

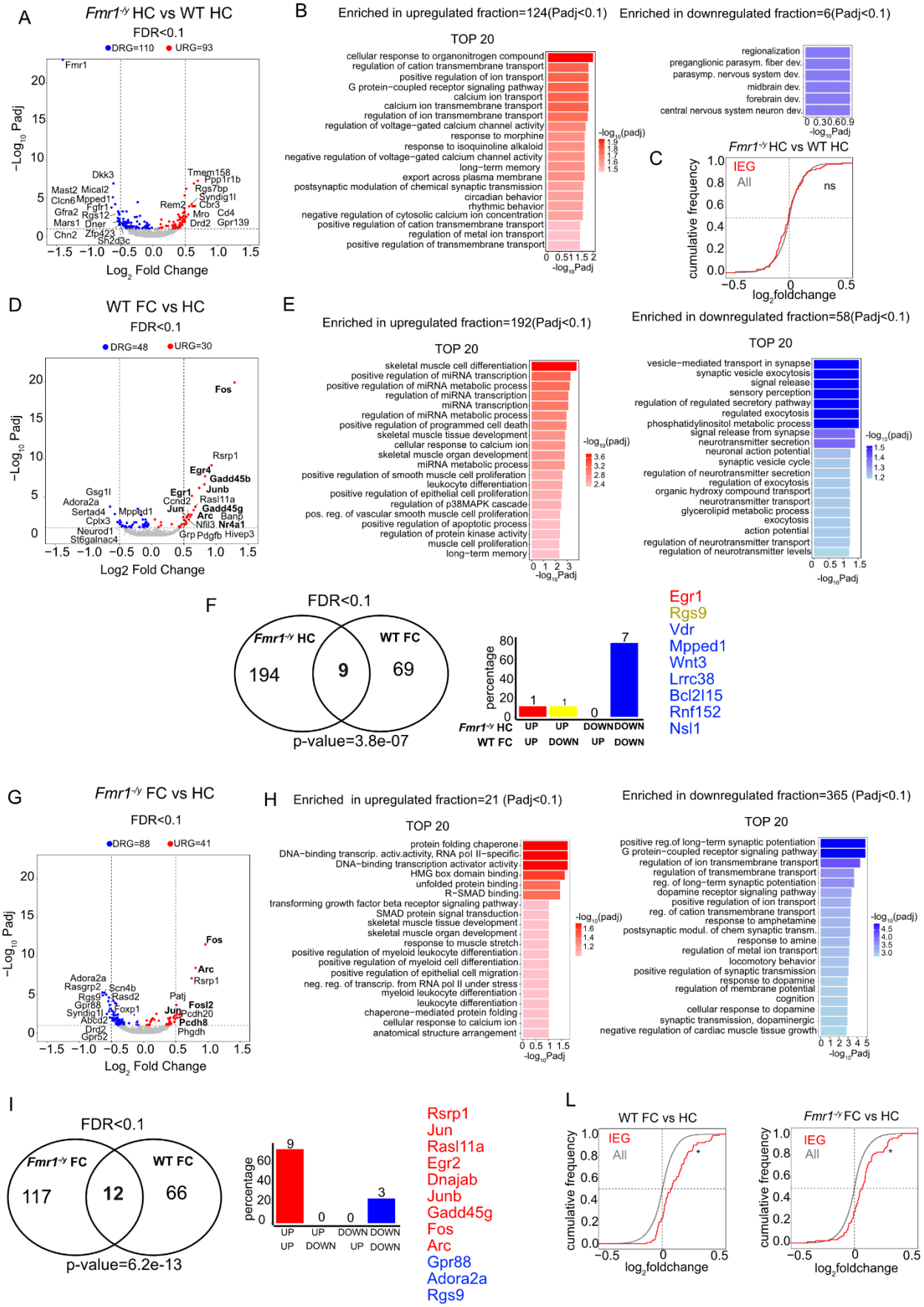

A
Fmr1-/y HC vs WT HC
FDR<0.1
DRG=110
URG=93
−Log10 Padj
Log2 Fold Change
Fmr1
Dkk3
Mast2
Mical2
Clcn6
Mpped1
Fgfr1
Gfra2
Rgs12
Mars1
Dner
Chn2
Zfp423
Sh2d3c
Tmem158
Ppp1r1b
Rgs7bp
Syndig1l
Rem2
Cbr3
Mro
Cd4
Drd2
Gpr139
B
Enriched in upregulated fraction=124(Padj<0.1)
TOP 20
cellular response to organonitrogen compound
regulation of cation transmembrane transport
positive regulation of ion transport
G protein−coupled receptor signaling pathway
calcium ion transport
calcium ion transmembrane transport
regulation of ion transmembrane transport
regulation of voltage−gated calcium channel activity
response to morphine
response to isoquinoline alkaloid
negative regulation of voltage−gated calcium channel activity
long−term memory
export across plasma membrane
postsynaptic modulation of chemical synaptic transmission
circadian behavior
rhythmic behavior
negative regulation of cytosolic calcium ion concentration
positive regulation of cation transmembrane transport
regulation of metal ion transport
positive regulation of transmembrane transport
-log10(padj)
-log10Padj
Enriched in downregulated fraction=6(Padj<0.1)
regionalization
preganglionic parasym. fiber dev.
parasymp. nervous system dev.
midbrain dev.
forebrain dev.
central nervous system neuron dev.
-log10Padj
C
Fmr1-/y HC vs WT HC
IEG
All
ns
cumulative frequency
log2foldchange
D
WT FC vs HC
FDR<0.1
DRG=48
URG=30
−Log10 Padj
Log2 Fold Change
Fos
Rsrp1
Egr4
Gadd45b
Junb
Egr1
Jun
Ccnd2
Rasl11a
Gadd45g
Arc
Banp
Nfil3
Nr4a1
Pdgfb
Hivep3
Grp
Gsg1l
Adora2a
Sertad4
Mpped1
Cplx3
Neurod1
St6galnac4
E
Enriched in upregulated fraction=192(Padj<0.1)
TOP 20
skeletal muscle cell differentiation
positive regulation of miRNA transcription
positive regulation of miRNA metabolic process
regulation of miRNA transcription
miRNA transcription
regulation of miRNA metabolic process
positive regulation of programmed cell death
skeletal muscle tissue development
cellular response to calcium ion
skeletal muscle organ development
miRNA metabolic process
positive regulation of smooth muscle cell proliferation
leukocyte differentiation
positive regulation of epithelial cell proliferation
regulation of p38MAPK cascade
pos. reg. of vascular smooth muscle cell proliferation
positive regulation of apoptotic process
regulation of protein kinase activity
muscle cell proliferation
long−term memory
-log10(padj)
-log10Padj
Enriched in downregulated fraction=58(Padj<0.1)
TOP 20
vesicle−mediated transport in synapse
synaptic vesicle exocytosis
signal release
sensory perception
regulation of regulated secretory pathway
regulated exocytosis
phosphatidylinositol metabolic process
signal release from synapse
neurotransmitter secretion
neuronal action potential
synaptic vesicle cycle
regulation of neurotransmitter secretion
regulation of exocytosis
organic hydroxy compound transport
neurotransmitter transport
glycerolipid metabolic process
exocytosis
action potential
regulation of neurotransmitter transport
regulation of neurotransmitter levels
-log10(padj)
-log10Padj
F
FDR<0.1
Fmr1-/y HC
WT FC
194
9
69
p-value=3.8e-07
percentage
Fmr1-/y HC
WT FC
UP UP
UP DOWN
DOWN UP
DOWN DOWN
1
1
0
7
Egr1
Rgs9
Vdr
Mpped1
Wnt3
Lrrc38
Bcl2l15
Rnf152
Nsl1
G
Fmr1-/y FC vs HC
FDR<0.1
DRG=88
URG=41
−Log10 Padj
Log2 Fold Change
Fos
Arc
Rsrp1
Adora2a
Rasgrp2
Scn4b
Rgs9
Rasd2
Gpr88
Syndig1l
Foxp1
Abcd2
Drd2
Gpr52
Patj
Jun
Fosl2
Pcdh20
Pcdh8
Phgdh
H
Enriched in upregulated fraction=21 (Padj<0.1)
TOP 20
protein folding chaperone
DNA−binding transcrip. activ.activity, RNA pol II−specific
DNA−binding transcription activator activity
HMG box domain binding
unfolded protein binding
R−SMAD binding
transforming growth factor beta receptor signaling pathway
SMAD protein signal transduction
skeletal muscle tissue development
skeletal muscle organ development
response to muscle stretch
positive regulation of myeloid leukocyte differentiation
positive regulation of myeloid cell differentiation
positive regulation of epithelial cell migration
neg. reg. of transcrip. from RNA pol II under stress
myeloid leukocyte differentiation
leukocyte differentiation
chaperone−mediated protein folding
cellular response to calcium ion
anatomical structure arrangement
-log10(padj)
-log10Padj
Enriched in downregulated fraction=365 (Padj<0.1)
TOP 20
positive reg.of long−term synaptic potentiation
G protein−coupled receptor signaling pathway
regulation of ion transmembrane transport
regulation of transmembrane transport
reg. of long−term synaptic potentiation
dopamine receptor signaling pathway
positive regulation of ion transport
reg. of cation transmembrane transport
response to amphetamine
postsynaptic modul. of chem synaptic transm.
response to amine
regulation of metal ion transport
locomotory behavior
positive regulation of synaptic transmission
response to dopamine
regulation of membrane potential
cognition
cellular response to dopamine
synaptic transmission, dopaminergic
negative regulation of cardiac muscle tissue growth
-log10(padj)
-log10Padj
I
FDR<0.1
Fmr1-/y FC
WT FC
117
12
66
p-value=6.2e-13
percentage
UP UP
UP DOWN
DOWN UP
DOWN DOWN
9
0
0
3
Rsrp1
Jun
Rasl11a
Egr2
Dnajab
Junb
Gadd45g
Fos
Arc
Gpr88
Adora2a
Rgs9
L
WT FC vs HC
IEG
All
cumulative frequency
log2foldchange
Fmr1-/y FC vs HC
IEG
All
cumulative frequency
log2foldchange

Figure 6. Characterization of WT/*Fmr1*$^{-/y}$ dataset after conditioning. A) Differential expression analysis in basal state (*Fmr1*$^{-/y}$ HC) reveals upregulation of 93 transcripts vs downregulation of 110 transcripts. Significant population was determined as presenting a False Discovery Rate (FDR) <0.1. **B)** GO enrichment of differentially expressed population in *Fmr1*$^{-/y}$ HC TRAP reveals significantly upregulated and downregulated categories. **C)** Interrogation of IEG targets respectively to *Fmr1*$^{-/y}$ HC population reveals no change (z=-0.3093, p-value= 0.7571) **D)** Differential expression analysis in WT conditioned mice (WT FC) reveals upregulation of 48 transcripts vs downregulation of 30 transcripts. Significant population was determined as presenting a False Discovery Rate (FDR) <0.1. **E)** GO enrichment of differentially expressed population in WT FC TRAP reveals significantly upregulated and downregulated categories. **F)** Overlap analysis of genes differentially regulated in basal state and WT after conditioning reveals significant minimal overlap of 9 transcripts (pvalue=3.8e-07). Out of these 7 are convergently downregulated, 1 is convergently upregulated and 1 is divergently regulated. **G)** Differential expression analysis in *Fmr1*$^{-/y}$ conditioned mice (*Fmr1*$^{-/y}$ FC) reveals upregulation of 41 transcripts vs downregulation of 88 transcripts. Significant population was determined as presenting a False Discovery Rate (FDR) <0.1. **H)** GO enrichment of differentially expressed population in *Fmr1*$^{-/y}$ FC TRAP reveals significantly upregulated and downregulated categories. **I)** Overlap analysis of genes differentially regulated in *Fmr1*$^{-/y}$ and WT after conditioning reveals significant minimal overlap of 12 transcripts (pvalue=6.2e-13) and they are all convergently regulated. **L)** IEG targets are upregulated in WT FC and *Fmr1*$^{-/y}$ FC population (WT FC: z=6.8798, p-value= 5.995e-12; *Fmr1*$^{-/y}$ FC: z=5.2804, p-value= 1.289e-07).

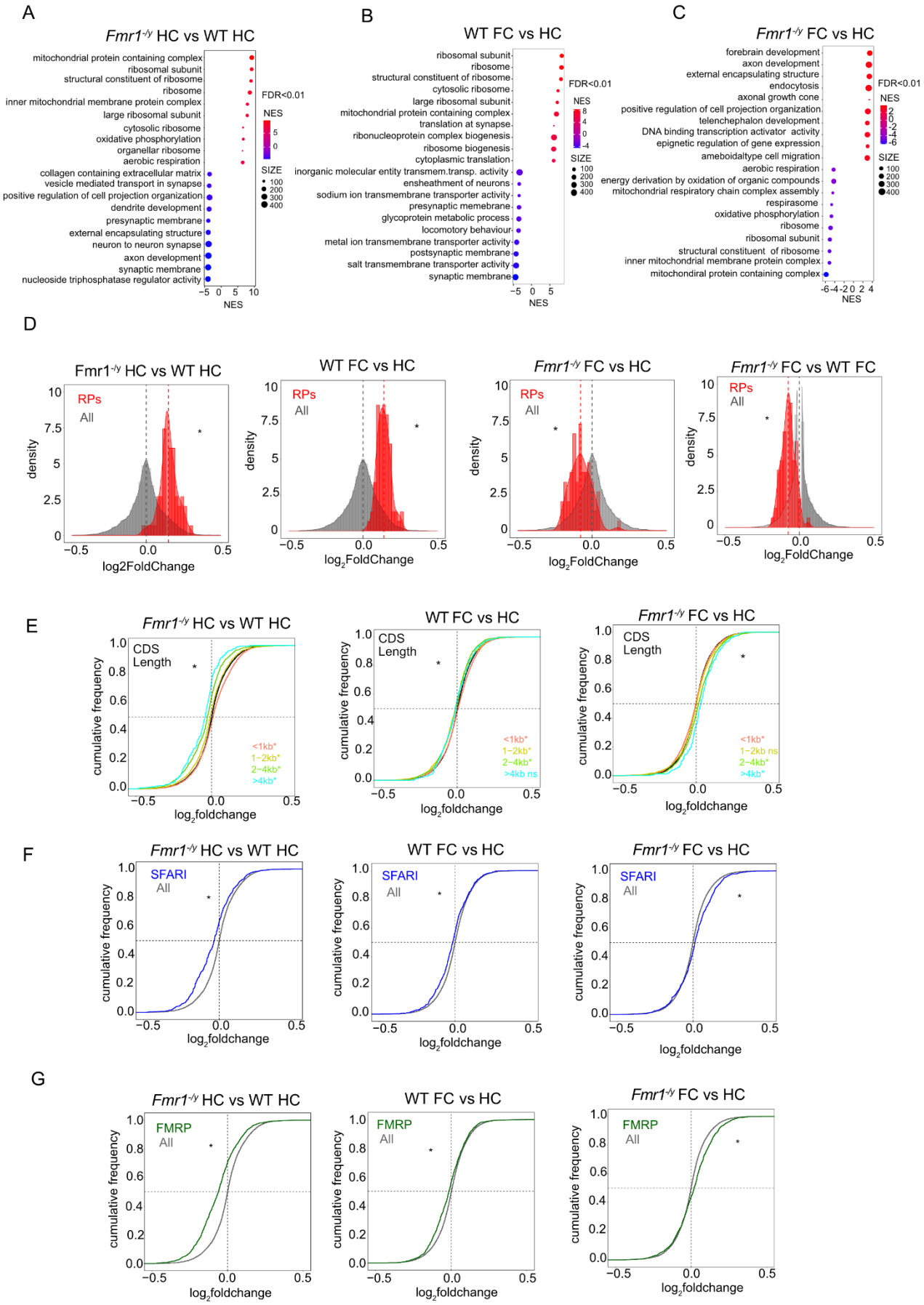

A
Fmr1-/y HC vs WT HC
mitochondrial protein containing complex
ribosomal subunit
structural constituent of ribosome
ribosome
inner mitochondrial membrane protein complex
large ribosomal subunit
cytosolic ribosome
oxidative phosphorylation
organellar ribosome
aerobic respiration
collagen containing extracellular matrix
vesicle mediated transport in synapse
positive regulation of cell projection organization
dendrite development
presynaptic membrane
external encapsulating structure
neuron to neuron synapse
axon development
synaptic membrane
nucleoside triphosphatase regulator activity
FDR<0.01
NES
SIZE
NES
B
WT FC vs HC
ribosomal subunit
ribosome
structural constituent of ribosome
cytosolic ribosome
large ribosomal subunit
mitochondrial protein containing complex
translation at synapse
ribonucleoprotein complex biogenesis
ribosome biogenesis
cytoplasmic translation
inorganic molecular entity transmem.transp. activity
ensheathment of neurons
sodium ion transmembrane transporter activity
presynaptic membrane
glycoprotein metabolic process
locomotory behaviour
metal ion transmembrane transporter activity
postsynaptic membrane
salt transmembrane transporter activity
synaptic membrane
FDR<0.01
NES
SIZE
NES
C
Fmr1-/y FC vs HC
forebrain development
axon development
external encapsulating structure
endocytosis
axonal growth cone
positive regulation of cell projection organization
telenchephalon development
DNA binding transcription activator activity
epigenetic regulation of gene expression
ameboidaltype cell migration
aerobic respiration
energy derivation by oxidation of organic compounds
mitochondrial respiratory chain complex assembly
respirasome
oxidative phosphorylation
ribosome
ribosomal subunit
structural constituent of ribosome
inner mitochondrial membrane protein complex
mitochondiral protein containing complex
FDR<0.01
NES
SIZE
NES
D
Fmr1-/y HC vs WT HC
WT FC vs HC
Fmr1-/y FC vs HC
Fmr1-/y FC vs WT FC
RPs
All
density
log2FoldChange
log2FoldChange
log2FoldChange
log2FoldChange
E
Fmr1-/y HC vs WT HC
WT FC vs HC
Fmr1-/y FC vs HC
CDS Length
cumulative frequency
<1kb*
1−2kb*
2−4kb*
>4kb*
<1kb*
1−2kb*
2−4kb*
>4kb ns
<1kb*
1−2kb ns
2−4kb*
>4kb*
log2foldchange
F
Fmr1-/y HC vs WT HC
WT FC vs HC
Fmr1-/y FC vs HC
SFARI
All
cumulative frequency
log2foldchange
G
Fmr1-/y HC vs WT HC
WT FC vs HC
Fmr1-/y FC vs HC
FMRP
All
cumulative frequency
log2foldchange

Figure 7. GSEA analysis and interrogation of distributions of specific populations of targets in WT/*Fmr1*$^{-/y}$ dataset after conditioning. **A)** GSEA analysis was performed on WT/*Fmr1*$^{-/y}$ FC TRAP dataset and plotted are the top 10 significantly upregulated and downregulated categories (FDR<0.01) respectively for *Fmr1*$^{-/y}$ HC, **B)** WT FC and **C)** *Fmr1*$^{-/y}$ FC comparisons. **D)** Interrogation of Ribosomal Populations respectively to *Fmr1*$^{-/y}$ HC (z=21.666, p-value<2.2e-16); WT FC (z=26.789, p-value<2.2e-16), RPs *Fmr1*$^{-/y}$ FC (z= -9.4161, p-value<2.2e-16) and RPs *Fmr1*$^{-/y}$ vs WT FC (z=-14.555, p-value<2.2e-16). **E)** Interrogation of binned populations of short and long genes as well as of shift in their translation respectively to WT FC (Ks <1kb vs >4kb, D = 0.092515, p-value = 0.03465; <1kb: z = 2.8647, p-value = 0.004174; 1kb-2kb: z = -4.8855, p-value = 1.032e-06; >2kb: z = -4.5851, p-value = 4.538e-06; >4kb: z = -1.531, p-value = 0.1258), *Fmr1*$^{-/y}$ HC (Ks <1kb vs >4kb, D = 0.30295, p-value < 2.2e-16, <1kb: z = 6.128, p-value = 8.901e-10; 1kb-2kb: z = -3.4216, p-value = 0.0006224; >2kb: z = -10.114, p-value < 2.2e-16; >4kb: z = -9.0996, p-value < 2.2e-16), *Fmr1*$^{-/y}$ FC (Ks <1kb vs >4kb, D = 0.18715, p-value = 8.368e-08, <1kb: z = -2.4913, p-value = 0.01273; 1kb-2kb: z = z = 0.52422, p-value = 0.6001; >2kb: z = 4.9508, p-value = 7.391e-07; >4kb: z = 5.624, p-value = 1.866e-08). **F)** Interrogation of SFARI targets respectively to *Fmr1*$^{-/y}$ HC (z=-8.6218, p-value<2.2e-16), WT FC (z= -4.2915, p-value=1.775e-05), *Fmr1*$^{-/y}$ FC (z=3.5145, p-value=0.0004406) populations. **G)** Interrogation of FMRP targets respectively to *Fmr1*$^{-/y}$ HC (z= -16.406, p-value<2.2e-16), WT FC (z= -5.3523, p-value=8.684e-08) and *Fmr1*$^{-/y}$ FC (z=4.5396, p-value=5.6362e-06) populations.

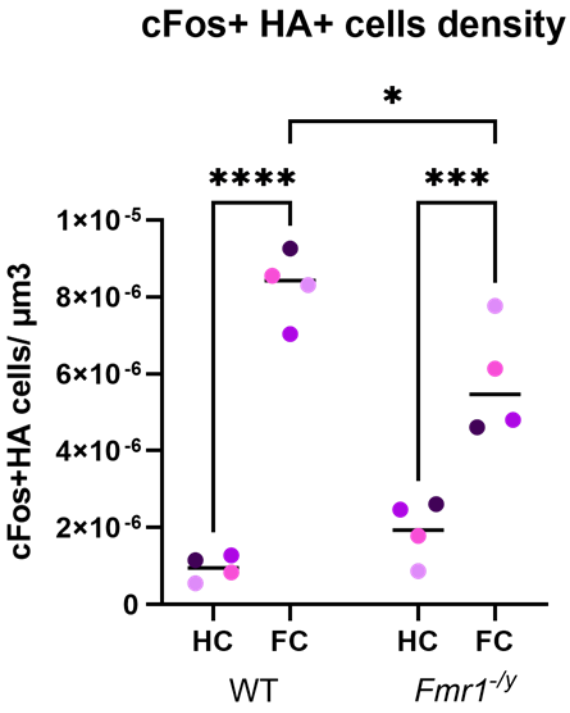


Figure 8. Cfos imaging in BLA after Fear Conditioning. Mice were habituated for 2 days to the contextual chamber and on the 3rd day the conditioned group underwent a training session of 3 shocks of 0.6 mA of the duration of 2 sec each 70sec, instead the home cage group was left in the cage. 90 minutes after the conditioning mice were sacrificed and perfused to image BLA specific ribosome bound fraction (HA antibody, Alexa Fluor® 488 anti-HA.1,16B12, 1:500), total population of neuons (NeuN, Merck; 1:500) and activated cells (Cfos, Abcam; 1:1000). Conditioning induces significant activation in both WT and *Fmr1*$^{-/y}$ mice at the level of BLA-specific ribosome bound fraction as represented by the higher density of Cfos cells overlapping with HA+ cells found in Conditioned mice vs Home Cage. The level of activation is significantly different between the two genotypes, with a decrease in activation for *Fmr1*$^{-/y}$ mice (2 way RM Anova, Genotype x Condition: P= 0.0330; Genotype: P= 0.0376; Condition: P<0.0001; Multiple comparisons FDR corrected. FC VS HC WT: P= 0.0002, FC VS HC *Fmr1*$^{-/y}$, P=0.0045; *Fmr1*$^{-y}$ vs WT HC: P=0.1769; *Fmr1*$^{-y}$ vs WT FC 0.0073).

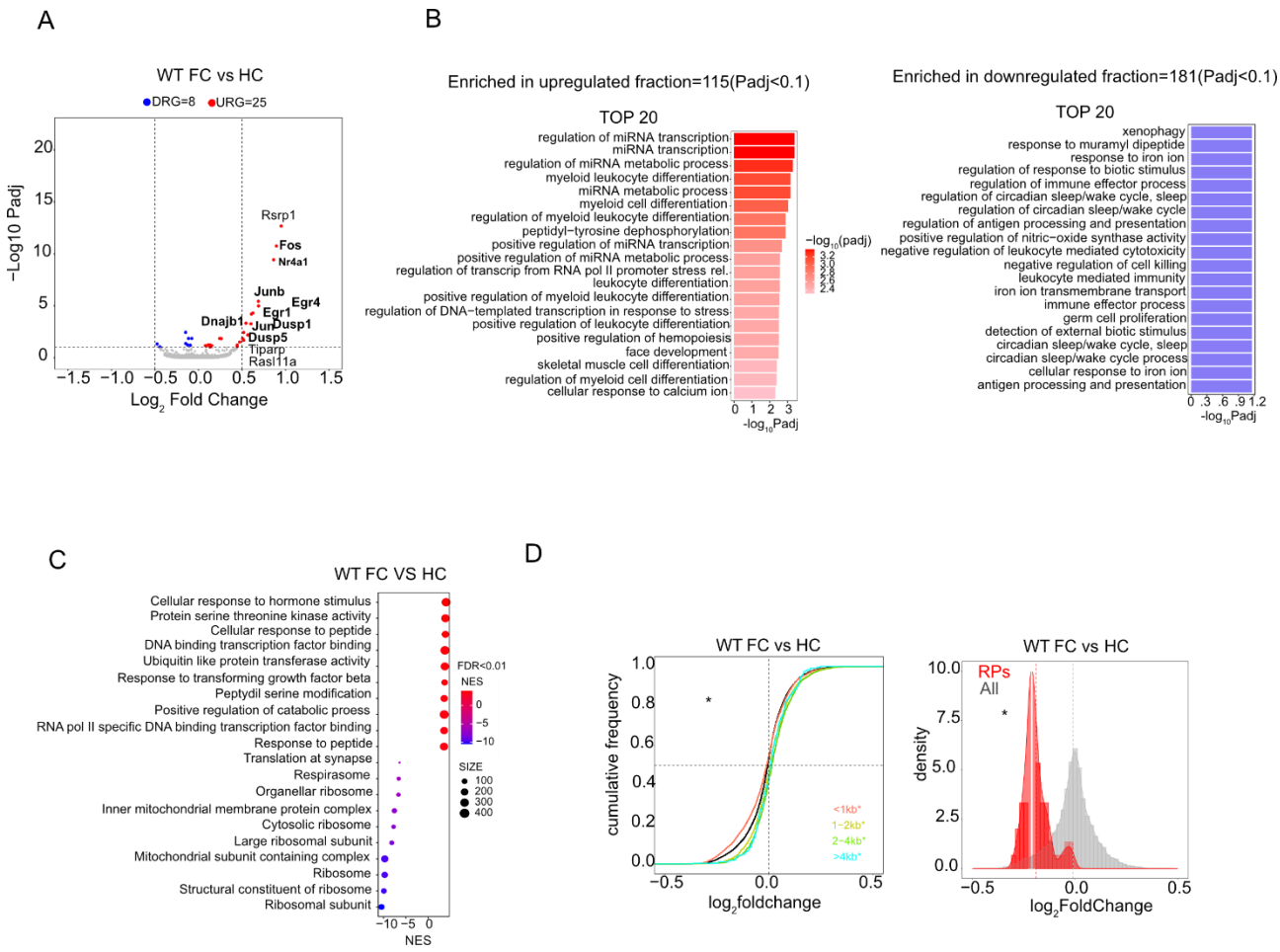


Figure 9. Characterization of WT profile after conditioning relative to Syngap$^{+/-}$ littermates. A) Differential expression analysis in WT after conditioning (WT FC) reveals upregulation of 25 transcripts vs downregulation of 8 transcripts. Significant population was determined as presenting a False Discovery Rate (FDR) <0.1. **B)** GO enrichment of differentially expressed population in WT FC TRAP reveals significantly upregulated and downregulated categories. **C)** GSEA analysis was performed on WT FC dataset and plotted are the top 10 significantly upregulated and downregulated categories (FDR<0.01). **D)** Ribosomal population is downregulated in WT FC (z= -26.056, p-value < 2.2e-16). Information about length for most abundant transcripts expressed in BLA specific neurons was retrieved and populations were binned accordingly to define a population of short (<1kb) and long (>2kb) genes that served to interrogate WT FC datset. Relative to WT FC population shift is present in translation of long vs short transcripts with short genes being downregulated and long ones being upregulated (Ks <1kb vs >4kb, D = 0.16658, p-value = 1.376e-06). Binned population are significantly shifted (<1kb: z = -5.858, p-value = 4.684e-09; 1kb-2kb: z = 8.4792, p-value < 2.2e-16; >2kb: z = 9.1557, p-value < 2.2e-16; >4kb: z = 4.0481, p-value = 5.163e-05).

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Thomson, S. R., S. S. Seo, S. A. Barnes, S. R. Louros, M. Muscas, O. Dando, C. Kirby, D. J. A. Wyllie, G. E. Hardingham, P. C. Kind, and E. K. Osterweil. 2017. 'Cell-Type-Specific Translation Profiling Reveals a Novel Strategy for Treating Fragile X Syndrome', *Neuron*, 95: 550-63 e5.

ESR13 – Loredana Cumpana

ESR13: overview of results towards the objective of the action

Project title – The effect of Corticotropin-releasing hormone on a thalamic hub for neurodevelopmental disorders

WP3 – Modulators of neuropsychiatric disorders

Early Stage Researcher 13 – Loredana Cumpana

Supervisors – Carmen Sandi and Simone Astori

Host Location – Brain Mind Institute (BMI), École Polytechnique Fédérale de Lausanne – Switzerland

The corticotropin-releasing hormone (CRH) is a peptide associated with stress and anxiety that acts as a potent modulator throughout the nervous system. The CRH receptor 1 (CRHR1) has been described to have a high expression in the thalamic reticular nucleus (TRN) neurons[1]. The TRN has a well-known role in sleep, as part of the NREM-promoting thalamo-cortical circuit. Particularly, it is important for generating sleep spindles, brief 10-15Hz oscillations occurring during NREM sleep, which in tandem with other oscillations have been related to sleep stability and memory consolidation[2,3].

We have confirmed the expression of CRHR1 in the TRN through a highly specific in situ mRNA hybridization method (RNAscope), and found that CRHR1 mRNA has the highest expression in parvalbumin positive TRN neurons (~97% of total TRN neurons), which comprise the sleep spindle generators[4,5]. In brain wide CRH manipulation studies, CRH has been described as a regulator of wakefulness, decreasing total sleep[6], whereas new studies begin to show its region-specific effects, such as the CRH neurons in the subthalamic nucleus increasing REM sleep as a stress-induced compensatory mechanism[7]. Considering the high expression of CRHR1 in spindle generating TRN neurons, our aim is to investigate the modulation of NREM sleep and sleep spindles by CRH through the activation of its receptor in the TRN.

Both male and female mice were used in most experiments, and no significant sex differences were found. CRHR1 mRNA expression in the TRN was also not different between males and females, therefore the data were pooled.

Objectives

Task 13.1 Impact of CRH on TRN cell excitability

We investigated the effects of CRH on TRN neuron firing modalities (low threshold calcium bursts vs. tonic firing) using ex-vivo electrophysiology, recording in whole-cell configuration from mouse acute brain slices containing the TRN. Bath application of CRH (200nM-1µM) significantly decreased the number of bursts and increased tonic firing in response to somatic depolarizations (Fig. 1).

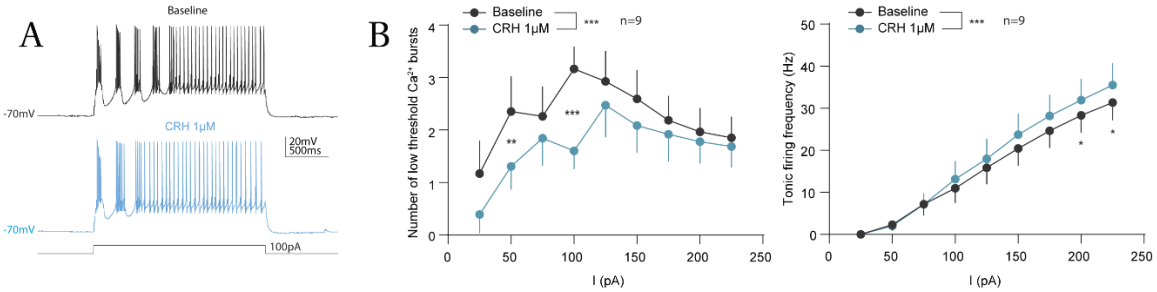


Fig. 1 – CRH reduces burst firing in TRN neurons. (A) Example traces of current-clamp recordings showing the low-threshold bursting activity followed by tonic firing of TRN neurons with and without CRH application. **(B)** Bath-applied CRH decreases low-threshold bursting, and consequently promotes tonic firing (for Bursts: Between groups ***p<0.0001, RM 2way-ANOVA, Sidak's multiple comparisons). **(C)** Dose-response curve of CRH effect on TRN bursting (*p=0.01, One-way ANOVA between groups, post-hoc Tukey's multiple comparisons).

