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Periodic Technical Report
Part B

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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, we aim to train a 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 are 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 first reporting period 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 progress

The first reporting period for Syn2Psy, refers to the period from March 2019 to February 2021. 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 experiments and led to a substantial the delay of the individual projects, delay in training, postponement of network-wide events, several deliverables and milestones as well as uncertainty regarding the secondments.
In this report, the consequences of the SARS-CoV-2 pandemic will be referred as "COVID-19-related restrictive measures".

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 is 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 aims 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 are being 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 this first reporting period 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 exploitable results and an explanation about how they can be exploited (when applicable). No deliverables nor milestones were planned for this reporting period for WP1.

To establish the role of Stargazin in psychiatric disorders (**Task 1.1**), **ESR1**, **Orsolya Antal**, under the supervision of Ana Luísa Carvalho at CNC, is characterizing two knock-in mouse models of neuropsychiatric disorders, each carrying a disease-associated variant of the AMPAR-auxiliary protein Stargazin, encoded by the CACNG2 gene. ESR1 is currently performing behavioural and morphological analysis in these animals, which will be complemented by further functional characterisation, all focusing on alterations in synaptic function in the prefrontal cortex and its behavioural consequences. Furthermore, since exposure to chronic stress is a major risk factor for neuropsychiatric disorders, ESR1 aims to assess its impact on these structural, functional and behavioural phenotypes in the disease-associated models. 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, has conducted genetic manipulations in mice to induce loss of function of Mitofusin 2 (Mfn2) in the Nucleus Accumbens (NAc) and has assessed the impact thereof on motivated behaviour. Downregulation of Mnf2 in medium spiny neurons expressing the dopamine receptor 1 (D1-MSNs) induced an impairment in effort-related behavior, as shown by the adoption of a passive coping behavior in the forced swim test, indicative of depression-like behavior. ESR2 performed cFos mapping of neuronal activation during the behavioral task, which revealed decreased engagement of NAc neurons. Based

on these results, it was decided to first study the cellular and synaptic consequences of Mfn2 downregulation in accumbal cells, before exploring the impact in NAc downstream regions. ESR2 performed electrophysiological recordings in mice with Mfn2 downregulation in D1-MSNs, which indicate aberrant intrinsic excitability, altered excitatory synaptic inputs and reduced dendritic complexity in D1-MSNs. ESR2 will extend this analysis by exploring the reciprocal inhibitory connections between D1- and D2-MSNs. Ribosome-associated mRNA (Ribotag) and mitochondrial isolation (Mitotag) approaches were set up to perform an unbiased cell-type and brain region specific RNA sequencing, that will allow the identification of the molecular mechanisms responsible for the dendritic and synaptic defects induced by Mfn2 deregulation. Full description of the achievements: Annex 1 – ESR2 Alessandro Chioino.

To produce human-derived neurons and brain organoids (**Task 1.3**), **ESR3, Giuseppe Cammarata**, supervised by João Peça, chose a contingency plan that involved the reprogramming of dental stem cells instead of direct differentiation into neurons. Human Induced Pluripotent Stem Cells (HiPSC)-derived human brain organoids have been shown to be more stable and consistent in the generation of neurons when compared to the direct differentiation of dental stem cells. Stem cells from patients and control subjects have been collected. ESR3 has contributed to the reprogramming of 3 cell lines into hiPSC from the ProTeAN biobank. These lines will be characterized to test their pluripotency capability and, if they show positive outcomes, they will be used as a source for the development of 3D brain organoids. ESR3 is currently testing valproic acid (VPA) exposure on human brain organoids derived from hiPSCs of the control pool and is testing the neurophysiological, morphological and network activity properties of both control and VPA-treated organoids. Preliminary data suggest that exposure to VPA leads to the development of hyperactive network. These results may contain hints of a possible impact of VPA on the inhibitory system formed in the organoids treated with this drug, and more investigations are needed to assess what is the cause of this excitatory/inhibitory 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 is 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 focuses 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 this first reporting period 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 exploitable results and an explanation about how they can be exploited (when applicable).

No deliverables nor milestones were planned for this reporting period for WP2.

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 it was decided that new models should be developed: human pluripotent cells (hPS) and Shank3 knock-out (ko) mouse. This task is in stand-by since former ESR4, Diogo Soares resigned in October 2020, until the official start of the new recruited ESR4, Veronica Villeri, in May 2021. During this waiting time, the ko Shank3 mouse model was established and hPS was implemented on another pathological model. Full description of the achievements: Annex 1 – Diogo Soares.

To monitor the cortical E/I balance *in vivo*, (**Task 2.2**), **ESR5, Marcos Sintes**, formerly under the supervision of Thomas Knöpfel and currently under the supervision of Vincenzo De Paola at ICL, 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, ESR5 used animals prenatally treated with valproic acid (VPA) 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. ESR5 analysed the obtained videos 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, ESR5 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. In a second set of tests, ESR5 generated the VPA and control animals by injection of two doses of VPA or saline, respectively, into female mice during pregnancy. At 8 weeks of age, ESR5 first tested the behavioural traits of the offspring using the three-chamber test. A week later, psilocybin or saline was injected, and head-twitch events were counted. The following day and after one and two weeks, ESR5 tested the mice again using the three-chamber test and performed both software-based analysis of travel distance, time of approach and time in each chamber, and observer-based analysis of time of nose-to-nose interactions. Description of the tasks: 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 studying the impact of patient-derived anti-NMDA receptor autoantibodies (NMDAR-Abs) on $GABA_A$ receptor-mediated inhibitory signalling and on AMPA receptor-mediated hippocampal excitatory signalling and observed a disruption of both networks.

To investigate the structure and global regulation of excitatory and inhibitory synapses after exposure to NMDAR-Abs, ESR6 transfected fluorescently-tagged synaptic scaffolding proteins of excitatory and inhibitory synapses: Homer1c and Gephyrin, respectively. ESR6 observed reduced density and size of

inhibitory synaptic clusters, but no effect on the excitatory synapses was apparent. Similarly, ESR6 investigated the synaptic organisation of $GABA_A$Rs and AMPARs, and observed discrepant disturbance of inhibitory and excitatory synaptic organisation. Furthermore, ESR6 analysed preparations exposed to NMDAR-Ab for indicators of activation of the immune system, which did not reveal an activation of astrocytic or microglial cell populations. Full description of the achievements: Annex 1 – ESR6 Daniel Hunter.

To examine alterations of the synaptic properties in Fragile X Syndrome (**Task 2.4**), a condition related to the transcriptional silencing of the Fmr1 gene, **ESR7, Elisa Corti**, supervised by Carlos Duarte, Ramiro Almeida and Paulo Pinheiro at CNC, has mimicked what happens in this disease, by knocking down (KD) Fmr1 expression using two different sets of shRNAs in hippocampal neurons. ESR7 observed that Fmr1 KD was sufficient to increase the synaptic content of GluN2A and GluN2B-containing NMDA receptors, and to impair BDNF-induced synaptic clustering of such receptors. ESR7 is currently testing the efficacy of the above mentioned shRNAs in axons as well, in order to study presynaptic differentiation. Moreover, ESR7 has established a protocol for autaptic cultures, which will be useful to study the dynamics of synaptic vesicle exocytosis and recycling in Fmr1 KO neurons. Full description of the achievements: Annex 1 – 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, has performed experiments to dissect the mechanisms of the increased synaptic density in DS. ESR8 performed surgeries and 2-photon imaging for the evaluation of synaptic bouton density, survival, turnover, and size. The dataset is close to completion with the current DS clone set and shows a trend increase in survival and decrease in turnover rate in trisomic cells. Additionally, a significant increase in bouton size has been found in trisomic cells compared to isogenic controls, indicating potentially increased strength of synapses. This dataset will be built on with the findings resulting from dissecting the mechanisms of the functional network defects in DS. Surgeries for this dataset have recently be optimized for GCaMP 2-photon activity imaging of somas. Imaging is ongoing in a large group of animals at 1 month, 3 months and 7-8 months post-transplantation. Analysis is currently underway to determine if the data fits with the hypothesis, but imaging of activity (both low-level and across multiple somas) has been successfully performed. It is expected that electrophysiological data and super-resolution data from future secondments will further expand on this dataset and its findings. 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, 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. Mutations in the gene GLRA2, encoding the glycine receptor α2 subunit, have been reported in patients with autism. Glra2 knockout mice exhibit deficits in learning and memory in the novel object recognition test and impaired long-term potentiation in the PFC. First, the cellular correlates of PFC synaptic dysfunction in Glra2-deficient mice were explored. ESR9 quantified excitatory and inhibitory neuron populations in the PFC using fluorescent in situ hybridisation and immunofluorescence. Although there were no differences in the density of total excitatory or inhibitory neurons in Glra2−/Y mice, ESR9 observed an increase in the number of somatostatin interneurons, with no significant differences in the other subpopulations of interneurons studied (parvalbumin, calretinin and cholecystokinin). Immunofluorescence experiments were also performed at postnatal day 14 to rule out a

developmental effect, and the results were similar to those obtained in adults. Quantification of excitatory and inhibitory synapses in the PFC via immunofluorescent co-localisation of pre-synaptic and post-synaptic proteins showed a decrease in inhibitory synapses with no change in excitatory synapses, pointing to an excitatory/inhibitory imbalance in the PFC of Glra2−/Y mice. Analysis of spine density and dendritic arborisation in PFC pyramidal neurons after biocytin injection revealed increased spine density and dendritic arborisation, with increased dendritic length and branching. These findings indicate that there is altered connectivity of the pyramidal neurons in the PFC of Glra2-deficient mice, which is expected to result in perturbations of synaptic function and disruption of normal cortical network dynamics. In addition, ESR9 mapped the brain regions implicated in the novel object recognition deficit observed in Glra2-deficient mice, using c-fos. ESR9 observed a decrease in c-fos-positive cells in the infralimbic PFC of mutant mice following novel object recognition, with no significant differences in the other regions or under basal conditions. These results suggest that there is hypoactivation of the PFC of Glra2−/Y mice, which may underlie the cognitive deficit. This experiment is currently being repeated in a larger cohort of mice. Full description of the achievements: Annex 1 – Laura Upton.

1.2.3 Work Package 3 – Modulators of neuropsychiatric disorders

WP3 has 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 focuses 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 this first reporting period 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 exploitable results and an explanation about how they can be exploited (when applicable).

No deliverables nor milestones were planned for this reporting period for WP3.

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, collected the brains to evaluate the basal levels of the two main endocannabinoids and the two receptors, CB1 and CB2. Endocannabinoid levels will be measured by our collaborators at the University of Bordeaux. ESR10 learned how to perform Western blots and tested the antibodies for the receptors; these experiments will be completed in the next few months. To study the effects of administration of endocannabinoid modulators on the phenotype of Shank3-deficient mice, ESR10 performed behavioural experiments to evaluate the effect of the CB1 receptor antagonist rimonabant (1 mg/kg/day i.p. for 7 days) in male and female heterozygous (Het), knock-out (KO) and wild-type littermates. After the last injection, mice

were tested to evaluate locomotion, motor coordination, learning and memory, and anxiety-like behaviour. KO males exhibited hypolocomotion, which remained unchanged after rimonabant. No significant locomotion deficits were observed in Het males or in females. KO males exhibited a coordination deficit in the rotarod that was rescued by rimonabant. In females, both Het and KO mice had a coordination deficit, which was only partially improved by rimonabant. In two novel object recognition tasks, with novel objects differing from the familiar object either by texture or shape, rimonabant rescued deficits in both Het and KO mice. ESR10 is currently finishing the evaluation of anxiety-like behaviour in the dark-light box. In addition, ESR10 recently completed a pilot test to evaluate the effects of another drug, AEF0217 (Aelis Farma), a signal specific inhibitor of CB1 receptors, which unlike rimonabant, has no side effects, and is thus suitable for clinical use. AEF0217 was administered continuously through the drinking water for at least 7 days; the results are promising, and we will soon decide how to continue to study the effects of this drug in our mouse model. 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 by Niels Plath and Kjartan Herrik at Lundbeck, performed experiments that identified SK channels as potential pacemakers of thalamocortical activity and thalamic reticular function. Positive and negative modulators of these channels elicited opposite effects on TRN firing, after burst afterhyperpolarization and time between bursts. These results allowed the project to move forward to the next step. Experiments to test the impact of pharmacological modulation of TRN neurons in thalamocortical rhythms during sleep and wakefulness in freely moving rats, are now ongoing. Full description of the achievements: Annex 1 – Ágata Silván.

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, started by characterizing Syngap+/- dataset precedently pre-processed in the lab. DESeq analysis revealed misregulated targets which were compared with the previously published Fmr1-/y CA1 dataset leading to the following observations: Syngap+/- CA1-TRAP revealed 218 upregulated transcripts and 248 downregulated transcripts; gene set analysis showed upregulation of terms for metabolic and RNA processing, downregulation of terms for GPCRs and serotonergic synapses; comparison of Fmr1-/y and Syngap+/- GO terms revealed common upregulation in rRNA processing, but opposite changes in several other gene sets. Interestingly, changes in Fmr1-/y CA1-TRAP were negatively correlated with changes in Syngap+/- CA1-TRAP. FMRP targets and SFARI transcripts, which are downregulated in Fmr1-/y CA1-TRAP, were upregulated in Syngap+/- CA1-TRAP. In addition to this dataset obtained for CA1 region, ESR12 started to originate a new TRAP line specific for amygdala BLA region of Syngap+/- and Fmr1-/y mouse models of disease. Through Immunofluorescence and Genotyping, recombination was spotted, which delayed the establishment of the line. However, amygdala BLA-specific TRAP line obtained through Ribotag technology, was validated through Immunofluorescence with HA antibody that showed BLA-specific staining. In addition, Rspo2+ population described to be BLA specific was showed to be present in the BLA region and colocalization with HA staining was present for 82.5% of the cells. 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, conducted Electrophysiological experiments in neurons of the thalamic reticular nucleus (TRN) from mouse slices. Exposure to the corticotropin-releasing hormone (CRH), either bath-applied or released by photoactivation of CRH-releasing fibers, was found to alter TRN cell excitability by decreasing low-threshold bursting via activation of the CRH receptor 1. The expression of this receptor in TRN neurons was confirmed by specific quantitative in situ hybridization of mRNA (RNAscope). ESR13 is further dissecting the ionic mechanisms involved in burst reduction, which will indicate molecular targets to be tested in experiments in vivo. By performing circuit mapping through viral infections, ESR13 has identified several extra-hypothalamic populations of CRH-releasing cells projecting to the TRN. These data provide the first demonstration that the stress-related CRH system affects TRN function. Based on progress achieved so far, ESR13 will soon set up polysomnographic recordings in freely-behaving mice and examine the effects of CRH on sleep rhythms through chemo- or optogenetic manipulations of TRN-projecting CRH-releasing cells. ESR13 has started pilot analyses of gene expression in TRN samples collected from mice exposed to early life stress. The results will guide further experiments in which the expression of genes relevant for psychopathology will be assessed after chronic exposure to CRH or stress. 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, 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, ESR14 has achieved to implement new protocols and methods to study robust social phenotypes. ESR14 has also obtained results that show a potential altered phenotype in social interaction with a new conspecific in the short-term in *Syngap1*+/ΔGAP and *Syngap1*+/- rats as well as potential alterations in social dominance. Furthermore, since these results are shown in young adults, ESR14 is in position (time-permitting) to characterize younger ages and define critical periods when these phenotypes are manifested. Once these potential phenotypes are confirmed, ESR14 will work on the second task of the objectives as described in the Individual Research Project. To study if cellular phenotype can be prevented, ESR14 first aims to define the specific brain circuits mediating the described social alterations. Full description of the achievements: Annex 1 – Vanesa Salazar.

1.2.4 Work Package 4 – Training and Doctoral Programme plan

WP4 has 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 synchronizes all training activities (local and network wide), 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 the COVID-19-related restrictions, some fellows had their doctoral courses either postponed or transferred to an online format. Nevertheless, all ESRs that are 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 1. List of courses taken by the ESRs.