The selective CRHR1 antagonist (NBI39565, 3µM) abolished the CRH-induced change in firing modality (Fig. 2).

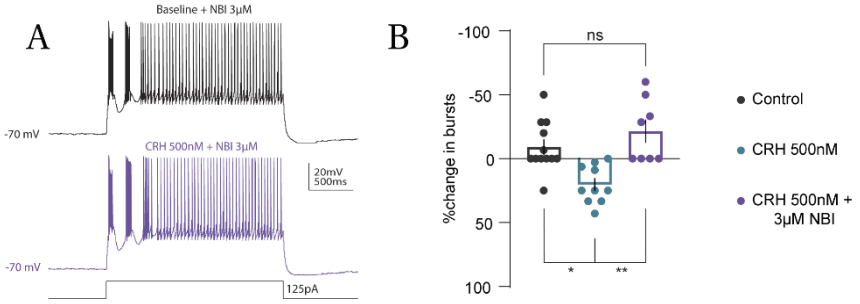


Fig. 2 – The decrease in burst firing is dependent on CRHR1. (A) Example traces of current-clamp recordings showing the low-threshold bursting activity followed by tonic firing of TRN neurons with and without CRH application under constant inhibition of CRHR1. **(B)** The selective CRHR1 antagonist NBI 35965 abolished the CRH-induced effects on the TRN firing (**p=0.001, One-way ANOVA between groups, post-hoc Tukey's multiple comparisons).

The decrease in bursting activity was replicated with endogenous release of CRH as well. CRH release was induced via photostimulation of axons contacting the TRN in transgenic mice expressing ChannelRhodopsin2 (ChR2) in all CRH neurons (Ai27DxCRH_IRES_Cre mice). A train stimulation at 20Hz induced a similar decrease in the number of bursts as seen with exogenous bath application of CRH (Fig. 3B,C). Inhibition of CRHR1 prevented the decrease in bursts in this condition as well (Fig. 3D).

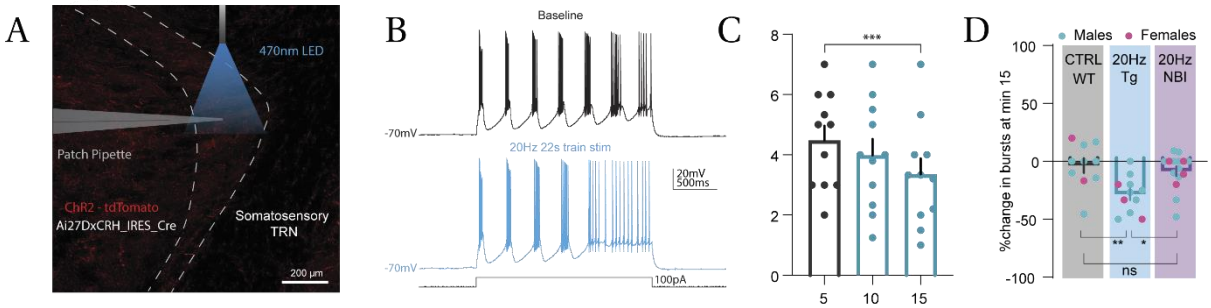


Fig. 3 – Replication of burst decrease with ex-vivo endogenous release of CRH using optogenetic activation. (A) Diagram depicting the ex-vivo stimulation protocol in transgenic mice expressing ChR2 in all CRH neurons with a blue LED to elicit the endogenous release of CRH in the axons contacting the TRN. **(B)** Example trace of bursts decreasing after photostimulation of the CRH axons. **(C)** Optogenetic stimulation of CRH afferents reproduced the effects seen with exogenous CRH application, by decreasing the number of bursts (***p<0.0001, RM 2way-ANOVA, Sidak's multiple comparisons). **(D)** CRHR1 inhibition by NBI abolished the effects of endogenous CRH (**p=0.003 One-way ANOVA, Tukey's multiple comparisons).

Next, we investigated the effect of CRH on TRN's synaptic input. The TRN receives the majority of its glutamatergic input from the thalamus and the cortical layers 5 and 6[8,9]. While electrically stimulating axons in the internal capsule, we saw that CRHR1 increased the excitatory postsynaptic potentials (EPSPs) of cortical origin only, differentiated from the thalamic inputs through a paired-pulse protocol. This effect was also replicated using endogenous CRH release in Ai27DxCRH_IRES_Cre mice (Fig. 4).

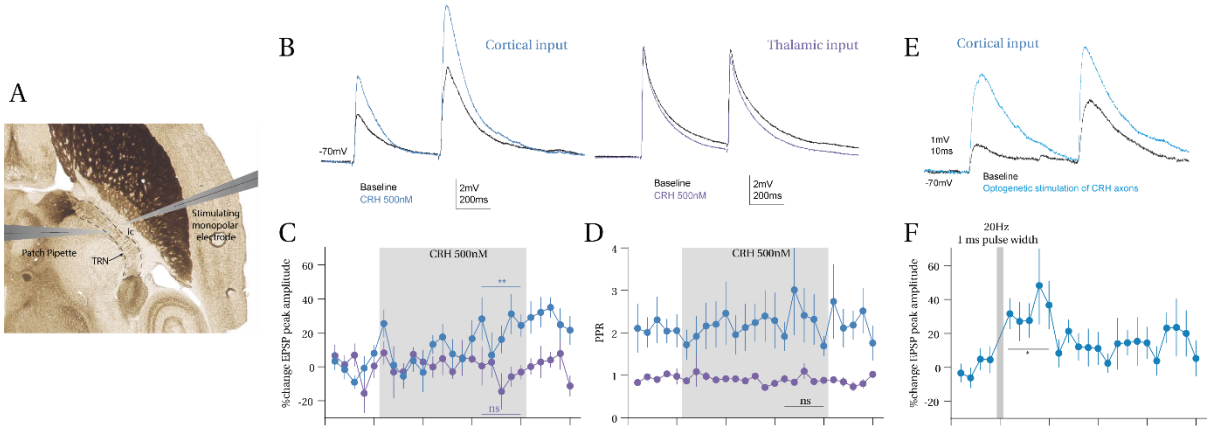


Fig. 4 - CRH increases cortical inputs to the TRN in electrically evoked EPSPs. (A) Diagram depicting the electrical stimulation protocol for inducing compound EPSPs in the TRN by stimulating afferent axons in the internal capsule. **(B)** Synaptic responses were sorted as either majority in cortical or thalamic origin based on a paired-pulse protocol (PPR), where cortical inputs show paired-pulse facilitation, whereas thalamic inputs induce paired-pulse depression. **(C)** Bath-application of CRH selectively increased the amplitude of cortical inputs (***<.0001 2way-ANOVA), **(D)** with no change in the PPR, suggesting a postsynaptic mechanism. **(E, F)** Endogenous CRH release via photostimulation of ChR2 in CRH_IRES_CrexAi27D mice replicated the effect on cortical EPSPs (*p=0.02, paired t-test, n=6).

To verify the CRH effect selectively on cortical inputs, we used transgenic mice (Ai27DxNtsr1_Cre) that express ChR2 selectively in layer 6 of cortical neurons that provide the cortical input to the TRN. In these mice, we can elicit EPSPs of purely cortical origin using photostimulation of the cortical afferents. When bath-applying CRH in this condition, the effect on cortical inputs was not observed (Fig. 5), indicating that an additional factor was being released when eliciting EPSPs using electrical stimulation, contributing to the previously observed effect. As noradrenaline (NE) is known to be released at the TRN during NREM sleep and is able to impair sleep spindles[10], we added NE to the bath, which reinstated the CRH-induced increase in EPSPs. NE by itself was not able to elicit an increase in the cortical EPSP amplitude, but only when co-applied with CRH. In addition, the selective α1-receptor antagonist (Prazosin, 5µM) was able to abolish the CRH effect, demonstrating that concomitant CRH and NE receptor activation is needed to increase synaptic transmission.

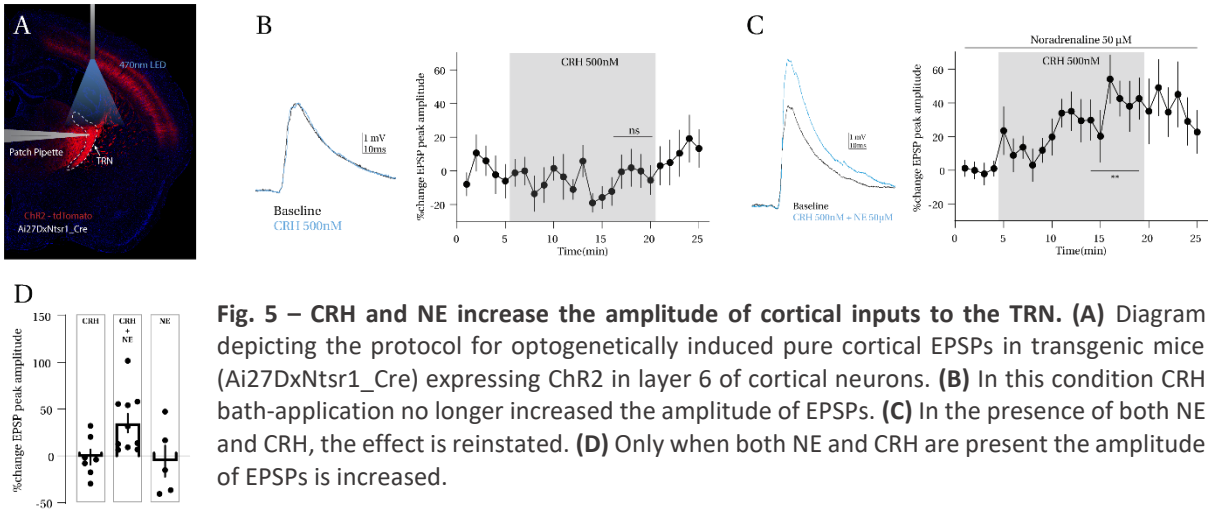


Fig. 5 – CRH and NE increase the amplitude of cortical inputs to the TRN. (A) Diagram depicting the protocol for optogenetically induced pure cortical EPSPs in transgenic mice (Ai27DxNtsr1_Cre) expressing ChR2 in layer 6 of cortical neurons. **(B)** In this condition CRH bath-application no longer increased the amplitude of EPSPs. **(C)** In the presence of both NE and CRH, the effect is reinstated. **(D)** Only when both NE and CRH are present the amplitude of EPSPs is increased.

In summary, we saw that CRHR1 is able to decrease the number of bursts in the TRN, which are the generating mechanism of sleep spindles[3]. Additionally, together with NE, CRHR1 impacted the weight of the cortical input to the TRN, which has been shown in computational models to be able to terminate sleep spindles[11]. These data suggest a potential effect of CRH on sleep spindles, which we explored further in vivo in Task 13.3.

Task 13.2 Identification of the endogenous sources of CRH onto TRN

Employing transgenic mice (CRH_IRES_Cre) and viral methods, we mapped the source of endogenous CRH projections to the TRN. Using a retrograde Cre-dependent AAV2 stereotactically injected in the somatosensory TRN, we labelled all CRH projecting somas and axons contacting the TRN, which showed a diversity of candidate regions. To anatomically confirm the regions identified through retrograde tracing, we injected an anterograde Cre-dependent AAV1 in the CRH projecting regions and identified labelled axons going to the TRN. Our data indicate that the CRH afferents have diverse origins, contacting the TRN from different brain regions: anterior baso-lateral amygdala (aBLA), dorsal zona incerta (ZID), medial and superior geniculate nucleus (MGM and SGN). Other regions that have not been anterogradely confirmed yet but are promising candidates from retrograde tracings are: the ventrolateral geniculate nucleus, the parvocellular region (VLGPC), and layer 6 of the medial entorhinal cortex. No projections were found from classical CRH-releasing hypothalamic regions. This suggests that the TRN is not directly influenced by CRH release within the hypothalamic-pituitary adrenal axis that governs the neuroendocrine response to stress, but by diverse extra-hypothalamic regions throughout the central nervous system in majority of subcortical nature (Fig. 6).

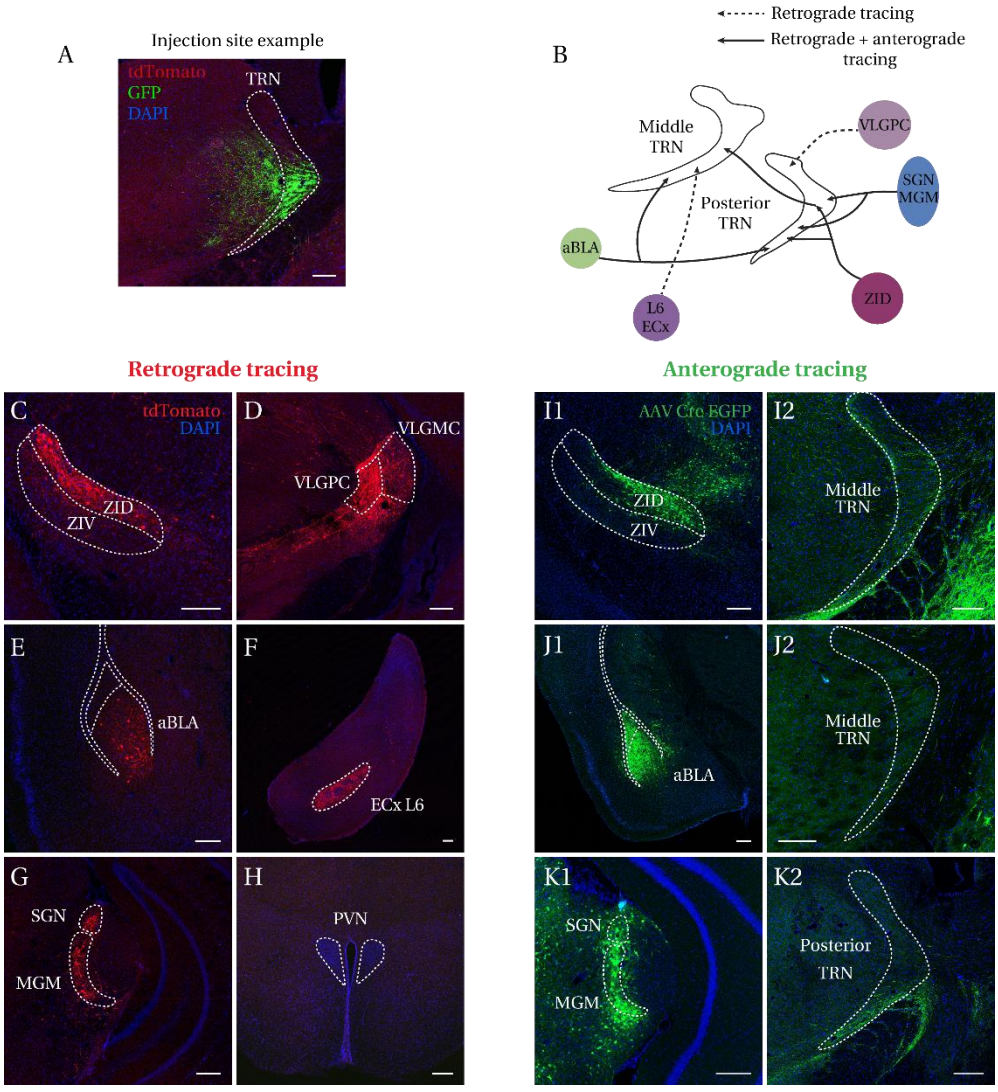


Fig.6 – Sources of CRH projections to the TRN. (A) Example of injection site in the TRN for retrograde tracing, visualized with green (GFP) through a synapsin dependent AAV, co-injected with the retrograde Cre-dependent AAV which labels CRH projections with red (tdTomato). **(B)** The CRH projections were found to be diverse and region-specific, as depicted in the diagram showing a summary of the anatomically confirmed regions (filled arrows) as well as the other candidate regions identified through retrograde tracing (dotted arrows). The different sources of CRH appeared to project to anatomically defined regions within the TRN, based on its known connectivity with the respective brain structures. (All scale bars are 200µm). **(C-G)** Identified candidate sources of CRH through retrograde tracing include the dorsal zona incerta (ZID), anterior basolateral amygdala (aBLA), superior and medial geniculate nucleus (SGN, MGM), ventrolateral geniculate nucleus containing parvocellular cells (VLGPC), and the layer 6 of the medial entorhinal cortex (ECx). **(I1-K2)** Three of the sources of CRH were confirmed anatomically using anterograde tracing in the same mouse line. **(H)** No projections were found from hypothalamic regions, such as the paraventricular nucleus of the hypothalamus (PVN).

Task 13.3 Impact of CRH on thalamocortical sleep rhythms

We investigated the impact of CRH on TRN-related sleep rhythms by increasing or decreasing CRH release at the TRN via optogenetic manipulations in vivo. The mice were implanted with EEG (2 fronto-occipital derivations) and EMG electrodes, as well as with optic fibers bilaterally over the TRN to deliver the optogenetic stimulation. All mice from any EEG experiment underwent behavioural phenotyping for anxiety in two timepoints (before and after surgeries corresponding to pubertal and adult life stages), which will be used to correlate their anxiety state with different parameters from the EEG experiments when the final dataset is reached. To increase the CRH release at the TRN, we used the transgenic mouse line Ai27DxCRH_IRES_Cre, which expresses ChR2 in all CRH neurons. To decrease the CRH release, we use a novel inhibitory opsin Parapinopsin (PPO), a modified G-protein coupled receptor with an inhibitory G-protein (Gi) that is able to inhibit neurotransmitter release presynaptically[12]. PPO was virally expressed bilaterally in the TRN using a Cre-dependent retrograde virus in CRH_IRES_Cre mice. The expression of PPO shows labelled CRH positive axons in the TRN, and labelled CRH positive neurons in the CRH projecting regions identified in Task 13.2 (Fig.7). All optogenetic manipulation experiments have 2 days of baseline recordings of undisturbed sleep performed on the days before the optogenetic manipulation day. Then, all optogenetic manipulation recordings were performed using a closed-loop stimulation paradigm based on threshold rules for sigma (10-15Hz) and slow (0.5-1Hz) oscillation powers, to detect and stimulate selectively during consolidated NREM sleep (ChR2-20-10Hz, 5ms pulse width, 10-50s, ~1.5mW at the fiber tip; PPO-10Hz, 10ms pulse width, 10s, ~1.0mW at the fiber tip).

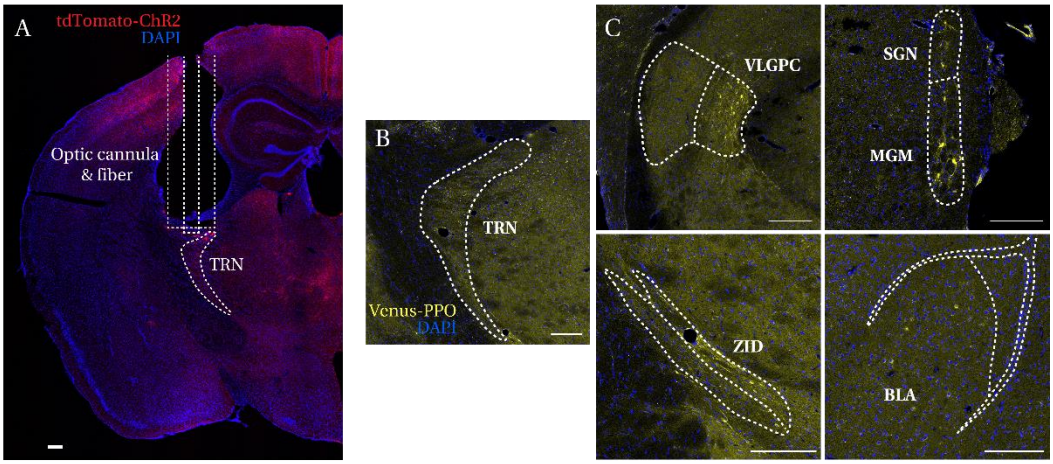


Fig.7 – Opsin expression and optic fiber placement. (A) ChR2 (tdTomato-red) endogenously expressed in all CRH producing neurons in the mouse line Ai27DxCRH_IRES_Cre used for increasing CRH release at the TRN. Dotted lines represent the optic fiber placement over the TRN **(B)** Close-up of axons in the TRN labelled with Venus (yellow) indicating the viral expression of PPO, used to inhibit CRH release. **(C)** Labelled soma from the virally expressed PPO showing the same CRH sources as described in Task 13.2. All scale bars are 200µm.

When increasing CRH release at the TRN, the number of microarousals during NREM sleep were increased. On the other hand, when inhibiting CRH release at the TRN, NREM sleep was more consolidated, showing increased bout length and decreased bout count, with no change in total NREM sleep (Fig.8A). The relevant frequency band for NREM are the slow, delta and sigma oscillations, which show no change in average power with either opsin (Fig.8B).

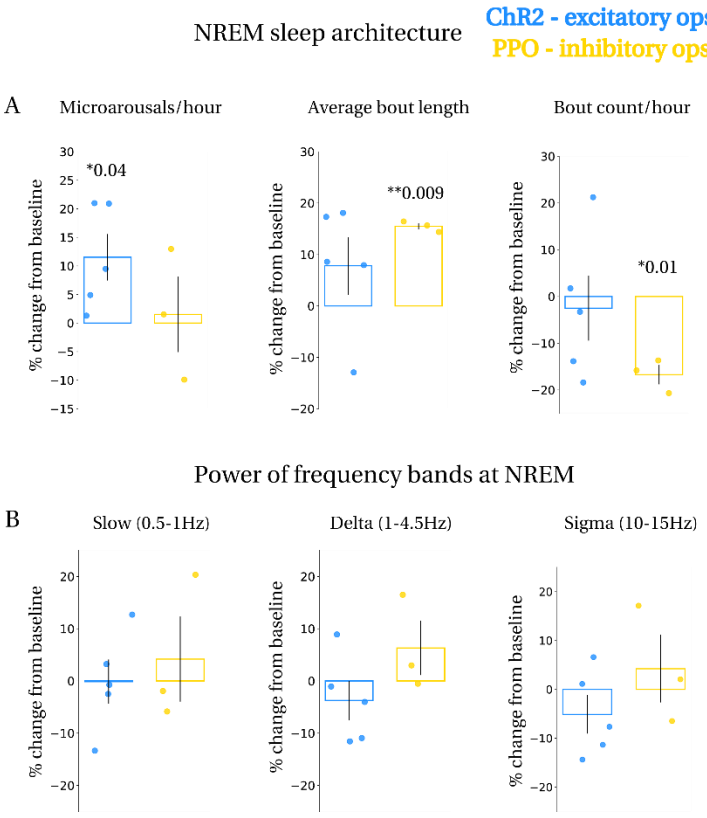


Fig.8 – Effect of CRH manipulations of NREM sleep architecture and frequencies. (A) ChR2 increased the number of microarousals, whereas PPO increased the average bout length and decreased the bout count, consolidating NREM sleep. **(B)** No significant changes in frequency bands relevant for NREM sleep. All statistics are paired t-test against each recording's baseline.

During the last 50-25 seconds of NREM sleep before a switch to REM sleep, also known as the transition to REM sleep or intermediate sleep, the sigma power band, used a proxy for sleep spindles (peaks indicating clusters of sleep spindles), shows a large surge. The surge in the sigma oscillation in the transition to REM sleep showed a larger latency to REM sleep with ChR2, whereas the peak amplitude of the surge was increased with PPO (Fig.9).

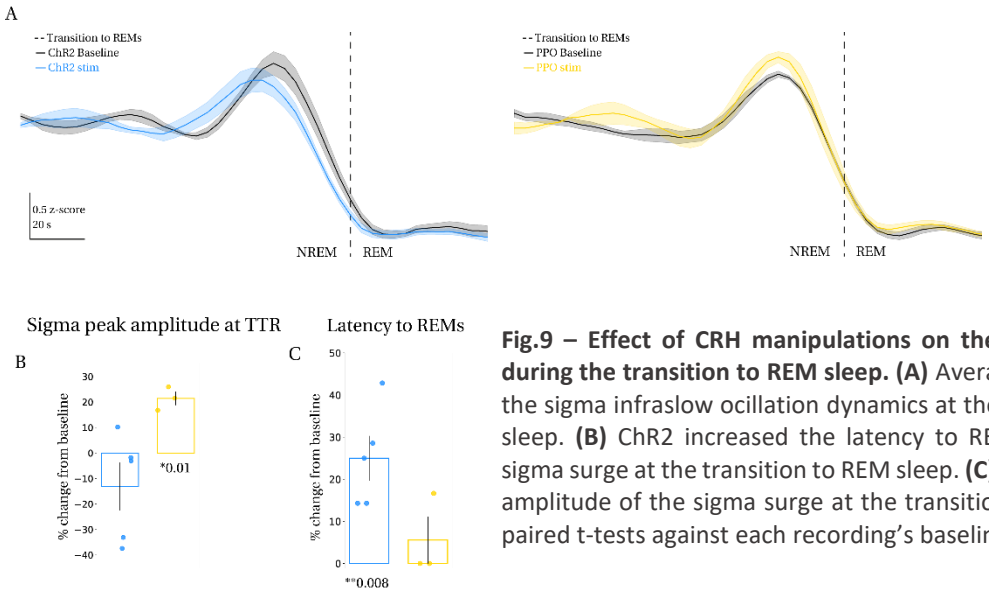


Fig.9 – Effect of CRH manipulations on the sigma oscillation during the transition to REM sleep. (A) Average over all mice of the sigma infraslow ocillation dynamics at the transition to REM sleep. **(B)** ChR2 increased the latency to REM sleep from the sigma surge at the transition to REM sleep. **(C)** PPO increased the amplitude of the sigma surge at the transition. All statistics are paired t-tests against each recording's baseline.

Task 13.4 Impact of stress on TRN gene expression profile

We conducted an exploratory study on gene expression in the TRN using samples from animals exposed to peripubertal stress (PPS). Wild-type mice underwent a PPS paradigm that aims to model exposure to unpredictable stressful experiences of non-social nature during late childhood and puberty[13]. On 7 days scattered between PN28 and PN42, animals from the stress group were exposed to innate and physical stressors in a repetitive but unpredictable manner. An aged-matched control group was exposed only to handling. At PN70, the mice underwent behavioural characterization, such as open-field/novel object, and social interaction/social preference tests. At PN77 the mice were sacrificed and the brains were collected. The tissue containing the TRN was dissected and processed for qPCR. The samples were quantified for 17 genes, such as the CRH receptors, GABAergic makers present in the TRN, potassium and calcium channels, as well as genes related to neurodevelopmental disorders. The stress group was further split into vulnerable and resilient based on the results from the social preference test (Fig.10). We found no difference in the relative gene expression between the control and stress group for CRHR1, but an increase in CRHR2 only in the mice vulnerable to the PPS. The gene GRIN2C, associated with schizophrenia, was increased in the vulnerable group, whereas the gene CHD2, associated with Autism Spectrum Disorder (ASD), was decreased in the vulnerable group and increased in the resilient group. No other significant changes were found between groups in the tested genes related to the expression of channels and receptors in the TRN that are responsible for burst rhythmogenesis.

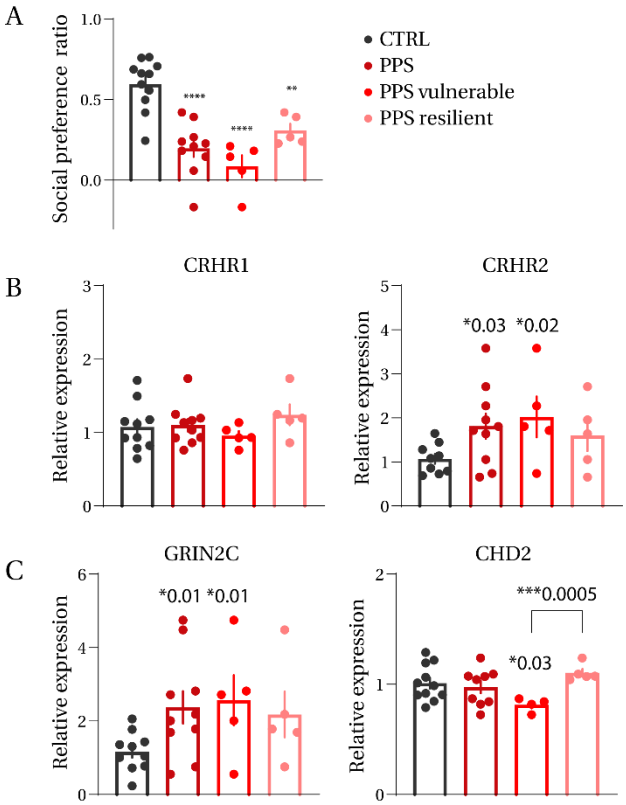


Fig.10 – Impact of peripubertal stress on gene expression in the TRN. (A) The PPS group was segregated into vulnerable or resilient based on the social preference score. **(B)** CRHR1 relative expression showed no change, whereas the vulnerable PPS group showed an increase in CRHR2. **(C)** GRIN2C and CHD2 levels were found to be significantly different compared to controls. All statistics are unpaired t-tests.

Task 13.5 Dynamics of CRH release at the TRN during sleep

Using a genetically-encoded CRH sensor, GRAB_CRH, combined with EEG recordings, we investigated the CRH release at the TRN throughout vigilance state. Fiber photometry recordings of the GRAB_CRH showed that surprisingly, CRH release at the TRN is higher during NREM sleep, compared to wakefulness or REM sleep. Control recordings performed with a mutated variant of the GRAB_CRH that is CRH-insensitive showed decreases during wakefulness and REM sleep in the same direction as

the CRH-sensitive sensor but with a smaller amplitude, indicating that there are other factors influencing the signal readout particularly during those vigilance state, such as blood flow or pH changes. During NREM sleep, the CRH sensor showed a large oscillatory pattern which was not present in the controls, that anticorrelated with the sigma infraslow oscillation, where peaks represent clusters of sleep spindles (Fig.11). These data further indicate a relationship between CRH release at the TRN and sleep spindles, providing additional insight into how CRH may modulate NREM sleep.

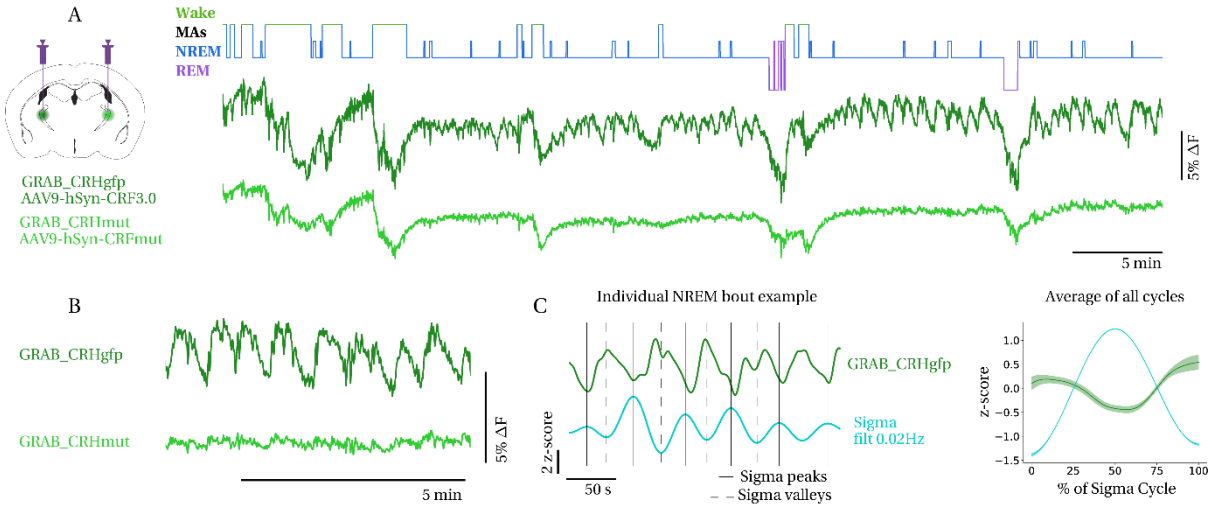


Fig.11 – Dynamics of CRH release at the TRN during NREM sleep. (A) Mice were injected in one hemisphere with the GRAB_CRH sensor, and with a control GRAB_CRHmut, that is CRH-insensitive, in the contralateral hemisphere. On the right is an example trace of concomitant recordings from both hemispheres matched against the synchronised sleep recordings, showing that there are other factors influencing the signal readout especially during wakefulness and REM sleep. **(B)** During NREM sleep, CRH shows an oscillatory pattern that is not apparent in the control. **(C)** The CRH release during NREM anticorrelated with the sigma infraslow oscillation.

In summary, we show that CRH modulates NREM sleep homeostasis by acting on spindle generating mechanisms in TRN neurons. In particular, decreasing CRH levels can stabilize NREM sleep and improve the sigma oscillation, which has been shown to increase cognitive abilities during wakefulness and mitigate the symptomatology of various psychiatric disorders[2,14]. Revealing the contribution of the stress and anxiety related peptide CRH on sleep rhythms contributes to our understanding of the fundamental modulation of sleep but can also be used to explore further its implications in pathology. Upon the completion of this data set in the near future we will understand if there are correlations between anxiety and the vulnerability to CRH-induced changes in NREM sleep. Additionally, as the TRN has been described to be part of a circuit endophenotype altered in neurodevelopmental disorders[15], our findings could have implications for sleep impairments often associated with such disorders but also various other psychiatric conditions.

Potential of results for exploitation

The mechanisms we describe could be used as novel targets for pharmacologically treating stress or anxiety induced sleep impairments. Furthermore, potential associations between CRH modulation of sleep or NREM sleep parameters and anxiety might serve as new biomarkers in clinical studies and contribute to the identification of individuals at risk for developing sleep impairments or psychiatric disorders.

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ESR14 – Vanesa Salazar

ESR14: overview of results towards the objective of the action

Project title – Circuit deficits associated with ASD/ID

WP3 – Modulators of neuropsychiatric disorders

Early Stage Researcher 14 – Vanesa Salazar

Supervisor – Peter Kind

Host Location – Centre for Discovery Brain Sciences (CDBS), University of Edinburgh – Scotland

Introduction and goals

SYNGAP1 haploinsufficiency is one of the most common monogenetic causes of intellectual disability (ID) cases and autism spectrum disorder (ASD). In addition, approximately 80% of people with *SYNGAP1* haploinsufficiency present generalized epilepsy(Michaelson et al., 2018; Mignot et al., 2016). *SYNGAP1* haploinsufficiency presents with an autosomal dominant mode of inheritance and is triggered by *de novo* mutations in the *SYNGAP1* gene resulting in a 50% reduction in *Syngap1* protein expression(Mignot et al., 2016). Alterations in social behaviour, especially social interaction and communication impairments, are one of the major traits in *SYNGAP1* haploinsufficiency and in general in ASDs(*American Psychiatric Association*, 2019; Mignot et al., 2016). As the precise cellular and circuit dysfunction that underlie abnormal social behaviours are difficult to ascertain from human studies due to high degrees of variability, using rodent models can help us gain novel and useful insights. In this project I will be using two novel rat models of this project I will be using two novel rat models of *SYNGAP1* haploinsufficiency.

Historically, *SYNGAP1* haploinsufficiency has been modelled using mice(Kim et al., 2003; Komiyama et al., 2002; Nakajima et al., 2019). However, ethologically, male mice social interactions are limited and predominantly antagonistic since they live in single-male territorial structures (Van zegeren, 1979). In contrast, rats live in complex hierarchical colonies with more dynamic dominance structures and exhibit complex social behaviours such as social play(Schweinfurth, 2020). Therefore, I aim to study social interactions in ASDs rat models to take advantage of their complex social repertoire.

SynGAP is a RasGAP protein highly expressed in glutamatergic synapses, specifically in the post-synaptic density PSD-95 complex with NMDA receptors(Kim et al., 1998). The main functional domains in SynGAP are a RasGAP domain, a C2 domain and a PH domain. The RasGAP domain is responsible for the SynGAP negative regulation of Ras/Rap via upregulation of GTP hydrolysis of Ras/Rap(Kim et al., 1998; Zhu et al., 2002). The calcium dependent lipid binding or C2 domain is essential for the normal SynGAP Rap-GTPase activity in addition to mediating calcium binding(Pena et al., 2008). The pleckstrin homology or PH domain is positioned in the N-terminus and its sequences is only truncated in the SynGAP - C isoform. The PH domain is suggested to act in membrane trafficking and protein-protein interactions by binding the phosphatidylinositol of cell membrane(Chen et al., 1998; Gamache et al., 2020).

Most pathologic mutations of SYNGAP1 detected in individuals lead to half expression of the total quantity of SynGAP protein and therefore to an *haploinsufficiency*. *Syngap1*$^{+/-}$ rats were generated to

study the consequences of *Syngap1* protein reduction in heterozygosis. On the other hand, although most identified mutations in individuals cause 50% reduction of *Syngap1* protein, several pathogenic missense mutations are identified in the C2 and GAP domain(Mignot et al., 2016). To model these conditions, rats with a selective deletion of the C2 and GAP domain (*Syngap1*$^{\Delta/GAP}$ rats) were generated.

A recent study in the lab showed on a version of the 3-chamber task that both *Syngap1*$^{\Delta/GAP}$ and wildtype (WT) rats, spend more time sniffing the social than the empty cage. However, the *Syngap1*$^{\Delta/GAP}$ rats spend less time sniffing the social cage than wildtype rats (Katsanevaki et al., 2020). These results suggest social impairments in the *Syngap1*$^{\Delta/GAP}$ rat model. These results suggest some impairments in social behaviour, but the task does not allow us to determine the precise characteristics of these impairments. Therefore, alternative, and better designed paradigms need to be used. Furthermore, to gain a complete understanding of the underlying biology that leads to impairments in social interaction we will need to study the underlying brain circuits mediating these altered behaviours. Moreover, the development of new therapeutic strategies for *SYNGAP1* and NDDs disorders highly rely on the study of synapse-circuit duality during development(Frye, 2018). However, little is known about the specific circuit deficits causing social interaction alterations in *SYNGAP1* haploinsufficiency.

Hypothesis: SYNGAP protein is necessary for the normal expression of social behaviour

- Characterization of social behaviour in two rat models of *SYNGAP1* haploinsufficiency
- Which are the circuits' deficits underlying social alterations in *SYNGAP1* haploinsufficiency?

Results overview

I designed a task to address how social interactions are altered with a new conspecific (stimulus), whether a subject can habituate to new conspecific and discriminate in a short period of time. A conspecific habituation dishabituation task (Figure 1A). Briefly, after 10 min on a previously habituated arena, the subject is presented a first stimulus rat (non-familiar and non-related) and they are let to freely explore each other for 5 min (exposure 1). This is repeated for a total of 4 times with 5 min inter-exposure. On the fifth exposure, the subject is presented a second stimulus (non-familiar and non-related).

Both *Syngap1*$^{+/\Delta GAP}$ and *Syngap*$^{+/-}$ rats display a lower social exploration time (SET) than wildtype littermates (Figure 1B, 1C) while their ability to habituate to the first stimulus and discriminate it from a second stimulus remains unaltered (Figure 1D, 1F). Furthermore, we addressed whether the speed at which *Syngap* rats habituate and encode the first stimulus was different from wildtypes. Social exploration time was normalized to their initial performance on the first exposure (% of habituation) and demonstrated there are no differences at the rate of habituation (Figure 1E). Performance of stimuli rats was analysed and showed no differences between stimuli rats, regardless of the genotype they interacted with (Figure 1G, 1H). These results provide evidence differences in *Syngap* subjects are not due to different behaviour from their stimuli group. All in all, these results highlight the enzymatic domain of SYNGAP as necessary for the normal expression of social interest while it might not be essential for social memory.

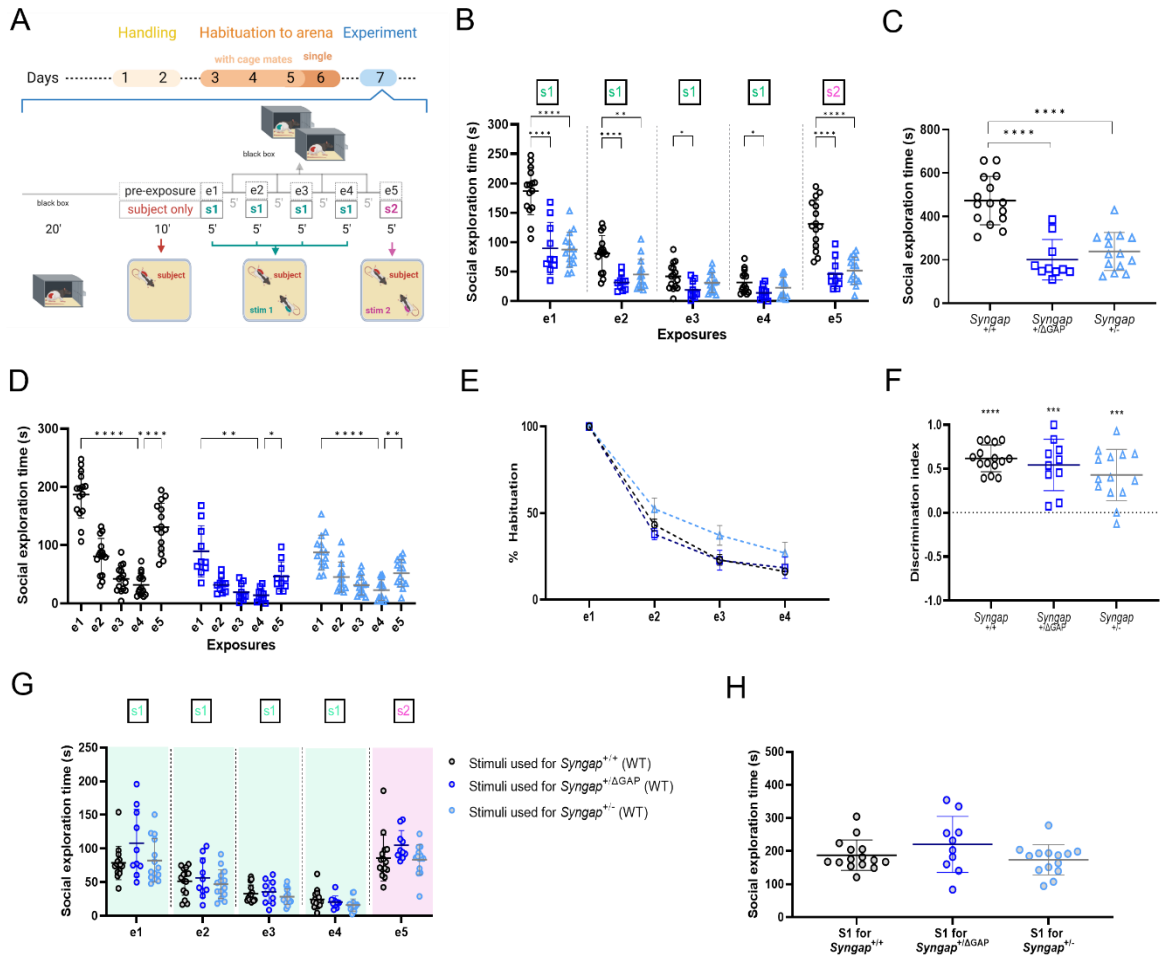


Figure 1 **A. A conspecific habituation-dishabituation task. B. Comparison by genotype of SET among exposures on subjects.** Syngap$^{+/\Delta GAP}$ and Syngap$^{+/-}$ present less SET than WT on exp 1, exp 2 and exp 5 (Two-way ANOVA, multiple comparisons, post-hoc Tukey corrected). **C. Total SET on subjects.** Syngap$^{+/\Delta GAP}$ and Syngap$^{+/-}$ present less SET than WT (One-way ANOVA).**D. SET among exposures on subjects.** Syngap$^{+/\Delta GAP}$ and Syngap$^{+/-}$ as well as WT habituate to the S1 conspecific since they show a significant decreased in SET between exp 1(S1) to exp 4 (S1). Two-way ANOVA, multiple comparisons, post-hoc Tukey corrected. **E. SET normalized by SET on exp 1 and expressed as a percentage.** Syngap$^{+/\Delta GAP}$ and Syngap$^{+/-}$ don't present statistical different % of habituation when compared to WT or between them. Plotted mean with SEM. Two-way ANOVA, multiple comparisons, post-hoc Tukey corrected. **F. Discrimination index.** (DI = SET e5-e4/e5+e4) WT, Syngap$^{+/\Delta GAP}$ and Syngap$^{+/-}$, they all present significant difference from chance (DI=0) (one sample t-test, Benjamini, Krieger and Yekutieli correction). There is no difference between Syngap lines and WT nor between Syngap$^{+/\Delta GAP}$ and Syngap$^{+/-}$ (One-way ANOVA). **G. SET among exposures on stimuli.** SET of S1/S2 used for Syngap$^{+/\Delta GAP}$ and Syngap$^{+/-}$ is not different from SET of S1/S2 used for WT for any exposures. (Two-way ANOVA, multiple comparisons, post-hoc Tukey corrected. **H. Total SET on stimuli 1.** S1(WT) used for Syngap$^{+/\Delta GAP}$ and Syngap$^{+/-}$ present no different SET than S1 (WT) used for WT nor between them (One-way ANOVA). Unless specified mean with SD plotted. Significance considered when $p \leq 0.05$, (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$) (n_{WT} =15, $n_{+/\Delta GAP}$ =10, $n_{+/-}$=14, $n_{S1\text{-}WT}$ =15, $n_{S1\text{-}+/\Delta GAP}$ =10, $n_{S1\text{-}+/-}$=14, $n_{S2\text{-}WT}$ =15, $n_{S2\text{-}+/\Delta GAP}$ =10, $n_{S2\text{-}+/-}$ =14)

Exploration of different body parts has been linked to different communication signal, having a main role in communication of hierarchy (Wesson, 2013). This analysis and results are especially relevant from a translational point of view since allow to compare results to the time spent on different face parts (e.g. eye, mouth) carried out on patients. Therefore, we analysed this aspect of social interaction over the whole task duration. Both *Syngap1*$^{+/\Delta GAP}$ and *Syngap*$^{+/-}$ rats showed an altered preference hierarchy between body parts compared to wildtype subjects (Figure 2A) as well as a decreased interest in anogenital exploration (Figure 2B, 2C).

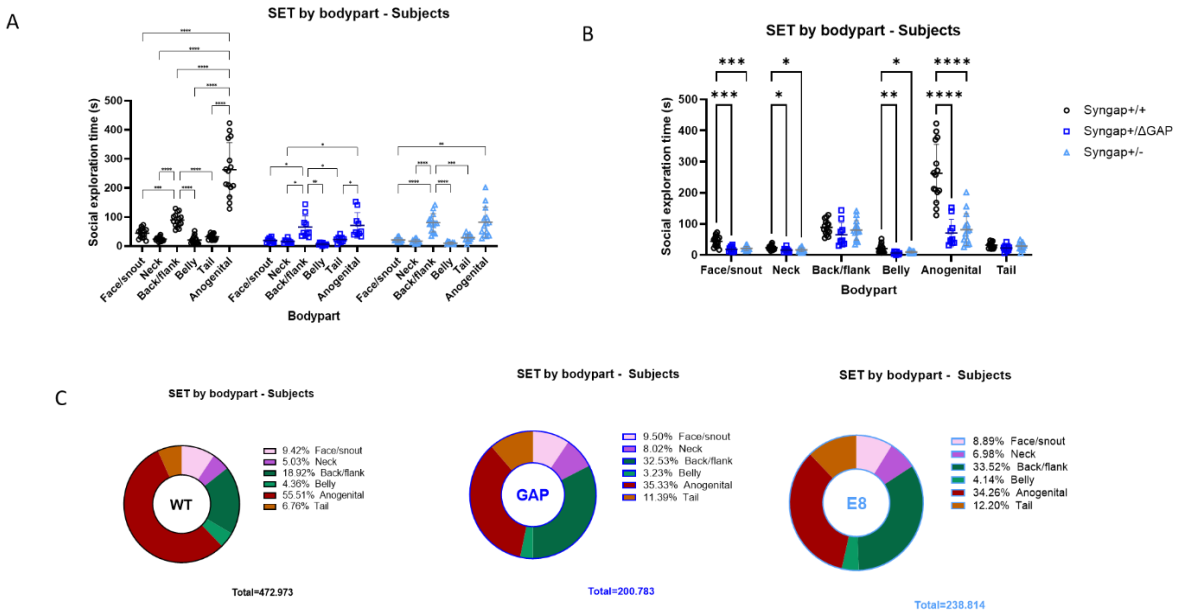


Figure 2. **Distribution of Social exploration time (SET) by bodypart. A)** Comparison between bodyparts SET of the total task on subjects. Other significant comparisons not shown on graph are: Syngap$^{+/+}$: face vs neck (**), face vs belly (**) | Syngap$^{+/\Delta GAP}$: face vs belly (*), neck vs belly (*), belly vs tail (***) | Syngap$^{+/-}$: face vs belly (****), neck vs belly(*), neck vs ail (*), belly vs tail (***) (Two-way ANOVA, multiple comparisons post-hoc Tukey corrected). **B)** Comparison between genotype of SET by bodypart (Two-way ANOVA, multiple comparisons post-hoc Tukey corrected) **C)** Bodypart percentage of the total SET for each genotype group (subjects).

Unless specified mean with SD plotted. Significance considered when $p \leq 0.05$, (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$) ($n_{+/+}$=15, $n_{+/\Delta GAP}$ =10, $n_{+/-}$=14, $n_{S1for+/+}$ =15, $n_{S1for+/\Delta GAP}$ =10, $n_{S1for+/-}$=14, $n_{S2for+/+}$ =15, $n_{S2for+/\Delta GAP}$ =10, $n_{S2for+/-}$=14). SET = Social exploration time.

Regarding the study of communication during social interaction, ultrasonic vocalizations (USVs) were analysed. Ultrasonic vocalizations are emitted by rodents to communicate their internal states (Portfors, 2007). Generally 22 kHz are associated to aversive states and 50 kHz to appetitive states (Portfors, 2007). I analysed the recordings during the first exposure. Given that calls couldn't be assigned to a particular individual since both rats are being recorded, the unit of measure was the dyad. There were no differences on the 22 kHz range (Figure 3A, 3B) which can indicate *Syngap* rats don't feel social interactions stressful. Regarding the 50 kHz range, there are no differences on the number of calls (Figure 3C) and a slight trend (although not significantly) on *Syngap*-wildtype dyads showing a longer duration (Figure 3D). However, given the fact these results are by dyads and not individually, we cannot confirm *Syngap* rats don't display differences on the expression of positive calls associated to social interaction since wildtype stimuli rats could be overcompensating.

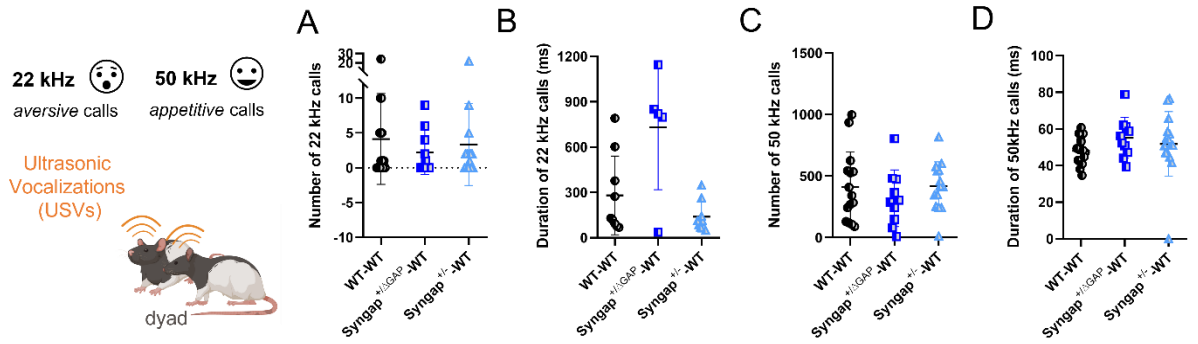


Figure 3. **A. Number of 22 kHz calls.** No difference between heterogeneous and homogenous dyads (Kruskal-Wallis Test, $n_{WT\text{-}WT}$ =15, $n_{+/\Delta GAP\text{-}WT}$ =10, $n_{(+/-)\text{-}WT}$=14). **B. Duration of 22 kHz calls.** No difference between heterogeneous and homogenous dyads (Kruskal-Wallis Test, $n_{WT\text{-}WT}$ =9, $n_{+/\Delta GAP\text{-}WT}$ =5, $n_{(+/-)\text{-}WT}$=9). **C. Number of 50 kHz calls**. No difference between heterogeneous and homogenous dyads (One-way ANOVA, $n_{WT\text{-}WT}$ =15, $n_{+/\Delta GAP\text{-}WT}$ =10, $n_{(+/-)\text{-}WT}$=14). **D. Duration of 50 kHz calls**. No difference between heterogeneous and homogenous dyads (One-way ANOVA, $n_{WT\text{-}WT}$ =15, $n_{+/\Delta GAP\text{-}WT}$ =10, $n_{(+/-)\text{-}WT}$=14). Unless specified mean with SD plotted. Significance considered when $p \leq 0.05$, (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$)

Travelled distance was analysed on subjects during habituation days and prior to social exposure. Results on subjects rats show no significant difference between *Syngap* subjects and wildtype subjects on the last habituation day (Figure 4B) and either on the pre-exposure (Figure 4C. These results discard that *Syngap* rats display hyperactivity on the arena.

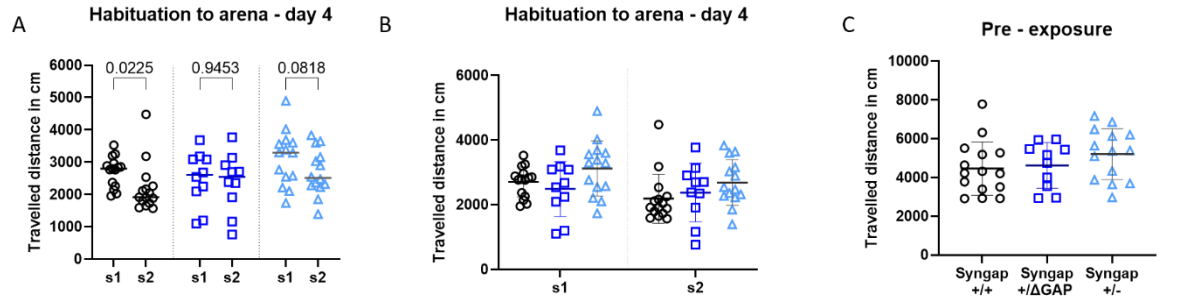


Figure 4. **Travelled distance on subject rats on last day of habituation on pre-exposure on day of experiment. A)** Decrease in travelled distance from session 1 to 2 on the 4th day of habituation. Two-way ANOVA, multiple comparisons post-hoc Tukey corrected **B)** No differences between genotype on the travelled distance on the 4th day of habituation. Two-way ANOVA, multiple comparisons post-hoc Tukey corrected. **C)** No differences between genotype on the pre-exposure before introducing the 1st stimulus rat. One-way ANOVA.

Unless specified mean with SD plotted. Significance considered when $p \leq 0.05$, (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$) ($n_{+/+}$=15, $n_{+/\Delta GAP}$ =10, $n_{+/-}$=14).

In order to discard olfaction deficits as the cause of decreased social interaction I carried out a habituation dishabituation task to non-social odours using spices as odorants (Figure 5A). *Syngap* rats are able to detect, discriminate and habituate to non-social odours (Figure 5A, 5D,5E). The speed at which they habituate to a new non-social odour in Syngap rats is no different to wildtype (Figure 5B). These results demonstrate that *Syngap* rats don´t have olfactory deficits identifying non-social odour cues. Furthermore, *Syngap* rats total time spent sniffing during the task is lower than wildtypes (Figure 5C). These results indicate that *Syngap* lack of interest is not restricted to a social valence.

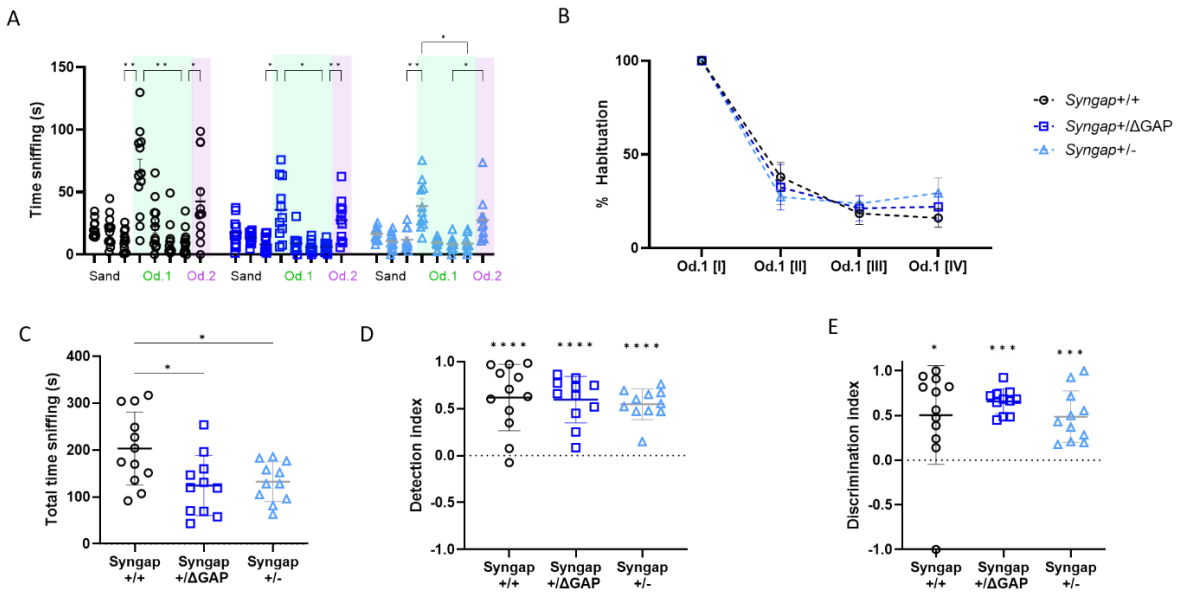


Figure 5. **Syngap rats can detect, habituate and discriminate non-social odours. A)** Time sniffing the pot with unscented sand (session 1 to 3), first non-social odour (session 4 to 7) and second non-social odour (session 8). Other significant comparisons not shown are: Syngap$^{+/+}$: sand I vs od.1 I (**), sand I vs od.1 IV (*), sand II vs od. 1 I (*), od.1 I vs od.1 II (**), od.1 I vs od.1 III (**), od.1 I vs od.1 III (*) | Syngap$^{+/\Delta GAP}$: sand II vs od.1 III (*), sand II vs od.1 IV (*), sand III vs od.2(*), od.1 I vs od. 1 II (*), od. 1 I vs od.1 (iii) (*), od.1 I (II) vs od.2 (**), od.1 (II) vs od.2(**) | Syngap$^{+/-}$: sand II vs od.1 I (**), od.1 I vs od. 1 II (**), od. 1 I vs od. 1 III (**). Two-way ANOVA, multiple comparisons post-hoc Tukey corrected. **B)** Syngap rats habituate at the same rate than wildtype based on percentage of Habituation. Two-way ANOVA, multiple comparisons post-hoc Tukey corrected. **C)** Syngap rats spend less time sniffing the pot than wildtype during the task. One-wayANOVA. **D)** Syngap rats can detect a new non-social odour (One-sample t test with FDR for multiple comparisons) and their DeI score is no different from wildtype (One-way ANOVA) **E)** Syngap rats can discriminate non-social odours (One sample Wilconxon test with FDR for multiple comparisons) and their DI score is no different from wildtype (Kruskal-Wallis test).

Unless specified mean with SD plotted. Significance considered when $p \leq 0.05$, (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$) ($n_{+/+}$=12, $n_{+/\Delta GAP}$ =11, $n_{+/-}$=11).

In order to test for deficits identifying and recognizing social odorant cues as the cause of decreased social interaction I carried out a habituation dishabituation task to social odours using urine of individual male rats as odorants (Figure 6A). *Syngap* rats are able to detect, discriminate and habituate to social odours of two different conspecifics (Figure 6A, 6D, 6E). The speed at which they habituate to a new social odours in Syngap rats is no different to wildtype (Figure 6B). These results demonstrate that *Syngap* rats don´t have olfactory deficits and social alterations previously described are not due to alterations in olfaction.

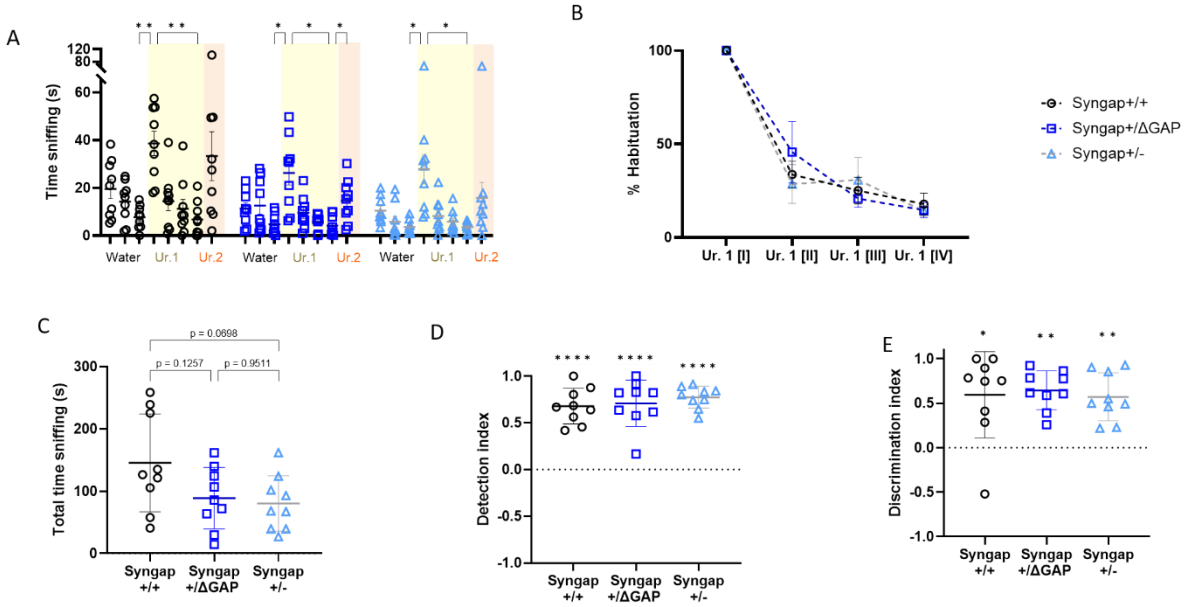


Figure 6. **Syngap rats can detect, habituate and discriminate social odours. A)** Time sniffing the pot with destilled water (session 1 to 3), urine 1 odour (session 4 to 7) and urine 2 odour (session 8). Other significant comparisons not shown are: Syngap$^{+/+}$: water I vs ur.1 I (**), water II vs ur.1 I (*), ur.1 I vs ur.1 II (**), ur.1 I vs ur.1 III (**) | Syngap$^{+/\Delta GAP}$: water I vs ur.1 IV (**), water III vs ur.2 (*), ur.1 I vs ur.1 III (*), ur.1 III vs ur.2 (*) | Syngap$^{+/-}$: water I vs water III (*), water I v ur.1 IV (*). Two-way ANOVA, multiple comparisons post-hoc Tukey corrected **B)** Syngap rats habituate at the same rate than wildtype based on percentage of Habituation. Two-way ANOVA, multiple comparisons post-hoc Tukey corrected **C)** Syngap rats display a non-significant tendency to spend less time sniffing than wildtype during the task. One-way ANOVA **D)** Syngap rats can detect a new social odour (One-sample t test with FDR for multiple comparisons) and their DeI score is no different from wildtype (One-way ANOVA) **E)** Syngap rats can discriminate non-social odours (One sample Wilconxon test with FDR for multiple comparisons) and their DI score is no different from wildtype (Kruskal-Wallis test).

Unless specified mean with SD plotted. Significance considered when $p \leq 0.05$, (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$) ($n_{+/+}$=9, $n_{+/\Delta GAP}$ =9, $n_{+/-}$=9).

Early environmental and social enrichment ameliorates behavioural abnormalities in other rodent models of ASD such as fragile-X syndrome (Oddi et al., 2014; Restivo et al., 2005). Therefore, rearing these animals in a highly socially-enriched environment could prevent the emergence of the observed lack of interest in *Syngap* rats. Animals were reared in the Habitat, an environmentally enriched set-up mimicking the natural burrow system of rats and after 3 months old they were tested on the conspecific habituation dishabituation task (Figure 7A). Results show that social exploration time in *Syngap* rats is no different from wildtype (Figure 7B, 7C).