ESR	**Courses**
ESR1 Orsolya Antal	Laboratory animal science; Core technologies; Connecting researchers with the society; Portuguese language course; Laboratory rotation module.
ESR2 Alessandro Chioino	State-of-the-Art Topics in Neuroscience Vlll. 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.
ESR3 Giuseppe Cammarata	Cellular and molecular biology; Experimental Biology and Biomedicine 1; Portuguese language course; Science Communication course; Core technologies; Laboratory Rotations module.
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.
ESR6 Daniel Hunter	Research integrity in scientific professions; Regulatory training for staff developing experimental procedures and projects; Surgery training course.
ESR7 Elisa Corti	Core technologies; Animal handling course; Portuguese language course; Laboratory Rotations module; Synapse Biology course.
ESR8 Elizabeth Brockman	Introduction to Learning & Teaching; Introduction to Assessment & Feedback for Learning; Research Computing: Introduction to LaTeX.
ESR9 Laura Upton	Regulatory course for project designers (rodents & lagomorphs).
ESR10 Flavio Tomasi	Regulatory course for project designers (rodents & lagomorphs).
ESR11 Ágata Silván	Neurograd Winter School; Introduction to Matlab.
ESR12 Manuela Rizzi	Autumn school for PhD students; Animal course; Tick@lab; Bioinformatic for Genomics course.
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.
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;

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.

Three **wide-ranging Network Schools** (NS), were planned for the first reporting period (**Task 4.2**). The initial plan included theoretical and hands-on sessions for all three Network Schools: NS1 - Basic concepts on imaging tools and data analysis in Coimbra, Portugal; NS2 - Genetic approaches for the study of neuropsychiatric disorders in Paris, France; and NS3 - Drug discovery and development: from idea to product in Copenhagen, Denmark.
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 (Senior Manager Application Sales Specialists - Carl Zeiss Microscopy GmbH). 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 "First Network School Report" (D4.2 D24), submitted via continuous reporting on the EC Portal in October 2019.

The COVID-19-related restrictions and confinement measures, which included a strict lockdown for several months and impossibility to travel, led to the postponement of Network Schools 2 and 3. NS 2, initially planned for Paris in June 2020, was postponed to the end of 2021, to be held on-site if the pandemic situation allows. If this is not the case, it will be held remotely on similar dates. NS 3, initially

planned for January 2021 in Copenhagen, was rescheduled to May 5-7 (2021) and changed to an online format. These modifications were included in the amendment to the Grant Agreement, approved in March 2021.

Syn2Psy is committed to provide training opportunities that lead to the development of the ESRs professional careers. As such, a **plan for training in transferable skills** was approved by the Supervisory Board and will take place in 2021 and 2022.
This training will be provided in a virtual format by a specialised company (Chaperone - https://chaperone.online/) that offers personalized career development for scientists. The approved plan includes eight group sessions, four to be held in 2021 and four to be held in 2022. The topics for 2021, were chosen by the fellows, and are as follows: Project Management; How to Write a Scientific Article; How to Prepare for a Scientific Conference and Wellbeing/Work-Life Balance.
These group sessions will be held in September and October 2021 and will further be complemented by individual one-to-one sessions for each ESR, according to each fellow's need.
The group sessions for 2022 will be chosen from the following list: Thesis Writing; CV Writing; Academia or Industry?; Creating my Own Business; How to Prepare for an Interview?; How to Commercialize my Ideas?; How to Choose a Lab for a Postdoc?; Conflict Management; Networking Skills Boost and Dealing with Uncertainty.

One of the major topics in the Syn2Psy project is the creation of proper science contents for multiple audiences. Within Syn2Psy the ESRs have the opportunity to develop transferable skills in outreach, communication and public engagement, as well as in **bridging the science-society gaps** through several initiatives ongoing (discussed in WP5 - Dissemination & Communication and Exploitation).
These initiatives include the creation of scientific articles for children (to submit to the journal Frontiers for Young Minds), with the support and expertise of Syn2Psy partner PIN; the creation of a script for a theatre play on neuropsychiatric disorders fostered by Syn2Psy partner Marionet; opinion articles for the general public (blog-posts) and the creation of short-videos describing each of the fourteen projects adapted for the general public.

All ESRs have a defined plan to undertake **secondments** in other beneficiaries and partner organisations (**Task 4.3**) for a total duration of a maximum of 30% of the recruitment period. The secondment plan was designed to expand their international, intersectoral and interdisciplinary research and training, as well as to build working relationships. These secondments are planned for 2021 and 2022, thus have not taken place during the first reporting period.

One ESR (ESR11 Ágata Silván) had the plan to start her first secondment at EPFL, under the supervision of Carmen Sandi and Simone Astori, in March 2020. This coincided with the start of the pandemic-related restrictions in Europe which led to the closure of the laboratories at EPFL. Due to this, Ágata Silván was unable to perform the experimental work initially planned for this secondment. Alternatively, Drs. Simone Astori and Carmen Sandi provided material to work remotely in sleep scoring and EEG analyses. During the 3 months of lockdown, Ágata Silván was able to learn about the Sirenia Sleep Pro software system and worked on these analyses. Although this was highly beneficial for Ágata Silván's training, together with her supervisors at Lundbeck and the responsible researchers at EPFL, it was decided that an in-person secondment would benefit the fellow and the project as a whole.

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 during the first reporting period:

Table 2. Deliverables submitted for WP4.

Deliverable		**Date of submission**
D4.2 D24	Report on network school 1 (NS1)	16. Oct. 2019
D4.6 D32	Career development plan of ESRs	13. Fev. 2020
D4.8 D37	Report on PhD courses	07. Set. 2020
D4.9 D42	Supervisory Board	09. Ago. 2019

The following **WP4 milestone** was achieved during the first reporting period:

Table 3. Milestones achieved for WP4.

Milestone		**Means of verification**
MS9	Personal career development plan	Written report concerning the ESRs personal career development plan.

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, 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 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 have been 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 intends to reach a high number of people and 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 first reporting period, Syn2Psy website had a total of 2 536 visitors and 7 170 visits.

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



Figure 2. Print screen of the *Home* area of Syn2Psy website.

The **blog-post area** of the website has the purpose of allowing the ESRs to share their thoughts regarding sciece, their projects and/or the PhD experience in an informal and personal point of view. During this first reporting period, 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:

Table 4. Titles of blog-posts uploaded to the blog area of the website during the first reporting period.

ESR	Topic
ESR1 Orsolya Antal	An alternative approach to writing protocols;
ESR2 Alessandro Chioino	A typical day in science;
ESR3 Giuseppe Cammarata	Loneliness: the importance of social bonds;
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	Email exchange with Ágata Silván;
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 first reporting period, the Blog Series had a total of 621 visits.
For the next round of blog-posts the ESRs will focus on the ways in which science/ research/ neuroscience can solve societal challenges (present or future).

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.

Three episodes have been released during the first reporting period. 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 5. SimpleSci episodes released during the first reporting period.

SimpleSci Episode	ESR
Episode 1	ESR12 Manuela Rizzi;
Episode 2	ESR1 Orsolya Antal;
Episode 3	ESR9 Laura Upton.

Together, these videos reached a total of 583 views on Youtube and Instagram platforms.

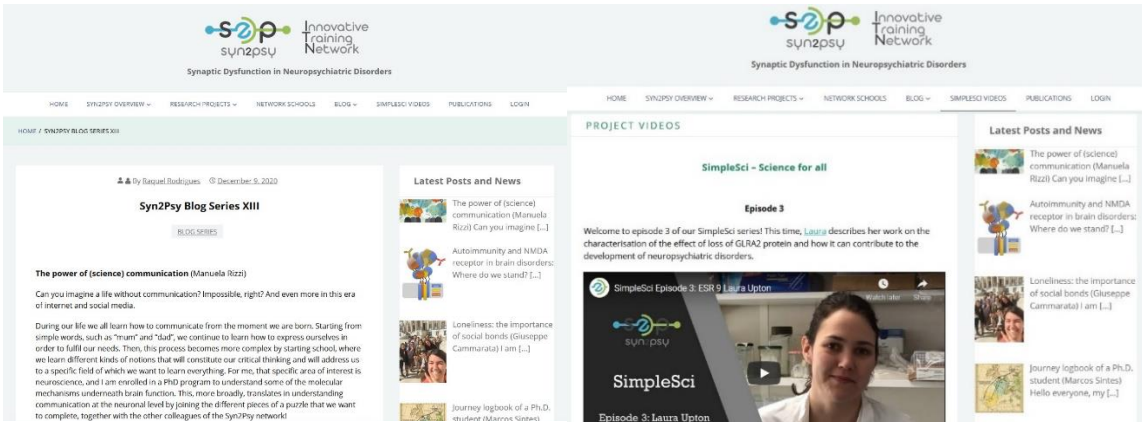


Figure 3. Print screens of the Blog and SimpleSci areas of the website.

Syn2Psy ESRs are 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, this first reporting period 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 have not had the opportunity to present in conferences or publish in scientific journals during the first reporting period. Furthermore, the conferences scheduled for this period were cancelled or transferred to an online format.

Publications by the ESRs:

- **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.

- **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.

Presentation of results in scientific events:

- **ESR9, Laura Upton** presented the poster "Altered prefrontal cortex assembly in mice deficient in the autism-associated gene Glra2", at the *Scientific days ED3C*, an online event organized by the doctoral school.

- **ESR10, Flavio Tomasi** presented the poster "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.

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: 3 Postdocs, 2 PhD candidates and one Assistant Professor.

An important aim 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.
Within the framework of the Brain Awareness Week, **ESR3, Giuseppe Cammarata** and **ESR7, Elisa Corti** participated in the 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. The fellows participated in this event with the publication of an article in Italian (Annex 2) and subsequent discussion with questions from the public. The topics of the articles and discussion were respectively "Organoidi di cervello – L'emancipazione etica degli studi sullo sviluppo cerebrale umano" (Brain organoids – The ethical emancipation of studies on human brain development) and "Ma la libertà di scelta esiste?" (But, does freedom of choice exist?).
This event was held on the Facebook page of *Biologi per la Scienza*, which has over 50 000 followers and the specific video had over 3 000 visualisations and 36 comments, which included questions from the participants.
The articles together had 379 views on the website, 747 interactions on Facebook, and were shared 82 times.



Figure 4. AperiNeuro event during the Brain Awareness Week. Bottom picture: ESR3 Giuseppe Cammarata; Top-left picture: ESR7 Elisa Corti.

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.
FYM is a scientific journal edited by and for children and the goal of this journal is to bridge between scientists' high-quality work and the children's scientific interest. The fellows sent seven abstracts (Annex 3) and all the topics were deemed suitable. Currently the ESRs are in the process of writing the full articles, with the support of Syn2Psy's partner, PIN.

Topics and authors are the following:

Table 6. Authors and titles of the articles to be sent to the journal Frontiers for Young Minds.

ESRs	Title
ESR2 Alessandro Chioino ESR13 Loredana Cumpana	Reaching for the top: the role of mitochondria.
ESR9 Laura Upton ESR10 Flavio Tomasi	Fantastic beasts and where to find them: how can we use animals to study neuropsychiatric disorders?
ESR7 Elisa Corti ESR12 Manuela Rizzi	The play-doh brain: shapeable neurons make you learn!
ESR1 Orsolya Antal ESR4 Diogo Soares ESR11 Ágata Silván	Schizophrenia: what causes hallucinations and distorted thinking?
ESR8 Elizabeth Brockman	Exploring the living mouse brain using 2-photon microscopy.
ESR5 Daniel Hunter ESR14 Vanesa Salazar	A brain composed by multilingual neurons: A story of coded messages and crosstalking.
ESR3 Giuseppe Cammarata ESR5 Marcos Sintes	How to build a mini human brain.

Under the guidance of Marionet, Syn2Psy fellows are in the process of writing a **script for a play on the activities of Syn2Psy** (**Task 5.6**), to be performed in the Syn2Psy final meeting.

The following **WP5 deliverables** were submitted during the first reporting period:

Table 7. Deliverables submitted for WP5.

Deliverable		**Date of submission**
D5.1 D29	Syn2Psy public website	19. Nov. 2019
D5.3 D34	Public media dissemination	07. Out. 2019

The following **WP5 milestones** were achieved during the first reporting period:

Table 8. 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

1.2.6 Work Package 6 – Management

WP6 has 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 is 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 will also oversee the quality and quantity of supervision of the ESRs.
- **Research Coordination Committee** is responsible for oversight of the recruitment of ESRs and shall report and be held accountable by the Supervisory Board. This board is 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** will provide advice to the Supervisory Board on all training and transfer of knowledge issues and shall report to and be held accountable to the Supervisory Board. The Training Board is responsible for approving all training plans/reports and secondment reports, ensuring implementation of the training program.
- **D&C Committee** will be responsible for managing the dissemination and outreach activities of the Project and for oversight of protection and exploitation of the research results.
- **Management Board** is composed of the Project Coordinator and the Project Manager. The Management Board is responsible for 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** is composed of three ESRs. The ESR Board will change annually, and will represent ESRs in all issues related to the work and contract conditions. The ESR Board will provide feedback to the Supervisory Board. Feedback from each ESR will be obtained through the annual progress monitoring meetings and the ESR Board.
- **External Advisory Board** consists of two to four European and non-European top researchers in the research field of the Project, who will be appointed to observe and advise on the strategic direction of Syn2Psy research training programme. The External Advisory Board will also embrace independent members to afford guidance on transferable training skills, including links with industry and technology. The External Advisory Board will be appointed at the kick-off meeting.

A **project manager**, based at CNC, has been recruited and is responsible for handling administrative, financial and legal management. The project manager provides direct assistance to the project coordinator, oversee the ESRs recruitment process, the logistical preparation of the network-wide events, the production of technical and financial reports and manages the website as well as the outreach initiatives.

One **project meeting** was held during the first reporting period (**Task 6.2**): Syn2Psy Kick-off meeting. It 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 Syn2Psy.

The main topics covered by the discussions were the following:

- Project structure, goals, timeline and events;
- Financial aspects, management structures, reporting, open access policies and ethics;
- Implementation strategy and plans for future work;
- Network Schools, Secondments and Personal 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. The participants agreed that this meeting was a success.
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 5. Group photography taken on September 26th, 2019 in Coimbra, during the Syn2Psy Kick-off meeting.



Figure 6. (Left) Group photograph during the Kick-off meeting dinner. (Right) Early stage researchers during a guided visit to University of Coimbra.

Due to the cancellation of all the Syn2Psy events planned for 2020 and early 2021, and given the uncertainty regarding traveling in the months that followed, the consortium members agreed to participate in an **online PhD day** (October 5th 2020, Zoom) to promote scientific discussion and interaction between the consortium members, as well as to give the opportunity to the ESRs to receive scientific feedback.
On this occasion, the ESRs described their progress (including training) in a 15 min presentation, followed by 15 min of discussion. Despite the delay in the projects as compared to the original plan, there was a unanimous opinion that the projects are on the right track and moving forward. The consortium members agreed that this event was fruitful and constructive.
The Training and Doctoral Studies Committee (TDSC) had an active role in assessing the ESRs' progress (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.



Figure 7. Photo taken during the online PhD day.

For the same motives as described above for the online PhD day, 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 this first reporting period, regular **internal communication** (**Task 6.2**) was achieved through electronic correspondence, dedicated mailing lists and virtual meetings.
The internal area of the website is used to share Syn2Psy documents, including ESRs presentations/data and information on progress.

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.

It was up to each PI to make sure that local recruitment rules were followed and that students applied to the local doctoral programs in due time.

In total 141 candidates applied to the Syn2Psy ESR positions. Initially, 16 candidates (11,3%) were considered ineligible due to reasons such as: being already a PhD holder, having stayed for more than 12 months in the last 3 years in the host country, or having longer research experience than 4 years.
The PIs received a list of the eligible candidates and their 3 project preferences. This list also contained candidates which eligibility was uncertain - some of these candidates were considered ineligible later due to the fact that they haven't finished their Master programme at the time of recruitment.
The PIs applied the evaluation criteria to shortlist the candidates, using the initial screening evaluation form (table 3):

Most shortlisted candidates were interviewed on Skype by a panel of 2-4 evaluators, in which the chair was the PI. The candidates were evaluated using criteria decided by the RCC (table 5). The highest scored candidate was officially offered the position (table 6).

The highest scored candidates were shortlisted by the PIs mostly taking in consideration the candidate's first option. It is worth mentioning that ultimately the best candidate was chosen (even if the specific position was 2nd or 3rd preference).
In total 50 eligible candidates were shortlisted as their first choice (table 4). Some candidates were shortlisted by more than one PIs (24 candidates). If the same candidate was selected for interview by more than one PI, and offered a position in more than one laboratory, the candidate had the ultimate choice regarding the laboratory and project (independently on the candidate's initial preference).

The selected candidates have an age comprised between 23 and 28 years old, come from 8 different countries and have in the majority a Neurosciences background. Both genders are nearly equally represented (table 7 and figure 1).
The employment contracts of the ESRs started between September and October 2019 and have a duration of 36 months in full time and exclusive work for the action (100%) (table 8). Additionally, the ESRs are all enrolled in a PhD programme.
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.