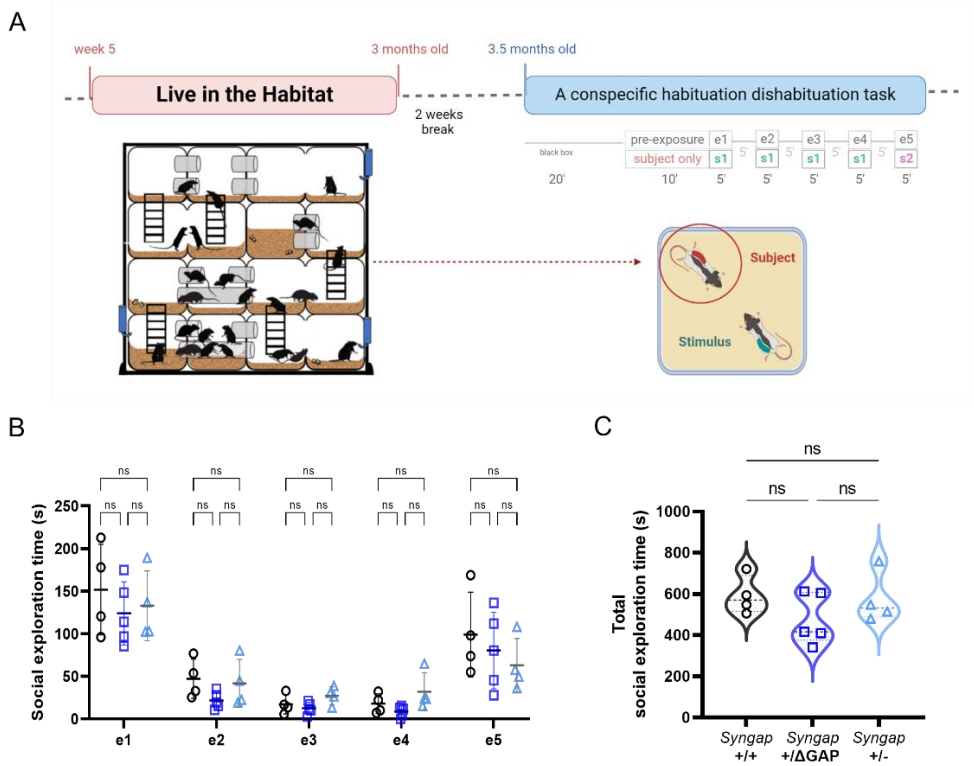


Figure 7. **A. Schematics of the timeline of experiments.** Syngap male rats grew up in the Habitat since week 5 until they were adults (3months old). After 2 weeks break, they went through the conspecific habituation dishabituation task as subjects. Stimuli rats didn't grow up in the Habitat but in standard cage housing. **B. SET is no different between Syngap rats and wildtype rats for any exposure**. Two-way ANOVA, multiple comparisons post-hoc Tukey corrected. **.C. The total SET in the whole task is no different on Syngap rats compared to wildtype rats.** One-way ANOVA.

Unless specified mean with SD plotted. Significance considered when $p \leq 0.05$, (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$) ($n_{+/+}$=4, $n_{+/\Delta GAP}$ 5, $n_{+/-}$=4, $n_{S1for+/+}$ =4, $n_{S1for\ +/\Delta GAP}$ =5, $n_{S1for\ +/-}$=4, $n_{S2for+/+}$ =4, $n_{S2for\ +/\Delta GAP}$ =5, $n_{S2for\ +/-}$=4). SET = Social exploration time.

The lack of interest observed in both *Syngap* rats models could be caused by an altered functional connectivity between reward-linked brain areas. Within the mesolimbic system, coherence between prefronal cortex to amygdala is been shown to be higher during social encounters (Kuga et al., 2022) and high-gamma oscillations in the nucleus accumbens are higher during social interaction (). Therefore alterations on these frequency bands could be underlying the behavioural phenotype. In order to address this hypothesis while keeping the same naturalistic approach of the habituation dishabituation task I implemented a wireless recording of local field potential *in vivo*. I am currently on the process of analysing these recordings at the social interaction epochs (Figure 8). A lower coherence between prefrontal and amygdala and prefrontal and nucleus accumbens is predicted during social interaction epochs. These experiment set a progress on the field since they allow *in vivo* electrophysiological recordings in a wireless way. Furthermore, this set-up is able to record other features such as acceleration (movement) and ultrasonic vocalizations (sound). All the recorded data is properly synchronized with the movement-free expression of behaviour by the animal via TTL pulses sent by infrared emitters to the wireless device and displayed LED flashes on the video.

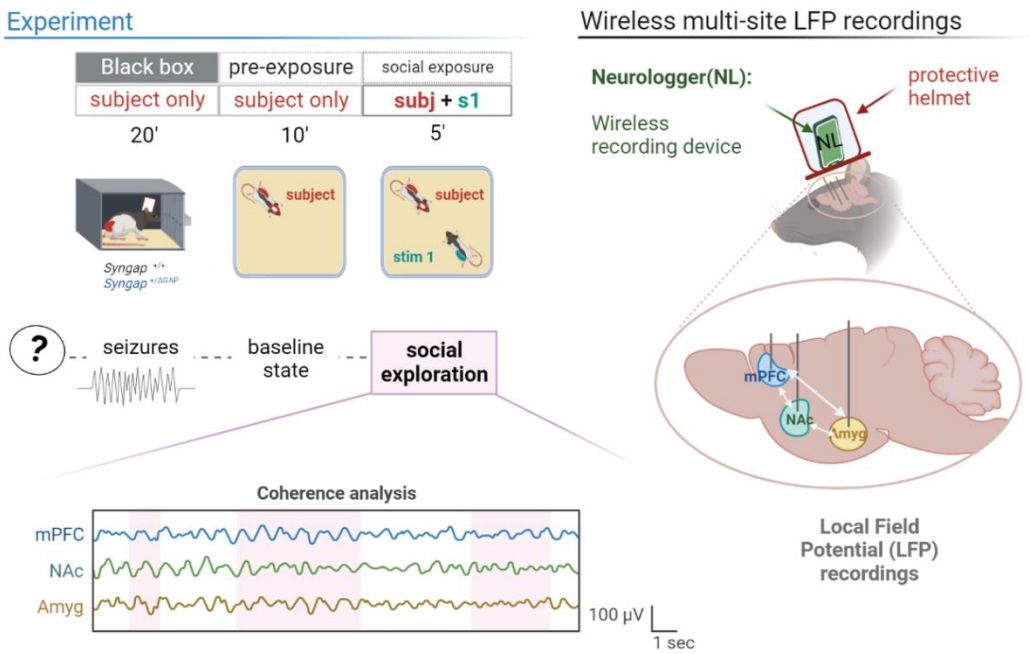


Figure 8. **Social task for in vivo electrophysiological recordings (left).** A shorter version of the habituation dishabituation task is performed only until exposure 1. Experiments are focused on Syngap$^{+/\Delta GAP}$ only since there are no major differences between the two SYNGAP lines for the social phenotype. The main goal is to look at the coherence between regions of interest during social exploration events. In addition, potential seizures events during quite wakefulness period ((black box) can be analysed and correlated to the performance during social interaction. **In vivo electrophysiology recording approach (right).** Recording electrodes are implanted on brain regions of interest linked to motivational and exploratory behaviour: prefrontal cortex (mPFC),Nucleus Accumbesn (NAc) and Amygdala (Amyg). The implant includes a 3D-printed baseplate for future attachment of a protective helmet. Recordings are performed using a wireless device: Neurologger (NL) (Evolocus). In addition to local field recordings, Neurologger also carries a microphone and accelerometer that allows to record ultrasonic vocalizations (USVs and movement data. During the experiment, NL is protected with a 3D-printed protective helmet (iHelmet: Saxena et al., 2021) .

Progress beyond the state of the art

The research conducted during my PhD has made significant progress beyond the state of the art in understanding and addressing social and circuit deficits associated with SYNGAP1 haploinsufficiency in Autism Spectrum Disorder (ASD) and Intellectual Disability (ID). By creating a new robust paradigm to address social behaviour in rodents, elucidating the lower social interaction and lack of interest in SYNGAP1 rat models, identifying the potential of environmental enrichment to rescue these phenotypes, and developing novel approaches for studying functional connectivity *in vivo*, my work has contributed novel insights into the molecular and circuit-level mechanisms underlying social behaviour impairments as well as experimental designs applicable to other scientific questions of similar nature.
These advancements collectively push the boundaries of current knowledge and pave the way for innovative therapeutic strategies and a deeper understanding of the complex interplay between genetics, neural circuits, and social behaviour in neurodevelopmental disorders.

Potential of the results for exploitation

The results obtained from my research on two rat lines of SYNGAP1 haploinsufficiency hold promising potential for exploitation in various areas. Firstly, the finding that these rat models display reduced

social interaction and general lack interest provides valuable insights into the underlying mechanisms of social behaviour deficits observed in individuals with SYNGAP1 mutations, which could potentially contribute to the development of targeted therapeutic interventions. Additionally, the identification of a potential rescue of this phenotype through rearing in a highly enriched environment and social stimulation opens up avenues for designing interventions that focus on play and social stimulation as potential coping strategies during development. Moreover, the future results on the study of altered connectivity between reward-like brain regions in these rat models present opportunities for further exploration of neurobiological pathways and potential pharmacological targets in the mesolimbic system. Overall, these results provide a foundation for future studies and the exploitation of novel approaches for addressing social behaviour and circuit impairments associated with SYNGAP1 haploinsufficiency.

ESR15 – Veronica Villeri

ESR15: overview of results towards the objective of the action

Project title – Identifying differences in the level of neuronal activation in the basolateral amygdala of Fmr1-/- and Syngap1 +/- mice after encoding for a fear-related memory

WP3 – Modulators of neuropsychiatric disorders

Early Stage Researcher 15 – Veronica Villeri

Supervisor – Emily Osterweil

Host Location – Centre for Discovery Brain Sciences (CDBS), University of Edinburgh – Scotland

Fragile X and SYNGAP1 syndromes are among the most frequent forms of monogenic autism disorders. These disorders are caused by mutations of respectively the *Fmr1* and the *Syngap1* gene. Mice mutant models of these neurodevelopmental disorders show excessive neuronal protein synthesis, suggesting the hypothesis that defective synaptic mRNA translation may be a point of convergence of the two syndromes.
A project in the lab is currently investigating whether these two mouse models share common translational dysregulations in some brain regions involved in specific phenotypes characteristic of both models, namely hippocampus and basolateral amygdala. Indeed, both mouse models show an impaired capacity of acquiring fear-related memory, a process mediated by the basolateral amygdala (BLA).
To this aim, mice were subjected to a fear conditioning protocol, in order to induce the encoding of a fear-related memory. Ninety minutes after the shock, the brains were either fixed or processed for TRAP sequencing. At this time point, we expect that the number of c-Fos positive cells is correlated to the capacity of encoding for the fear memory, thus changes in c-Fos levels in mutants would suggest a possible difference in the encoding mechanism.
The mouse line used for this experiment expresses an HA-tagged RPL22 (Ribosome Protein Large subunit 22) under the promoter for RASGRP1, a gene that in the amygdala is expressed only in the BLA glutamatergic neurons. This allows to isolate BLA cells for the TRAP-Seq and better recognize the BLA after staining for the HA-tag.
In this context, I performed a series of confocal acquisitions of the brain fixed after the shock delivery.
Each experiment comprised four conditions: a wild-type home-caged, a wild-type fear-conditioned, a mutant home-caged and a mutant fear-conditioned. For each brain, three coronal slices were selected and stained for Neun as a neural marker, c-Fos and HA.
Images were acquired using confocal microscopy and subsequently tridimensional image-analysis were performed using the IMARIS software.
The experiment showed that, in all conditions, fear conditioning induced an increase of c-Fos expression**(Fig.1)**, including, more specifically, in HA+ cells **(Fig.1,2)** . The observed differences were not due to differences in the number of HA-tagged cells **(Fig.3).**

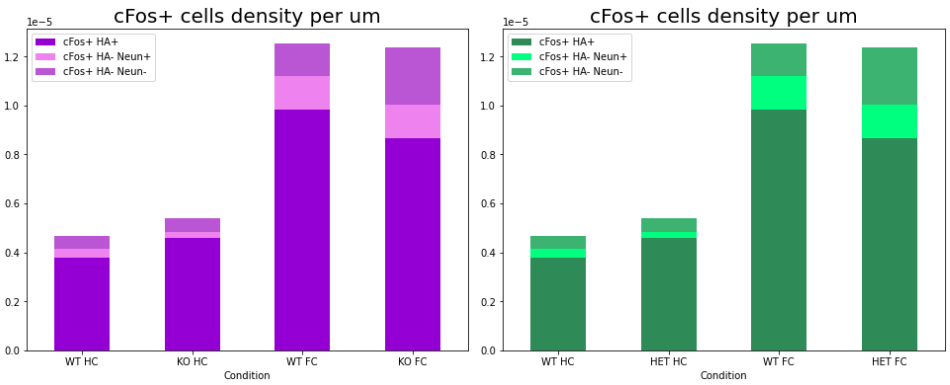


Figure 1. *C-Fos+ cells density for Fmr1$^{-/y}$ (left) and Syngap1 $^{+/-}$ (right)and the respective wild-type. HC=Home-caged. FC=Fear-conditioned.*

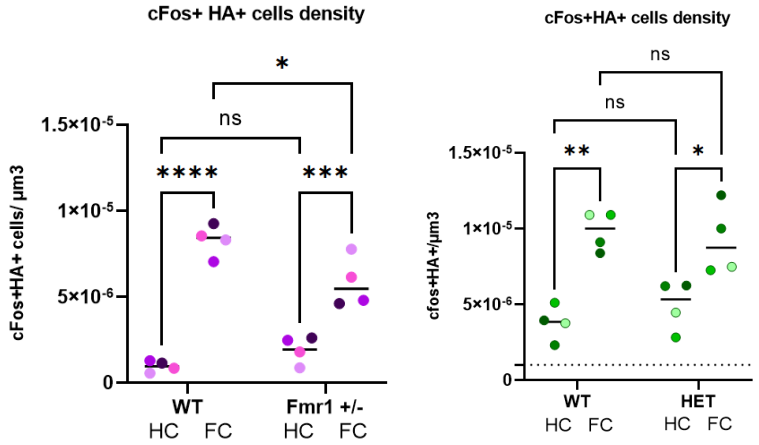


Figure 2. *C-Fos+HA+ cells density for each experimental condition for Fmr1 +/y (left) and Syngap1+/- (right). Two-way ANOVA. Tukey multiple comparison test. Left: p-values WTHC:WTFC<0.0001 ; KOHC:KOFC=0.0005 ;WTFC:KOFC=0.0166. Right: p-values WTHC:WTFC=0.0015 HETHC:HETFC=0.0195*

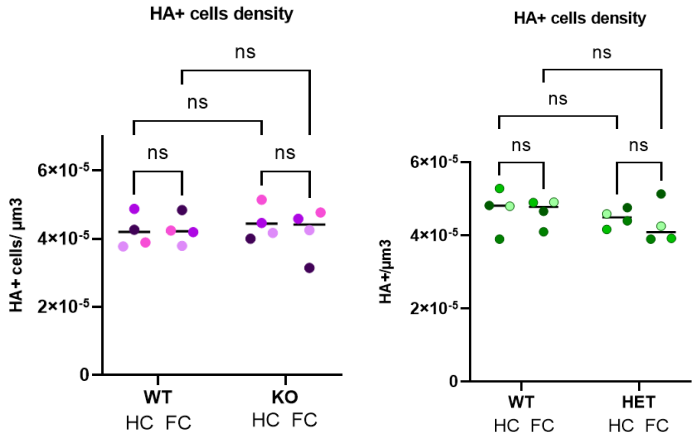


Figure 3. *HA+cells in BLA in Fragile X (left) and Syngap1 (right) mutants and their respective wild-types*

Annex 2 – Post-workshop report by Chaperone – group workshops 2021

chaperone

Workshop for Syn2Psy

Organised by: Pedro Resende from Chaperone
Event date: September and October 2021
Location: Zoom

1. Description of the event

A series of four workshops covering different topics (see next section) with individual and group activities, to provide tools and guide scientists to further develop and manage their careers.

2. Event program and trainers

The topics covered and trainers of the workshops are detailed below:

- ➔ How to write a paper, 24 Sep - Joana Moscoso;
- ➔ Preparing for a scientific conference, 1 Oct - Rob Hardwick;
- ➔ Project management, 8 Oct - Pedro Resende;
- ➔ Wellbeing & work-life balance, 15 Oct - Hannah Roberts.

3. Who participated in the event ("demographics")

A total of 14 people participated in the workshop. They were all PhD students.
According to the **pre-workshop questionnaire:**

- **51 %** of the participants **positively evaluated their career management skills.** This number **increased to 67 %** after the workshop;
- **56 %** of the participants **feel encouraged by their supervisors or line managers** to attend career development courses/events;
- **42 %** of the participants said that their work/course colleagues **participate in career development courses/events**.

4. Workshop evaluation and feedback

A post-questionnaire form was sent after the 4 workshops. According to the 12 responses obtained:

- the **overall rating** of the workshop was **4 out of 5**;
- **78 %** found the **workshops to be relevant** to their future;
- **76 %** reported that the workshop contributed to **enhancing their skills**.

Data from the pre and post-workshop questionnaires show that the **participants' perception** regarding "I know how to manage my career" **improved from 2.5** (before the workshop) **to 3.3** (after the workshop) on a scale from 1 to 5, showing that **the workshop was successful in reaching its goals**.

5. What did the participants say about the event

76 % of participants would **strongly recommend the workshop** to their colleagues.

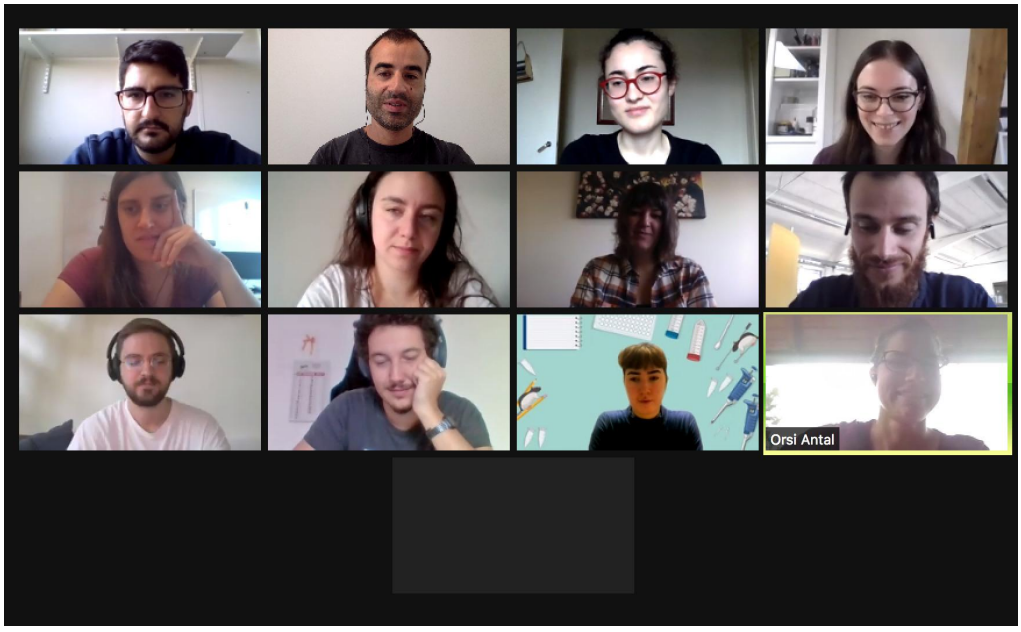
6. Challenges and recommendations for future events

The workshop on "Wellbeing and work-life balance" was the most appreciated by the participants. Given the broad nature of the topics of the other workshops, we recommend that future workshops have a more specific title and a short description of the content. This should be provided in advance to the potential participants in order for them to better assess their interest in the workshop and manage their expectations.

8. Materials used at the event

Please see here.

9. Representative photos of the event (Please keep it private. Do not share.)



Annex 3 – Post-workshop report by Chaperone – group workshops 2022

chaperone

Workshops for Syn2Psy

Organized by: Pedro Resende and Rita Brás
Event date: June 2022
Location: Zoom

1. Description of the event

A series of three workshops covering different topics (see next section) with individual and group activities, to provide tools and guide scientists to further develop and manage their careers.

2. Event program and trainers

The topics covered, trainers, and respective dates of the workshops are detailed below:

- Thesis Writing and Thesis Defense, June 8th - Raquel Oliveira;
- CV and Cover Letter Writing, June 15th - Rob Hardwick;
- Interview Preparation, June 22nd - Filipa Nunes.

3. Who participated in the event ("demographics")

An average of 10 Ph.D. students participated in all three workshops.
According to the **pre-workshop questionnaire:**

- **45 %** of the participants **feel supported by their supervisors or line managers** to attend career development courses/events;
- **18 %** of the participants stated, "**I know how to manage my career**". This number **increased to 70 %** after the workshop;
- **73 %** of the participants stated, "**Career planning and management is important to me.**". This number **increased to 90 %** after the workshop;

4. Workshop evaluation and feedback

A post-questionnaire form was sent after the three workshops:

- the **overall rating** of the workshops was **4 out of 5**;
- **90 %** found the **workshops to be relevant** to their future;
- **80 %** reported that the workshop contributed to **enhancing their skills**.

Data from the pre and post-workshop questionnaires show that the **participants' perception** regarding "I know how to manage my career" **improved from 2.7** (before the workshops) **to 3.7** (after the workshops) on a scale from 1 to 5, showing that **the workshops were successful in reaching their goals**.

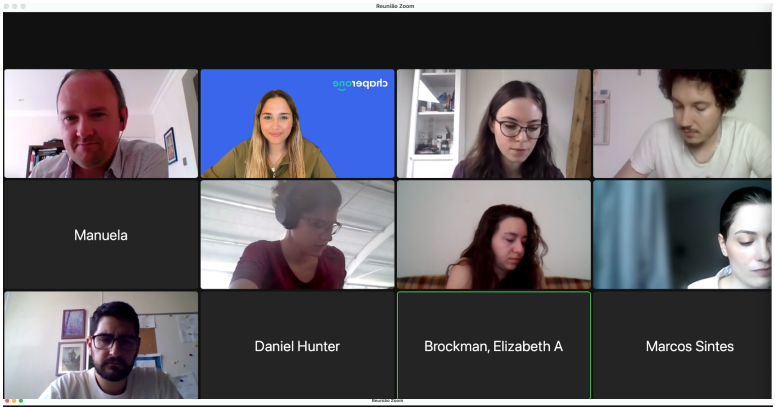
5. What did the participants say about the event

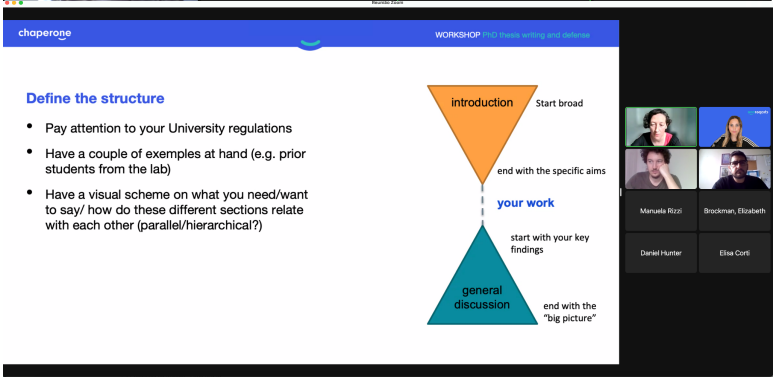
100 % of participants would **strongly recommend the workshop** to their colleagues.

6. Materials used at the event

Please see here.

7. Representative photos of the event (Please keep it private. Do not share.)





Annex 4 – Post-workshop report by Chaperone – individual workshops

chaperone

www.chaperone.online

Personalized Career Consultancy Sessions

SYN2PSY

Brief Description

In addition to group training provided in workshops (separate report), PhD researchers from the SYN2PSY program, were offered an intro call to better understand their specific individual career needs, and received recommendations for consultants on our platform, to support them with 1-to-1 1h online sessions. Before and after sessions, researchers received continuous support from our team.

Starting date: 17-09-2021
End Date: 08-08-2022

Overall balance

A total of 14 PhD Students were considered eligible and contacted to be offered our services. **12 registered on our platform, and booked their individual sessions**. Sessions feedback was excellent. PhD students rated their sessions with an average of **4.9** (1-5), and testimonials gathered suggest a very high satisfaction and effectiveness of the sessions. Most of the PhD were looking for support regarding their next career step, on **exploring different career options / paths** or getting **support on job applications for specific roles,** and the second most common need was related to the **development of time management and wellbeing skills**. Consultants mentioned that they really enjoyed working with SYN2PSY students. Overall, we feel the goals of this pack of sessions were fully-achieved and we are very happy with the outcomes and impact generated in the personal and professional development of the students.

Future recommendations

Students' high satisfaction in their individual sessions lead us to conclude that it would be worth considering allowing each student to do more than one individual session. Students manifested feeling very busy with extra-projects activities, so being able to book their session for the best time of the year was a big plus for them.

Sessions dates

25/11/2021; 26/11/2021; 27/11/2021; 29/11/2021; 14/12/2021; 20/12/2021; 12/01/2022; 26/01/2022; 10/02/2022; 11/02/2022; 21/06/2022; 08/08/2021.

Testimonials

(name of consultants was removed to respect privacy policy agreement)

#1 My session with <consultant name> was very helpful! I chose her because I saw she had a lot of experience in communication with the non-scientific public and requested some tips on how to do that better (as we have a lot of such tasks that are part of Syn2Psy). I was surprised to be able to achieve so much during only 1h. She gave me great general tips on how to improve my writing and presentation aimed towards the non-scientific public, and also had the time to go with me through a piece I am writing for Syn2Psy, where I am giving a pitch on my project!

#2 Amazing! It was really helpful to understand what I want for my future career and what I need to do after my PhD.

#3 It was a great experience, she was very supportive and encouraging, and I learned a lot from her!

#4 <consultant name> gave me really useful advice about the transition from PhD to postdoc. She provided me with practical tools in relation to looking for labs and grants, but also I valued that she shared her own personal experience and she could identify my scientific and personal needs. PhD students are sometimes uncertain about what to do in the future, and having PIs like <consultant name> willing to help makes things a bit easier! Thanks a lot.

Annex 5 – Report by Desiree Dickerson – psychology webinars

Desiree Dickerson. PhD

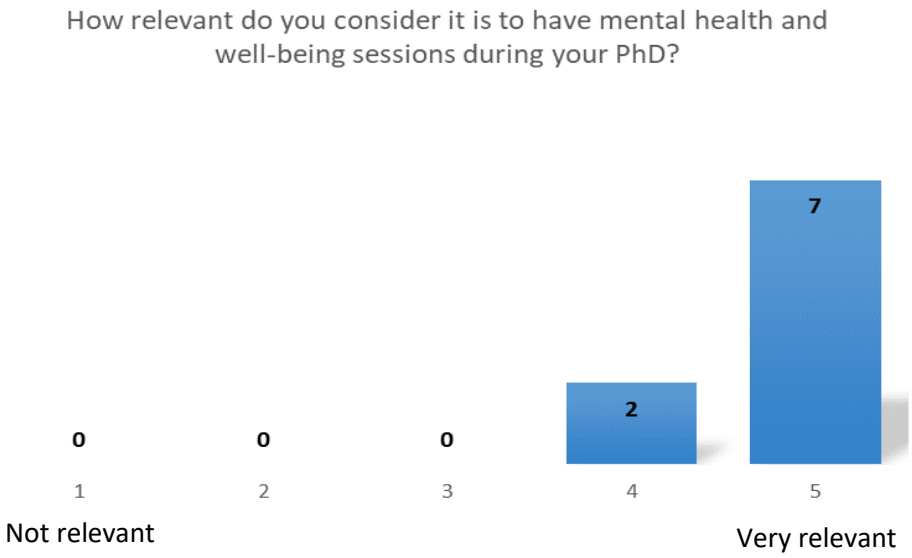
Mental health and well-being @ Syn2Psy

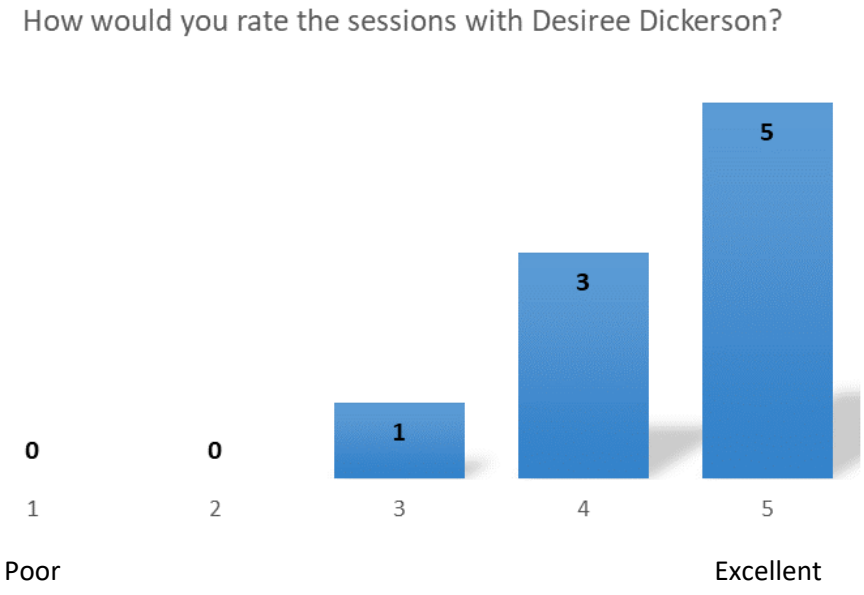
Syn2Psy
April 2022

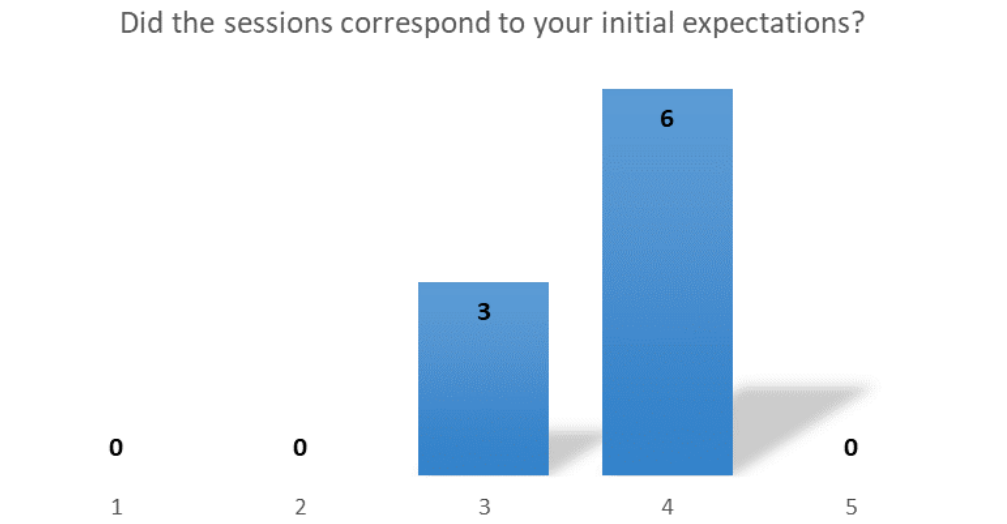
Over the course of course of three months (Feb – April, 2022), the Syn2Psy PhD cohort have begun building a foundation for well-being and mental health in their PhD programme. Through the interactive delivery of five webinar sessions exploring various themes related directly to student mental health, well-being and performance. Students have explored how to navigate the uncertainty of living the academic experience through a pandemic and whilst having our close neighbours at war. They have explored how to manage worry, stress, doubt and fear; how to combat procrastination and low motivation; to recognise and work to alleviate isolation and loneliness; as well how to tackle thoughts that drive perfectionism and imposter syndrome. They have discussed how to navigate challenges in their supervision experience, how to advocate for their needs and better manage conflicts in an open and transparent manner. Overarching goals spread throughout the sessions, were to begin to shift student mindset to create adaptive, resilient individuals who know how to use the resources at their disposal.