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 during the first reporting period:

Table 9. 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. Fev. 2020
D6.5 D31	Annual progress report 2	01. Mar. 2021
D6.8 D41	Consortium Agreement	30. Oct. 2019
D6.9 D43	Data Management Plan	13. Dec. 2019

The following **WP6 milestones** were achieved during the first reporting period:

Table 10. 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 has been established.
MS5	Annual progress report 1	Written report was produced.
MS9	Personal career development plan	Written report concerning the ESRs personal career development plan 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 4).

The following **WP7 deliverables** were submitted during the first reporting period:

Table 11. 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.

1.3 Impact

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

2. 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.

3. Update of the data management plan

The Data Management Plan (DMP) has been updated: ESR14, Vanesa Salazar included an extra type if rats being used in her experiments. The Data Management Plan – v2 is included in Annex 5.

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

In October 2020, the ESRs had a (remote) private meeting with the REA Project Officer. The feedback comments provided by the Project Officer and the information on how they have been followed up are described below.

- **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 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.**

Syn2Psy is in the process of stablishing a fall-back procedure, in case the pandemic will last for a long period.

5. Deviations from Annex 1 and Annex 2

During the first reporting period, Syn2Psy submitted a Consortium Requested Amendment (AMD-813986-3) to the REA, which was accepted on March 9th 2021. This 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.

Apart from the changes included in the amendment, four other projects went through modifications either due to a change in supervision, due the constraints caused by COVID-19-related restrictive measures 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 will not affect the implementation of the action.

ESR5

ESR5, Marcos Sintes went through a change in supervision which led to a change in the project description. ESR5 is now under the supervision of Vincenzo de Paola. Therefore, this project was adapted to target some of the original scientific questions of the project from a new perspective that will allow to use the tools that this group has developed. The main aims of the project will be the characterisation of the abnormalities in the regulation of synaptic structural plasticity and network activity in ASD and the exploration of the therapeutic potential of psychedelic 5HT2AR agonists in such context. As genetically modified and drug-induced mouse models do not represent some of the characteristics of human idiopathic ASD, which is found in a majority of patients, IPSCs derived from human donors with ASD will be implanted into the mouse cortex to generate a cortical graft.

Published research about the potential of psychedelics to treat ASD is for the moment very limited. Experiments carried out in the sixties before psychedelic drugs were banned in most countries (Mogar et al., 1969), clinical trials with MDMA (an empathogen amphetamine derivate with some psychedelic properties) (Danforth et al., 2018) and anecdotal reports from individuals provide an optimistic perspective for the therapeutic potential of these compounds. The results obtained during the previous project provide a first account on the effects of psilocybin in an ASD mouse model, and, in this new project, it will be useful for the experimental design and as complementary results. The future experiments will help to expand our knowledge about the neuronal plasticity mechanisms that could play a role in the positive effects of psychedelics in the treatment of ASD.

ESR6

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.

ESR12

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.

ESR14

This project originally intended to explore two rat models, Fragile X syndrome and *SYNGAP1* haploinsufficiency. However, the COVID-19 pandemic and subsequent severe lockdown in Scotland interrupted ESR14's experimental work for a minimum of 6 months. Given the time lost to the pandemic, the scientific interests were prioritized to ensure the progress of the project and to meet the objectives in the Grant Agreement in time. Therefore, the project focused on one of the two conditions (*SYNGAP1* haploinsufficiency). Depending on the scientific progress of the project and time-permitting, the same goals and experiments may be carried out on a rat model of fragile X syndrome.

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

Neuropsychiatric disorders are among the leading causes of disability, with alterations in (glutamatergic) synaptic function a common contributor to their pathophysiology. We have generated two knock-in mouse models of neuropsychiatric disorders, each carrying a disease-associated variant of the AMPAR-auxiliary protein stargazin, encoded by the *CACNG2* gene. We are currently performing behavioural and morphological analysis in these animals, which will be complemented by further functional characterisation, all focusing on alterations in synaptic function in the prefrontal cortex and its behavioural consequences. Furthermore, since exposure to chronic stress is a major risk factor for neuropsychiatric disorders, we aim to assess its impact on these structural, functional and behavioural phenotypes in our disease-associated models.

State of the art

Disturbances in cognitive function is a common feature of several, highly prevalent neuropsychiatric disorders, with these diseases presenting a large healthcare burden worldwide. Even though there are some existing therapeutic options for neuropsychiatric disorders, these often fail to improve the full spectrum of symptoms present [1]. The exact pathophysiological background of neuropsychiatric diseases is often complex, as both genetic and environmental factors can converge to lead to alterations in synaptic function. In fact, recent evidence has highlighted a substantial number of susceptibility genes that are involved in the normal functioning of synapse, plasticity, and the structural characteristics of neurons [2–5]. A key site with such alterations is the prefrontal cortex (PFC), an area commonly impaired in neuropsychiatric disorders [5–7]. Furthermore, there is significant evidence for some environmental risk factors leading to similar alterations in synaptic function. Exposure to chronic stress has long been implicated in driving or worsening neuropsychiatric disorders, with the prefrontal cortex being especially vulnerable to such insults. The impact of chronic stress on prefrontal neurons and circuitry has been extensively investigated and commonly found to lead to alterations in the architecture and function of PFC neurons [8,9].

Mutations in the *CACNG2* gene - coding for stargazin, an auxiliary protein of AMPA-type glutamate receptors - has been found to be associated with intellectual disability and schizophrenia [3]. These mutations have been predicted to be highly damaging to the function of the protein, suggesting that the disrupted function of stargazin may lead to synaptic dysfunction contributing to the pathophysiology of these diseases. We have generated two knock-in mouse models, each carrying one of the disease-associated stargazin variants (Stg^{ID} and Stg^{SCZ}).

In this project, we hypothesize that the impact of chronic stress and genetic susceptibility may summate to generate aberrant alterations in the PFC morphology and function that give rise to cognitive behaviour phenotypes characteristic of intellectual disability and schizophrenia.

Objectives

Our main goal is to assess how genetic susceptibility, as conferred by the disease-associated stargazin variants, converges with insults like exposure to chronic stress, leading to synaptic dysfunction in the prefrontal cortex and giving rise to the behavioural phenotypes consistent with cognitive impairments. Particularly, we aim to:

- Assess alterations in PFC-dependent cognitive function and the impact of chronic stress in Stg^{ID} and Stg^{SCZ} mice using a battery of behavioural tests
- Evaluate morphological/neuroanatomical alterations in PFC (pyramidal) neurons and dendritic spines of Stg^{ID} and Stg^{SCZ} mice and assess the impact of chronic stress
- Perform electrophysiological analysis to assess synaptic transmission and excitability in PFC neurons and the impact of chronic stress on these phenotypes
- Evaluate alterations in mitochondrial function in the PFC as a target for reversing behavioural and neuronal phenotypes

Description of ongoing work and future prospects

Alterations in PFC-dependent cognitive function and the impact of chronic stress in Stg^{ID} and Stg^{SCZ} mice using a battery of behavioural tests

Previous experiments from our lab have shown that Stg^{ID} mice present impairments in contextual and spatial memory (Fig.1). Preliminary results also suggest similar alterations in Stg^{SCZ} animals (in tests such as the object displacement task for spatial recognition memory or the T-maze test for working memory). Further PFC-dependent tasks are currently being optimised for use with our models, including a fear conditioning paradigm to assess the recall and extinction of contextual fear memory, as well as a temporal order recognition task (Fig.2)

Further behavioural characterisation includes the open field and elevated plus maze tasks, which will also be used to perform an initial characterisation of anxiety-related behaviours, followed by exposure to a chronic stress protocol. After this, cohorts will undergo a repeated assessment of anxiety-like behaviours, followed by cognitive tests, such as the T-maze and temporal order recognition tasks to assess working memory and PFC-dependent recognition memory.

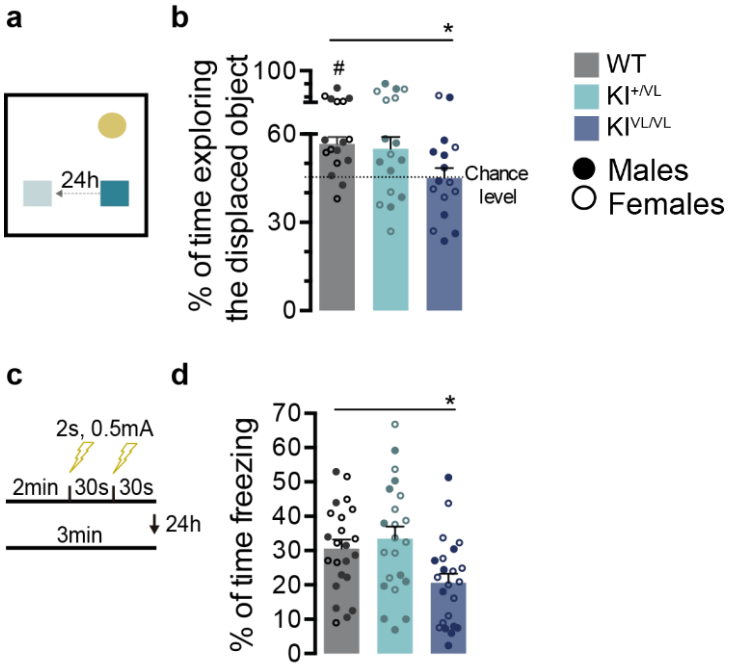


Figure 1: Stg^{ID} knock-in mice show cognitive deficits in contextual learning and spatial memory. When subjected to the object displacement recognition test (a), homozygous Stg^{ID} ($KI^{VL/VL}$)mice spent less time exploring the displaced object when compared to their wild-type (WT) counterparts (b). Data is presented as mean ± SEM.*p≤0.05, One-way ANOVA followed by Dunnet's multiple comparison post hoc test. N≥16 (males and females) for all genotypes. Homozygous Stg^{ID} mice present significantly less freezing behavior than WT counterparts in the contextual fear conditioning test (c), which is suggestive of impairments in associative learning ability (d). Data is presented as mean ± SEM. * p≤0.05, One-way ANOVA followed by Dunnett's multiple comparison post hoc test. N≥22 (males and females) for all genotypes.

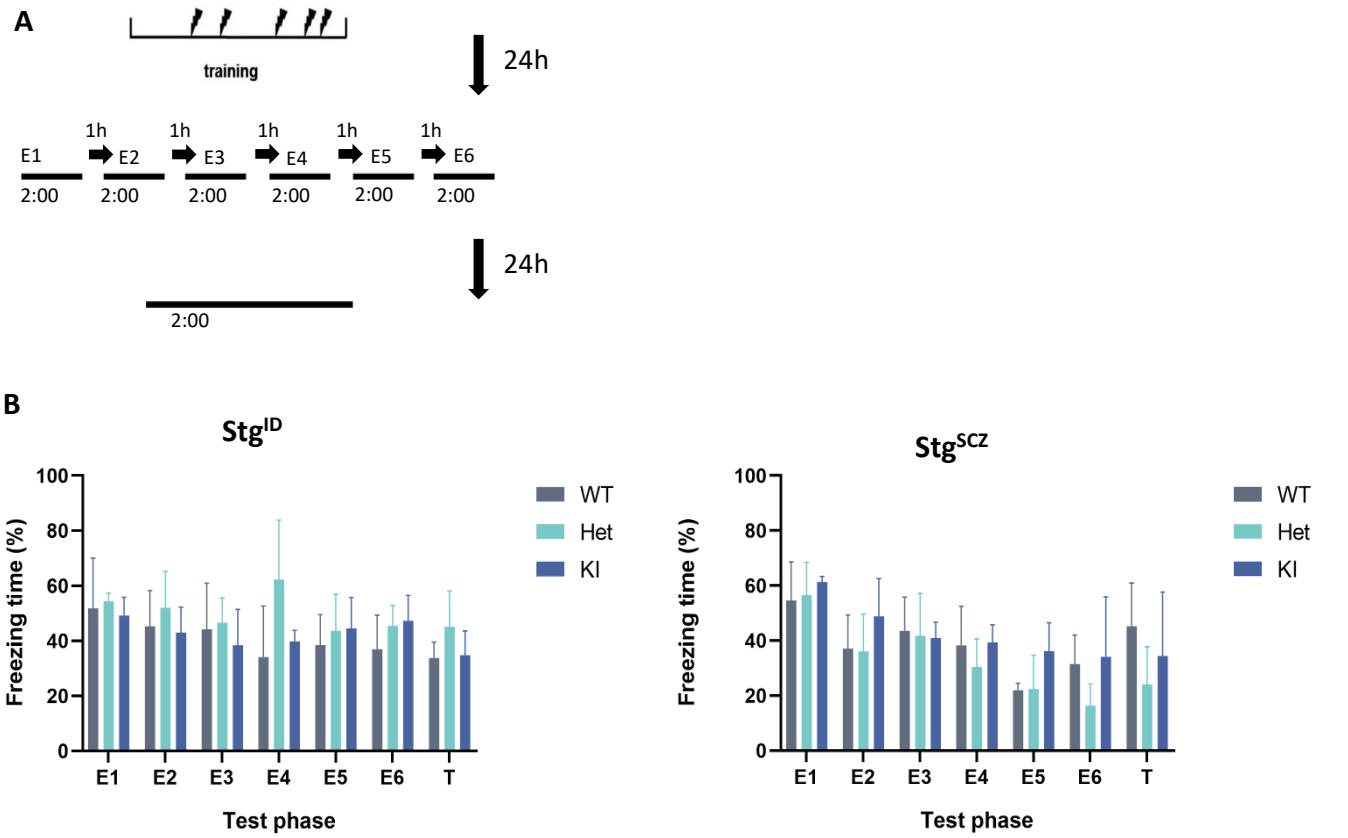


Figure 2: Stg^{ID} and Stg^{SCZ} knock-in mice show trends of altered extinction of fear memory. A) Schematic of the fear conditioning and fear extinction paradigms, adapted from Santos and colleagues [10]. In short, animals were trained to associate a neutral context (the fear conditioning apparatus) with an aversive stimulus of 5 mild footshocks, received at varying intervals during a training session of 5 minutes. 24 hours later, the animals were subjected to the fear extinction paradigm consisting of six extinction trials (E1-E6), separated by 1h delay each time, during a single extinction session. A further test of the retention of fear memory was carried out 24 hours later (T). B) Heterozygous (Het) and homozygous (KI) Stg^{ID} and Stg^{SCZ} mice appear to present alterations in the extinction of contextual fear memory compared to their WT counterparts. However, since the test is still under optimisation, larger sample sizes are required (currently sample sizes are: Stg^{ID} WT n=5, Stg^{ID} Het n=3, Stg^{ID} KI n=3; Stg^{SCZ} WT n=3, Stg^{SCZ} Het n=5, Stg^{SCZ} KI n=2).

Morphological/neuroanatomical alterations in PFC (pyramidal) neurons and dendritic spines of Stg^{ID} and Stg^{SCZ} mice and assess the impact of chronic stress

Since alterations in neuronal morphology (and specifically, alterations in spine density and spine morphology) have been reported in neuropsychiatric disorders, as well as being characteristic of chronic stress exposure, we aim to assess morphological alterations in Stg-KI animals. Our previous results have also shown altered dendritic complexity and spine density in the hippocampus of Stg^{ID} animals (Fig.3). Both studies focusing on neuropsychiatric disorders and chronic stress have highlighted the prefrontal cortex (and especially layer 2/3) as a site particularly vulnerable [6,9,11,12], therefore our main focus will be on this area. We are evaluating potential alterations in neuronal morphology and spine density in the PFC, and to evaluate the impact of chronic stress on such changes, focusing primarily on layer 2/3 pyramidal neurons. To this end, we are using the Golgi-Cox staining method to achieve sparse, high-contrast labelling of PFC neurons, which is currently being optimised to achieve complete, high-quality staining of prefrontal neurons. The stained neurons are visualised using confocal microscopy, followed by 3D-reconstruction of the complete dendritic arbor. We assess dendritic complexity using Sholl analysis, and changes in dendritic spines will also be evaluated based on the density, type and volume of spines of apical and basal dendrites.

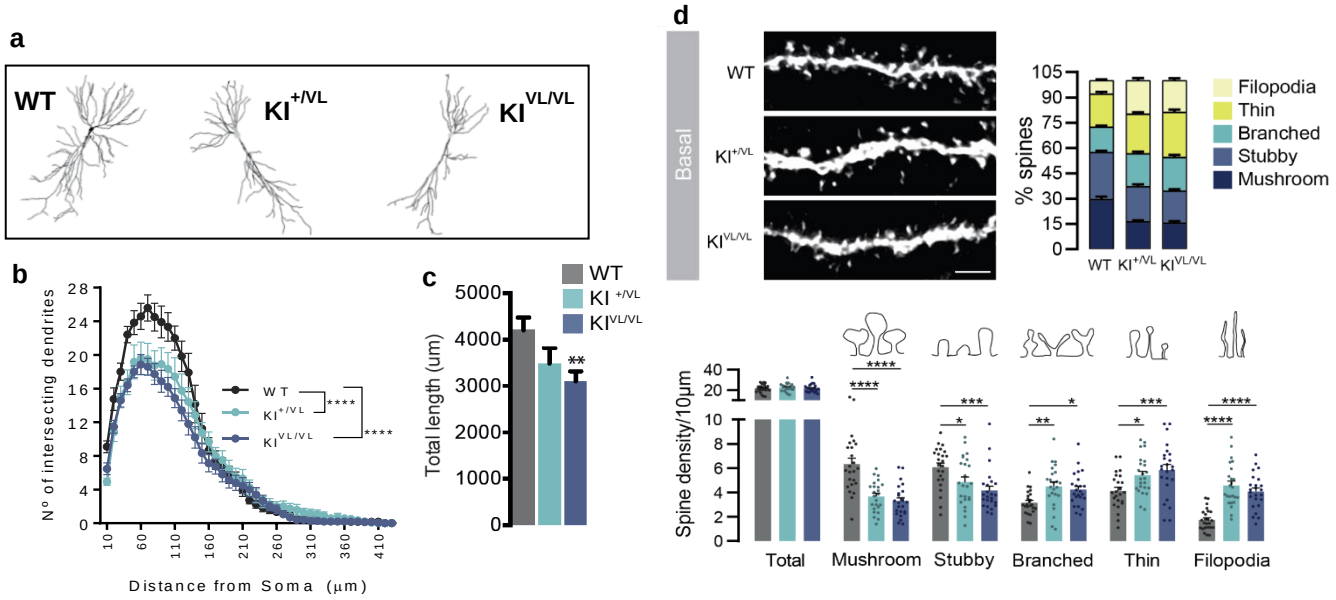


Figure 3: Stg^{ID} knock-in mice present alterations in dendritic arbor complexity and spine morphology in the hippocampus. Representative images of CA1 pyramidal neurons from WT and Stg^{ID} knock-in mice that were traced according to eGFP expression, reconstructed from AAV9.hSyn.eGFP-labelled hippocampal neurons (a). Sholl analysis reveals reduced dendritic complexity in Stg^{ID}-KI mice (b). Data is presented as mean ± SEM. ****p≤0.0001, Two-way ANOVA followed by Dunnett's multiple comparison post hoc test (shell radius genotype interaction p<0.0001, genotype effect p<0.0001 and effect of the shell radius p<0.0001). n = 13 cells/1 animal (male) for WT mice, n = 12 cells/1 animal (male) for KI$^{+/VL}$ mice, n = 15 cells/1 animal (male) for KI$^{VL/VL}$ mice. Total dendritic length was also significantly decreased in Stg^{ID}-KI animals (c). Data is presented as mean ± SEM. **p≤0.01, One-way ANOVA followed by Dunnett's multiple comparison post hoc test. Analysis of dendritic spines revealed altered spine morphology in Stg-KI mice (d). Data is presented as mean ± SEM, ****p≤0.0001, Two-way repeated measures ANOVA, followed by Dunnett's multiple comparisons post hoc test.

Electrophysiological analysis to assess synaptic transmission and excitability in PFC neurons and the impact of chronic stress on these phenotypes

We aim to evaluate any functional changes in synaptic function in the PFC in Stg^{ID} and Stg^{SCZ} animals, especially as previous assessment of Stg^{ID} mice have revealed changes in functional characteristics of hippocampal neurons (Fig.4). We also aim to assess how these phenotypes are impacted by exposure to chronic stress. We will perform whole-cell patch clamp recordings in PFC pyramidal neurons in acute coronal brain slices containing the cingulate, prelimbic and infralimbic cortices, primarily focusing on layer 2/3 pyramidal neurons. We will record miniature- and spontaneous excitatory postsynaptic currents (mEPSCs and sEPSCs) and inhibitory postsynaptic currents (sIPSCs and mIPSCs) in pyramidal neurons. Since stargazin is also crucial for synaptic plasticity, we will assess LTP by recording fEPSPs following high-frequency stimulation. The impact of chronic stress will also be investigated, leveraging the same experimental approach after exposing animals to chronic restraint stress.

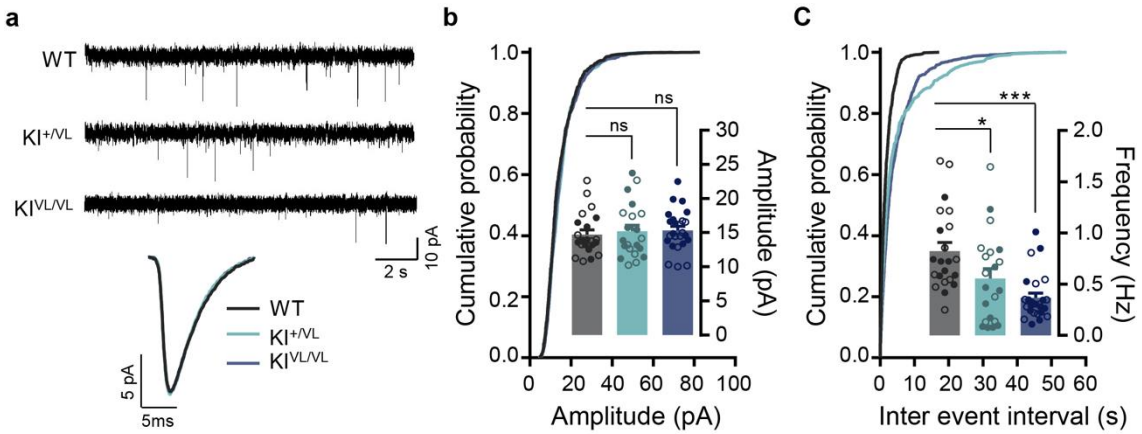


Figure 4: Stg^{ID} knock-in animals show decreased mEPSC frequency in hippocampal CA1 neurons. Representative traces of mEPSCs recordings and single average event of CA1 pyramidal neurons in acute hippocampal slices from WT and Stg^{ID}-KI mice (P15-P20) (a). Cumulative probability distribution and average mEPSCs amplitude (b) and frequency (c) plots, showing a reduction in frequency but not amplitude of mEPSCs. Data is presented as mean ± SEM. *p < 0.05, *** p < 0.001, ns, not significant, Kruskal-Wallis followed by Dunn's multiple comparison post hoc test. n = 22 cells/10 mice (4 male and 6 female) for WT mice, n = 21 cells/8 animals (4 male and 4 female) for KI$^{+/VL}$ mice, n = 25 cells/6 animals (3 male and 3 female) for KI$^{VL/VL}$ mice.

Alterations in mitochondrial function in the PFC as a target for reversing behavioural and neuronal phenotypes

Mitochondrial dysfunction has been extensively linked to neuropsychiatric disorders, with evidence for its involvement in schizophrenia, depression, or anxiety disorders. Even subclinical alterations to mitochondrial function can confer increased vulnerability to other genetic or environmental insults and contribute to the diverse phenotypes seen in neuropsychiatric disorders[13,14] Importantly, rescuing mitochondrial function has been shown to reverse the behavioural and neuronal alterations associated to disease[15].

Preliminary analysis from our lab has highlighted decreased expression of several mitochondrial proteins in cortical postsynaptic densities (Fig. 5), therefore we find it of interest to explore potential alterations in mitochondrial function in Stg-KI animals. We will first validate our previous mass spectrometry data, followed by evaluating mitochondrial function by analysing mitochondrial respiratory capacity. We will also assess potential alterations in the structure of mitochondria by performing electron microscopy analysis, within the framework of one of the secondments, the details of which are being planned at present.

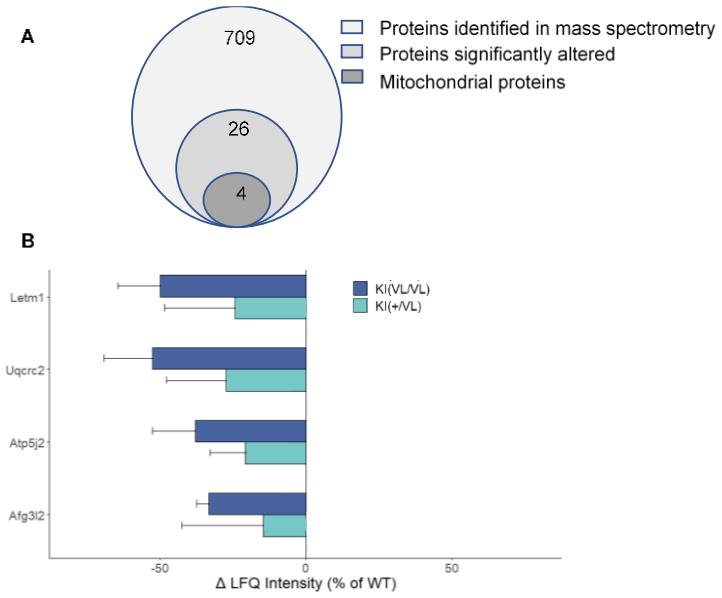


Figure 5: Mass spectrometry analysis suggested altered expression of 4 mitochondrial proteins in Stg^{ID} knock-in mice. Venn diagram summarising the results of mass spectrometry analysis in PSDs isolated from cortices of Stg^{ID} knock-in mice (**A**). 709 proteins were detected in the samples, 26 of which were found to be significantly altered. 4 of those 26 proteins, Letm1, Uqcrc2, Atp5j2 and Afg3l2 were mitochondrial proteins (**B**).

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ESR2 – Alessandro Chioino

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

Mitochondrial dysfunction is increasingly being implicated in the aetiology of psychiatric conditions, but the underlying cellular and molecular mechanisms have not been fully identified. Previous work in our lab has established a link between trait anxiety and the expression of the mitochondrial fusion protein Mitofusin 2 (Mfn2) in the Nucleus Accumbens (NAc) (Gebara et al., 2020), a part of the ventral striatum essential for motivation and goal-directed behavior. Highly anxious rats display increased passive coping behavior when exposed to a stressful situation requiring exertion of effort, such a forced swim test (FST). They also show lower Mfn2 levels in the NAc and reduced dendritic arborization and synaptic input in NAc medium spiny neurons (MSNs). The behavioral and cellular deficits could be reverted by overexpressing Mfn2 in the NAc.

This project aims at investigating the mechanisms whereby alterations in Mfn2 expression levels in NAc lead to morphological and synaptic alterations in MSNs and to impairments in motivated behavior. More specifically, by using genetic deletion of Mfn2 in mice targeting the MSNs expressing the dopamine receptor 1 (D1-MSNs), we address the following specific aims:

1. Evaluation of the activation of NAc circuits during an effort-related behavior: in mice with Mfn2 deletion in D1-MSNs and wild-type mice, neuronal activation is evaluated via cFos mapping after exposure to the behavioral task; the ongoing engagement of the NAc during behavior is evaluated by in vivo fiber photometry.

2. Analyses of electrophysiological and morphological alterations in NAc MSNs: patch-clamp recordings are conducted to measure intrinsic excitability and synaptic connectivity of D1- and D2-MSNs in mice with Mfn2 deletion in D1-MSNs. Biocytin-filling is performed in order to obtain morphological reconstructions of the recorded neurons.

3. Identification of the molecular determinants of morphological and synaptic alterations in NAc MSNs: cell-type specific isolation of ribosome-associated mRNA (Ribotag) and cell-type specific mitochondria isolation (Mitotag) in Mfn2 deficient mice in D1-MSNs and wild-type mice before and after behavioral exposure will be implemented. RNAseq analysis will be conducted on the isolated biological material.

4. Investigation of the impact of accumbal Mfn2 deficiency on glutamate receptors dynamics: electrophysiological, molecular and microscopy approaches will be applied in both naïve and stress-exposed animals to unravel changes in surface dynamics of AMPA and NMDA receptors.

Initial results for aims 1-2

1. Initial experiments in the lab have been conducted with a cell-type specific local deletion of MfN2 in D1-MSNs by injection of an AAV-Cre expressing a dynorphin promoter (DynCre) (Darvas and Palmiter, 2015) into the NAc of Mfn2lox mice. We exposed AAV-Cre ($Mfn2^{Dyn}$) and AAV-GFP (WT) injected mice to the forced swim test (FST). This standard paradigm evaluates the coping strategies (swimming vs. floating) adopted when test animals are facing an inescapable stressful condition, such as being placed in a beaker full of water that cannot be exited (De Kloet & Molendijk, 2016; Molendijk & de Kloet, 2015, 2019). The higher %immobility time indicates the MfN2 deletion in D1-MSNs promoted the adoption of a passive coping behavior (Fig 1a).

To evaluate the engagement of NAc cells during FST by cFos staining, we employed a tamoxifen-inducible genetic mouse line: Mfn2lox-Tg(DrD1a-CreERT2)-R26-LSL-TdTom. This mouse line was chosen because it induces a uniform downregulation of Mfn2 in D1-expressing cells ($Mfn2\text{-}D1^{KO}$), thus overcoming the issues related to inhomogeneous viral expression and/or mistargeting that might complicate cFos analyses. Interestingly, despite the less evident difference in %immobility time between wild-type (WT) and $Mfn2\text{-}D1^{KO}$ mice (Fig. 1b), the latter showed a significant reduction in neuronal activation in the NAc medial shell (Fig. 1c-d).

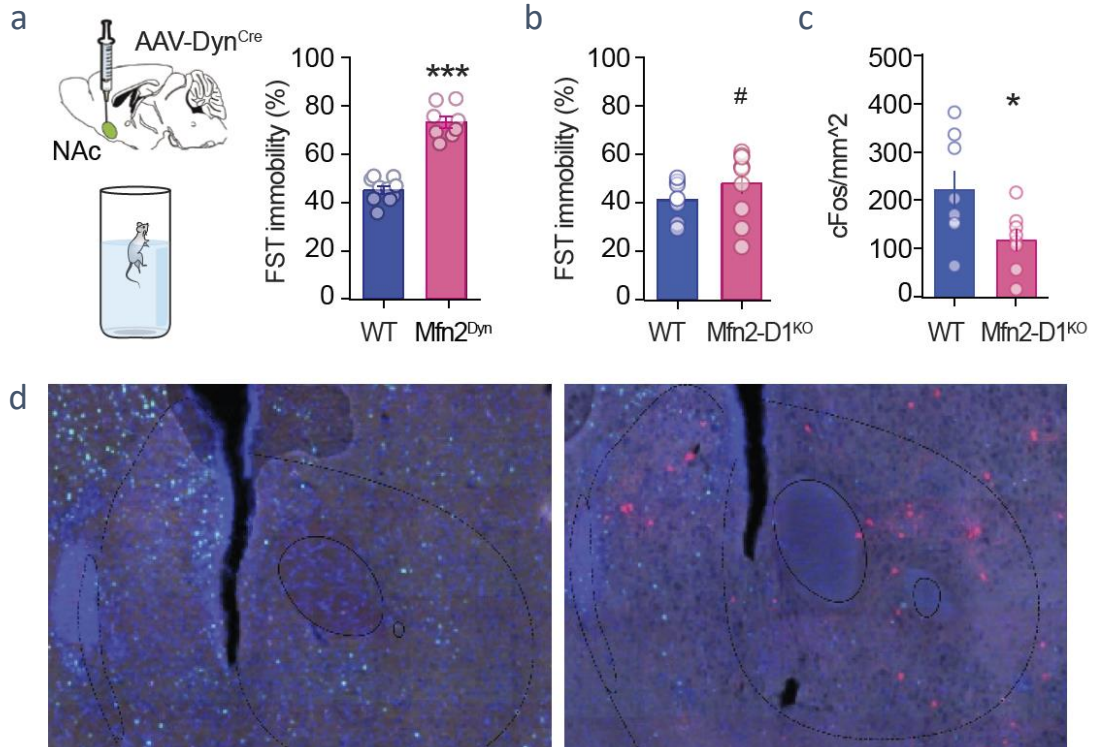


Figure 1: a) Viral strategy for Mfn2 downregulation in NAc D1-MSNs and subsequent test of effort-related behavior using forced swim test (FST). Immobility time (%) during FST was higher in AAV-Cre-injected mice ($Mfn2^{Dyn}$) as compared to AAV-GFP injected mice (WT), indicating greater passive coping behavior. b) Tamoxifen-inducible $Mfn2\text{-}D1^{KO}$ mice showed tendency to higher immobility compared to WT mice, p=0.059; c) cFos quantification as number of cFos positive cells/area shows significant reduction in NAc medial shell activation in $Mfn2\text{-}D1^{KO}$ mice, p=0.037. Unpaired Mann-Whitney t test; d) Representative cFos staining images for NAc medial shell. Blue=nuclear staining; Red=D1 cells marker; Light Blue= cFos staining.