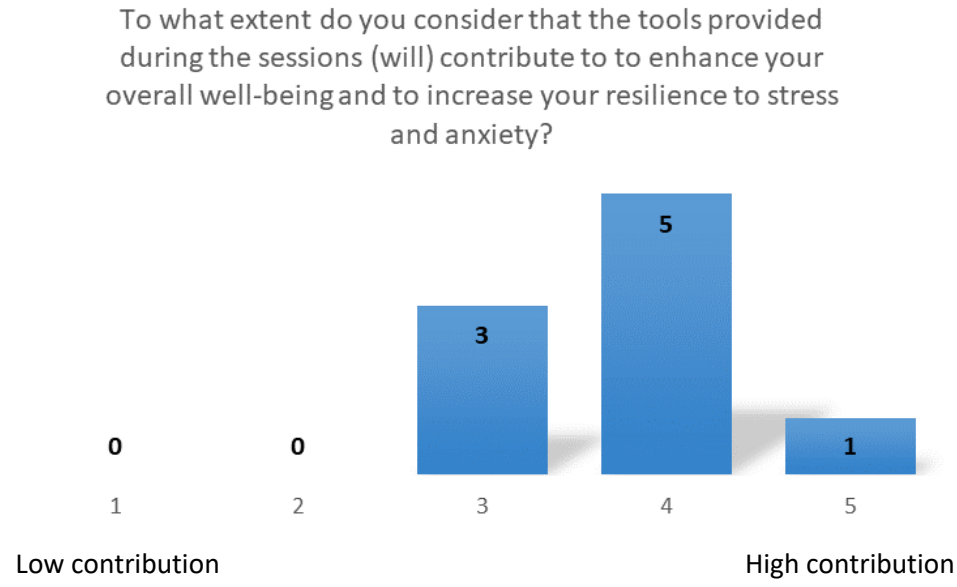
Annex 6 – Satisfaction Survey – psychology webinars

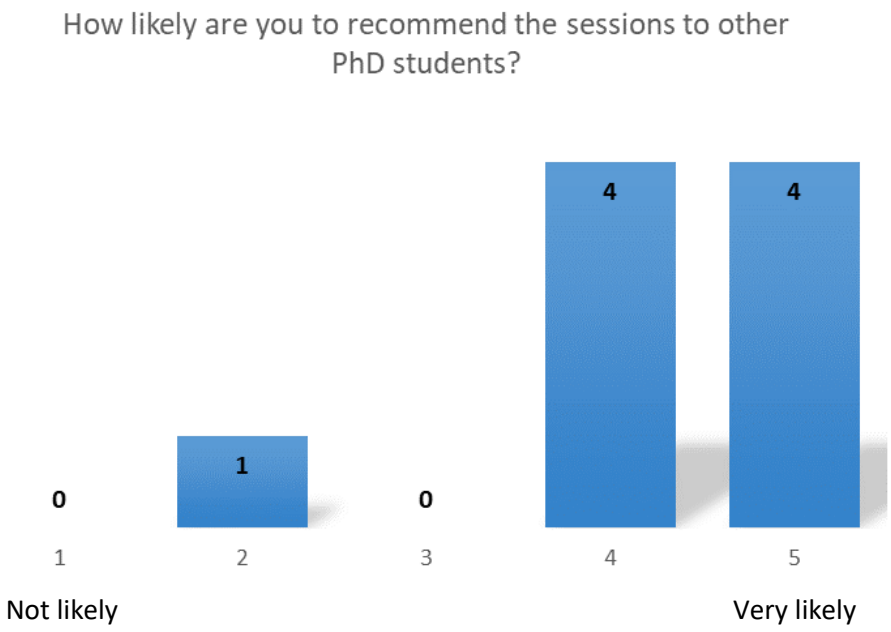
Results of the Satisfaction Survey for the Mental Health and Well-being sessions provided by Desiree Dickerson






Did the sessions correspond to your initial expectations?
0
0
3
6
0
1
2
3
4
5
Did not correspond
Corresponded completely


To what extent do you consider that the tools provided during the sessions (will) contribute to to enhance your overall well-being and to increase your resilience to stress and anxiety?
0
0
3
5
1
1
2
3
4
5
Low contribution
High contribution


How likely are you to recommend the sessions to other PhD students?
0
1
0
4
4
1
2
3
4
5
Not likely
Very likely

Annex 7 – 3rd PhD day programme

3rd PhD day

Coimbra | 18-19 October 2022

Tuesday, 18th October 2022

9:00 – 9:10 Welcome – Biblioteca do Rómulo
Ana Luisa Carvalho
9:10 – 12:30 Session with Marionet – Biblioteca do Rómulo
Mário Montenegro
12:30 – 14:00 Lunch – Cafetaria do Museu
14:00 – 18:00 Science Communication workshop – Biblioteca do Rómulo
14:00 – 14:45 *Science for all: democratizing knowledge*, Mariana Alves
14:45 – 18:00 *Storytelling and media-training*, Sara Amaral, Marta Costa and Karine Paniza

Wednesday, 19th October 2022
Auditorium - Department of Life Sciences, University of Coimbra

8:30 – Introduction
Ana Luísa Carvalho

8:45 – 17:00 ESRs' Scientific presentations (15 min + 15 min)
Chair: Peter Kind
08:45 – 09:15 ESR8 Elizabeth Brockman
09:15 – 09:45 ESR7 Elisa Corti
09:45 – 10:15 ESR5 Marcos Sintes
10:15 – 10:45 Break
Chair: Catalina Betancur
10:45 – 11:15 ESR3 Giuseppe Cammarata
11:15 – 11:45 ESR2 Alessandro Chioino
11:45 – 12:15 ESR1 Orsolya Antal
12:15 – 14:00 Lunch – Loggia
Chair: Kjartan Herrik
14:00 – 14:30 ESR14 Vanesa Salazar
14:30 – 15:00 ESR13 Loredana Cumpana
15:00 – 15:30 ESR12 Manuela Rizzi
15:30 – 16:00 Break
Chair: Laurent Groc
16:00 – 16:30 ESR10 Flavio Tomasi
16:30 – 17:00 ESR9 Laura Upton
17:00 – Final remarks
Carlos B Duarte

19:30 Dinner – Papa

Annex 8 – Abstracts of the articles (to be) submitted to FYM

Abstracts sent to the journal Frontiers for Young Minds

Authors: Alessandro Chioino and Loredana Cumpana

Title: Reaching for the top: the role of mitochondria.

Abstract: Humans, like most animals, live in social groups that naturally form ranks, following an order from dominant to subordinate that we call social hierarchy. What makes individuals more likely to fall into a certain rank? To study brain functions that contribute to this, social hierarchies are studied in laboratory rodents and identified, through social competition tests. Recent studies have shown that mitochondria, the powerhouse of the cells that produce the energy necessary for cells and organisms to work, play a role in social behaviour. Subordinate rats have lower mitochondrial capacity than dominant rats in a brain region -the nucleus accumbens- important for effort and motivation. If subordinate animals are energized with a booster of mitochondrial function, their behaviour can shift from subordinate to dominant. We will discuss what this animal research means for humans and how it may help us find new ways of treating troubled social behaviour.

Authors: Laura Upton and Flavio Tomasi

Title: Fantastic beasts and where to find them: how can we use animals to study neuropsychiatric disorders?

Abstract: A psychiatric disorder is a mental illness that disturbs a patient's behaviour, mood or ability to think, and can get in the way of them leading a normal life. Even though studying human patients might seem like the most obvious way to understand these illnesses, doing so is not always practical, affordable, or even possible. Because of this, other more roundabout methods have to be found. Fortunately, we can create animal models of some psychiatric disorders, to better understand their biological causes. Genetic differences are a common cause for these kinds of diseases. Because of this, genetic animal models are a very common way to study how these illnesses modify brain function and behaviour, and can even help us to find treatments for these disorders. Did you know that mice can imitate the symptoms of depression, schizophrenia or autism? In this article, we will discuss what kinds of information we can obtain from animal models, and what are the pros and cons of using these models to understand human diseases.

Authors: Elisa Corti and Manuela Rizzi

Title: The play-doh brain: shapeable neurons make you learn!

Abstract: Imagine your mum just baked pizza and you immediately eat a piece of it. Bad decision, it is really hot! Now you will wait for it to cool down before continuing to eat. The burning sensation reaches the brain and the neurons communicate with each other so that you stop eating. Next time your mum will cook pizza you will have learnt to be more patient. This is possible thanks to a process called synaptic plasticity, in which neurons increase the connection already formed in the first communication and prevent you from hurting yourself again.

In this article we will describe some properties of neurons, as well as what happens to these cells when you create new memories in a more detailed way. We hope that you will learn something new by reading this piece, and share it with others, as neurons would do!

Authors: Orsolya Antal, Diogo Soares and Ágata Silván

Title: Schizophrenia: what causes hallucinations and distorted thinking?

Abstract: Imagine living without being sure if what you see, hear or think is real or not. Schizophrenia is a mental disorder characterized by distortions in thinking, perception, emotions, language, sleep, and behaviour. It affects 20 million people worldwide, and typically becomes evident during late adolescence or early adulthood. While medication can be used to control hallucinations, most symptoms are resistant to treatment. As a result, patients and families have serious difficulties in their daily lives. But what do we know about what is happening in the brain of people afflicted with this disorder? Which parts of the brain may be altered and how can we develop better treatments based on this knowledge? In this article we will explore how the brains of people with schizophrenia are different and how gaining more knowledge can help them.

Authors: Elizabeth Brockman

Title: Exploring the living mouse brain using 2-photon microscopy.

Abstract: The human brain is a highly complex system comprised of billions of cells, called neurons, interconnected to each other in functional neural circuits. Normal brain function is dependent on effective communication between neurons, a process that requires synapses, the point of contact between nerve cells. Several diseases result from impairments in the communication between neurons, which is why it is essential to study synapses in the living brain. Since the human brain is not accessible, scientists use simpler organisms like mice.

To see mouse neurons, a piece of skull is replaced with a transparent glass window. Powerful microscopes called 2-photon microscopes, can then be used to capture snapshots of neurons deep inside live brains! Clever use of lasers to excite tags placed inside the cells make neurons emit fluorescent light and glow in the dark. Join us as we illuminate the inner workings of neuron communication in the living brain.

Authors: Vanesa Salazar and Daniel Hunter

Title: A brain composed by multilingual neurons: A story of coded messages and crosstalking.

Abstract: Everyday, we communicate with each other using language. To do this effectively requires two things: 1) the speaker to be clear and precise, and 2) the listener to understand the language and respond.

Similarly, in our brains, neurons communicate with each other, at a location called the synapse where one neuron "talks" to another neuron by releasing a particular chemical message. The “listening” neuron understands this message by receiving the chemical at receptors on their surface. Every neuron speaks to hundreds of others, simultaneously and using different languages. The brain works as a social network which integrates information and responds to hundreds of “chats” arriving at the same time.

This article focusses on synapses that use a chemical messenger (or language) called glutamate and two particular receptors, called AMPARs and NMDARs, that decode the message. This language is key to how we interpret the message and for learning and memory.

Authors: Giuseppe Cammarata and Marcos Sintes

Title: How to build a mini human brain.

Abstract: Growing miniature brains in a laboratory may sound like science-fiction, but advances in technology have made it possible. Scientists can collect human cells from different tissues, like the skin, culture them in a dish and reprogram them to make stem cells, like the ones in an embryo. These reprogrammed stem cells have the ability to multiply and convert into all the kinds of cells that form our body, which lets us study the development of human organs. Under the right conditions, stem cells can aggregate and grow into small brain-like structures, called brain organoids. The study of brain organoids has allowed us to expand the possibilities of recreating the human brain and can reproduce reliably many features of its function and development. In this article, we would like to explore how these brain organoids are created and how they can help us make new discoveries on diseases related to the development of the brain, such as autism.

Annex 9 – Newsletter 1 and 2

Newsletter

Issue 1 – August 2020

Innovative Training Network

Synaptic Dysfunction in Neuropsychiatric Disorders



All Syn2Psy members at the Kick-off meeting, held in Coimbra, Portugal. September 27th 2019.

Welcome to Syn2Psy!

By Ana Luísa Carvalho, Syn2Psy Coordinator.

Here is the first Newsletter on Syn2Psy – the Innovative Training Network on Synaptic Dysfunction in Neuropsychiatric Disorders. The Early Stage Researchers in Syn2Psy joined their laboratories last September, and were in for a very challenging first year of their project. We are very proud of the diverse group of excellent PhD students in Syn2Psy! They told us about meeting in Coimbra, their adventures in discovering a new country, joining a laboratory, finding out about their project, learning new methods, and adjusting to life under lock-down. Much has changed in their lives in the last year.

Despite inevitable delays in projects and course work caused by the COVID-19 pandemic, the Syn2Psy Early Stage Researchers have managed to make progress, read the literature, analyze data and even start joint projects such as the articles for Frontiers for Young Minds. Syn2Psy activities have been rescheduled, and it will be possible to make up for the lost time.

Enjoy this Newsletter, make the most out of your time as PhD students, and keep up the good work!

European Commission

MARIE CURIE ACTIONS

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska- Curie grant agreement No 813986.

Syn2Psy KICK-OFF IN THE EYES OF AN ESR

Diary of an early stage researcher.

Article by Manuela Rizzi, ESR.

September 24, 2019: Tomorrow is the day: I am flying to Coimbra to attend the first kick-off meeting and microscopy course! I am really looking forward to meet the other students that will share this incredible experience with me as well as all the professors that allowed all this to be possible. I feel anxious and excited at the same time. I don't know what to expect but I hope to be overwhelmed by positive vibes!

September 27, 2019: These two past days have been amazing! Anxiety and fear were accompanying me the first day but as soon as I entered the conference room they left place to wonderful feelings: everyone was welcoming, friendly and especially this looked as the right place and time to be in for me. Professor Ana Luísa Carvalho welcomed all and started to present the Syn2Psy program followed by the other professors that briefly presented their research outputs. It was interesting to come across the so many different angles you could choose to look at similar research topics.



Syn2Psy members at the NS I, Coimbra, Portugal. October,

Moreover, it is not all about conducting experiments and be locked up in a laboratory environment. Communication and dissemination would give an added value to our research and to this aim, collaboration with different associations close to the people affected with the diseases would be a great opportunity to do so. A real surprise to me is the involvement of arts as another mean to achieve this. This was presented by MarioNet theatre company committed in connecting science with theatre with which we will collaborate and end up with a theatre script at the end of our PhD. Amazing! I am really looking forward to start this new experience and to share it with other 13 researchers divided across Europe but connected by the same purpose: passion for science and a need for contribution in an active way to ameliorate people's life. This will represent a great opportunity for my personal and professional growth. But first: let's hang out and explore places and flavours of Portugal together!! An exciting microscopy week is yet to come!



ESRs in Coimbra, Portugal. October, 2019.

October 5, 2019: Here we are at the end of this experience, the first of many! I have to sadly say goodbye to Portugal and all of my new friends and colleagues, but only for now! This past week has been amazing: we explored the fascinating and mysterious Coimbra, spent a nice day at the beach in Figueira da Foz, and visited the iconic Porto. But it was also instructive! We had a deep outlook in microscopy and the hands-on sessions allowed us not only to take lots of high-resolution images with different imaging tools but also to let these images express themselves after data analysis. I am looking forward to meet all again next summer in Paris!!

Manuela

FIRST EXCHANGES BETWEEN ESRs ELISA AND ÁGATA



Elisa in Coimbra.

Hi Bonita!

Cómo estás? How are you in Copenhagen?

Here in Coimbra everything is ok, it's been raining for a whole month and now I really miss the sun. Fortunately, it's not as cold as in Copenhagen, but I couldn't imagine Portugal to be THAT rainy. I hope it'll be sunny as soon as possible because I want to visit every single spot, every beach and city, I want to learn how to surf and take this great opportunity to enjoy.

And also, you won't believe, I can say something in Portuguese! My favourite sentence is "this is not yellow", that might seem quite useless but, who knows? I will start a Portuguese class next month and I'm really enthusiastic, I think it's fundamental to know the language to integrate in what will be my home for four years and I can't wait to get really contaminated by Portugal. On the other hand, I'm scared as hell, I'm far from home and everything here is different and quite challenging, from the food to the streets and the habits of people. I miss home but I'm happy and determined in cultivating my passion.

I can't wait to see you again in Paris!

Kiss,

Elisa



Ágata in Copenhagen.

Hi, Elisa bonita!

It is good to hear that everything is going well in Coimbra, and I really hope that sunnier days will come soon for you! I am afraid that here in Copenhagen the weather is not much better so far. To be honest, I really miss some light hours during the day (here it gets dark at 15:30 now!!). It is a great place to live though, and during these days people traditionally place candles in almost every corner of the city, so it warms the spirit a bit. I can't wait for you and the others to come here and share the experience of Copenhagen with you, and I already miss the feeling of being altogether there learning and enjoying Coimbra. Hopefully, Paris will be even more exciting, as we might be able to present some data from our projects! I am sure we can learn so much from each other. I understand perfectly how you feel about home and the challenges to face in a new environment, lab and culture. The fact of starting a PhD it is quite challenging itself but doing it in a new country and in a complex (but also fascinating) topic as neuropsychiatric disorders it is just crazy. It feels both stimulating and scary at the same time! Fortunately, all of us are full of passion and ready to prepare for becoming independent researchers in neuroscience. Sounds exciting, right?

I am really looking forward to hearing from your advances in the project, and please keep me updated with all the details of your experience in Coimbra!

Hugs,

Ágata

The Center for Neuroscience and Cell Biology (CNC) is a nonprofit research institute founded in 1990, aiming to foster biomedical research and multidisciplinary graduate teaching at the University of Coimbra. CNC was the first established "Laboratório Associado" in Portugal, and it has steadily increased the scope of scientific competences over the years, with a strong focus on the exploitation of the fundamental mechanisms of aging and brain diseases.

1290 UNIVERSIDADE D COIMBRA

CNC CENTER FOR NEUROSCIENCE AND CELL BIOLOGY UNIVERSITY OF COIMBRA PORTUGAL

Lundbeck is a global pharmaceutical company highly committed to improving the quality of life of people living with brain diseases. For this purpose, Lundbeck is engaged in the research, development, manufacturing, marketing and sale of pharmaceuticals across the world. The company's products are targeted at the disease areas within psychiatry and neurology.

Lundbeck

ADAPTATION TO THE PhD LIFE

Chilling adventures of an early stage researcher

Article by Vanesa Salazar, ESR.



Manuela and Vanesa in Edinburgh.

Once upon a time in Edinburgh… October, it was a rainy evening, two young ladies waited at the bus stop at the airport. They have just arrived from Coimbra (Portugal) after a long day travelling. However, they weren´t tourists. Their names were Manuela and Vanesa, two early stage researchers about to start their PhD. For Vanesa, it was the first time in Scotland. She was about to discover a whole new world. In fact, she had no idea of all the crazy stories she would live through, in just her first months in Scotland. How do I know? I am Vanesa.

Time to give my closet (and my head) a makeover: new country, new life. Coming from the Mediterranean country of Spain, it makes sense to be shocked by the constant rain and early sunsets at 4 p.m. Although Scotland offers a very cold weather, people offered me a very warm welcome (especially in the lab). One of the things I struggled with was the first one had to do: find a place. When I arrived, I had rented an Airbnb for just two weeks: no time to lose! I was surprised to see how many scams there were and, sadly detected several in my attempt to visit them. Also, there is such a high demand, it is not rare to visit a place and find that a previous visitor has already taken your chance, no way!! Running out of time, no place to stay, what would I do? An ad replied to me at the very last minute: it was a beautiful house in a calm neighbourhood. I was saved and just in time!!!Time for shopping: no fresh fish, no seafood, no unwrapped meat, no uncanned legumes and no makeup section at the supermarket… This was an emergency!! I had to start my own research on alternative shops where to find my beloved ingredients as well as to try Scottish traditions like haggis. Early, I started to take a cup of tea and porridge daily (almost a total Brit, huh? except for my accent hahaha).

What is it like to do PhD in Edinburgh? It is thrilling and challenging. If you already follow me on twitter @weird_neuron, it is very likely that this question is the one that interests you more. In my case, I am part of a very big and international lab. It is also a very multidisciplinary lab. For first months, I learnt the different studies on monogenetic mutations related to ASD/ID that are carried out in the lab. I also had the chance to meet many other PhD students of first, second and last year during different workshops organized by the University of Edinburgh. We spend a lot of time together, in the lab and outside as well. I remember how much I laughed in one of our 'Mario Kart competition' Saturdays, the Halloween party or the good pints at 'The Pear Tree' pub after work. What do I find special about doing a PhD here? Good question. From my point of view, the main point is the strong collaborative environment. Collaboration is enhanced from the very beginning between PhD students but also it is something you can observe within and between labs. Another thing I like is the wide range of training the university offers, both online and in-person. I have had the possibility to learn topics from programming to writing skills. During these first months of my PhD, I have also planned my first experiments and I was properly trained for working with animals when inducted into centre facility. Also, I attended the UK-Japan Symposium where I learnt about cutting-edge imaging techniques and current neuroscience research lines in each country. I met and discussed my ideas with other researchers from the field and received feedback. Research, networking, discussion, socializing, learning, training, planning and all over again. In addition, I have just started to run my first behavioural experiments…exciting times to come!

The Centre for Discovery Brain Sciences (CDBS) explores fundamental physiological mechanisms and pathways, from single genes to complex behaviour. CDBS is located at Central Campus and Little France Campus and its research consists of a broad variety of disciplines and cutting edge techniques. Members of the Centre for Discovery Brain Sciences (CDBS) carry out research at molecular, cellular, systems and behavioural levels to understand fundamental mechanisms and pathways relevant to brain and body function in health and disease.

CDBS
Centre for Discovery Brain Sciences

Edinburgh: the city of mysteries, myths & legends. Just in the middle of Edinburgh there is a Sleeping Dragon. It used to circle the skies but one day, it rested on a peak and it hasn´t woken up yet. It is better known as Arthur's seat today. When climbing this famous hill, I tried to be careful and not to awake the dragon (we all know what happens when you awake the dragon by Game of Thrones…). Luckily, it keeps sleeping.

Friday night is for going from pub to pub with some friends. During one of those nights, a crazy wind blew against us and, in the twinkling of an eye, my friend's glasses were missing. The ghosts of Edinburgh went to party tonight!? Cars on the road, people crossing from one pub to another…where were they? We looked for them in the middle of the street with no clue of whether we were going to find them and if so, in which state. After walking some minutes searching, the wind calmed down and we found the glasses next to the road in a puddle. It looks like the ghosts didn't want to play anymore. What a crazy night!



On the left, the castle of Edinburgh. On the right, Vanesa at Loch Ness.

If you come to Scotland, you come to the nation of legends, myths, and mysteries. From Nessie to kelpies (a mythical water horse), Scotland is full of mythical creatures and ghosts. Pay attention when you come, you don´t want to miss them or much worse, to disturb them!

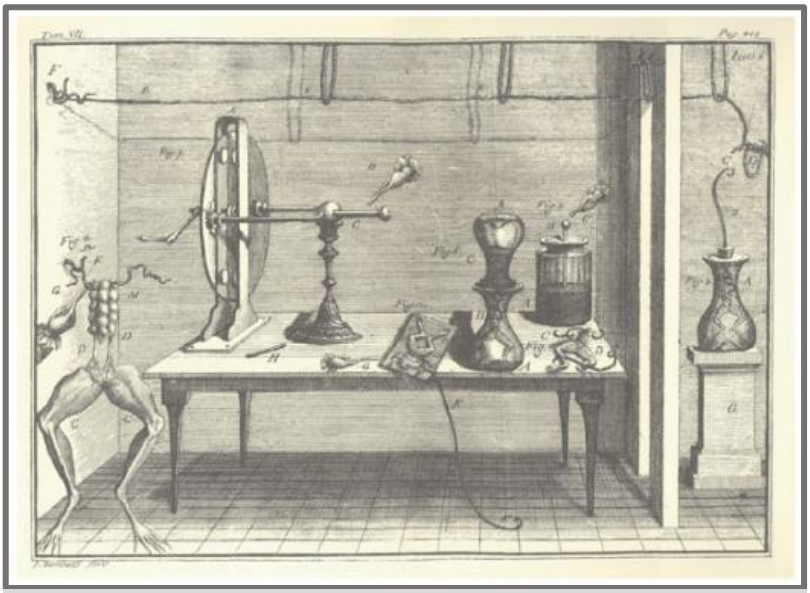
Vanesa

A typical day in science.

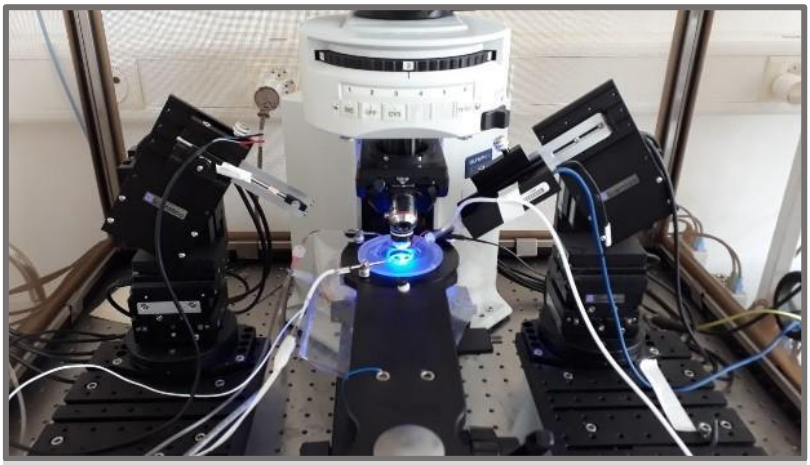
Article by Alessandro Chioino, ESR.

Hey there, I am your friendly neighbourhood Early Stage Researcher 2, but my friends call me Alessandro. Seven months ago, I moved to Lausanne to start my new life as PhD student and a few things changed since then. I would love to give you all the details about how difficult it was to find a place to live along the Lake Geneva (or Lac Léman if you like), but time is running.

I set some important experiments for today and I can't stay to talk…you know what? Join me for the day, but don't tell anyone! What? You wonder how it is to work in a laboratory and what we are up to during the day? Well, it really depends on each laboratory and every day ends up being different from the others. You need to organize your schedule early in advance and carefully plan all the steps of your experiments: order reagents, materials, be sure the instrument you need is available on that day, etc. Oh, and don't forget to be resilient, the passion for what you do is important, but you feel like your experiments will never work. Me and my group are mainly working on understanding how stress events impact brain function and behaviour, with a focus on hormones and cellular metabolism.



Galvani's experiment, from "History of Electrophysiology and the Patch Clamp" Verkhratsky, Pampura 2014.



A typical electrophysiology set-up nowadays. Picture provided by Simone Astori, LGC laboratory.

Hey, we arrived at my department. Please, follow me into the laboratory and be ready for a world of wonders.

Welcome to the electrophysiology room! This is where scientists prepare their magic solutions with one aim in their minds: capture the activity of single neurons. What? You don't know what electrophysiology is? Luckily for you I am here to help you with that.

We can say that electrophysiology was born in the 18th century thanks to the work of an Italian academic in Bologne, Luigi Galvani, on the muscular contraction of frogs. Galvani observed that when a spark was applied to the muscular preparation, a robust contraction was triggered in the sample. This first simple observation led him to conduct further experiments and to postulate the existence of an "electrical force" that governs the muscular and cellular excitation. Science is not an easy environment and his theory was not immediately accepted, the myth says he was even called "the frogs dancing master".

Fortunately for us, other scientists kept working on this initial theory, changing it and improving the tools to provide more fine observations of the bioelectrical properties of living tissues. Think about that: more than two-hundred years divide Galvani from us but we still are walking on the path he first drew. With the knowledge we accumulated and the technical expertise we now have, it is possible for us to use equipment for electrophysiology that allows us to see how the cells behave inside the complex brain network, to modify their activity or to observe how external conditions shape their behaviour. Sounds crazy, doesn't it?

Let me prepare some solutions for my experiments now!

I am finally done with today's experiment! You are lucky, today is the day of the *aperitif* organized by the department: lots of free food, beers and singing scientists will be there, you cannot miss it!

Hope you had fun following me today!

Alessandro

EPFL Founded in 1853, the École Spéciale de Lausanne was renamed École Polytechnique Fédérale de Lausanne in 1969. EPFL is the most cosmopolitan of all European technical universities. It welcomes students, professors collaborators of more than 120 nationalities. With both a Swiss and international vocation, EPFL focuses on three missions: education, research and innovation.

Animal Handling course and visit to the Musée Pasteur.

Article by Laura Upton, ESR.

It has been 4 months now since I moved to Paris to embark on this exciting, challenging, sometimes hectic but always rewarding experience that is a doctorate. In the process of joining a new lab, there is always a period of discovery and uncertainty, as you adjust to the conventions of the new space you are moving into. You have to figure out where the extra pipette tips can be found, where to bring the hazardous waste when it needs disposing of, how to book large equipment when it is needed, and so on. Moving to a new country adds an extra layer of complication: you have to deal with the fact that many of the lab systems and safety protocols that are in place may not be in the language you are used to, or the procedures may be slightly different between countries.

Sometimes, procedural differences are so crucial that it is important to be trained specifically to be sure to carry out tasks correctly and in keeping with the local regulations. For this reason, much of the first few months of my doctorate has involved safety training and training for the use of certain techniques. One such course, was a training program for the use of animals for experimentation. My experiment involves studying a mouse model of autism, and thus I will be performing experiments on mice. As such it was essential that I receive training to be able to do so safely and legally in France. On the 26th of November, Flavio Tomasi (the other Syn2Psy early-stage researcher in my lab) and I travelled to the Institut Pasteur in Paris to complete two weeks of training. The training program & certification was for project designers who need to use rodents or lagomorphs in their experiments. The course was the only one of its type run in English in all of France, and so it regrouped a large number of international researchers & doctoral students that were just starting their research here. Some had even had to cross continents to complete this course for their research, as there was at least one person present who was carrying out their research in a French overseas territory (DOM-TOM). It was exciting and inspiring to see the cultural diversity of the scientific community here in France.



Institut Pasteur. Photo by Flavio Tomasi.

The course was made up of interactive seminars, workshops, and hands-on sessions. The seminars covered a range of topics; from the legal and regulatory framework that is imposed on the use of animals in research and how to navigate this from both legal and ethical stand-point, to the functioning of animal research facilities & animal care, justifications for the use of various species as models, rodent husbandry, safe & effective use of anaesthesia, and humane methods of euthanasia. Discussion was encouraged, especially with regards to the ethical considerations and suggesting ideas for limiting harm to the animals. The workshop series focused on the importance of statistics in the effective design of experiments involving animals, and how pilot studies followed by accurate power calculations can drastically reduce the unnecessary harm to animals and loss of animal lives in experiments. To my surprise, this was the portion of the course that grabbed me the most. I am always awed at the ways in which mathematics, statistics and modelling can help us as biological researchers to improve the quality of our data, optimize our experiments and, in this particular case, make experiments more ethical.


Pasteur Museum (image from https://www.pasteur.fr)

As part of the course, we also had to take part in a practical session, to be introduced to a number of procedures described as minimally invasive procedures. The session involved a staff demonstration of these techniques on an awake mouse, followed by a practice session in which we were able to attempt some or all of these procedures on anaesthetized animals, under the supervision of animal technicians. At the end of the course, we were assessed and received our certifications. The course does not certify that we are proficient in the handling of animals in experimental procedures, but it certifies that we have the necessary basic knowledge to be able to develop some or all of these skills in our institution, with the assistance of experienced researchers or technicians.

We were also given a private guided tour of the museum of the Institut Pasteur. Since the terrorist attacks that took place in Paris in 2016, it is, for safety reasons, very difficult to visit the Musée Pasteur without invitation and/or prior approval, so this was a fantastic opportunity to see something really unique and special.



Pasteur Museum (image from https://www.unjourdeplusaparis.com).

The museum is found in the oldest building of the institute, and was also where Monsieur Pasteur himself lived, and was eventually buried alongside his wife, in a purpose-built crypt. An offer was made to Pasteur to be buried in the Panthéon – the burial place of some of France's most significant and distinguished citizens – however he refused, preferring to be buried alongside his work. The tour began with the scientific history of Pasteur, from his beginnings as a chemist, to his numerous groundbreaking discoveries in molecular biology – including the concept of "germs" resulting in disease, which resulted in the development of hygienic practices, and his most famous scientific feat, the discovery and development of vaccines. This explanation was illustrated by the hundreds of genuine experimental samples and vials from actual experiments of his. Some of these vials were from attempts that Pasteur had made to find vaccines to attenuate the effects of serious infectious diseases such as rabies & cholera. The room was a testament to Pasteur's unwavering passion and dedication to his work. The visit continued with a tour of the house, in which we were able to observe his living space & home life. The home is lavishly decorated, but in that slightly heavy, suffocating style that that was so popular in the Victorian era. The tour ended with a visit of the crypt, which was the clear high point of the visit. The crypt is hauntingly beautiful. It is kept under lock and key, sealed with a massive metal gate. Inside, the feel could not be more different from the austere, asphyxiating atmosphere of the family quarters. The walls are made of a white dappled marble, and the floor and ceiling are covered with Byzantine mosaics, depicting the life and achievements of this French hero. The tomb itself is very simple, made of a jet black marble and very little in terms of inscriptions or flourishes. His wife, Marie Laurent Pasteur, was laid to rest in a discrete tomb at the foot of the small altar situated at the far end of the room. It is said that she wanted her tomb to be as subdued as possible, as she did not feel she deserved the reverence that her husband was owed. The room in its entirety is a formidable tribute to a man who was at the origin of some of the most pivotal discoveries for medical practice in recent history.