To further assess the engagement of the NAc during behavior we will combine FST recordings with *in vivo* fiber photometry in mice with Mfn2 downregulation in D1-MSNs that will also be injected with a virus to drive expression of the Ca^{2+} indicator CGaMP6 in the NAc. Bulk Ca^{2+} signals, used as a proxy of neuronal activity, will be compared with a fine-grained scoring of the FST behavior performed using DeepLabCut (Sturman et al., 2020). This will allow us to correlate NAc neuronal activity with the different behavioral components of the FST (swimming, floating, movement initiation, vigor etc.) with a sub-second resolution.

2. To measure the impact of MfN2 downregulation in D1-MSNs, we performed patch-clamp recordings in NAc shell MSNs in acute slices from mice with either conditional (Mfn2-D1^{KO}) or virally (Mfn2^{Dyn}) mediated MfN2 downregulation. We present here preliminary data from the first recordings, that need to be confirmed by increasing the sample size.

In MSNs from Mfn2-D1^{KO} and WT mice, we elicited cell firing by somatic injections of depolarizing current steps (Fig. 2a). Interestingly, D1-MSNs show higher excitability when Mfn2 is downregulated. D2-MSNs did not show difference between genotypes, confirming that the Mfn2 deletion in D1-MSNs does not affect the intrinsic properties of other NAc cell types.

We then examined synaptic inputs by recording spontaneous excitatory synaptic currents (sEPSCs) in Mfn2^{Dyn} mice with virally mediated downregulation in D1-MSNs, and in mice injected with a control virus. In D1-MSNs, we observed an increase in the peak amplitude on sEPSCs (recorded at -70 mV holdig potential) and a decrease in the inter event interval (IEI)(Fig. 2b). Similar analyses on D2-MSNs are ongoing, as well as analyses of inhibitory currents.

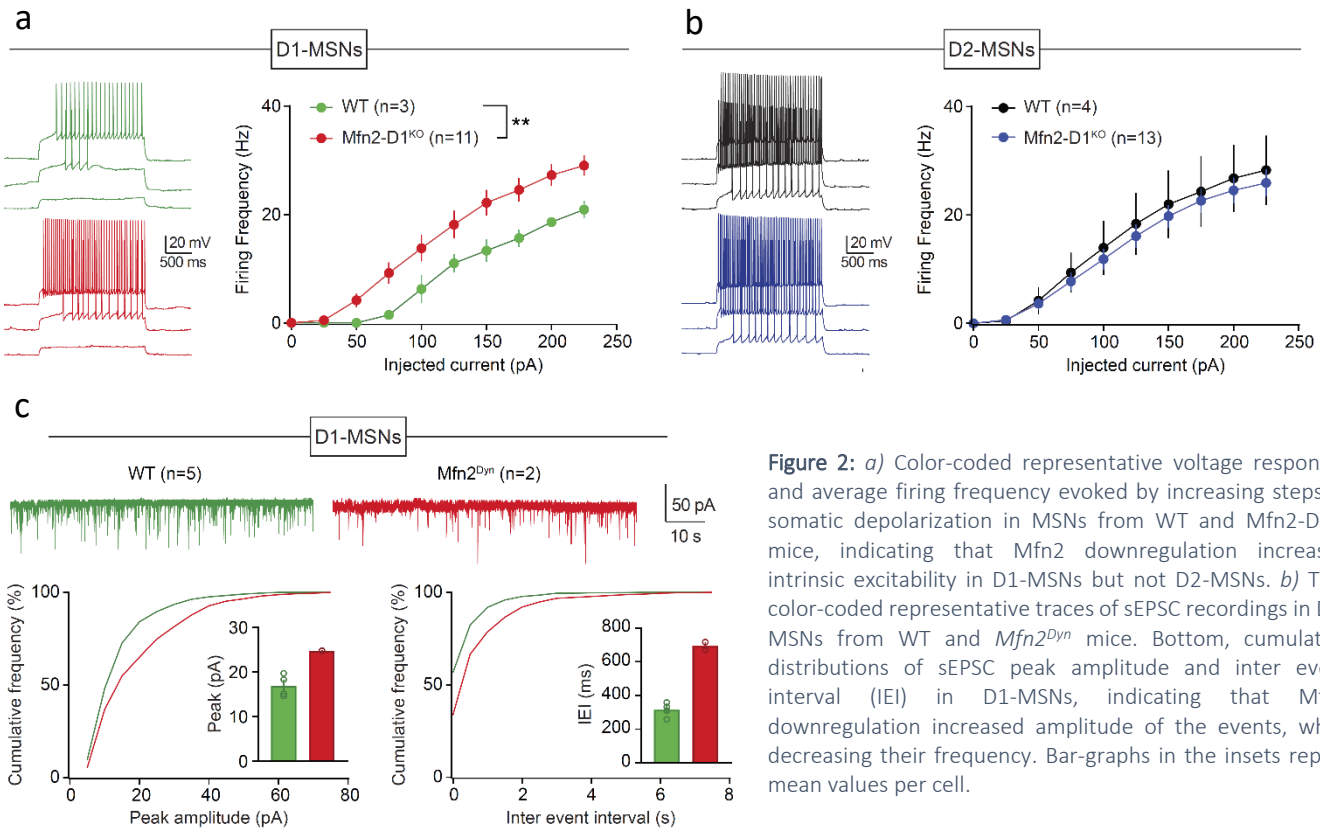


Figure 2: *a)* Color-coded representative voltage responses and average firing frequency evoked by increasing steps of somatic depolarization in MSNs from WT and Mfn2-D1^{KO} mice, indicating that Mfn2 downregulation increased intrinsic excitability in D1-MSNs but not D2-MSNs. *b)* Top, color-coded representative traces of sEPSC recordings in D1-MSNs from WT and *Mfn2^{Dyn}* mice. Bottom, cumulative distributions of sEPSC peak amplitude and inter event interval (IEI) in D1-MSNs, indicating that Mfn2 downregulation increased amplitude of the events, while decreasing their frequency. Bar-graphs in the insets report mean values per cell.

Initial morphological analysis was carried out in cells recorded in electrophysiological experiments. Cell were infused with biocytin via the patch pipette. D1-MSNs with Mfn2 downregulation appear to have reduced dendritic complexity, as indicated by Sholl analysis of dendritic arborization (Fig. 3). This dataset will be expanded to include more cells, and complemented with the analysis of spine density and morphology.

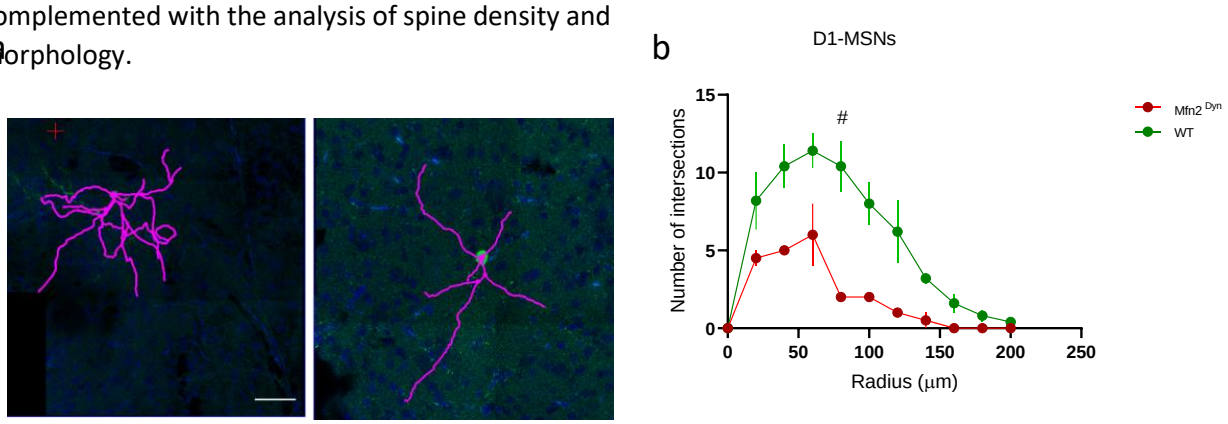


Figure 3: *a)* Examples of reconstructed D1-MSNs in a WT (left) and a *Mfn2^{Dyn}* (right) mouse. Scale bar 60 µm. b) Sholl analysis showing a tendency towards reduced dendritic complexity for Mfn2-deficient neurons, p=0.01, 2-Way ANOVA.

Overall, these cellular data indicate that Mfn2 downregulation in NAc D1-MSNs affects the intrinsic excitability, synaptic inputs and the dendritic complexity of this cell type.

Outlook for aims 3-4

3. To understand the molecular underpinnings of the morphological and dendritic remodeling, we will perform an unbiased RNA sequencing using a ribosome-associated mRNA (Ribotag) approach. Multiple gene families will be screened, with cell-type and brain region specificity. We expect to appreciate differences in D1-MSNs upon Mfn2 downregulation in genes associated with dendritic and synaptic growth.

As Mfn2 is a nuclear gene exerting its function on mitochondria, we will analyze if downregulation in this protein leads to alteration in the mitochondria genome expression by performing an unbiased cell-type and brain region specific RNA sequencing, using an approach that will allow us to isolate mitochondria (Mitotag). The results from this analysis will help understand the specific contribution played by mitochondria in the behavioral and electrophysiological differences previously observed.

4. Based on the preliminary electrophysiological results, we hypothesize that differences in glutamate receptor composition and dynamics might contribute to behavioral differences observed upon Mfn2 downregulation in D1-MSNs. We will perform electrophysiology experiments to assess the contribution of the two major glutamate receptors (AMPARs and NMDARs) to the synaptic transmission onto MSNs from specific inputs (e.g. from the prefrontal cortex). We will employ super resolution imaging approaches to detect the receptors dynamics at the membrane surface (during a secondment in the laboratory of Prof. L. Groc in Bordeaux).

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ESR3 – Giuseppe Cammarata

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

Understanding the exact consequences of valproic acid (VPA) exposure during human brain development has been a challenge over the past years. VPA is a widely prescribed anticonvulsant and mood stabilizer and its antiepileptic properties have been attributed to inhibitory effects on the degradation of gamma-Aminobutyric acid, modulation of ion channels and inhibitory action on histone deacetylases. Usage of VPA is now significantly restricted during pregnancy due to the high risk of the child later developing autism spectrum disorder (ASD). Data from animal models have suggested that an imbalance between excitatory and inhibitory neuronal populations may occur as a consequence of VPA administration. However, little is known about the direct effects of this drug on the developing human brain. Using brain organoids, we aim to address how exposure to VPA affects neural differentiation, neuronal physiology and emerging network properties leading to ASD.

Ongoing work and preliminary results

To reach the aforementioned goal, we are adopting a multidisciplinary approach focused on human brain organoids to model VPA exposure during neurodevelopment. Furthermore, we are pioneering the study of the consequences in the inhibitory system using the innovative and reliable organoid model.
To generate human brain organoids, we are following the protocol by Lancaster and colleagues. For this protocol, the initial plan was to use organoids differentiated directly from dental stem cells which are known to be derived from the neural crest and have been shown to readily differentiate into neurons. Unfortunately, the direct differentiation of dental stem cells into organoids was not generating mature organoids both at the physiological and molecular level. For this reason, a reprogramming step has been added before the organoid generation. The reprogramming process has been implemented with the use of the ReproRNA™-OKSGM, from Stem Cells Technologies, which uses a non-integrating, self-replicating RNA-based reprogramming vector to generate induced pluripotent stem cells (iPSCs) (Figure 1).

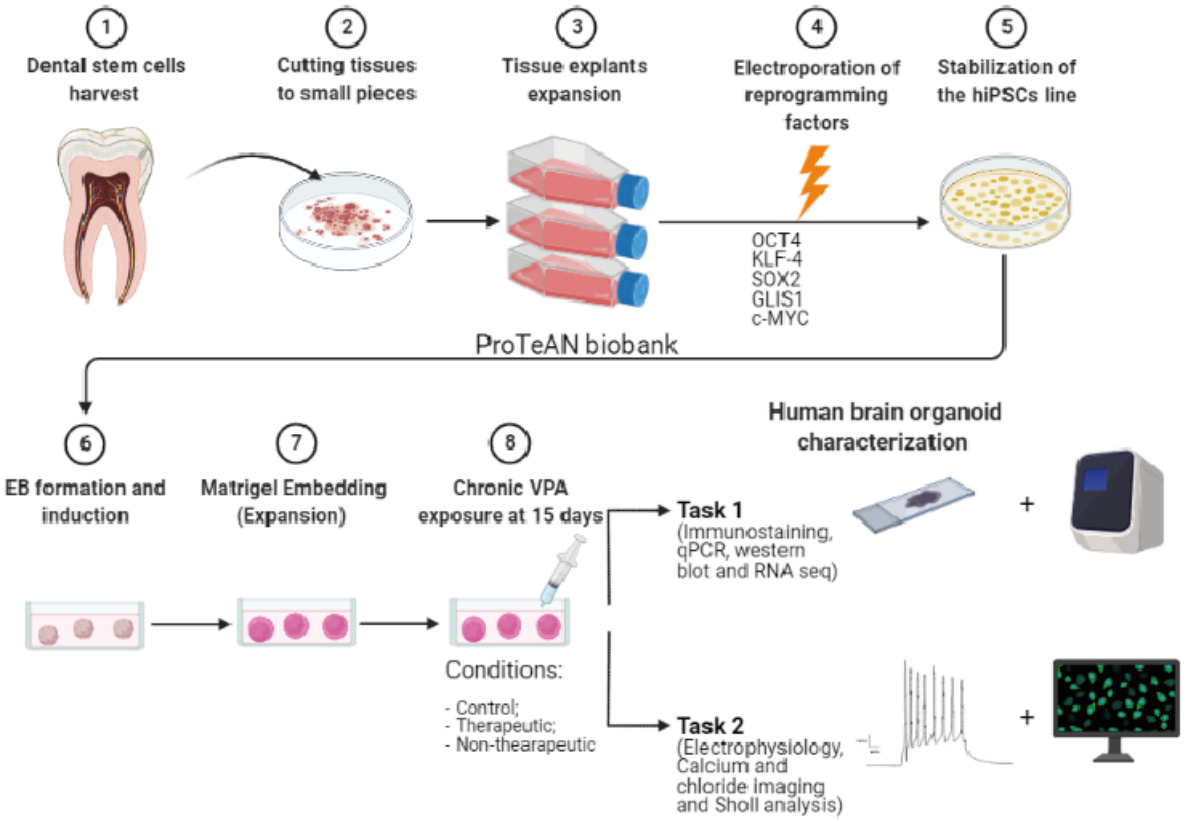


Figure 1. Experiment Scheme. Schematic representation of the pipeline of work. From the harvesting of dental stem cells to the reprogramming phase to produce human induced stem cells; and to the generation of human brain organoids used for neurodevelopmental experiments.

Human iPSCs-derived human brain organoids will be used to pursue the following tasks:

-Task 1- Perform a molecular and cellular characterization of human brain organoid development under VPA exposure.

The human brain organoids give us the possibility to observe how VPA affects structural organizations present in this model, such as separations of the ventricular-like zone, the appearance of upper- and deep-layer neurons in late development. As VPA has been shown to cause neurodevelopmental impairments, we established three conditions of human brain organoid development: 1) a control condition (sham exposure), 2) organoids exposed to a therapeutic concentration of VPA (0.5 mM), and 3) organoids exposed to a high concentration of VPA (1.5 mM) (Figure 1). The therapeutic concentration is within the concentration range found in patients treated with VPA (0.3 to 0.6 mM VPA). The higher concentration will accentuate any observable changes to better assess dose-dependent effects.
We have optimized the protocols for molecular analysis on these three conditions at different stages of development and we plan to use different hiPSCs patient-derived lines in order to validate our observations.

This task aims to analyze the cell type populations generated by organoids at 1-month, 4-months and 6-months post-differentiation both in control and VPA treated conditions. Exposure to VPA is known to trigger E/I balance shift and neurodevelopmental delay. We are planning to perform immunohistochemistry targeting specific markers to observe the excitatory and inhibitory populations

generated by the three experimental conditions. Antibody anti-vGLUT1, for vesicular glutamate transporter, will identify the excitatory population, and anti-GAD65, for glutamic acid decarboxylase, will identify the inhibitory population as in our preliminary data (Figure 2c).

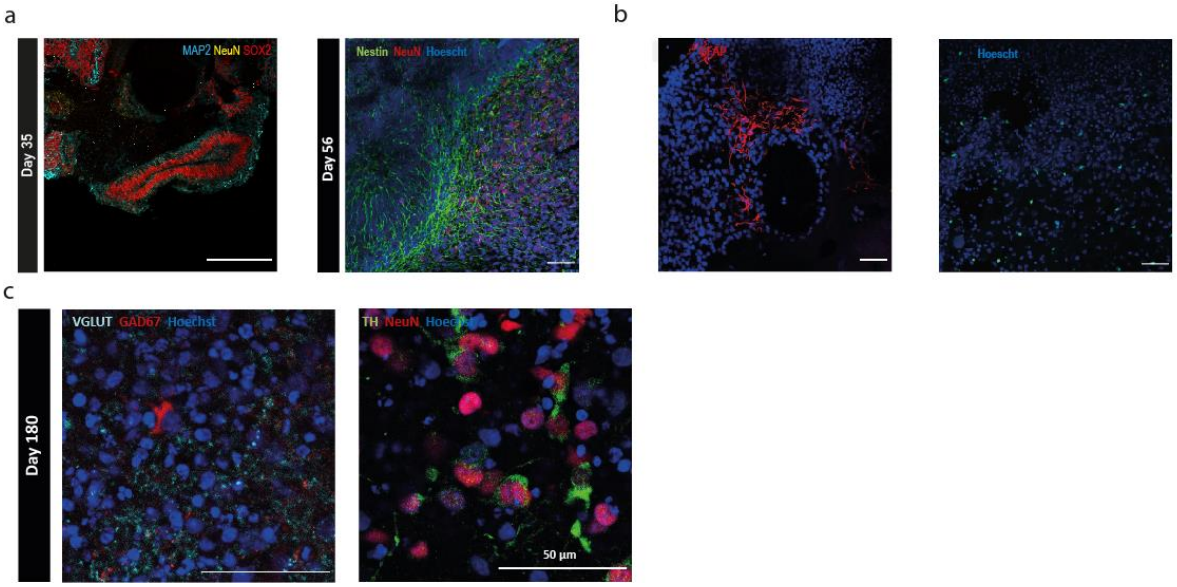


Figure 2. Human brain organoid Immuno characterization. a) In the first month, human brain organoids start to form ventricular-like zones. Structures formed by neural progenitor cells (SOX2 positive) from which neurons migrate (NeuN positive cells) forming additional layers. b) At day 70, astrocytes (GFAP positive) and oligodendrocyte (Olig2 positive) can be identified in our brain organoids. c) At around 6 months in development, some sub-types of the neuronal population can be identified: excitatory neurons (positive to vGLUT), GABAergic neurons (positive to GAD67) and dopaminergic neurons (TH positive cells) (Scale bars from a b and c are 50um).

Additionally, we are investigating the effect of VPA on neurodevelopment in terms of impairments in layer organization, following markers to characterize different cell types in brain organoids: neural progenitor cells (SOX2 and Nestin), deep-layer neurons (TBR1) and upper-layer neurons (SATB2) (Figure 2a-b). We will quantify and compare the percentage of cells positive to the abovementioned markers relative to the positive DAPI cells found in VPA-treated and control organoid slices. In addition to that, we will address the ability of the neurons to form synaptic connections using PSD95 (postsynaptic density marker) and Synapsin presynaptic markers.

-Task 2 - Define the effects of VPA on the maturation of the human neurons and organoid network activity, in particular the inhibitory system.

Even though it is well known that exposure to VPA leads to a shift in the E/I balance, the specific effects on the inhibitory system have not been addressed yet. Therefore, we will pioneer these experiments in brain organoids.

To dissect the alterations in neuronal membrane properties, synaptic communication and overall network activity we will perform electrophysiology recordings and calcium imaging in mature brain organoids of 4-months and 6-months of development. Neurons derived using brain organoids have been observed to be functional as they can maintain a physiological membrane potential, fire action potentials and integrate synaptic activity. In our lab, we have perfected the techniques to assess these levels in control brain organoids and our preliminary data indicate that at those time points, there is sufficient maturation to observe neuronal activity (Figure 3 and Figure 4).

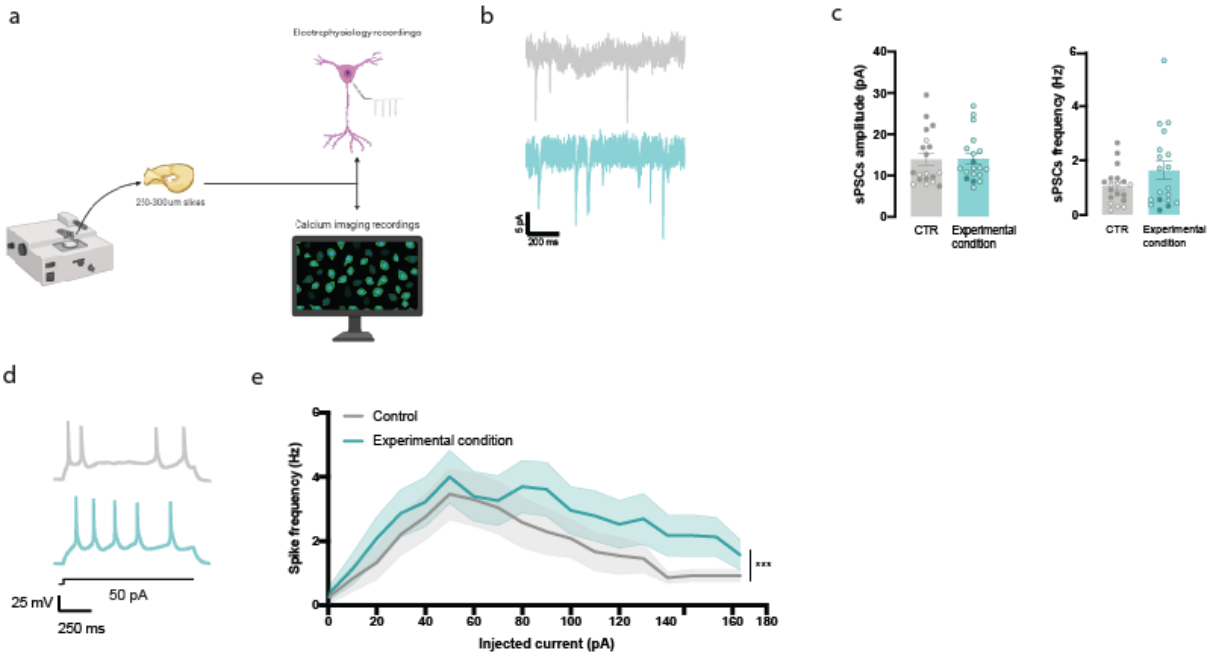


Figure 3. Example of recordings from whole-cell patch-clamp in human brain organoids. a) Slices from 4- and 6-months-old organoids are collected for physiological analysis. b) Representative traces of spontaneous postsynaptic currents (sPSCs) recorded from control and an experimental condition. c) Amplitude and frequency of spontaneous postsynaptic currents (sPSCs) from control and environmental condition. d) Representative traces of action potentials fired per current injected. e) Firing frequency of action potentials, in response to current injection.

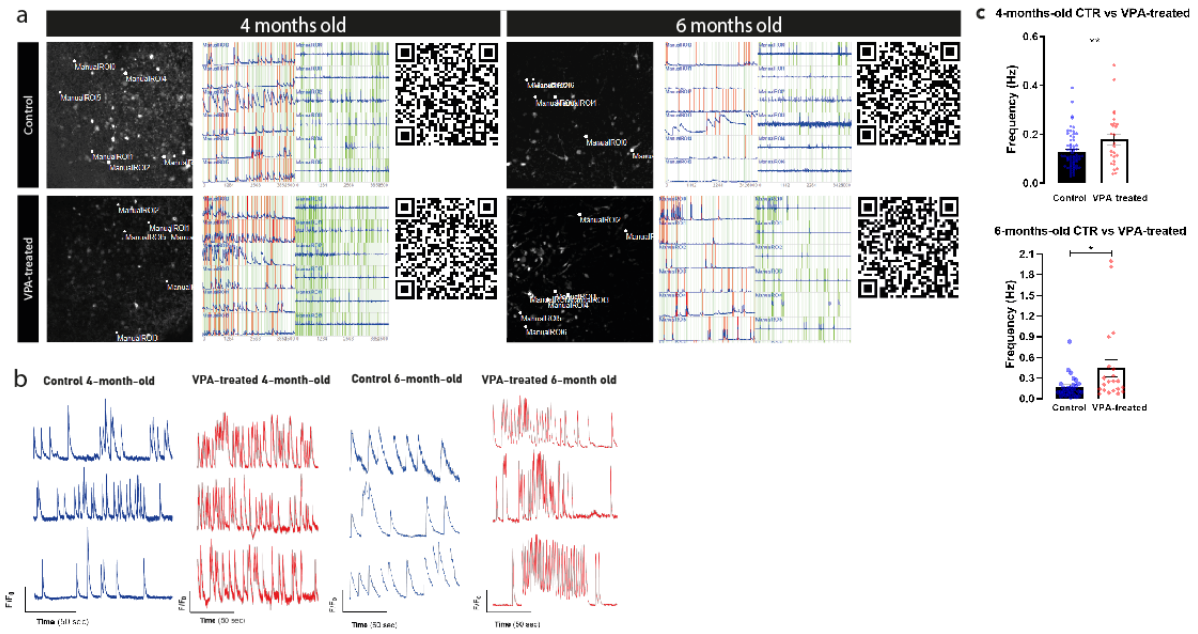


Figure 4. Calcium imaging preliminary data. a) Control and VPA-treated organoid slices labelled with Fluo-4-AM at 4 and 6 months. On the side Calcium imaging traces recorded from the respective ROIs detected by CALIMA. QR codes of the videos (60fps) from Control and VPA-treated available. b) Example of traces recorded from Control (blue trace) and VPA-treated (red trace) cells. c) Frequency comparison between Control and VPA-treated organoids (4 months analysis: one organoid per group, 65 ROIs for the control and 26 ROIs for VPA-treated; 6 months analysis: 4 organoids Control and 1 VPA-treated, respectively 24 and 21 ROIs, analysis ongoing) respectively p=0.0042 and p=0.0140, Mann Whitney test, two-tailed.

Using organoid slices and spinning-disk confocal microscopy we will assess network activity using fluorescent probes. Currently, we are using a calcium-sensitive probe, Fluo-4-AM, to observe the activity of the networks in control and VPA-treated organoids. Videos are recorded with a 20x objective and a 2x2 binning and 25ms of exposure to increase the sampling frequency at around 30hz. Recordings are processed using ImageJ with the following procedures: background subtraction, x and y movement correction and bleach correction. The traces acquired from the cells recorded are analyzed in Clampfit.

Our preliminary data, from 4- and 6-month-old organoid slices, suggests VPA-treated organoids show hyperactive network properties (Preliminary results in Figure 4a-b-c). Observations will be repeated with organoids derived from different hiPSCs lines and at later stages of development to assess if the difference is consistent.

Using whole-cell patch-clamp recordings in voltage-clamp and current-clamp mode, we will evaluate membrane properties (resting membrane potential, capacitance, membrane resistance, rheobase), action potential characteristics in single firing versus sustained firing mode (kinetics, duration), firing properties (adaptive vs non-adaptive firing; fast- or burst-spiking), as well as spontaneous postsynaptic excitatory and inhibitory activity (sEPSC and sIPSC). Our preliminary results show that 6-month old organoids are competent to fire action potentials in response to current injections (Figure 3). If robust transmission deficits are observed in VPA-treated neurons, we will apply pharmacological treatments to isolate the underlying channels potentially driving the alteration (for example, GABA-blockers or AMPAkines).

To comply with FAIR data principles, the raw data from neuronal traces and electrophysiological properties will be deposited in open-source archives and we will endeavour to publish only in gold open access journals.

To conclude, these two tasks will give us the possibility to highlight the effects of VPA on the development of brain organoids thereby modelling prenatal VPA exposure. By focusing on the molecular and physiological effects of this drug in the developing inhibitory system, we expect to shed light on the role of this system in neurodevelopmental disorders such as ASD.

Future steps and improvements

Together with the ongoing work, we are also planning the addition of a few improvements that will allow us to address the effects of VPA on molecular pathways and the inhibitory system generated by brain organoids:
As in Task 1, we are planning to analyze the expression levels of proteins and genes of interest using western blot and qRT-PCR, respectively, including vGLUT1, NMDA subunits, NR2a and NR2b, voltage-gated channels (NaV1.1 and Kv1.1) and GAD65 in 1-month, 4-months and 6-months old organoids. To identify the biological pathways that are affected upon VPA exposure, we will perform transcriptome analyses (RNA-seq) in collaboration with Dr Emily Osterweil at Edinburgh, a partner of the Syn2Psy Network. The candidate genes will be validated using qRT-PCR.

In parallel to the calcium imaging recordings in Task 2, we are optimizing the protocol involving the AAV-GCamp7 virus. The use of this virus will significantly increase the resolution of our calcium imaging recordings at the action potential level and will help us to distinguish the activity of neurons from the other cell types that are generated in the brain organoids.
Moreover, the introduction of chloride sensitive probes, such as Clomeleon, for chloride imaging recordings will allow us to focus on the role of the inhibitory system which seems to be the main target of VPA.

During neuronal recording experiments in Task 2, neurons will be filled with an internal solution containing biocytin to perform post hoc neuronal reconstruction of recorded cells using Neurolucida-assisted neurite tracing. The reconstructed neuron skeletons will be used to perform Sholl analysis, dendrite branch order analysis and convex hull analysis to assess if there is neuronal atrophy or, conversely, dendritic exuberance in a neuron from VPA-treated organoids. Neuronal morphology and electrophysiology parameters will then be cross-referenced to segregate cells into a profile of excitatory and inhibitory neurons according to their neuronal firing patterns, cell body morphology capacitance and action potential shape and kinetics (waveform analysis) and firing rate properties.

These further improvements will allow us to uncover the neurobiological properties that are associated with the development of ASD in the context of prenatal VPA exposure and with an unprecedented focus on the inhibitory system in human brain organoids.

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.

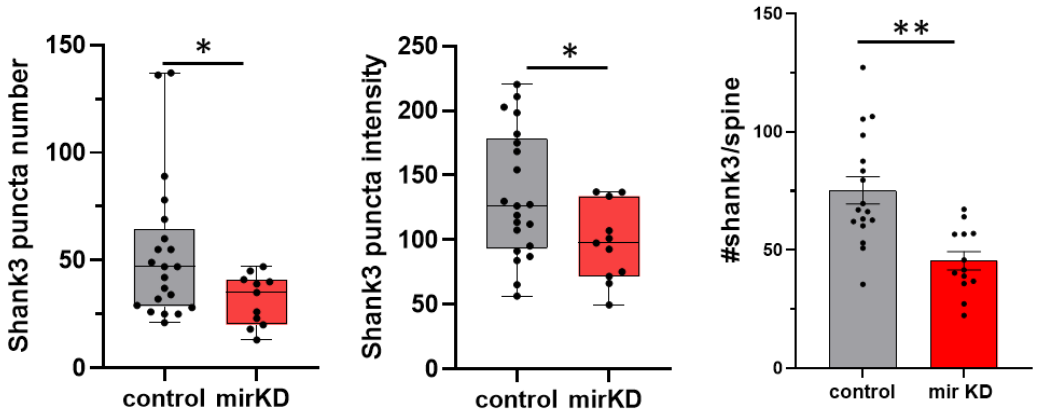


Figure 1. Effect of the shank3 shRNA expression on both spine density (right panel) and the intensity (middle panel) or the amount (right panel) of shank3.

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.