Laura

The Paris-Seine Institute of Biology (IBPS - FR3631), created on January 1, 2014, concentrates all of the biology research on the Pierre and Marie Curie campus, within Sorbonne University (SU), where this discipline has occupied always a central place. Located in the Quai St-Bernard building on the banks of the Seine, the IBPS brings together more than 500 people in 5 units and 7 technological platforms.

INSTITUT DE BIOLOGIE PARIS SEINE

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ESRs IN LOCKDOWN

A Note to Self: Reflecting on the Past and Present, Towards a Better Future.

Article by Elizabeth Brockman, ESR.



Elizabeth in London.

Hello! My name is Elizabeth. I am a PhD student currently working at Imperial College London studying how Down Syndrome affects the brain. I have been living in the UK for 18 months, having moved here from Canada last year to do my Master's degree. I have thoroughly enjoyed the changes and challenges that being an international graduate student have offered. While I suspected there would be many obstacles along the way, nothing could have prepared me for our current circumstances. I, like many, have been blindsided by this frightening new reality. Be that as it may, living through this pandemic has provided me with a few new and useful insights.

The first is that people are good. Truly, honestly and wonderfully good. We are surrounded by a community of neighbours, coworkers, friends, family, partners, and strangers that we often forget about in the hustle of the day-to-day. It has been in the face of this tragedy that I have been reminded of how much good there is around us. Healthcare and critical workers putting their safety on the line for the needs of others. People distancing from their loved ones to keep others healthy. Neighbours reaching out to support one another. Strangers becoming friends by way of smiles from afar, encouraging posts online, or just by making us laugh for a while.

The second is that life can change so quickly! That what you once knew can so easily be replaced by a different truth. As our lives change, so do we. We grow, learn and develop into better versions of ourselves. It is my hope, and my belief, that surviving this will help us become more compassionate people. I know for sure that I have changed, not just due to this situation, but slowly over the course of many years. My education, my travel, my friendships and my experiences have changed the way I think about my whole world. So, I write the remainder of this blog post as an address to my many past selves, as a way to reflect on my growth and on how change can make us all stronger.

To Elizabeth, age 9, the nervous smarty-pants. I know you spend your school assemblies staring up at the "Big Kids" in the back row, wishing you were up there with them. Wishing you were all grown up and that it was your turn to face the world. Don't worry, your time will come. In the meantime, enjoy it! Be a little more wild! Getting perfect grades at your age isn't as important as living joyfully.

To Elizabeth, age 16, the high school student, the determined young woman. You are working so incredibly hard and doing so well! I am proud of how far you've come and how much you've learned in so few years. Remember to love these experiences. It will be one of the few times left in your life with so much comfortable structure. Life gets crazier later on. Friends live a little further away, life becomes your own to plan and figure out (which is as difficult as it is exciting), and things will be far less straight forward. Grab life with both hands and hold on! It's going to get a little bumpy for a bit, but sometimes bumpy is just a rodeo ride.

To Elizabeth, age 18, the first-year undergraduate student crying after failing your first calculus exam. It's okay not to be perfect. No one ever is, and I know it sucks, but you aren't an exception. Take this lesson and learn from it, you'll find your way through this and come out the other side laughing.

To Elizabeth, age 21, about to embark on your first international adventure on a work term in Japan, you're going to be GREAT! This experience will be the best thing in the world for you, not only because of how much science you're going to learn, but also because of how many wonderful people you'll meet. You will cherish this experience for the rest of your life. Keep the lessons you learn from the beautiful Japanese culture close to heart. They are lessons passed on over generations, full of wisdom. Remember what it's like to spend a year living as a foreigner, not speaking the language of the people around you. This will humble you in the best sort of way.

To Elizabeth, ages 23 and 24, applying for grad programs, YOU WILL GET SOMETHING! IT'S OKAY! Rejection is an important part of life. Take your rejections with a smile, learn from your mistakes, and don't forget to keep on trying. You'll find in time that what you do get will be exactly what you needed. After all, you miss 100% of the shots you never take (thanks Wayne Gretzsky).

And finally, to all of you out there reading this (however few that number may be), take this moment to reflect on how much you have struggled through. Remember all of your successes and all the wonderful people that have helped you chase your dreams. Remember your failures and what you have learned from them. And most importantly, remember that this situation will be something we all look back on in the future. Make this a time where we know we did the best that we could and more. A time that we used to plan for a better future. One where we came together and helped one another to get through something globally frightening. Make this a time that will change our world for the better.

Stay safe out there! We'll get through this together.

Your friendly neighbourhood PhD student,



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BACK TO POST-PANDEMIC PhD LIFE

Back to the future.

Article by Flavio Tomasi, ESR.

10/05/2020 About 9 months ago I started my PhD at the Sorbonne University, in Paris. The aim of my project is to evaluate whether the modulation of the endocannabinoid system has beneficial effects on a mouse model of autism. The first months of my PhD have gone very well, I learned a lot and I met wonderful people.

Until a few weeks ago, I was thinking of dedicating this post to the organization of the second Network school of our Syn2Psy program – about the genetic approaches for the study of neuropsychiatric disorders – that was planned for mid-June in Paris. I was so much looking forward to seeing again that group of passionate and crazy people that are the other Syn2Psy early stage researchers.

Unfortunately, as you know, our plans have changed, and our network school has been postponed. France, as many other states in Europe and worldwide, responded to the Sars-CoV2 emergency with a lockdown.

The lockdown, that started in France on the 17th of March, will officially finish tomorrow. I went to the lab a couple of times during the lockdown, basically to maintain my mouse colony. My mice are the beginning of every single experiment of my project, so I really need to take care of them. And they need some care during a pandemic too! But tomorrow things are going to change! I know that we are not coming back to our normal life and that the deconfinement (this is how they call it here) is going to be stepwise, but at least I start to believe that this too shall pass.

11/05/2020 Today I went to the lab. I couldn't take the metro because during the deconfinement you need a special permission to take public transport, so I walked. I love to walk in Paris, and, to go from my place to the campus where the lab is, I decided to walk down Boulevard Saint Michel, one of the most beautiful streets in Paris, running alongside the Luxemburg gardens and the old Sorbonne University, reaching the Seine river and the Île de la Cité, where the Notre Dame Cathedral is. The weather was typical Parisian, with a grey sky and a light rain, a bit melancholic, the opposite of my state of mind. Notre Dame was full of scaffolding for the work that started after the fire that destroyed the roof over a year ago. I really hope that the works will start again soon!



Notre-Dame Cathedral, Paris.

I continued my walk alongside the river finally reaching the campus. Nobody was in the lab. Even if the lockdown is officially finished in France, our University decided to delay it for a few more weeks. Moreover, since my next experiment will be the evaluation of the effects of an endocannabinoid modulator on the behaviour of my mice I will have to wait until the animal facility will be open again to start my beloved scientific work.
In the meantime, I will continue to come from time to time, just to genotype my mice. You must know that they are characterised by a mutation on the Shank3 gene, associated in the human to the Phelan-McDermid syndrome, a genetic disorder characterised by intellectual disability and autism (among other manifestations). When new-borns are ready to be weaned, I have to check if they have inherited the mutation from their parents, through a PCR. This is a molecular biology technique that allows to rapidly make millions to billions of copies of nucleic acid fragments with known initial and terminal nucleotide sequences, even starting from a very small sample of DNA. Thanks to this technique I can distinguish between mice that have or not the mutation on Shank3. Nowadays it may seem an outdated technique but when it was conceived, in 1983, was a revolution for molecular biology. Kary B. Mullis, in 1993, was awarded with the Noble Prize for Chemistry for this invention. He has been a very eccentric scientist. During my bachelor I read his autobiography, a book called "Dancing Naked in the Mind Field" (that's what I mean with eccentric). This book inspired me a lot, with its advices and its strong support to interdisciplinarity and creativity, both allow us to see things from different points of view, in the scientific fields, but also in our personal lives. In this book, Mullis gave, I think, one of the best definitions of our work: "When you get the hang of it, science, like everything else people do for a living, is pretty straightforward. You are in the business of solving puzzles. The way to approach a puzzle is to think about it for a while, look at all the facts you can find out about it, and then take a guess. Propose a solution. The next step is to try your best to disprove your solution. Show that the pieces don't fit together in the way that you have proposed. If you can do that, then propose another solution." Well, I hope to restart solving puzzles again soon.

P.S. Tomorrow, the 25th, the animal facility will be open, and I will be able to start again my experiments! I'm so happy! I will keep you updated about the progress of my project.

Take care!

Flavio

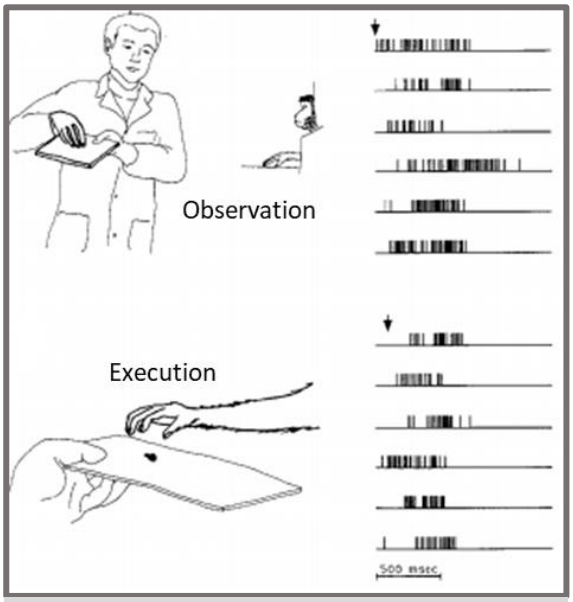
BOOK REVIEW

The Myth of Mirror Neurons: A Review of Hickok, 2014

Article by Daniel Hunter, ESR.

Thinking back, to when I first began to discover the world of neuroscience, I remember distinctly learning about the concept of mirror neurons from some popular science paperback that I picked up in a second-hand bookshop in my hometown. The notion that a small bunch of neurons with one simple function – they fire both when we perform an action and when we observe the same action in others – could potentially explain a whole host of complex human behaviours: empathy, language, theory of mind, is one of the reasons I was initially so drawn to this field of academia. I suppose there is an odd comfort in believing that these historically complex problems, that have plagued brilliant minds and philosophers for centuries, may not require comparably perplexing answers after all. However… If there is one thing that I have come to realise during my studies, and particularly so since beginning my PhD, it is that – in reality – nothing is truly simple. Reflecting on this recently I decided to read a little more about this once seemingly elegant hypothesis of mirror neurons, and thought it may be a good opportunity to see what lessons I might take away from it a second time around. For the sake of nostalgia I decided to go full circle and buy another pop-science book (except this time fully-armed with university journal subscriptions for tracking down original sources). So I hope you will enjoy this short synopsis and review of The Myth of Mirror Neurons by Gregory Hickok…

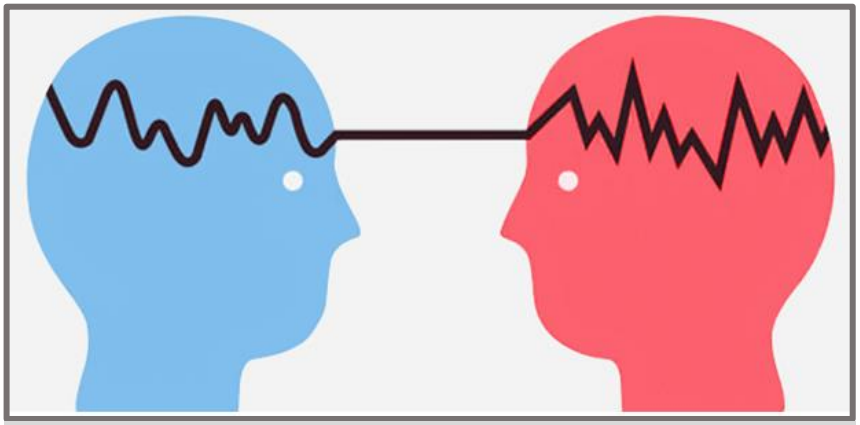
The serendipitous discovery of mirror neurons by Di Pellegrino et al. (1992)[1], sparked a speculative wildfire that has since permeated into several subfields of cognitive neuroscience – particularly the neurobiology of linguistics, theory of mind and action understanding.[2-4] Here, we review 'The Myth of Mirror Neurons'[5] with particular focus on Hickok's deconstruction of mirror neuron-based theories that have not demonstrated resilience in light of subsequent experimental investigation. The suggested alternative role for mirror neurons in imitation-learning and proposed future investigations are also briefly considered.



Mirror neurons: neurons that fire in response to action, as well as to the observation of the action. Image adapted from Di Pellegrino *et al.* (1992).

A flawed premise that has developed the supposed involvement of mirror neurons in many diverse – and complex – cognitive challenges, is rooted in their simplicity: they fire in response to the observation of action and during action execution.[1] This lead the Rizzolatti team to consider that this mechanism of internal simulation, when observing the action s of others, allows us to understand their actions – both what they are doing and why they are doing it.[1,6] Hickok suggests, that this interpretation of mirror neurons as the correlate for action understanding stems from the motor theory of speech, a theory which he claims to have long since been abandoned by the linguistic community, however the author of the motor theory published a revision shortly before the discovery of mirror neurons.[7] Thus, it does not seem as far a leap as Hickok suggests, that the entirety of mirror neuron theories are based on an archaic residual of linguistics in the 1950s. In fact, this speaks to a larger point that, while Hickok's arguments are scientifically justified and compelling, at times they seem unnecessary, dismantling mirror neurons myths that simply did not exist in the scientific community (beyond known and cautious speculation).

If mirror neurons are indeed the basis of action understanding, including the action of speech as proposed by the motor theory, then this lends itself to various testable hypotheses. Namely, that damage to the mirror neuron system in humans (including Broca's area, the homologue to mirror neuron-containing premotor cortex area F5 in macaques), will induce difficulty in both speech and language perception.[5] This however, is not the case. Many studies have shown that patients with Broca's aphasia (damage to the human mirror and linguistic-motor system) are significantly impaired in speech production but display no deficits in language perception.[8,9] Although it should also be pointed out that the ability of Broca's aphasics to comprehend language in more naturalistic circumstances (for example with background noise present), and the relative speed of their comprehension has been questioned and remains highly debated, while conveniently omitted by Hickok in this instance.[10-12]



Mirror neurons were thought to represent our ability to "simulate" the minds of those around us. Image from www.designneuro.com.

Moreover, there is a more intuitive failure of the mirror neuron explanation of action and speech perception, being that we are capable of understanding action we cannot ourselves perform – and thus have no motor system to simulate. Here we consider a study in which participants are asked to categorise dog barks into contextual groups, with significantly high levels of accuracy.[13] This study counters the mirror neuron based theory in two respects: (i) humans are able to infer a level of action understanding (why the dog barks, be that fear, aggression, playfulness etc.) from vocalisations that we cannot simulate in our own motor areas and (ii) an equally well performing subset of participants in this study were blind, and thus have no entrained observational experience for mirror neuron systems to employ.

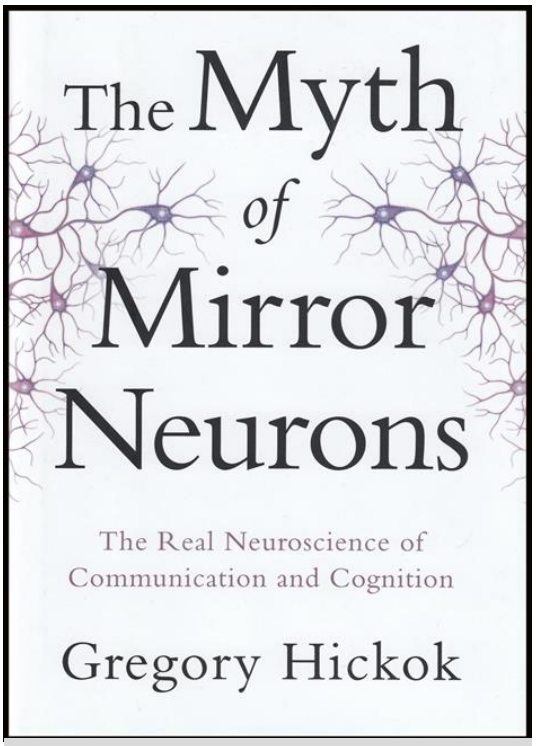
This argument extends beyond that of auditory perception, into the realms of emotional cognition. Given that mirror neuron theorising has affected research regarding empathy and emotional intelligence, it is reasonable to test whether they may play a role in understanding the emotions of others.[14] Again, the theory collapses. A rare congenital condition known as Moebius syndrome causes complete facial paralysis, hence a mirror neuron enthusiast would likely expect that such an individual – without the motor ability to instruct facial muscles into typical emotional conformations – would show deficits in reading the emotions of others by expression alone. However, this is not the case, those with moebius syndrome are equally capable as controls in this regard.[15] Given at this point that mirror neurons have failed to explain understanding of either emotional signals or motor actions, it is sensible to re-evaluate their role in *understanding*, period. There is no doubt that these truly are fascinating neurons, and likely have some elegant potential applications, however we must concede that this appears not to lay in the realms of traditionally higher-level cognitive understanding.

Ironically, the proposed new direction for mirror neuron research can be identified by yet another interesting failure of these neurons to explain action understanding. Consider an experiment in which muscle evoked potentials are greater in the index finger while observing the same finger moving on another's hand.[16] Initially, this seems like long anticipated good news for mirror neuron theorists, as it shows that the observation of an action in humans likely causes some motor simulation. However, this experiment went further and found that this mirror system can dissociate the observed preference with the motor action. For example, training participants to move the little finger when observing index finger movement shifted this increase in excitability initially seen in the index to the little finger.[16] Of course, one would not expect that the effect of this training caused participants to misunderstand the observed index movement as a little finger, thus action understanding is not defined by mirror systems. Instead their actions most likely reside in associative or imitation learning.

Furthermore, this adaptability and learning of the mirror system has since been demonstrated to occur in macaques, becoming sensitised to the grasping actions by tools (without an associated understanding of its usefulness).[17]

A concluding point of note, the narrative throughout *The Myth of Mirror Neurons*[5] should not be considered one of simple cynicism, but the scientific voice-of-reason that ought to be striven for and is required for scientific progression. The trajectory of mirror neuron research since their discovery in 1992[1], generally highlights the importance of speculative caution in scientific writing. Moreover, it is vital that as a research community, we can indeed celebrate discovery *and* recognise failures. Hence now – after exhaustive attempts to redeem what may appear as a temptingly elegant hypothesis – we must move our consorted research efforts to more promising horizons.

Daniel



The Myth of Mirror Neurons by Gregory Hickok. Image from www.amazon.fr.

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FRONTIERS for YOUNG MINDS

By Raquel Rodrigues, PM.

One of the major topics in the Syn2Psy project is the creation of proper science contents for multiple audiences, beginning from young publics. With this in mind, and with the will of producing communication articles shaped for younger audiences, we approached the journal Frontiers for Young Minds. After the presentation of our consortium and our projects, were invited to submit several abstracts for the Neuroscience section of this journal.

Frontiers for Young Minds is a scientific open access journal edited by and for kids. Their goal is to "connect curious minds to the experts and to information that will motivate them to ask informed and critical questions about real science throughout their lives." Frontiers for Young Minds bridges the scientists' high-quality work with the kids' scientific interest, which helps "foster curiosity both in and out of the classroom and engage the next generation of citizens and scientists."

Keep reading and you will find the seven abstracts submitted by Syn2Psy ESRs. You will also find some information about the authors!

Reaching for the top: the role of mitochondria

<u>Abstract by Alessandro and Loredana</u>

Humans, like most animals, live in social groups that naturally form ranks, following an order from dominant to subordinate that we call social hierarchy. What makes individuals more likely to fall into a certain rank? To study brain functions that contribute to this, social hierarchies are studied in laboratory rodents and identified, through social competition tests. Recent studies have shown that mitochondria, the powerhouse of the cells that produce the energy necessary for cells and organisms to work, play a role in social behaviour. Subordinate rats have lower mitochondrial capacity than dominant rats in a brain region -the nucleus accumbens- important for effort and motivation. If subordinate animals are energized with a booster of mitochondrial function, their behaviour can shift from subordinate to dominant. We will discuss what this animal research means for humans and how it may help us find new ways of treating troubled social behaviour.

ALESSANDRO



Alessandro comes from Italy, where he obtained a MSc in Neuroscience at Trieste University.

"The position offered within the Syn2Psy network is an ideal environment to unravel fundamental questions regarding the circuit structure of the brain and the pathologies arising from its imbalance. Furthermore, the network schools offered by the program cover most of the cutting-edge technology needed to solve the students' scientific questions."

LOREDANA



Loredana is from Romania, where she obtained her BSc in Biology and MSc in Neurobiology from the University of Bucharest. Her training focused on receptors and ion channels in the peripheral nervous system.

"Understanding cognition and emotions through the lens of Neuroscience sparked my passion for this field. In particular, I want to contribute to the way we view and treat neuropsychiatric disorders through a better understanding of their underlying mechanisms as they represent an increasing challenge in our society."

LAURA



Laura is a British and New Zealand dual citizen, who grew up in New Zealand and France, before moving to Bristol to study Pharmacology, followed by Molecular Neuroscience. She recently came back from a genetics-focused internship in New Zealand.

"I am delighted to be back in Paris and to be pursuing this doctoral project which combines my love of genetics and neuroscience. The prospect of working on a disease model is particularly exciting, given the possibility that the research may result in a greater understanding of the disorder, and consequently more effective treatment for affected individuals further down the line."

FLAVIO



Flavio is from Italy. He graduated in Neurobiology at Sapienza, University of Rome.

"I do believe in the importance of research, not only to contribute to knowledge, but especially to improve society and people's lives."

Fantastic beasts and where to find them: how can we use animals to study neuropsychiatric disorders?

Abstract by Laura and Flavio

A psychiatric disorder is a mental illness that disturbs a patient's behaviour, mood or ability to think, and can get in the way of them leading a normal life. Even though studying human patients might seem like the most obvious way to understand these illnesses, doing so is not always practical, affordable, or even possible. Because of this, other more roundabout methods have to be found. Fortunately, we can create animal models of some psychiatric disorders, to better understand their biological causes. Genetic differences are a common cause for these kinds of diseases. Because of this, genetic animal models are a very common way to study how these illnesses modify brain function and behaviour, and can even help us to find treatments for these disorders. Did you know that mice can imitate the symptoms of depression, schizophrenia or autism? In this article, we will discuss what kinds of information we can obtain from animal models, and what are the pros and cons of using these models to understand human diseases.

frontiers FOR YOUNG MINDS

The play-doh brain: shapeable neurons make you learn!

Abstract by Elisa and Manuela

Imagine your mum just baked pizza and you immediately eat a piece of it. Bad decision, it is really hot! Now you will wait for it to cool down before continuing to eat. The burning sensation reaches the brain and the neurons communicate with each other so that you stop eating. Next time your mum will cook pizza you will have learnt to be more patient. This is possible thanks to a process called synaptic plasticity, in which neurons increase the connection already formed in the first communication and prevent you from hurting yourself again.

In this article we will describe some properties of neurons, as well as what happens to these cells when you create new memories in a more detailed way. We hope that you will learn something new by reading this piece, and share it with others, as neurons would do!

ELISA



Elisa is from Italy and she studied Neuroscience at the University of Trieste.

"Here I have become more and more conscious about the fact that doing science means trying to solve problems and to answer questions and considering that this is a never-ending process stimulates my mind."

MANUELA



Manuela is from south Italy and she obtained her BSc degree from the University of Ferrara, followed by a MSc degree in Cellular and Molecular Biotechnology at Trento University. After graduation, she went to Lund University, Sweden, where she joined the Behavioural Neuroscience Laboratory and focused on Parkison's Disease.

"These experiences and the huge desire to contribute to science, pushed me towards academy and to start a PhD program in Edinburgh University, part of a fascinating network such as the Syn2Psy which will offer me the opportunity to be in an international environment."

ORSOLYA



Orsolya is from Hungary and she completed her undergraduate and MSc degrees in Pharmacology, at University College London, UK.

"During my studies, I took interest in neuropharmacology and specifically In the role of AMPARs and their auxiliary subunits in cognition and disease. This interest drove me to write my undergraduate and master's dissertations on auxiliary subunit-selective antagonism of AMPARs and I'm extremely excited continue exploring this area and to join the Syn2Psy programme with my ESR1 project."

ÁGATA



Ágata comes from Spain and she obtained her BSc degree at UCM - Universidad Complutense de Madrid and her Neuroscience MSc at UAM - Universidad Autónoma de Madrid.

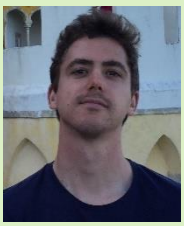
"I am particularly interested in studying the function of E/I balance and how this is disrupted in neuropsychiatric diseases. Understanding the implication of both excitatory and inhibitory systems in cortical and subcortical microcircuits could help us to unravel the mechanisms that underlie higher brain cognitive functions. Restoring this balance might constitute a promising therapeutic strategy against disorders such as schizophrenia."

Schizophrenia: what causes hallucinations and distorted thinking?

Abstract by Orsolya, Diogo and Ágata

Imagine living without being sure if what you see, hear or think is real or not. Schizophrenia is a mental disorder characterized by distortions in thinking, perception, emotions, language, sleep, and behaviour. It affects 20 million people worldwide, and typically becomes evident during late adolescence or early adulthood. While medication can be used to control hallucinations, most symptoms are resistant to treatment. As a result, patients and families have serious difficulties in their daily lives. But what do we know about what is happening in the brain of people afflicted with this disorder? Which parts of the brain may be altered and how can we develop better treatments based on this knowledge? In this article we will explore how the brains of people with schizophrenia are different and how gaining more knowledge can help them.

DIOGO



Diogo is originally from Lisbon, Portugal. He obtained a Bachelors degree in Biology from EKU in the U.S. and a Masters degree in Neuroscience from the University of Lisbon.

"My motivation for my PhD is to study how synaptic organization influences synaptic function and how its dysfunction may be related to the development of neurological disorders."

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Exploring the living mouse brain using 2-photon microscopy.

Abstract by Elizabeth

The human brain is a highly complex system comprised of billions of cells, called neurons, interconnected to each other in functional neural circuits. Normal brain function is dependent on effective communication between neurons, a process that requires synapses, the point of contact between nerve cells. Several diseases result from impairments in the communication between neurons, which is why it is essential to study synapses in the living brain. Since the human brain is not accessible, scientists use simpler organisms like mice.

To see mouse neurons, a piece of skull is replaced with a transparent glass window. Powerful microscopes called 2-photon microscopes, can then be used to capture snapshots of neurons deep inside live brains! Clever use of lasers to excite tags placed inside the cells make neurons emit fluorescent light and glow in the dark. Join us as we illuminate the inner workings of neuron communication in the living brain.

ELIZABETH



Elizabeth is Canadian and has recently completed a MSc Clinical Neuroscience at University College London.

"My work focuses on studying the mechanisms of neural oscillatory patterns in a novel in vivo model of Down Syndrome. "

VANESA



Vanesa is Spanish and she studied a bachelor in Basic and Experimental Biomedicine in Seville followed by a Master in Neuroscience in Alicante.

"My main motivation is to find out which/how circuitry disfunctions cause attention disorders and explain social behaviour features."

DANIEL



Daniel grew up in the South-East of the UK and studied at the University of Sussex, where during his Masters studies, he worked in the lab of Dr. Andrew Penn.

"I am particularly fascinated by the way that disruption of synaptic function – at the level of individual receptors – can translate throughout the levels of neuroscience, from the molecular through to a behavioural or clinical phenotype."

A brain composed by multilingual neurons: A story of coded messages and crosstalking.

Abstract by Vanesa and Daniel

Everyday, we communicate with each other using language. To do this effectively requires two things: 1) the speaker to be clear and precise, and 2) the listener to understand the language and respond.

Similarly, in our brains, neurons communicate with each other, at a location called the synapse where one neuron "talks" to another neuron by releasing a particular chemical message. The "listening" neuron understands this message by receiving the chemical at receptors on their surface. Every neuron speaks to hundreds of others, simultaneously and using different languages. The brain works as a social network which integrates information and responds to hundreds of "chats" arriving at the same time.

This article focusses on synapses that use a chemical messenger (or language) called glutamate and two particular receptors, called AMPARs and NMDARs, that decode the message. This language is key to how we interpret the message and for learning and memory.

frontiers FOR YOUNG MINDS

How to build a mini human brain.

Abstract by Giuseppe and Marcos

Growing miniature brains in a laboratory may sound like science-fiction, but advances in technology have made it possible. Scientists can collect human cells from different tissues, like the skin, culture them in a dish and reprogram them to make stem cells, like the ones in an embryo. These reprogrammed stem cells have the ability to multiply and convert into all the kinds of cells that form our body, which lets us study the development of human organs. Under the right conditions, stem cells can aggregate and grow into small brain-like structures, called brain organoids. The study of brain organoids has allowed us to expand the possibilities of recreating the human brain and can reproduce reliably many features of its function and development. In this article, we would like to explore how these brain organoids are created and how they can help us make new discoveries on diseases related to the development of the brain, such as autism.

GIUSEPPE



Giuseppe obtained his BSc degree from the Tuscia University in his home country, Italy. His interest for developmental disorders in human brain led him to apply for a Research MSc in Neuroscience at the Erasmus University Rotterdam, Netherlands.

"As an early stage researcher of the Syn2Psy Innovative Training Network (ITN), I would like to broaden my knowledge in human neurodevelopment and give an insightful contribution on the mechanisms that affect this process at the synaptic, cellular and structural levels."

MARCOS



Marcos is from Spain, where he studied a BSc in Biotechnology and a MSc thesis at the laboratory of Vertebrate Central Nervous System Development at the Centre of Molecular Biology Severo Ochoa in Madrid.

"My main area of interest is cognitive neuroscience and neurodevelopment, and my goal is to contribute with my research to the treatment and understanding of neuropsychiatric disorders."

FORTHCOMING EVENTS

Due to the recent SARS-CoV-2 crisis, the network-wide events planned for June this year were postponed. While we are on the process of rescheduling the Syn2Psy Network School II, here you have a summary of the events happening in the months to come:

Scientific PhD day – online, October 5, 2020.
Half-day remote meeting of ESRs with the EC Project Officer – online, October 20, 2020.
Mid-term meeting – Copenhagen, May 3-4, 2021.
Network School III – Copenhagen, May 5-7, 2021.
Network School II – Yet to be defined.

Newsletter

Issue 2 – August 2021

Innovative Training Network

Synaptic Dysfunction in Neuropsychiatric Disorders



Syn2Psy Early Stage Researchers during Network School 3, held online in May 2021.

Welcome to the second issue of the Syn2Psy newsletter!

By Ana Luísa Carvalho, Project Coordinator.

So much has happened since the first Syn2Psy Newsletter! The world faced several waves of COVID-19 and most of us have been through a second confinement, having to adapt to the restrictive measures put in place in the labs. It has been an extraordinarily challenging year, full of uncertainties and the need to re-think and re-arrange Syn2Psy's activities. Despite this, thanks to the great efforts made by all of you, Syn2Psy is on the right track and moving forward steadily. I would like to thank Early Stage Researchers, Principal Investigators and Partners for coping with this difficult situation in such a positive manner.

During this year we had our first online PhD day, where the ESRs presented their results within the consortium for the first time. The ESRs delivered excellent scientific talks and the lively discussions with the consortium's PIs resulted in new ideas arising for the progress of the projects. In May, our ITN was reviewed by the REA during the online Mid-term meeting, which was followed by Network School 3. During this online school, the fellows learned the process of developing new drugs, from idea to patients, from the experts at Lundbeck.
Furthermore, the ESRs have promoted Syn2Psy through several outreach activities including the release of four episodes of the SimpleSci series, where the ESRs describe their project in a language adapted for the general public; and the publication of several entries for the blog in topics ranging from the importance of science communication to the impact of loneliness, the adaptation to PhD life and creative writing.

We are now looking forward to our next network-wide events: the second online PhD day in September, with the participation of the two members of the External Advisory Board; and Network Schools II and IV to be held in 2022.

Keep reading for more details about all this and more.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska- Curie grant agreement No 813986.