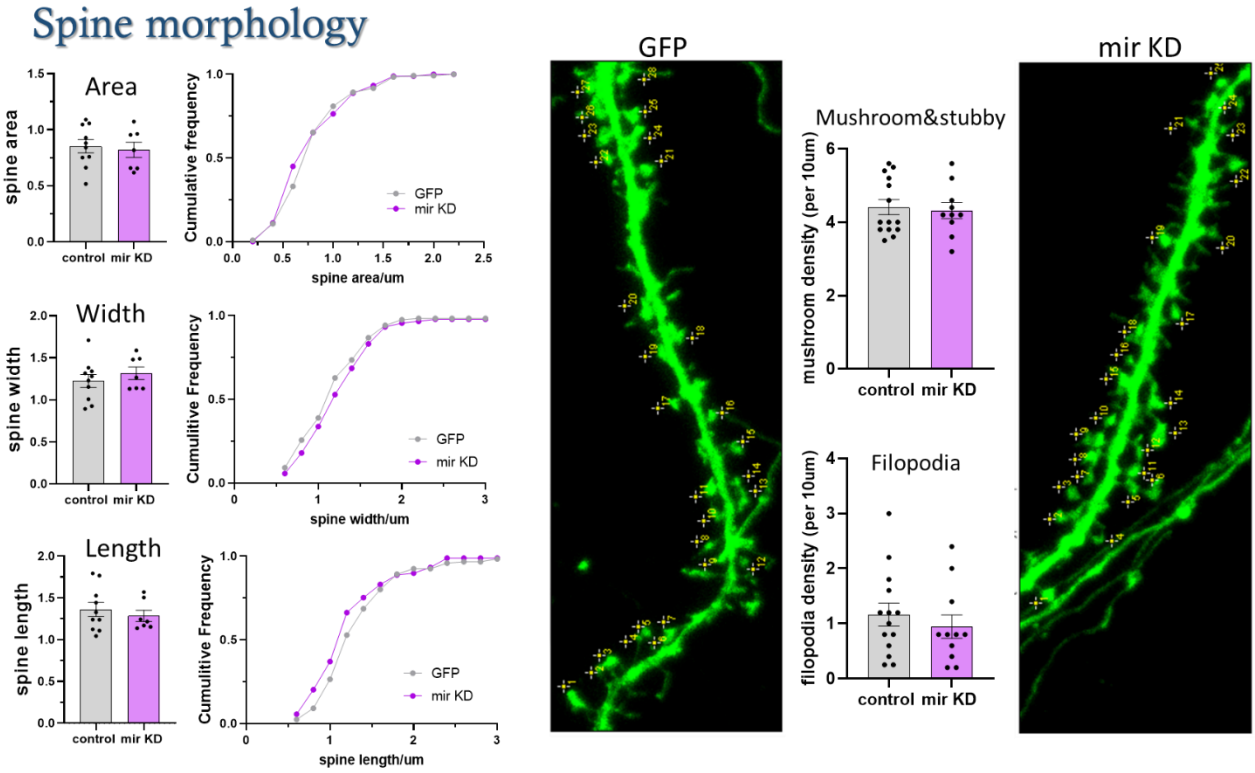


Figure 2: Determination of the spine morphology characteristic in control condition and when the shank3 shRNA is expressed. No significant differences are observed.

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.

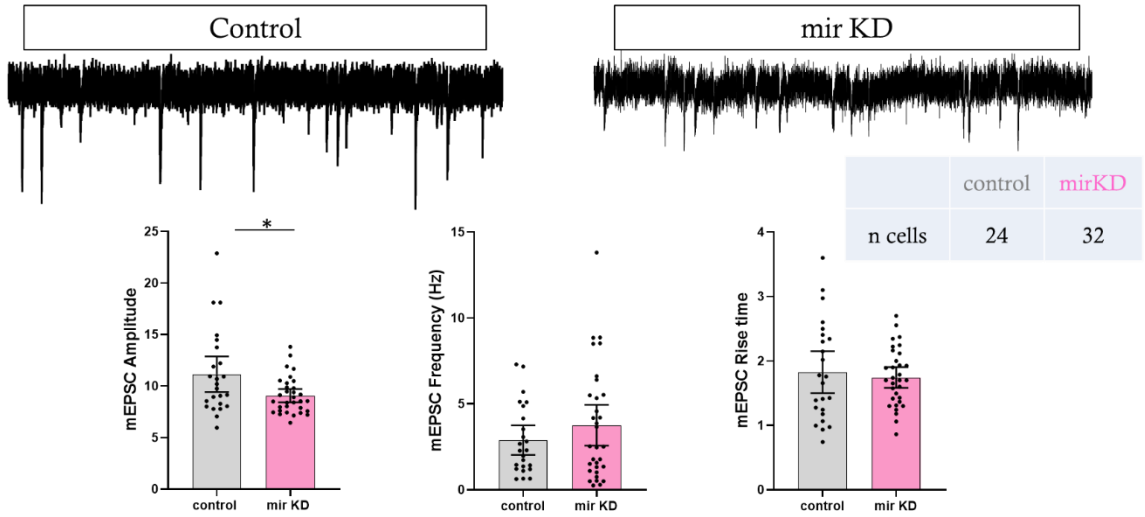


Figure 3: Shank3 shRNA expression decreases of 25% the amplitude of AMPAR dependent fast synaptic transmission currents (left panel) without affecting the frequency (middle panel) nor the rise time (right panel).

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.

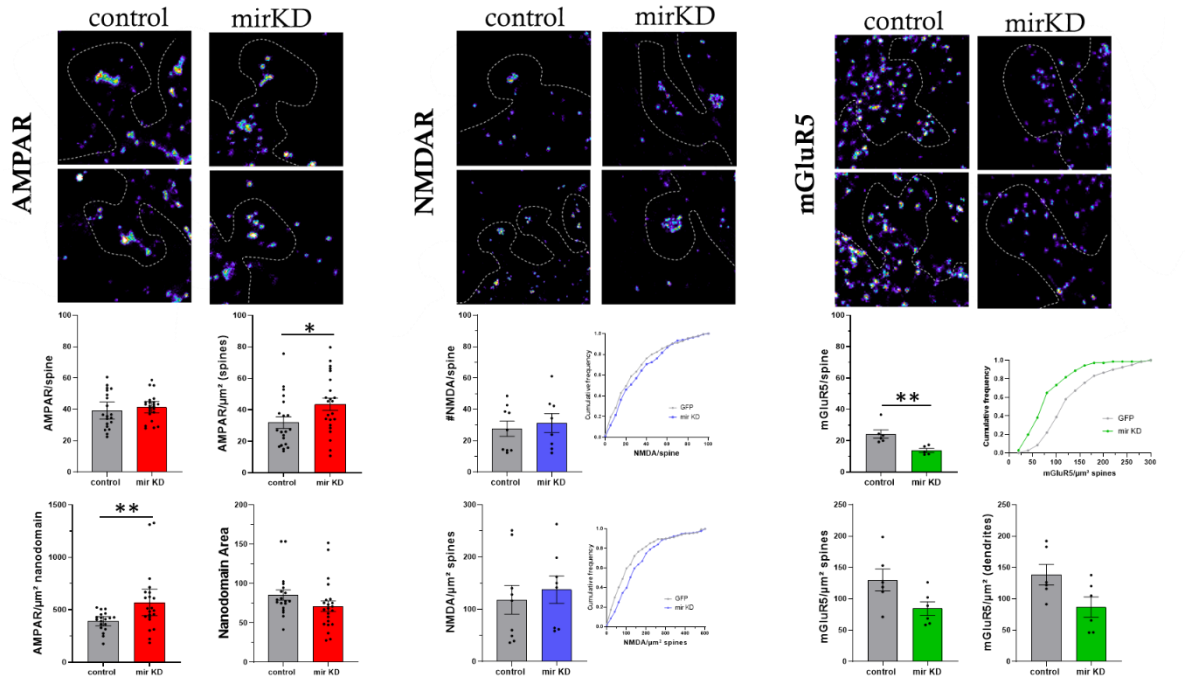


Figure 4: Single color d-STORM super-resolution experiments allow to decipher nanoscale organization and to determine the amount of both AMPAR (left panel), NMDAR (middle panel) and mGluR5 (right panel) at control and with shank3 shRNA expression. AMPAR amount per spine and inside nanodomain is increased, 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 – Mesoscopic monitoring and analysis of cortical excitation/inhibition balance in an animal model of synaptic dysfunction (title prior to the change in supervision)

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

Early Stage Researcher 5 – Marcos Sintes

Supervisors – Thomas Knöpfel (former) and Vincenzo De Paola (current)

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

The main aims of this project, as originally conceived, were (1) the characterisation of dysfunctional cortical excitation and inhibition (E/I) balance in a mouse model of ASD, using mesoscopic optical voltage imaging of cortical areas expressing cell-specific voltage sensitive fluorescent proteins (VSFPs), and (2) exploration of 5HT neuromodulatory effects on cortical activity using voltage imaging and 5HT2AR agonists and antagonists in the ASD model.

To achieve this, two main tasks were described:

- Task 5.1: *In vivo* monitoring of stimulus-free spontaneous activity and stimulus-evoked sensory processing of large cortical areas of control and ASD model mice, using mesoscopic voltage imaging on mice previously assessed individually for ASD-like behavioural traits.
- Task 5.2: Application of 5HT2AR agonist and antagonist drugs to the ASD model and characterisation of the effects on E/I balance using voltage imaging cortical activity monitoring.

The experiments I carried out were focused on developing the necessary experimental requirements for carrying out the two tasks:

- Generation of an ASD mouse model and an experimental approach for characterising ASD features in it: a drug-induced model for ASD, using mice prenatally treated with valproic acid, was selected as the main candidate for this project instead of the genetically modified one initially proposed due to practical and theoretical reasons. Valproic acid (VPA), a frequently prescribed antiepileptic drug, has been shown to cause developmental abnormalities in human children when given to pregnant women, including ASD symptomatology (Bromley *et al.*, 2008). Injection of VPA in pregnant female mice has been extensively described in the literature as a way of generating ASD-like features in the offspring. We generated mice treated prenatally with valproic acid (VPA), a well-established and extensively described model for ASD in the literature (Nicolini and Fahnestock, 2018). We treated female mice with VPA at a specific timepoint during pregnancy

and assessed the offspring at 8 weeks of age using behavioural experiments to characterise ASD-related phenotypical features.

To assess social behaviours, we used the three-chamber experimental approach, commonly used for detecting social interaction deficits in rodents (Nadler *et al*., 2004). Two tests, a sociability test and a social novelty test, are carried out sequentially. In the sociability test in this experiment, a subject mouse is placed in the centre of an arena consisting of three contiguous equally sized chambers. One of the lateral chambers contains an enclosure with a stranger mouse and the other lateral chamber contains an empty enclosure. The subject mouse can choose to move freely through the arena and interact with either enclosure for 10 minutes. Mice with normal sociability will usually prefer to spend time in the chamber containing the stranger mouse, while a mouse with ASD-like features may not show a preference for either chamber. In the social novelty test, a second stranger mouse is added to the empty enclosure, and the subject mouse is allowed again to interact with either mouse for 10 minutes. Mice with normal social memory will recognise the recently met mouse and will prefer to spend time with the new one. To quantify sociability and social memory both the time the subject mouse spent in each chamber and the time of direct nose-to-nose interaction between the subject and each stranger mouse were measured.

A set of preliminary experiments using a control cohort was carried out to evaluate the usability of the three-chamber test, which allowed to validate the test and make necessary modifications to the experimental design. A second cohort, made up of both control and VPA-treated mice, was then tested to compare potential deficits in sociability and social memory in the ASD model.

- Characterisation of the effects of treatment with psilocybin in the VPA mouse model of ASD: as the serotonergic system has been shown to play an important role in the pathophysiology of ASD (Muller *et al*., 2012), serotonergic modulators have been proposed as possible candidates for a drug-based therapeutic approach (Reddihough *et al*., 2019). As a collaboration with my co-supervisor as part of her postdoctoral project, along the animals from the second cohort just described a set of VPA and control animals were treated with the psychedelic drug psilocybin and tested using the same behavioural experimental approach. This drug, which acts mainly as a 5HT2A receptor agonist, has been described in the literature as a possible treatment of a variety of neuropsychiatric disorders (Rucker *et al.*, 2018, Carhart-Harris *et al*., 2020). My co-supervisor planned the experiments, generated the VPA treated mice and at 8 weeks of age injected either 1mg/kg or 3mg/kg psilocybin intraperitoneally and counted the head-twitch events after acute administration of the drug, which has been shown to be directly related to the effect of psychedelic compounds. Both my co-supervisor and I habituated the animals to the experimental arena and carried out the three-chamber experiments one day after the psilocybin injection. My co-supervisor manually analysed the direct nose-to-nose interactions while I performed the software-based analysis of the travel distance, the time in each chamber and time of approach of the test mice to the enclosures.
  The results of these experiments were made public as a BioRxiv preprint in September 2020: https://doi.org/10.1101/2020.09.09.289348 (Irene *et al*., 2020).

Afterwards I continued the psilocybin experiments, focusing on further characterising the effects of the drug by testing the animals before and after the treatment. VPA mice were generated by injection of two separate doses of VPA at different timepoints during pregnancy, as this could potentially create a stronger ASD-like phenotype in comparison to the previously used single injection. Control and VPA mice were tested for sociability and social memory. A week after, mice were treated with either 1mg/kg psilocybin or saline and the acute effects were measured by counting the number of head-twitch events. The day after, and the two following weeks, the social behaviour tests were repeated. For these tests, I generated the VPA mice, treated the animals and performed all the behavioural tests and analyses.

After switching to Vincenzo de Paola's supervision, my project was adapted to target some of the original scientific questions of the project from a new perspective that will allow to use the tools that this group has developed. The main aims of the project will be the characterisation of the abnormalities in the regulation of synaptic structural plasticity and network activity in ASD and the exploration of the therapeutic potential of psychedelic 5HT2AR agonists in such context. As genetically modified and drug-induced mouse models do not represent some of the characteristics of human idiopathic ASD, which is found in a majority of patients, IPSCs derived from human donors with ASD will be implanted into the mouse cortex to generate a cortical graft. Since starting this project, I have been mainly planning, preparing future experiments, and receiving the necessary training.

Published research about the potential of psychedelics to treat ASD is for the moment very limited. Experiments carried out in the sixties before psychedelic drugs were banned in most countries (Mogar *et al*., 1969), clinical trials with MDMA (an empathogen amphetamine derivate with some psychedelic properties) (Danforth *et al*., 2018) and anecdotal reports from individuals provide an optimistic perspective for the therapeutic potential of these compounds. The results obtained during my previous project provide a first account on the effects of psilocybin in an ASD mouse model, and, in this new project, will be useful for the experimental design and as complementary results. Our future experiments will help to expand our knowledge about the neuronal plasticity mechanisms that could play a role in the positive effects of psychedelics in the treatment of ASD.

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ESR6 – Daniel Hunter

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

In the current project we have aimed to elucidate the effects of NMDAR-encephalitic patient-derived autoantibodies on network scale synaptic transmission. Since the first description of anti-NMDA receptor encephalitis (NMDARE) in 2007, a significant volume of literature has been published, unravelling the mechanisms by which these pathogenic autoantibodies disrupt NMDAR surface dynamics and the surface expression of receptors on excitatory (principal) cells. However, the impact of this NMDAR loss on network scale neurotransmission is currently an open question. This is a particularly promising avenue of study given that the primary hallmarks of the disease, at the phenotypic level, are psychosis and seizure. Both of these phenotypes are thought to be induced by an alteration in excitation-inhibition balance in brain-wide networks.

We have employed whole-cell voltage-clamp electrophysiology to determine the impact of NMDAR-Abs on excitation and inhibition in an *in vitro* hippocampal system. We identified a significant reduction in AMPA-mediated spontaneous excitatory postsynaptic current (sEPSC) peak amplitudes, alongside a higher magnitude reduction in the peak amplitudes of GABAR-mediated spontaneous inhibitory postsynaptic currents (sIPSC). Interestingly, this reduction in peak amplitude, in both cases, was potentially offset by an increase in the frequency of both sEPSC and sIPSC events. The identification that NMDAR-Abs induce deficits in non-NMDAR mediated synaptic signalling is an intriguing finding, and demonstrated the necessity to further expand the current disease model to incorporate, not only network level disturbance, but also downstream impacts on the function of other major ion channels responsibly for the vast majority of fast excitatory and inhibitory signalling in the brain.