An alternative approach to writing protocols

Article by Orsolya Antal, ESR.

When I first had to choose my time for writing a blog, I thought this month would be just perfect. Back then, I planned to write this after our second meeting in France – cool science and Paris, what more do you need?

Then, of course, things changed, so I found myself revisiting an idea I had a while ago. The initial version of this short piece came to life on a February afternoon, when my attempts to learn as much as I could about experiments with mouse behaviour were suddenly interrupted by a fast-approaching deadline to submit a creative writing piece for my science communication course, so I combined the two, taking one or two artistic liberties. Despite not having done creative writing for a very long time, I ended up really enjoying it, but never quite made the time to revisit and finish it in a slightly more polished way. Until now, that is, and I was hoping to share it. I hope you'll enjoy an alternative perspective on a day in the life in the lab:

A day in the life

Days are calm here. There's a routine to everything, day after day after day. It's morning and then it's evening, always at the same time. You get used to the monotony and when it's all you've ever known; you might even find the structure comforting. There are no changes, no unexpected disturbances, nothing to fear. It's serene here in the lab, really.



Schematic of a laboratory mouse absolutely not thinking about biting the experimenter's hands (Who, them? Never.)

For as long as I can remember, it's just been the three of us. I've heard there are many other groups, even saw a few of them a couple times, but only very briefly. Shorttail and Skitter, however, have been here since we were born, and we've become a tight bunch over the weeks. It's good I'm not alone, too. Being alone is very scary: just imagine, you're all alone in that huge box with nothing to do all day.

Sure, you can run around and explore all the nooks and crannies and folds, but there's no Skitter to complain about Shorttail stomping on his tail again ("Whiskers, tell him to stop") and no Shorttail to brag how he's the toughest mouse around (he's not). I'm glad to have them around, even if they get on my nerves sometimes.

The space and company we have is not much, but it's enough for us. I guess you're okay with your world not being too big when you're so small. One day, however, this all changed. It started as just another day. We almost didn't even notice Coat come in. We had seen her before a few times. Initially she smelled weird, but then we ended up getting on. I like that one, especially if there are treats involved. For future reference, I myself am particular about peanut butter, Skitter is more of a Nutella-aficionado. Shorttail just eats whatever he's given.

Then suddenly it was as if the ground shook. Shorttail was the first to realise that we were being moved. But where? Why? For what purpose? Was it Coat? I was more curious than scared, but I must admit to being… cautiously apprehensive about the situation. Skitter, on the other hand, was frantic with worry. Thankfully, our sudden journey didn't last very long, and we arrived. To another floor? It smelled familiar, and we saw some of the others as well. If there were other mice around, we were probably not in danger. Besides, as the initial surprise wore off, I was starting to get excited. An adventure!

Soon after, Coat reappeared. All we heard was some faffing around in the room, I think I saw some bits of walls being moved around and then our roof was coming off and suddenly the world expanded. Forgetting how anxious he was becoming, even Skitter joined us in trying to climb and reach as high as we could so that we could sneak a look outside.

Shorttail spotted a route along the piece of paper towards the edge of the box, but before he could reach it, he was grabbed by Coat. Just like that, Shorttail was gone. Skitter kept asking me what was going on with him and was he going to be alright? Would he return? Was he gone forever? Were we next? Having no answers, I went to see if I could learn anything else about our surroundings. Shorttail returned (or, rather, was returned) soon after, and while he was slightly bamboozled about the sudden change in circumstances, he appeared unharmed. Before he could tell me what he saw, however, it turned out that I was the next to go off into the unknown.

The unknown ended up being a weird white room with two towers. It was slightly more spacious than our previous box, I saw no immediate threats. It was time to explore! First, I wanted to see those towers up close. After thorough examination, I found that while they were quite tall, they could be climbed on. Having no other option (because really, would you expect me to stay still? On the ground? While there's a tower to be climbed? Come on, now), I jumped on top and tried to see what was going on, this time from higher ground. You could almost see the edge of the box and the vast room beyond. Tragically, the edge was just a little too far to make a jump for it. Conceding that the large room was probably out of my reach, I climbed down and continued to roam around the smaller one. After a while, Coat must've had enough as I was transported back home. (I do not appreciate being picked up at all. Terribly uncomfortable and this time we're not even getting food. I was only in it for the food. Unhand me, human!)

As it turned out, all three of us had to go through this, so Skitter was next. He was more agitated than Shorttail and me, and he very much did not wish to go. He managed to evade Coat for quite a while, eliciting noises of exasperation from her, but was eventually caught. He returned in one piece as well, if only a bit annoyed at being put through such an ordeal. With that, we seemed to have been given some respite. Coat left and finally we had some time to rest and try to make sense of exactly what just happened.

The next few hours went by peacefully. Left to our own devices, we wondered what was going on. Shorttail appreciated the changes in scenery while Skitter appeared to be a bit disturbed by them. It was nothing special, really, just a new room for us to explore, even if Coat appeared to take us from one place to another at her leisure. For now, we revelled in having a break and not having to worry about who was going to be taken where next.

Our peace was disturbed again soon enough. Coat returned and pursued us again. As before, Shorttail was the first to go, followed by me and then Skitter. However, as soon as I was put in the white room again, I noticed that something was off.

It was almost the same as before, yet it was as if one of the towers had been moved. Weird. I went over to see if there was anything different about it, but it appeared to be the same tower as before (you could still climb on top of it, I was happy to note). Then why move it? Was it the other one that was changed? I ran back to examine that one as well, but just like before, no change. The view from the top of the tower was the same as well (as expected, but I just had to make sure). I kept trying to figure out what was new, but I must've run out of time as I was picked up and put back with the others. After Skitter had finished as well, I quizzed him and Shorttail to see if they could make any more sense of it.

Shorttail was similarly confused, while Skitter appeared nonchalant about it ("they were just towers in a room, what about it?"), as if he thought nothing had changed. Weird, though he has been a bit forgetful lately. Shorttail and I had started wondering if there might be something wrong with Skitter.

It turned out that had been the end of our day of adventure. Coat had replaced our roof and then the floor turned unstable yet again – we were travelling, back to our usual place. To our usual routine. As adventurous as the day had turned out to be, I was happy to go back to some peace and quiet (and Shorttail bragging and Skitter complaining). While munching on our dinner, we were wondering if this was a one-time occasion or there were others still to follow. I guess we would find out. For the time being, we went back to our serene routine and waited for what would come next – ready for just another day.

The Center for Neuroscience and Cell Biology (CNC) is a nonprofit research institute founded in 1990, aiming to foster biomedical research and multidisciplinary graduate teaching at the University of Coimbra. CNC was the first established "Laboratório Associado" in Portugal, and it has steadily increased the scope of scientific competences over the years, with a strong focus on the exploitation of the fundamental mechanisms of aging and brain diseases.

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CNC CENTER FOR NEUROSCIENCE AND CELL BIOLOGY UNIVERSITY OF COIMBRA PORTUGAL

Orsolya

One year of PhD in the eyes of Loredana and Marcos

Journey logbook of a Ph.D. student

Article by Marcos Sintes, ESR.

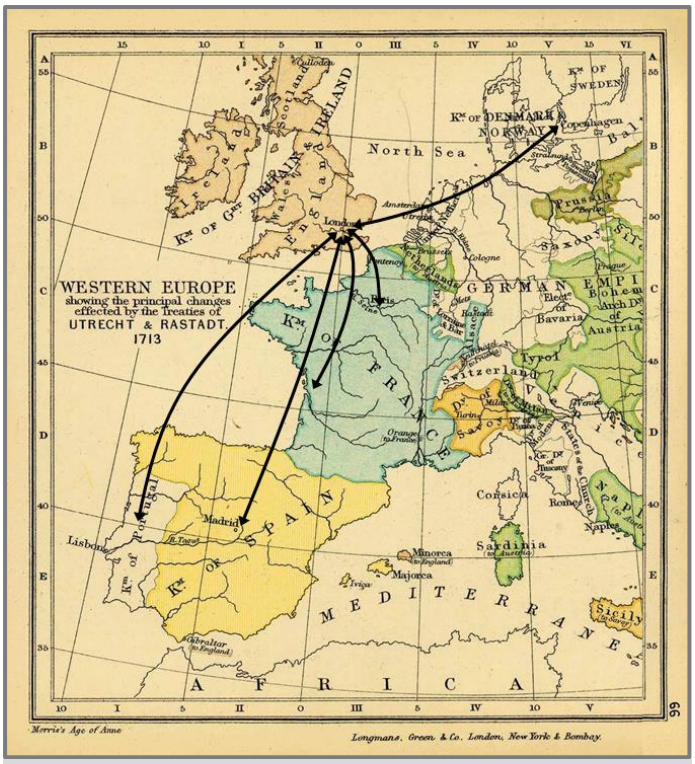
Hello everyone, my name is Marcos and I would like to tell you how my journey to become a Syn2Psy Early Stage researcher at Imperial College London began. I could not pinpoint the exact moment that my passion for science started, but I know I was very young. As a child I spent the mornings with my eyes glued to the ground, observing the daily life of the small creatures that crawled it and pretending to be a naturalist or explorer discovering the exotic species of a distant land. I would imagine myself following the steps of great scientist like Charles Darwin, setting foot on the Beagle, and navigating for years to unexplored shores, describing unknown ecosystems and facing all kinds of misadventures.

As time went on, these aspirations became more and more tangible. By stripping them of part of that childhood romanticism, science became a realistic career to pursue. I followed all the necessary steps: high school, university and master's degree. And finally, in 2019, the beginning of my Ph.D. I did not have to get on a boat and sail to unknown territories, but this adventure took me to the not-so-distant but kind of exotic land of England. Since then, I have been facing some of the expected challenges that come with moving to a different country and starting a new job, and some very unexpected ones, like a global pandemic. But most of all, I find myself doing what I always wanted, doing interesting scientific research.

I decided to document my odyssey across Europe, and I would like to share with you some of the most relevant entries:

September 17, 2019. Day 1:

All journeys have a beginning. Mine is leaving my home country, my loved ones and my whole previous life behind and embark on this new adventure. All my essential belongings are packed, carefully chosen to make the transition to my new home as smooth as possible. I said goodbye to my family at the airport and proceeded to go to the plane that would take me to the next chapter of my life. A short-rental accommodation in Kensington will be my temporary home for the next few weeks before leaving for Coimbra. After unpacking my luggage, I decided to make good use of the rest of the daylight time and go for a stroll to explore the neighbourhood. I am now going to bed early, equally nervous and excited. Tomorrow I will officially start my PhD and go to the lab for the first time.



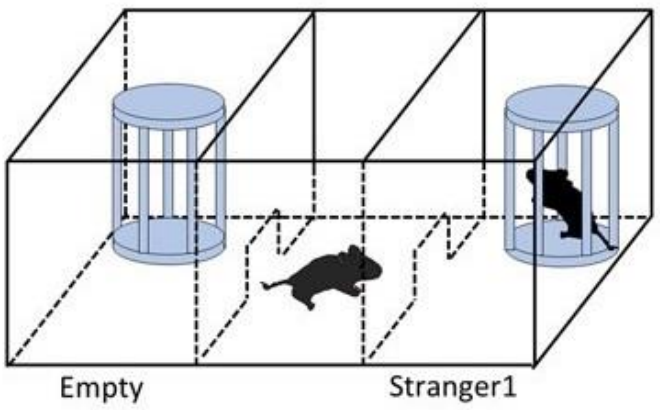
Planned itinerary across Europe during my Ph.D.

October 5, 2019. Day 19:

This last week was quite intense. The day after arriving in Coimbra I became acquainted with my travel companions: thirteen fellow passionate Early Stage Researchers from all over the world who decided to follow their love for science. We spent the first days getting to know each other and our unique personalities, sharing our background and motivations. The festive atmosphere made everything easier, as the start of the new academic year is quite an event in this city full of students. We also had the opportunity to live our first academic experience as Ph.D. candidates, attending to interesting classes at University of Coimbra. Overall, I found these last few days to be a thrilling introduction to what is yet to come. Now it is time to go back to London and truly start my new project.

November 25, 2019. Day 70:

Today I did the first round of behavioural experiments of the first part of my project. We used the three-chamber test to determine how sociable a group of control mice are. In this test, a subject mouse is placed in the centre of an apparatus consisting of three contiguous chambers, and over several minutes it can decide whether to spend time with a novel mouse situated inside an enclosure in one of the chambers, or with an empty enclosure in the opposite chamber. This simple tool, developed by the group lead by neuroscientist Jacqueline Crawley in 2004, will be instrumental in determining the sociability of our mouse model for autism.



Three-chamber sociability test (Lim et al. 2017).



What is a journey without a parrot on board?

December 31, 2019. Day 106:

Today is the last day of a year that has brought so many changes to my life. It is also the first time I will spend New Year's Eve far away from my family. This has served as a reminder of how important our connections are to us and how easy it is to take them for granted. Scientific work is very demanding and sometimes it is easy to get lost in it, but finding the time to take care of all the aspects of our life that are important to us, is key to avoid burning out.

June 15, 2020. Day 242:

Almost three months ago I had to say goodbye to my colleagues and my experiments and face the first big challenge that the new global situation would bring to my project: the closure of Imperial College. Being forced to stay away from the lab, I decided to spend that time with my family and travelled back to Madrid, where I stayed with my sister and my three cats. Despite being the first time in months that I was this close to my friends

June 15, 2020. Day 242:

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This concludes the beginning of my journey. It is not only my scientific journey, but also one of the milestones of my life. And it is a rocky beginning, I know. Nobody could have imagined we would have to face challenges like this so early in the millennium. We have spent decades envisioning all kinds of dystopic futures in books and movies, but reality has proven to be quite different from what we had ever thought. This experience has taught us that even in the worst global scenarios, life goes on and mundane things keep happening and filling our days. During these difficult times I have found comfort in my work. Having the opportunity to keep walking the path I chose in a time like this means a great deal to me. I hope this is just a bump in all our journeys, but even if it is not, I can say that this unusual situation has further consolidated my love for science. Watching healthcare professionals and scientists working together to minimise the impact of the pandemic has been at least as inspiring as the stories of 19th-century naturalists were to the five-year-old me. I feel now more motivated than ever to give all I can to help in the advance of science and I strive to contribute to a brighter future for all of us.

Imperial College London is a world top ten university with an international reputation for excellence in teaching and research. Consistently rated amongst the world's best universities, Imperial is committed to developing the next generation of researchers, scientists and academics through collaboration across disciplines. Located in the heart of London, Imperial is a multidisciplinary space for education, research, translation and commercialisation, harnessing science and innovation to tackle global challenges.

Imperial College London

Marcos

One year of Swiss cheese and chocolate

Article by Loredana Cumpana, ESR.

A year ago, in Romania, I packed minimum necessities into a small suitcase, said goodbye to my friends and family, and jumped on a plane to Switzerland to start a new chapter of my life. I felt awe, a mix of excitement and fear, as I dived into the unknown. Looking at the clouds, my mind was racing with the possibilities of the future, fully aware that it will be a difficult journey but nothing worth doing is ever easy!

The same applied to the lab too, getting started in a system that is quite different than what you're used to, implies an adjustment period. Now I can be more independent in the lab, being able to work my way around without always asking our technicians for help. I can move around the village that is the EPFL-UNIL campus without getting lost (most of the time, thankfully there's GPS). Having less day-to-day details to worry about allowed me to be able to dive into my PhD project and start exploring the wonderful topic that I feel lucky to have the opportunity to work on. But of course, as you get past one hurdle, the next comes. There are new techniques to learn, a pile of literature that you never seem to have the time to read, yet new articles keep being added to it.



EPFL Campus

You put long hours and a lot of effort into experiments to have a puzzle as results but you're not even sure they come from the same set. It can start to feel like you're not progressing, like your efforts are in vain and your time is lost. Sounds grim, you could ask, why bother? Well, this is exactly where Neuroscience can help you and the reason I am perpetually fascinated with it!

Understanding why we behave the way we do and how our brain may be biased, can help you find unexpected solutions to improve your life and wellbeing. For example, have you ever wondered how someone could obsessively play computer games with impressively sustained focus without seeming to get bored even when the tasks get increasingly repetitive and wondered why that type of focus is rarely applied to real-life situations? Games use a series of neuroscience hacks to catch our attention and maintain our motivation. This is mostly based on the fact that, evolutionarily speaking, our brains were not meant for long-term goals and are instead flooded by dopamine at the anticipation of a short-term reward. Using the tricks games use to motivate yourself in your everyday life is called "gamification". The main points that drive this concept are short-term reward and keeping track of your progression. Everyday tasks can be divided into small goals that are rewarded upon completion. Particularly, I find it very useful to acknowledge progression while keeping track of how that moves you closer to your end goal. Sometimes you can make mistakes or not achieve what you intended but that does not mean you did not improve that skill to be able to do better next time. Games do a great job of keeping track of these things for us, and adding some of these elements to your work can help you feel more motivated. I feel very lucky to be a part of my lab, the Laboratory of Behavioral Genetics.

Throughout my first year, I've had excellent supervision and training and great colleagues that maintain a wonderful and cheerful work environment.

Despite the normal difficulties associated with getting your PhD and the high-stress situations that can come with it, it has been surprisingly easy to deal with because of the support and encouragement that my supervisors, colleagues, and friends provide. Perhaps this is also aided by the fact that, in the lab, one of our main research topics is stress and how it can enhance the risk for psychiatric disorders. This was one of the reasons I wanted to join the lab, because I think this is an increasingly pressing issue in our society. Everyone experiences stress, but prolonged, intense stress can cause maladaptations. However, less mentioned and equally important is the fact that stress is also good. It's a physiological response that is meant to prepare you for a demanding situation. For example, when you are about to give a presentation you may feel your heart start to race and you can interpret that as anxiety, but you can also interpret it as excitement. Your perception of stress can influence how it affects you. So, don't be afraid of stress! In moderate amounts, it drives us forward!

Neuroscience research can be complex as it deals with biology which becomes more intricate the further down you go. Usually, the goal of neuroscience research is to understand how the brain works so that we can provide treatments for diseases related to this system. But often, this research also tells us about the fundamental inner workings of our minds. That knowledge can be used then by psychologists to provide new therapies or by anyone that is trying to understand themselves from a different perspective.



LGC Lab, EPFL 2019

Maybe I am biased to see everything through a neuroscience lens but because your brain is the one making up all your perceptions, thoughts, feelings, and wants, I do consider it the most important and fascinating part of our biology (according to the brain). So, I also think that understanding the new concepts that emerge from our research is not something just for other scientists but something everyone can use in their daily life.

Overall, this 1st year of my PhD has been very eventful. I've met so many wonderful people through the fellowship and at EPFL that walk the same path and share the same troubles which make the journey easier. I can't not mention the COVID-19 pandemic that changed the way we work and interact. I've had my candidacy exam, a milestone for the 1st year of PhD at EPFL, where I presented my work to a committee of experts in my field that provided valuable feedback for the future of my project. A considerable part of my duties also includes being a teaching assistant, which I found very rewarding to share my passion for neuroscience with students. I am looking forward to what the next years will bring, and I will be back with an update in exactly 1 year!

EPFL Founded in 1853, the École Spéciale de Lausanne was renamed École Polytechnique Fédérale de Lausanne in 1969. EPFL is the most cosmopolitan of all European technical universities. It welcomes students, professors and collaborators of more than 120 nationalities. With both a Swiss and international vocation, EPFL focuses on three missions: education, research and innovation.

Loredana

Loneliness: the importance of social bonds

Article by Giuseppe Cammarata, ESR.

I am writing this blog episode while thinking about the recent lockdown early this year. It was a tough time for everyone, a period in which people were, and still are, requested to keep a distance from one another, and to search for complete isolation in case of exposure to the virus responsible for coronavirus disease 19 (COVID-19).

This blogpost will be dedicated to one of the issues of serious concern of our modern society: loneliness. At the same time, I, Giuseppe Cammarata, will describe how this pandemic has worsened the loneliness problem, and how it can be faced.

As social animals, we humans crave for social connection. We naturally lean toward social behaviour to build relationships and belong to a group. Loneliness, on the other hand, is described as perceived social isolation and it is an unpleasant feeling which suggests unmet social needs and remains an ageless and universal experience.

Furthermore, loneliness has become one of the major issues resulting from the modern way of living. An increasing number of people do not live in extended families or even near each other. Likewise, people of all ages are living alone and often settle on the other side of the country or even across the world, far away from their relatives. Therefore, it seems that people are becoming more and more isolated [2, 4].

For instance, in modern countries like the UK, USA or Australia, a big slice of the population of different ages has reported to feel lonely in the last few years, so much that loneliness was declared to be a public health issue. Further studies performed on the same topic, put in evidence how dangerous loneliness can be. Especially when premature mortality, unhealthy behaviour and depression can manifest with a higher probability in lonely or socially isolated people. [2,3,4,5]. Unfortunately, with the recent outbreak of the COVID-19 pandemic, the issue of social isolation and loneliness has worsened due to the distancing policies introduced to restrain further spreading of the virus. An increase in anxiety and worsening of mental health conditions have shown up in both adolescents and adults. On the other hand, it is worth noticing how social connection and new relationships can positively affect health and our general well-being [6]. Indeed, the maintenance of social connections seems to be crucial to lighten the weight of social confinement. As shown by a study run in Australia, the reaction of the citizens to social distancing measures, focused on keeping people in contact and open to offer help [6]. As an example, people were offering to buy groceries or to bring essential medicines to those who were in strict confinement. Others were making phone calls only to have a friendly chat [1,6]. Similarly to what I have witnessed in Italy, people also started to play music both from their balconies and in public spaces. I believe that all these actions that positively affected the community should be examined in depth to eventually find a possible solution to tackle loneliness or social isolation experiences.

On a more personal note, I have experienced loneliness several times. From the moment I started my academic career, I have been travelling and living abroad rather regularly. I believe that the cause of my sporadic sense of loneliness is linked mainly to the distance between me and the people I love.

While being alone during these adventures made it easier for me to feel lost and in need of belonging, I always managed to build new bonds, so strong that they still last despite the distance.



First online meeting with my friends from the Research Master in Rotterdam. We endured together a long and stressful 2 years-long Master. Hope to meet them soon in person!

When I pursued my PhD studies here in Coimbra, it was no exception to the rule. In the beginning, I departed from my family and friends to face a whole new experience abroad and by myself. Even though all the chores and bureaucracies kept my mind busy for a while, I started to feel lonely again.

I believe that this feeling is not new for most of my friends either, because most of them have been studying abroad and had to leave their home for an extended time. In addition to that, living in a country with a different language and culture, can be both thrilling and scary.

However, I was pretty lucky since I had the support of people who cares about me and I believe that this is a precious thing. Additionally, I had the possibility to make new friends here in Coimbra, with which I am happy to share my passions and thoughts. Especially in the first month of my PhD, when I met all the colleagues involved in the Syn2Psy project who share my enthusiasm in studying Neuroscience and Neurodevelopmental disorders.

Six months after the start of my PhD project, the prime minister of Portugal issued a complete lockdown. The outbreak of the pandemic and the restrictions that came with it, increased the distance between people. Living together with my friends helped me to reduce that distance and to face this tough situation. We spent our time smart working, playing table games and cooking. I believe that living together also helped me to understand that we can find something positive, even during a worldwide pandemic in progress.

That is the reason which leads me to think that the lockdown challenged our very nature of social animals. Furthermore, I realized that the best way to face social isolation is through constant support and social connection, even if it comes from more virtual means.

As a take-home message, I would like this blogpost to be an invitation for you, my reader, to reach out to someone, perhaps a relative or an old friend (online, of course). Regardless of you being lonely or not, or simply thinking that you might improve someone else's day!



A pre-pandemic picture of my passionate colleagues from the Syn2Psy programme during NS I. The most sober picture that I found of us. Really nice to have you here in Coimbra. See you soon next year!

Write or have an online face to face interaction with them to share your thoughts, anecdotes of each other's life or play videogames. Stay open to new connections with new people and do these things remembering that we are all on the same boat and in need for connection.

Thanks to you, my reader, and a special thank you goes to those who support others in moments of need.

Giuseppe

The power of (science) communication

Article by Manuela Rizzi, ESR.

Can you imagine a life without communication? Impossible, right? And even more in this era of internet and social media.

During our life we all learn how to communicate from the moment we are born. Starting from simple words, such as “mum” and “dad”, we continue to learn how to express ourselves in order to fulfil our needs. Then, this process becomes more complex by starting school, where we learn different kinds of notions that will constitute our critical thinking and will address us to a specific field of which we want to learn everything. For me, that specific area of interest is neuroscience, and I am enrolled in a PhD program to understand some of the molecular mechanisms underneath brain function. This, more broadly, translates in understanding communication at the neuronal level by joining the different pieces of a puzzle that we want to complete, together with the other colleagues of the Syn2Psy network!

In order to define myself as neuroscientist there is a long path of study, which is for sure never ending: just think about how complex our brain is! What we know today is the fruit of several centuries of research. But research alone is not enough: without communicating it, there would be no basis on which to continue investigating and most of our work would be useless. In an environment as research and science, communication is fundamental. Imagine having teams of researchers discovering new theories but not letting anyone know. First of all, we would not be as many as we are today, since discovered therapies would still be hidden in scientists’ notebooks!

But the crucial question is: how to let the general public get closer to this world? During my studies I often realized how far people are from science, and the first example that comes into my mind is trying to explain to my family and friends what I was studying. I must have told them thousands of times, but they still don’t remember the name of my master’s degree! I bet most of my Syn2Psy colleagues faced this as well.



Science Communication

It seems to me that there is a deep-rooted belief that only people from science can understand science and I think today we can see a clear example of what this can lead to.

The inspiration for this post came from an interesting video that I saw on Facebook from an Italian youtuber, who explains science in a funny manner. The main topic was about COVID-19 pandemic and replying to people protesting against and denying measures taken by the governments by citing several scientific studies, but still doing so in an hilarious fashion.

This pandemic is an unforeseen situation we are facing, which is impacting our life in several ways. As for something we don’t know much about, it scares us and instils the need to know more, because the human mind cannot bear the uncertain and seeks explanations. This situation made me realise how important the way news are communicated is, and the impact of social media in our life. Although it is a powerful mean to boost all kinds of connections in a fast and simple way, it can also be a harmful weapon when not used correctly. You may think communication takes benefit from it.

This is certainly true. However, the easiness with which news are spread is inversely proportional to their reliability. This demands a higher ability to effectively discern between news that have a fundament, and on which to build critical thinking, from the increasing load of “fake news”.

If you think about surfing on Facebook for example, more and more public pages share posts with catchy headlines, which when you read the article, you realize that the content is completely different from what was announced, and often with no reference to sources. The problem is that we are relying almost exclusively on social media to be informed about what happens in the world and to answer to our questions. Quality newspapers with articles written by experts do not reach the general public as well as social media, and this creates a lot of misinformation.

This misinformation leads not only to the diffusion of hundreds of confounding theories, but also, sadly, it can have impact on other people’s lives. Unfortunately, this is an example from real life, although I wish it was fiction. And from this, I have gotten even more aware that during my path I should, in the first place, strengthen my communication skills, in addition to performing experiments in the lab.



Can you see the iguana?

During lockdown I had the chance to practice this by creating a short video where I present my research in simple words. Other videos from my colleagues will be available soon too. You can watch them on the website, on the “SimpleSci” section. In addition, I am writing together with my colleague Elisa Corti, an article to be published in a scientific review for kids. Although this is a really exciting activity, I realized that it is not that easy to make such a complex and still unknown world understandable for the general public, but it is necessary in order to let everyone feel close to science and not to think of it as an obscure and unreachable subject.

I also started joining events such as the “Researchers’ Night” which is a great opportunity to bring the public in contact to science through games and fun experiments. I had the pleasure to share this experience both in Trento and in Coimbra, with my Syn2Psy colleagues, during our kick-off meeting.

I hope to have even more occasions for you, the readers, to start being more familiar with science through our pieces of communication!

See you soon!

Manuela

The Centre for Discovery Brain Sciences (CDBS) explores fundamental physiological mechanisms and pathways, from single genes to complex behaviour. CDBS is located at Central Campus and Little France Campus and its research consists of a broad variety of disciplines and cutting edge techniques. Members of the Centre for Discovery Brain Sciences (CDBS) carry out research at molecular, cellular, systems and behavioural levels to understand fundamental mechanisms and pathways relevant to brain and body function in health and disease.

CDBS
Centre for Discovery Brain Sciences

Does free will exist?

Article by Elisa Corti, ESR.

Hi, I am Elisa and when I was asked to write a piece about something that I find interesting I chose to say "Yes!". Perhaps.

Each of us is faced daily with choices: what to wear, what to eat, whether to answer a phone call or go to the gym. We can, to some extent, control our own actions and we can choose to wear the blue t-shirt, to prepare coffee, to pick up the phone, or to rest on the sofa when we are tired. Our lives are literally full of decisions, and we think that we are fully conscious of our choices. But is that true?

Philosophers have been debating about the existence and the meaning of "free will" since forever. The vast majority of the opinions converge on a scenario in which humans are free: free of deciding what is good and what is bad, what is important and what is not, what to fight for and what to give up on.



In 1964, two German researchers opened the Pandora's box. Hans Helmut Kornhuber and Lüder Deecke did not agree with the widely accepted concept that the brain was just a machine able to produce thoughts and actions exclusively in response to external stimuli. To satisfy their desire of understanding how the brain can initiate an action in the absence of external stimuli, Kornhuber and Deecke invited a dozen of people and asked them to simply sit and perform a very simple task: flex the index finger whenever they wanted. The researchers were constantly recording the cerebral activity of the subjects thanks to a technique called electroencephalography (EEG), that basically consists in a cap containing various electrodes. Each electrode is able to record the activity produced by neurons underneath the electrode itself.

This activity is represented as wavy lines that resemble the movements of the financial markets. Taking a close look to such lines, the researchers realized that there was a particular pattern in the cerebral activity of the subjects (a raise followed by a sudden decay), and that this pattern usually took place half a second before the flex of the index finger. They called this Bereitschaftspotential, or readiness potential. This Bereitschaftspotential represented something that anticipates the action itself, the first measurable sign indicating that the decision of flexing the index finger has been taken. In other words, after the observation of the readiness potentials, scientists could infer that the subject would have moved the finger half a second later.

Two years later, the researcher Benjamin Libet tried to understand what was happening during this timeframe. He repeated the study of Kornhuber and Deecke, asking to the subjects to communicate the exact moment in which they decided to flex the finger. As expected, the Bereitschaftspotential appeared 500 milliseconds before the finger bending. The problem is that the subjects reported to have decided to flex the finger only 150 milliseconds before the actual flexion.

There was a gap of 350 milliseconds between the onset of the Bereitschaftspotential (that indicated that an action was about to be performed) and the moment in which the decision was taken. It seemed that the brain was autonomously able to initiate an action, even before the subject was aware of that decision.

A drastic paradigm shift occurred in 2010, thanks to researcher Aaron Schurger. He was using EEG to study the spontaneous fluctuations of brain activity at rest, which depend solely on the connections between billions of neurons. Schurger thought to combine the waves obtained by single electrodes to infer the general level of activity of the brain. He obtained something that really seemed like the Bereitschaftspotential. In this case no action was about to be performed, so the researcher concluded that the readiness potential does not have the specific function of initiating an action, rather it is something correlated to spontaneous fluctuations of the cerebral activity.

Two years later, Aaron Schurger, Jacobo Sitt and Stanislas Dehane proposed a theory: when we have to choose between option A and option B, there are groups of neurons that have to evaluate pros and cons of each option. To do so, they increase or decrease their activity, respectively. If option B is better than option A, the neurons that are involved in the judgment of option A will not be very “enthusiastic”, thus they will be less active. If option A is the best one, our neurons will be very animated, thus more active. When the activity of such group of neurons overcomes a certain threshold, we are ready to choose option A over option B.

However – and this is an important however – one can argue that in the experiments of Kornhuber, Deecke and Libet the subjects did not have to make any choice, but they had to spontaneously flex a finger. In this case there are no groups of neurons involved in judging, and no options to be judged at all! So, how is it possible to reach and overcome that threshold? This is where the spontaneous oscillations of brain activity at rest get back into the game: when, by chance, the neuronal activity is higher, it is closer to the threshold. Under this circumstance it would be easier for the subjects to decide to flex the finger. This does not mean, as Libet thought, that the brain decides for us. It rather means that these spontaneous oscillations are able to facilitate the attainment and overcoming of the threshold. This could be important if we have to take a decision in the absence of external factors on which to base ourselves.

Recently, Schurger repeated Libet’s experiment, including in the study a set of subjects that should not flex the finger. If Libet was right, namely if the brain decides autonomously when to flex the finger, the cerebral activity of people who had to flex the finger should be different from the one of people who did not have to flex the finger. This difference should pop up half a second before the flexion of the finger: in the first group of people we should see the Bereitschaftspotential, in the second case we should not. This did not happen. Actually, the first detectable differences between the cerebral activity of the two groups appear just 150 milliseconds before the movement, when the subjects declared to feel the will of flexing the finger. So, the awareness of the decision matches the decision-making process.

We still do not know if Schurger is right, we need additional studies to clarify the point and to understand if we are effectively capable of making a decision, of modifying the influence that the external world has on us, or if we are just the result of a threshold that is overcome as a consequence of the influence that the external world has on us.

Hi, I am Elisa and I do not know if I chose to write this piece anymore.



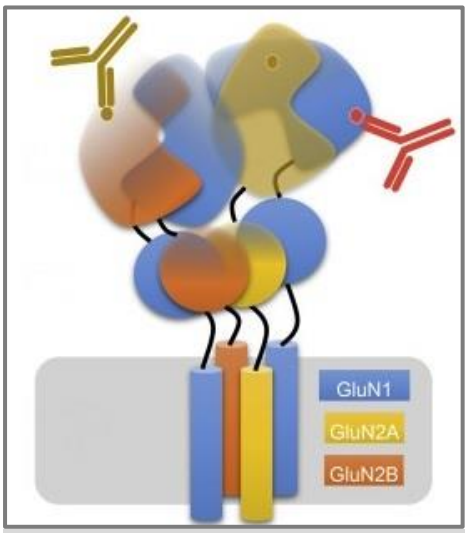
Dissemination

In this challenging first half of Syn2Psy project, a few ESRs had the opportunity to disseminate their results:



Daniel Hunter published, as first author, a review article under the title "**Autoimmunity and NMDA receptor in brain disorders: Where do we stand?**" in the Journal Neurobiology of Disease. DOI: 10.1016/j.nbd.2020.105161.

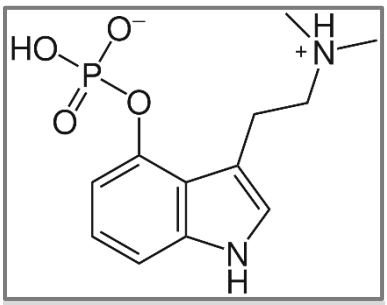
ABSTRACT: Over the past decades, the identification of autoimmune encephalitis in which patients express autoantibodies directed against neurotransmitter receptors has generated great hope to shed new light on the molecular mechanisms underpinning neurological and psychiatric conditions. Among these autoimmune encephalitides, the discovery of autoantibodies directed against the glutamatergic NMDA receptor (NMDAR--Ab), in the anti-NMDAR encephalitis, has provided some key information on how complex neuropsychiatric symptoms can be caused by a deficit in NMDAR signalling. Yet, NMDAR-Abs have also been detected in several neurological and psychiatric conditions, as well as in healthy individuals. In addition, these various NMDAR-Abs appear to have different molecular properties and pathogenicities onto receptors and synaptic functions. Here, we discuss the current view on the variety of NMDAR-Abs and, in particular, how these autoantibodies can lead to receptor dysfunction in neuronal networks. Since our mechanistic understanding on patients' NMDAR-Abs is still in its infancy, several complementary processes can be proposed, and further in-depth molecular and cellular investigations will surely reveal key insights. Autoantibodies represent a great opportunity to gain knowledge on the etiology of neuropsychiatric



NMDAR

Marcos Sintes posted as co-author on BioRxiv, a study on the **potential effects of psilocybin for the therapy of autism spectrum disorder**. Full posted article: https://www.biorxiv.org/content/10.1101/2020.09.09.289348v1.





Psilocybin (pngwing.com)

ABSTRACT: Autism spectrum disorder (ASD) is characterized by core deficits in social interaction. The classic serotonergic psychedelic psilocybin has been suggested as a therapeutic agent that may ameliorate in the core symptomology of ASD. We found that the acute response to psilocybin was attenuated in the prenatal valproic acid exposure mouse model of ASD, and importantly, psilocybin rescued the social behavioural abnormalities present in these ASD model mice.





Laura Upton and **Flavio Tomasi** presented, respectively, the posters entitled "**Altered prefrontal cortex assembly in mice deficient in the autism-associated gene Glra2**" and "**Assessing the endocannabinoid system as a therapeutic target in autism using Shank3-deficient mice**", at the Scientific days ED3C, an online event organized by the doctoral school.

Communication and outreach

Brain Awareness Week

Giuseppe Cammarata and **Elisa Corti** participated in an online event organized by *Biologi per la Scienza* (Biologists for Science), an Italian website dedicated to popularisation of science. For this event, entitled *AperiNeuro: fai la tua domanda!* (AperiNeuro: ask your question!), the ESRs published two articles and participated in an online session of Q&A with the public. The topic chosen by Giuseppe was "Organoidi di cervello – L'emancipazione etica degli studi sullo sviluppo cerebrale umano" (Brain organoids – The ethical emancipation of studies on human brain development) and by Elisa "Ma la libertà di scelta esiste?" (But, does free will exist?).

This event was held on the Facebook page of Biologi per la Scienza, and can be watched fully here. The articles can be found in Italian here and Elisa's article can also be found in English on Syn2Psy's blog (included in this newsletter, see above).



AperiNeuro event during the Brain Awareness Week. Bottom picture: ESR3 Giuseppe Cammarata; Top-left picture: ESR7 Elisa Corti.

SimpleSci series



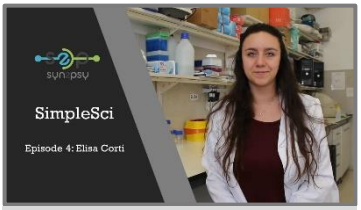
Episode 1: Manuela Rizzi



Episode 2: Orsolya Antal



Episode 3: Laura Upton



Episode 4: Elisa Corti

Four episodes of the SimpleSci series have been released. In these videos Manuela, Orsolya, Laura and Elisa describe each of their projects in a language adapted to the general public.

To accomplish this, the ESRs have to combine several skills: writing a script adapted to the general public, developing the visuals and animations, recording themselves on camera and recording the voice-over, so that in the end, everything fits together. Saying that this is a challenging task is an understatement, but the result so far speaks for itself! And in the process, the ESRs are learning a lot: how to explain such complex concepts in a simple manner is only one of many skills that they are developing. We could not have imagined that this would turn into such a fun experience and an excellent opportunity to create awareness about neuropsychiatric disorders, while contributing to the improvement of the population's scientific knowledge.

If you have not seen the videos yet, the four first episodes are available in the SimpleSci section of the Syn2Psy website and on the social networks.

Enjoy and stay tuned! More episodes coming soon!

QUICK NOTE

Divided in seven groups, all Syn2Psy ESRs are currently in the process of writing the full articles to submit to the journal Frontiers for Young Minds, with the help and expertise of our Partner PIN.

frontiers FOR YOUNG MINDS

Network-wide events

First online PhD day

On October 5th (2020) Syn2Psy had its first online PhD day. During this event, ESRs had the opportunity to share and discuss their results and get feedback from the consortium PIs. This event was considered a success and it was agreed that this should be an annual event. The second online PhD day will be held on the 23 – 24 of September (2021), this time with the participation of the External Advisory Board.



ESRs and PIs during the online PhD day.

Mid-term meeting

The Mid-term meeting was held remotely on May 2 – 3, 2021. This meeting, with the presence of the Syn2Psy Project Officer and the REA External Reviewer, had the goal of assessing the fulfilment of the administrative procedures and project progress. The Project Officer acknowledged the great efforts that Syn2Psy members have done in the past year and agreed that Syn2Psy is on the right track despite the COVID-19 situation.

On the second day of the meeting we had a scientific session under the theme "Synaptic dysfunction in neuropsychiatric disorders", with presentations from Syn2Psy PIs and the Syn2Psy External Advisor, Prof. Claudia Bagni.



Participants of the Syn2Psy Mid-term meeting.

Network School 3

Network School 3 (NS3), under the title "Drug discovery and development: from Idea to Patient" was held online on May 5 – 7, 2021. This school was organised by Kjartan Herrik at Lundbeck with the participation of Lundbeck's experts, as well as invited speakers from other pharmaceutical companies and the University of Copenhagen.

The ESRs gained theoretical understanding on how Lundbeck and other pharmaceutical companies develop scientific ideas and turn them into treatments to help patients with neuropsychiatric disorders.

NS3 was complemented with a workshop on the early signs and clinical variability of Autism Spectrum Disorder, given by Syn2Psy partner PIN.

As an evaluation, the ESRs were asked to pitch their project in the *Dragon's Den*. This enabled the ESRs to communicate the potential value of their project for patients, commercial interests and the scientific community, as well as to understand what is needed from them to achieve these goals. *Dragons* were invited from Psychiatry, Industry and the Lundbeck Foundation.

This school was a success and the training was deemed highly satisfactory!



Early Stage Researchers during the workshop with PIN.



Network School 3 global evaluation by the ESRs.

News

A new ESR4 has been recruited: Veronica Villeri



"I am deeply interested in the study of the molecular and synaptic underpinnings of neuropsychiatric disorders, as a window to both new treatments and the understanding of the bases of human cognition." Veronica Villeri.

We are pleased to welcome Veronica Villeri into our consortium! Under the supervision of Daniel Choquet and Eric Hosy, she will study the link between autism related mutations in Shank3, over synaptic, network and behaviour defects.

Veronica comes from Sicily, Italy, where she obtained her Bachelor's degree in Biology from the University of Catania and her Master's degree in Cellular and Molecular Biology, with a strong focus on neurobiology, from the University of Turin. After completing the Master's degree thesis in neurodevelopment, she got further experience in neuroscience research in Germany and Austria, focusing on local translation and on Autism Spectrum Disorder.

External Advisory Board

We are pleased to present the two members of the Syn2Psy Advisory Board: Prof. Claudia Bagni and Prof. Gavin Rumbaugh.

Prof. Claudia Bagni, Professor of Neuroscience and Head of the Department of Basic Neurosciences at the University of Lausanne, Switzerland, is a specialist in the structure and function of synapses, in particular the cellular and molecular mechanisms of synaptic plasticity and their dysfunctions, responsible for fragile X syndrome and autism spectrum disorders.



Prof. Gavin Rumbaugh, tenured Associate Professor at The Scripps Research Institute, Jupiter, Florida, has identified several genes that are critical regulators of synaptic function and increase the risk for developing neurodevelopmental disorders of cognition and sociability, such as intellectual disability and autism spectrum disorders.



Forthcoming events

Second PhD day – online, September 23-24, 2021.

Chaperone training in Career Development:

- Information session – online, September 17th, 2021;
- How to Write a Paper – online, September 24th, 2021. Trainer: Joana Moscoso;
- Preparing for a Scientific Conference – online, October 1st, 2021. Trainer: Rob Hardwick;
- Project management – online, October 8th, 2021. Trainer: Pedro Resende;
- Wellbeing & Work-life balance – online, October 15th, 2021. Trainer: Hannah Roberts.

Network School IV – Bordeaux, March 2022.

Network School II – Yet to be defined.

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Annex 10 - Ethical report by External Ethical Advisor

Grant Agreement number: **813986**

Acronym: **Syn2Psy**

Title: **Synaptic Dysfunction in Neuropsychiatric Disorders**

Deliverable date: 31.10.2023

Document Title	Final Ethical Report
Document Author:	External Advisor
Approving Body:	External Advisor
Date:	31.10.2023
Summary:	The Ethical Report ensures the project is in accordance with the relevant ethical principles in scientific research and that all researchers are aware of them.
Availability	This document will be made available to all research members of the Syn2Psy Team.

ETHICAL REPORT

1.1 PURPOSE OF DOCUMENT

THIS DOCUMENT IS THE FINAL ETHICAL REPORT AND INTENDS TO EVALUATE COMPLIANCE WITH ETHICAL AND LEGAL STANDARDS BY THE syn2psy PROJECT.

1.2 PROJECT GOALS

Syn2Psy ESRs were focused on the study of the therapeutic potential of the endocannabinoid system in an Autism Spectrum Disorder (ASD) model; on the identification of aberrant synaptic mRNA translation as a point of convergence for multiple genetic causes of ASD/ID; on the establishment of the impact of the corticotropic releasing hormone system on Schizophrenia (SCZ) - and ASD - related endophenotype; on the identification of circuit deficits associated with ASD; as well as on the identification of the role of the excitation/ inhibition (E/I) imbalance in the corticothalamic system in the development of neuropsychiatric disorders.

Syn2Psy aimed to inform better therapeutic strategies, to configure a new mindset for the research community devoted to the investigation of neuropsychiatric disorders, and to adequately prepare the next generation of researchers in the field. The project combined strong scientific with complementary know-how from the non-academic sector, and network-wide actions on scientific and complementary soft skills, to train a new generation of high achieving ESRs and provided them with the transferable skills necessary for thriving careers in a flourishing area.

The 14 studies were developed and can be summarized as follows:

ESR1

Results from work of this group show that genetic susceptibility, as conferred by the StgSN stargazin variant generates some alterations in synaptic function and dendritic spine morphology, as well as producing cognitive behavioural phenotypes characteristic of schizophrenia. As ways of potential exploitation of the results of the project, ESR1 propose that the StgSN knock-in model has significant construct validity in modelling the impact of synaptic dysfunction in schizophrenia and provides a valuable

tool for further investigation of the underlyin pathophysiological mechanisms of features of the disorder as conferred by a genetic risk factor. Under the supervision of Ana Luísa Carvalho, the group also propose that their model provides a platform to investigate how exposure to additional environmental factors may combine with preexisting genetic risk to exacerbate dysfunction and lead to disease-related phenotypes, leading to new insights on how disease states develop and revealing potential avenues for therapeutic intervention. Furthermore, in terms of generating new knowledge, investigating a variant of stargazin that impacts the normal functioning of the protein and its in vivo consequences on synaptic function also has the potential to provide a tool to further investigate the role of endogenous stargazin in regulating and maintaining normal glutamatergic transmission.

ESR2

ESR2 reported potential of the results for exploitation: the project will contribute to the dissection of nucleus accumbens function and dysfunction in the context of psychiatric conditions. We report here for the first time the causal role of mitofusin 2 deficits in a specific subpopulation of NAc during learned helplessness. This group provided novel information on the downstream morphological and molecular adaptations happening into the ventral striatum following reduced mitofusin 2 expression levels. Finally, ESR2 provided new insights on specific brain-regions adaptation and response to such change such as the prefrontal cortex, the amygdala and the thalamus: all areas related to stress and depressive-like conditions. Those findings can deepen the understanding of the mechanisms underlying motivational disorders and pave the way for the development of novel treatment strategies to depression.

ESR3

ERS3 drew some points that could be exploited in the future. For example, in performing chronic exposure to VPA for 6 months we explored the effects of VPA in long-term exposure. Surely, one of the next steps may be to also explore the short-term effects of the drug. Additionally, further experiments may be done on the physiological effects of VPA on the inhibitory system developed by the unguided neural organoids. The implications of the project's results for ASD research are particularly noteworthy. By establishing a platform that recapitulates the hallmarks of ASD using human in vitro models like unguided neural organoids, researchers can gain a deeper understanding also on other similar drugs and their effects. This knowledge can be utilized to advance diagnostic methods, identify potential biomarkers, and develop novel therapeutic strategies for ASD and other neurodevelopmental disorders. Moreover, the project's focus on identifying the mechanisms impacted by VPA, which are also associated with ASD phenotypes, opens up avenues for the development of targeted drugs. By designing drugs that act on these specific

mechanisms, it may be possible to develop more effective treatments for ASD and related neurodevelopmental disorders.

ESR4

To link autism related mutations in Shank3 gene over synaptic, network and behaviour defects (Task 2.1), ESR4, Diogo Soares, supervised by Daniel Choquet and Eric Hosy at IINS-CNRS, started the development of a pathological model with the use of a short hairpin RNA (shRNA), which decreases Shank3 expression of approximately 40%. Preliminary experiments did not reveal a noticeable modification of AMPA receptor organization nor currents after shRNA expression, thus, the development of two new models was planned: human pluripotent cells (hPS) and Shank3 knock-out (ko) mouse. In October 2020, after Diogo Soares resigned his position at CNRS, and while the new ESR4 fellow (Veronica Villeri) was being recruited, the ko Shank3 mouse model was established and hPS was implemented on another pathological model.

As soon as the new fellow was recruited, ESR4, Veronica Villeri, also supervised by Daniel Choquet and Eric Hosy at IINS-CNRS, started investigating the nanoscale organization of glutamate receptors in in vitro models of monogenic autism by means of d-Storm super resolution microscopy. More specifically, Veronica carried out four lines of experiments:

1) It is known that long-term potentiation (LTP) causes a reduction of SYNGAP1 at the spine, however, little information is available on the effects of long-term depression (LTD) on synaptic SYNGAP organization, as compared to long-term potentiation (LTP), which is known to cause a reduction of SYNGAP1 at the spine. Veronica investigated if and how LTD stimulation determines a change in the number of synaptic SynGAP1 and its colocalization with either AMPA receptors or PSD95 in WT rat hippocampal cultures. Preliminary data showed that there is 30% ca. reduction in Synaptic SynGAP1 following NMDA-mediated LTD.

2) SynGAP1+/- primary hippocampal cell cultures are characterized by an LTP-like basal state. Evidences from the Lab of Daniel Choquet showed that this may be caused by an increase in calcium-permeable AMPA receptors. Veronica investigated whether there is a parallel increase in synaptic NMDA receptors. Preliminary experiments showed no significant differences in NMDA levels.

3) Veronica started the optimization of the cell culture conditions and main line of investigation in Shank3 in vitro models, by studying whether changes could be observed in synaptic NMDA or AMPA receptors' density in primary culture of either cortical or hippocampal neurons of Shank3+/-, Shank3-/- and WT mice at DIV10 and DIV14.

4) Finally, Veronica optimized the culture and super-resolution imaging conditions of I3 human neurons in order to study changes in glutamate receptors organization in Syngap1+/- and Syngap1-/- human I3 neurons.

After a period of sick leave, Veronica Villeri was transferred to the University of Edinburgh (UEDIN).

ESR5

ESR5 results provided the first insights on the effects of psychedelics on neuronal plasticity in human iPSC-derived neurons. These models provide unique perspective into the effects of psychedelic drugs on human cortical circuits that is not attainable in the clinical, animal and animal-derived cell studies published to date. They are currently using these experimental approaches on both control and ASD patient-derived neurons, which could provide the first data on the potentially therapeutic mechanisms of psychedelic drugs for the treatment of neurodevelopmental disorders.

ESR6

The primary objective of the ESR6 thesis were to understand the functional mechanisms behind hippocampal pathophysiology caused by NMDA and GABAA receptor autoantibodies. The study aimed to investigate synaptic, cellular, and network deficits induced by autoantibody actions in hippocampal cell preparations. Overall, they emphasize the role of NMDA and GABAA receptor autoantibodies in altering hippocampal network activity and highlight the potential implications for seizures, cognitive dysfunction, and synaptic plasticity.

ESR7

ESR7 group described a wide range of synaptic proteins that are dysregulated upon Fmr1 knock out or knock down. Their findings will boost the research to understand how the phenotypes hat are caused primarily by the absence of FMRP can be rescued, ultimately to individuate new therapeutic targets.

ESR8

The ESR8 project's main aim was to 1) dissect of the mechanisms involved in the increased synaptic stability found in previous grafted Down Syndrome (DS) induced pluripotent stem cells (iPSCs) that had been differentiated into neuronal progenitors, injected into an immunodeficient mouse brain, and subsequently monitored with longitudinal multiphoton imaging over several months (Task 8.1 in the GA); and 2) monitor

these same grafts for calcium activity fluctuations to determine the mechanisms behind previously found deficits in oscillatory calcium activity in DS grafts (Task 8.2 in the GA). The results from this group provide a model and foundational knowledge upon which further DS research can be applied. This applies to research on DS neuron structure, neurodevelopment, calcium activity, and even pharmacological treatments. Future research could utilize this model for the study of the role of astrocytes in DS, as they are prevalent in our grafts. Furthermore, this body of work provides a secondary dataset in which the novel model from Real et al., 2018 was successfully used to study neurological development and activity. Future work on this model will allow for the expansion of the knowledge base on DS as well as a wide range of other neurological and neurodevelopmental disorders.

ESR9

To understand the role of glycinergic signalling in ASD, ESR9, Laura Upton, supervised by Catalina Betancur at IBPS-CNRS, is investigating the impact of the loss of the gene Glra2 on circuit assembly and function in the prefrontal cortex (PFC) of Glra2-deficient mice. The findings described in their report, observed in a mouse model deficient for Glra2, provide insights into the neurobiological impact of the loss of this gene. The characterization of the underlying brain dysfunction caused by the loss of Glra2 in this mouse model can contribute to a better understanding of the cellular and circuit mechanisms underlying ASD and intellectual disability in affected individuals and could point toward future therapeutic strategies. In addition, their studies uncover insights into the biological roles of GLRA2 in the brain.

ESR10

To assess the endocannabinoid system as a therapeutic target in the mouse model of autism Shank3-deficient mice and study the consequences of Shank3 deficiency on the endocannabinoid system, ***ESR10, Flavio Tomasi****, supervised by Catalina Betancur at IBPS-CNRS, collected the brains to evaluate the basal levels of the two main endocannabinoids and the two receptors, CB1 and CB2. Their work represents the first attempt to specifically modulate CB1 receptor activity in a Shank3- deficient mouse model, thus proposing a new pharmacological target for PMS. Importantly, given the characteristics of AEF0217 in terms of therapeutic profile, our results suggest that this compound is a potential pharmacological agent for the treatment of PMS and more generally of ASD. The results allowed them to file a patent application for the use of AEF0217 in PMS and to start planning a clinical trial in PMS patients. Results will be published in an article with Aelis Farma describing the development of AEF0217, including in vitro and in vivo preclinical studies and a phase 1 clinical trial in healthy volunteers. The manuscript is in preparation and will be submitted soon.*

ESR11

To investigate the excitation/inhibition imbalance hypothesis of neuropsychiatric disorders in the corticothalamic system, ***ESR11, Ágata Silván****, supervised by Niels Plath and Kjartan Herrik at Lundbeck, performed experiments that identified SK channels as potential pacemakers of thalamocortical activity and thalamic reticular function. The results obtained in their study point out to develop compounds targeting SK channels, particularly SK2, as a potential therapeutical strategy in the context of neuropsychiatric and neurodevelopmental disorders.*

ESR12

To identify convergent dysregulation of mRNA translation in multiple models of autism, ***ESR12, Manuela Rizzi****, supervised by Emily Osterweil at CIP-UEDIN, started by characterizing Syngap+/- dataset precedently pre-processed in the lab. They concluded that exploration of protein synthesis proved to be a potential target to retrieve key and relevant information for the design of new therapeutic strategies that can more precisely address subtle aspects of misregulation observed in disease state. In addition, these datasets represent a great resource that will be of public availability and allow scientists to build a far clearer picture of the molecular characterization interesting these complex disorders. They can be looked at from different perspectives depending on the focus of the analysis and this would allow to retrieve various pieces of information. Furthermore, the exploration at the level of multiple brain regions is an added point towards characterization of circuitry disfunctions. This would ultimately lead to generation of new experimental and scientific questions which pursue is targeted at the understanding of how the system works and how its manipulation could impact on healthy and disease state. The sets of results generated would potentially be the basis for new research projects to be carried out by future members of the lab.*

ESR13

In summary, ESR13, Loredana Cumpana, supervised by Carmen Sandi and Simone Astori at BMI-EPFL, shown that CRH modulates NREM sleep homeostasis by acting on spindle generating mechanisms in TRN neurons. In particular, decreasing CRH levels can stabilize NREM sleep and improve the sigma oscillation, which has been shown to increase cognitive abilities during wakefulness and mitigate the symptomatology of various psychiatric disorders. Revealing the contribution of the stress and anxiety related peptide CRH on sleep rhythms contributes to our understanding of the fundamental modulation of sleep but can also be used to explore further its implications in pathology. Upon the completion of this data set in the near future it will be possible to understand if there are correlations between anxiety and the vulnerability to CRH-induced changes in NREM sleep. Additionally, as the TRN has been described to be part of a circuit

endophenotype altered in neurodevelopmental disorders, their findings could have implications for sleep impairments often associated with such disorders but also various other psychiatric conditions. The mechanisms described could be used as novel targets for pharmacologically treating stress or anxiety induced sleep impairments. Furthermore, potential associations between CRH modulation of sleep or NREM sleep parameters and anxiety might serve as new biomarkers in clinical studies and contribute to the identification of individuals at risk for developing sleep impairments or psychiatric disorders.

ESR14

***ESR14, Vanesa Salazar**, supervised by Peter Kind at the CBDS-UEDIN, has the aim to determine whether robust social phenotypes are present in the rat models of SYNGAP1 haploinsufficiency that can be used for preclinical studies to determine efficacy of potential clinical strategies and their susceptibility for therapeutical intervention. In line with this first objective, the research conducted by ESR14 has made significant progress beyond the state of the art in understanding and addressing social and circuit deficits associated with SYNGAP1 haploinsufficiency in Autism Spectrum Disorder (ASD) and Intellectual Disability (ID). By creating a new robust paradigm to address social behaviour in rodents, elucidating the lower social interaction and lack of interest in SYNGAP1 rat models, identifying the potential of environmental enrichment to rescue these phenotypes, and developing novel approaches for studying functional connectivity in vivo, this work has contributed novel insights into the molecular and circuit-level mechanisms underlying social behaviour impairments as well as experimental designs applicable to other scientific questions of similar nature. These advancements collectively push the boundaries of current knowledge and pave the way for innovative therapeutic strategies and a deeper understanding of the complex interplay between genetics, neural circuits, and social behaviour in neurodevelopmental disorders. The results obtained from ESR14 research on two rat lines of SYNGAP1 haploinsufficiency hold promising potential for exploitation in various areas. Firstly, the finding that these rat models display reduced social interaction and general lack interest provides valuable insights into the underlying mechanisms of social behaviour deficits observed in individuals with SYNGAP1 mutations, which could potentially contribute to the development of targeted therapeutic interventions. Additionally, the identification of a potential rescue of this phenotype through rearing in a highly enriched environment and social stimulation opens up avenues for designing interventions that focus on play and social stimulation as potential coping strategies during development. Moreover, the future results on the study of altered connectivity between reward-like brain regions in these rat models present opportunities for further exploration of neurobiological pathways and potential pharmacological targets in the mesolimbic system. Overall, these results provide a foundation for future studies and the exploitation of novel approaches for addressing social behaviour and circuit impairments associated with SYNGAP1 haploinsufficiency.*

ESR15

ESR15, Veronica Villeri, supervised by Emily Osterweil, after being transferred to UEDIN, studied whether Fmr1-/- and Syngap1 +/- mice show differences at the level of neuronal activation in the basolateral amygdala after encoding for a fear-related memory. Indeed, both mouse models have shown an impaired fear-related memory, tested via the fear conditioning paradigm. If there is a defect at the level of memory encoding, a reduced activation of BLA cells and/or a change in their response can be hypothesised. To test the first hypothesis, mutant and WT mice were subjected to fear conditioning and then sacrificed in order to assess c-Fos expression by imaging. Veronica performed confocal imaging of brains stained for c-Fos, Neun and a basolateral amygdala (BLA) marker and the relative tridimensional image analysis and showed that in both mutants, as in the respective wild-type, there is a significant increase in the number of c-Fos+ cells, suggesting the formation of a fear-related memory engram. However, while there was no difference between the level of neuronal activation between Syngap1+/- and the respective wild-type, a reduced c-Fos expression in Fmr1+/- was observed, as compared to the respective wild-type.

All partners/researchers within the Syn2Psy Team were aware of the relevant ethical principles in scientific research, and thereby they had carried out all activities during the project in compliance with the ethical standards, scientific guidelines and with applicable international and national legislation.

The main object of Syn2Psy were to inform better therapeutic strategies, configure a new mindset for the research community devoted to the investigation of neuropsychiatric disorders, and create of innovative advanced training programs to adequately prepare the next generation of researchers in the field.

A. Participants

It was assured to the participants that they only participate if they want, if the research agrees with their values and interests. Moreover, the refuse to participate would not interfere with the medical care received. There were no payment of travels or pecuniary counterparts for their participation. The participation in the study and sample donation were voluntary and revocable and didn´t cause damage to the participants, who at any moment, by their free will, or by representation by their legal representative, had the right to remove their data, the sample and interrupt the collaboration, if they thought was convenient, without the need to justify and not being discriminated against.

Informed consent was a key principle of ethical research, ensuring that research participants were adequately informed of the risks of taking part in studies, that their participation is voluntary, and that the information about them gathered remained under their control.

We analyzed how Syn2Psy project data processing activities comply with the key principles on informed consent for the involved partners. Research participants had been informed fully about the purpose, methods and intended possible uses of the research, what their participation in the research entailed and what risks, if any, were involved. In addition, research participants would only participate in a voluntary way, free from any coercion.

The partners in the project, except for CNC, did not collect personal data for experiments, research, or any other purpose. Regarding partner CNC, all experiments that may result in data capturing were only carried out after approval from the Ethical Committee, whose standards not only respect informed consent principles but also the participants' right to confidentiality and the balancing of their involvement against the potential benefit of the conducted research to the overall community.

The CNC partner did not collect data directly from users, but only indirectly though the user-facing partners of the project. We foresee such collection was lawful under the existing Privacy Statements of the partners.

All the data collected were pseudonymized, at the point CNC receives it – in a very strong sense when it comes to aggregate data, and in a sufficient manner with respect to data protection when it comes to granular records.

In all cases, and in the spirit of the Ethics approval which required strong data minimization and purpose limitation.

B. Data Protection

The partners in the project, except for CNC, did not collect personal data for experiments, research or any other purpose within the Syn2Psy consortium. For this

reason, this report does not provide any information about the data management implementation procedures carried out by partners CNRS, EPFL, UEDIN, ICL, CNRS and Lundbeck.

Partner CNC, as part of Syn2Psy consortium, collected human adult stem cells (from teeth) and fibroblasts (from gingival biopsy, associated with the tooth extraction) from patients and healthy controls. Only in this part, the project involved collecting of personal data and samples from the participants (data subjects) for research purposes to generate experimental data in order to understand the neurobiological consequences of patients' genetic landscape in the context of autism spectrum disorder.

To comply with the General Data Protection Regulation (GDPR), personal data had been processed in line with the aim to "*protect all EU citizens from privacy and data breaches in today's data-driven world*", fowling the standards stablished in Data Protection Policy approved by CNC's DPO (Deliverable D7.3).

C. Incidental Findings

The Syn2Psy partners as defined an Incidental Findings Policy that ensures the project is in accordance with the relevant ethical principles in scientific research and that all researchers were aware of them. Regardless the Syn2Psy project involved a very low risk of occurrence of incidental findings, the original project approval granted from Ethics Committee at the University Hospital of Coimbra did not foreseen disclosure, nor not the non-disclosure of incidental findings, any such discovery with clinical relevance would have to first be submitted for Ethical Board review board on a one-to-one cases.

D. Animal experiments

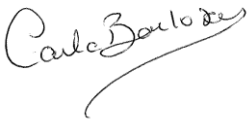
Several strains of transgenic mice and rats were used in Syn2Psy. Some experiments included transplantation of iPSC-derived neurons from human patients into mice. Studies have been performed in compliance with Directive 2010/63/EU (revising Directive 86/609/EEC). The European and national laws for animal experimentation have been addressed for all countries participating in animal experiments. The principle of 3Rs was well addressed. The partners had obtained the relevant authorizations (for breeders, suppliers, users, and facilities) for animal experiments, which are kept on file.

CONCLUSIONS

All personal data accessed by the researchers for the Syn2Psy research project were pseudonymized. The confidentiality of participants information was protected, and the nature of the proposed studies did not give rise to stigmatization for those bearing the diseases under study. As such, the existing data protection and incidental findings agreements and procedures for the project do not require amendment. The partners had obtained the relevant authorizations (for breeders, suppliers, users, and facilities) for animal experiments.

Ethics Advisor Statement. The Syn2Psy project is unusual, in some senses. As ethics advisor to the project, I am satisfied that the research in the project had been designed a developed with full consideration for ethical concerns, and that the appropriate measures were in place for compliance with data protection and informed consent of users to participate in the research. All personal data accessed by the researcher for the Syn2Psy project was pseudonymized. The confidentiality of participant information had been protected and the nature of the proposed studies did not give rise to stigmatization for those bearing the diseases under study. Animal experiments were compliant with the European and national laws for animal experimentation and the principle of 3Rs was well addressed. The partners had obtained the relevant authorizations for animal experiments.

As ethical advisor I am satisfied that all ethical considerations have been fundamentally incorporated into the research of the project.



Carla Barbosa
External Advisor