To further investigate whether these alterations in sEPSC and sIPSC could be explained by some indirect inflammatory processes, elicited by autoantibody exposure, we quantified the levels of Iba1 and GFAP, microglial and astrocytic intracellular proteins that are known to be upregulated in response to inflammatory processes and immune cell activation. We found that the levels of these proteins does not differ between cultures exposed to NMDAR-Abs or no-antibody controls. Hence, we assume that the regulatory mechanisms that provoke reduction in AMPA- and GABA-mediated currents are independent of complex immune-responses. Therefore, we shifted our focus onto structural features of excitatory and inhibitory synaptic scaffolds, and the synaptic organisation of both AMPA- and GABARs. Employing immunocytochemistry on dissociated hippocampal cultures transfected with fluorescently-tagged homer1c (a principal synaptic scaffold of glutamatergic synapses) and gephyrin (the predominant synaptic scaffold of inhibitory synapses), we

identified that while the size and density of excitatory synapses remains unaltered, there is a significant reduction on both the size and density of gephyrin puncta in cells exposed to NMDAR-Abs. Further, while global AMPAR organisation appears intact, exposure to NMADR-Abs shows a moderate but significant disruption of AMPAR localisation to the excitatory synapse, offering an explanation for the reduced sEPSC current amplitude, previously observed. Similarly, while overall GABAR organisation is moderately impacted by NMDAR-Ab exposure, observing a moderate reduction in cluster density; synaptic GABAR populations are strikingly disorganised, displaying a potent reduction in both size and density. Together, these results show consistently that NMDARs are not the only synaptic proteins impacted by exposure to patient autoantibodies and demonstrate that downstream impacts of NMDAR internalisation may also contribute to disease etiology. Increasing our understanding of the plethora of downstream pathogenic impacts of NMDAR-Ab induced synaptic disorganisation is vital to build a more robust disease model. Moreover, understanding the finer complexities of these network scale disorganisations, particularly those which alter the excitation-inhibition balance will be instrumental in determining the underpinning pathogenicity of NMDAR-Abs in disease states, and may provide novel avenues for therapeutic intervention.

Progress

Anti-NMDA receptor encephalitis (NMDARE) is a rare neurological condition diagnosed by the identification of production and/or circulation of patient-generated IgG autoantibodies directed against NMDARs (NMDAR-Abs). Encephalitic patients experience a complex and progressive display of symptoms, generally encompassing psychiatric syndromes comorbid with neurological complications. Typically, psychiatric presentation of NMDARE is remarkably comparable to non-organic psychotic disorders, including schizophrenia and bipolar disorder. In fact, this resemblance to the diverse array of symptoms present in these conditions can commonly lead to misdiagnosis of NMDARE. However, acutely following presentation, NMDARE progresses to neurological complications, including pronounced dyskinesia and heterogonous seizure phenotypes. Strikingly, these two hallmark characteristics of disease progression: psychosis and seizure, are consistent with a dysregulation of excitation-inhibition (E/I) balance in the brain, yet this avenue of disease pathogenesis remains unexploited.

Since the first published characterisation of NMDARE in 2007, a great deal of work has provided significant insight into the pathogenic mechanisms of patient-derived autoantibodies. Namely, the increased synaptic displacement of antibody-bound NMDARs, via disruptions in extracellular interactions with anchoring partners such as ephrinB2 receptors. This synaptic displacement is followed by pathological receptor stabilisation at extrasynaptic sites, mediated by the crosslinking capability of IgG. Subsequently, the antibody induced internalisation of stabilised receptor-antibody complexes results in an overall surface loss of NMDARs from the neuronal membrane. While these insights have provided a solid base to understand the pathogenesis of NMADRE, these investigations have focussed primarily on cell-autonomous mechanisms of pathogenesis, with little focus on the network level interaction of these alterations in cell surface receptor mobility and expression. Intriguingly, the lack of investigations into network scale disruptive action of anti-NMDAR autoantibodies has limited our current understanding of disease etiology to their action solely on excitatory (principal) cells. In this project we aim to characterise network scale disruption, encompassing both excitatory and inhibitory network dysfunctions, induced by patient derived monoclonal antibodies; with the overarching objective of developing a more robust model of disease pathogenesis that may provide alternative therapeutic strategies in the future, alongside a clearer depiction of autoantibody action as it relates to patient phenotypes.

To investigate E/I balance and network scale alterations elicited by NMDAR-Abs we performed whole cell voltage clamp experiments on rodent hippocampal slice cultures, exposed for 16-24 hours to NMDAR-Abs or

a non-reactive monoclonal human IgG (Control-Ab), to quantify the relative frequency and amplitudes of spontaneous excitatory and inhibitory postsynaptic currents (sEPSC and sIPSC, respectively) onto principal calls in the CA1 subfield. This organotypic culture system was chosen due to the relative maintenance of network architecture in hippocampal structures, with the added benefit of rendering the slices easy to manipulate during the experimental application of monoclonal antibodies. Cultures were prepared from Sprague-Dawley rat pups at postnatal day 5, and maintained in vitro for 12-15 days. The day before recording, slices were incubated with either Control- or NMDAR-Abs by placing a 10µl droplet of antibody supplemented culture media (0.5µg/ml) directly on top of the slice. Slices were taken the following day for whole-cell recording, transferred to a 32°C bath filled with aCSF of the following composition: 126mM NaCl, 3.5mM KCl, 2mM $CaCl_2$, 1.3mM $MgCl_2$, 1.2mM NaH_2PO_4, 25mM $NaHCO_3$ and 12.1mM glucose. Glass capillary micropipettes were pulled to have a resistance of 4.5-6.5 MΩ when placed into the recording bath and filled with intracellular solution composed of: 130mM $CsMeSO_4$, 20mM HEPES, 2mM NaCl, 0.2mM EGTA, 5mM QX-314·Cl, 4mM Na_2-ATP and 0.4mM Na-GTP. Series resistance (R_S) was 15.5 ± 1.0 MΩ, and not compensated for. Recordings were excluded from analysis if R_S was greater than 30 MΩ or fluctuated by more than 20% during 20-minute recording intervals. To obtain sEPSC recordings, cells were voltage-clamped at -70mV for 10 minutes, in each case sIPSC recordings were subsequently obtained by clamping at 0mV.

The peak amplitudes of AMPA-mediated sEPSC recordings of slice cultures exposed to NMDAR-Abs (M = -60.58pA, SD = 13.97pA), compared to control-antibody treated cultures (M = -90.86pA, SD = 27.03pA), were found to be significantly reduced ($t(16) = 2.99$, $p = 0.0087$). However there was no change observed in the rise tau ($t(17) = 1.01$, $p = 0.3265$), or decay tau ($t(17) = 1.3$, $p = 0.2078$) of the events. Together, this indicates a non-subunit specific loss of AMPA receptors from synaptic compartments, resulting from exposure to NMDAR-Abs. Similarly, GABA-mediated sIPSC in slices treated with NMDAR-Abs (M = 113.8pA, SD = 92.4pA), compared to control-Ab treated cultures (M = 240.6pA, SD = 73.5pA), showed a significant reduction in peak amplitude ($t(17) = 3.29$, $p = 0.0044$), with no significant change in either rise or decay kinetics.

We further investigated the alterations in synaptic signalling, by analysing the frequency of recorded excitatory and inhibitory events. We uncovered a significant increase in the frequency of both excitatory ($t(17) = 2.60$, $p = 0.0186$) and inhibitory events ($t(17) = 3.83$, $p = 0.0013$). The increase in excitatory drive is not surprising given the epileptogenesis associated with NMDARE, however, the increase in inhibitory drive requires further investigation. We hypothesise that this increase inhibitory event frequency may reflect a homeostatic regulation by the network, to compensate for both the increased frequency of excitatory events and reduced peak amplitude of inhibitory currents, but this remains an open question.

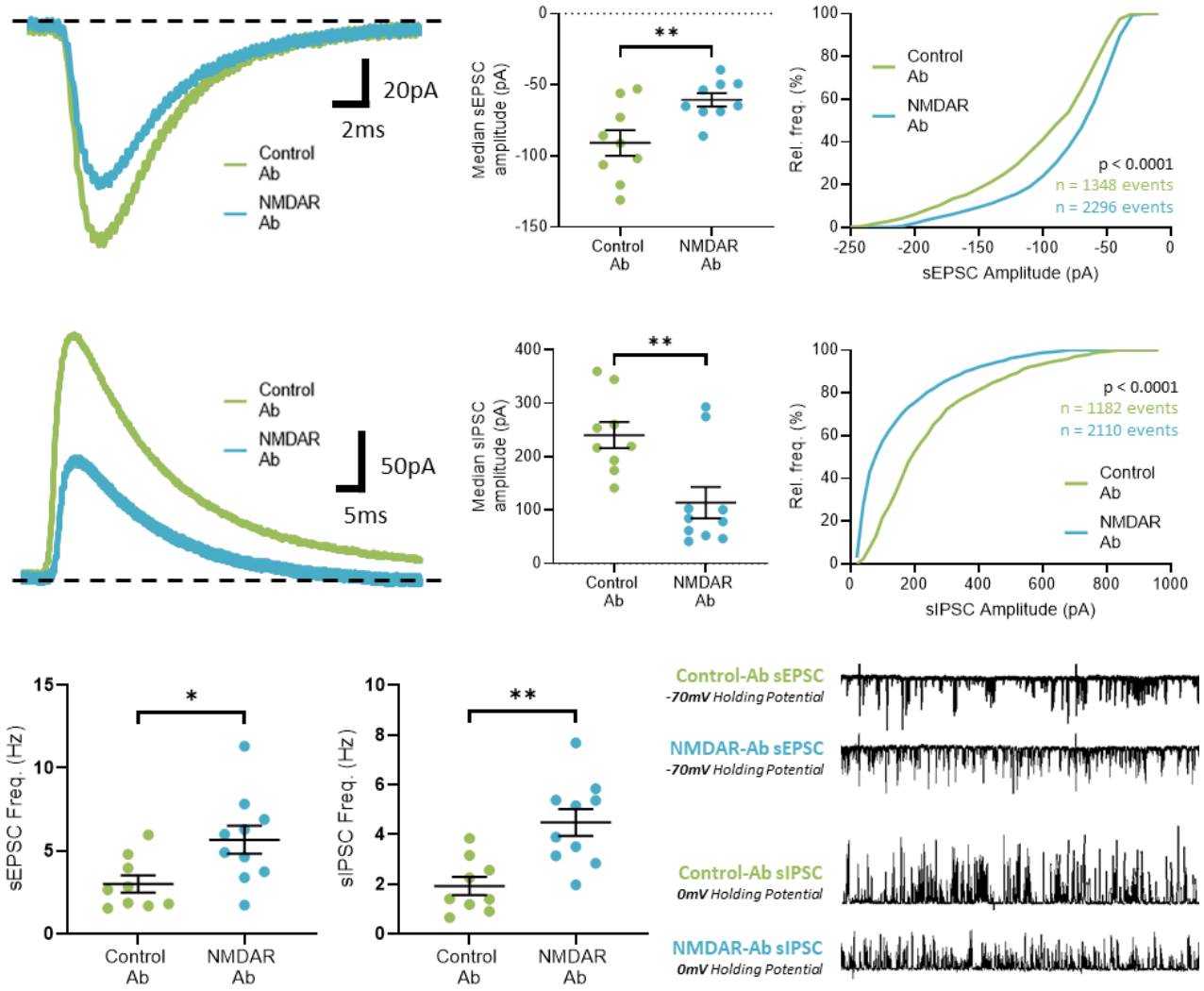


Figure 1. Top left shows example average traces of sEPSC and sIPSC from individual CA1 pyramidal hippocampal neurons, centre, median peak amplitudes and right, cumulative frequency distributions of all spontaneous events pooled. Bottom left, shows sEPSC and sIPSC event frequency and right, example traces. Error bars represent the mean +/- SEM.

Since NMDARE is autoimmune syndrome, and given that such autoimmune activation is often associated with auxiliary immune responses, including micro-inflammatory responses upregulating the activation of local microglia and astrocytes; we aimed to test if NMDAR-Abs alone may induce such processes. There is evidence to suggest that low level chronic inflammation can disrupt network homeostasis and differentially impact excitatory and inhibitory drive. Hence, we wanted to explore this avenue.

Organotypic hippocampal slice cultures were prepared as for the previous electrophysiology work, treated with either NMDAR-Abs or antibody-free culture media. This control condition was selected to identify whether the application of human IgG, regardless of reactivity with the tissue, was sufficient to induce immune-mediated activation of local microglial or astrocytic cells. Activation of these cell types was investigated by immunostaining for Iba1 or GFAP, cytoplasmic proteins of microglia and astrocytes, respectively. Slices were counterstained with a mounting media solution containing DAPI. To quantify the level of protein expression in cultures slices, 20µm thick z-stacks were taken from the CA1 hippocampal subfield and maximum intensity projections were generated. A uniform threshold was applied across all projection images to identify the relative ratio of Iba1+ or GFAP+ pixels per stack projection, with the expectation that an increased activation of microglia or astrocytes will result in an increased expression of these intracellular

proteins, and thus an increased positive-pixel ratio. We found that control and NMDAR-Ab treated sliced demonstrate equivalent levels of Iba1 and GFAP protein expression ($t(24) = 0.04$, $p = 0.966$ and $t(21) = 0.91$, $p = 0.375$, respectively), suggesting no detectable upregulation of micro-inflammatory processes, at least at the scale of microglial or astrocytic activation. This suggests that the disruptions observed in spontaneous excitatory and inhibitory synaptic signalling are not mediated by indirect immune response-dependant mechanisms.

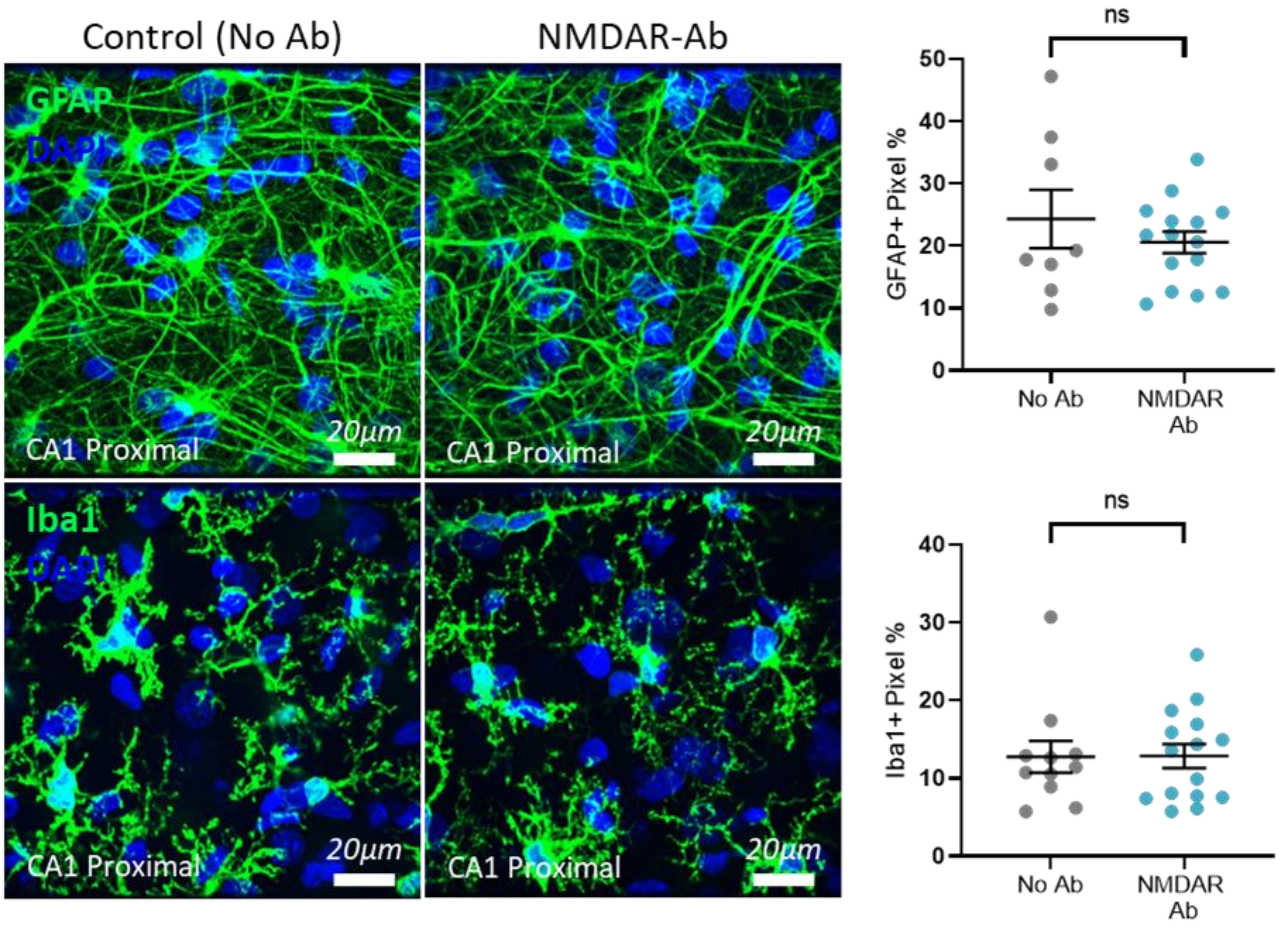


Figure 2. Left panel shows example max intensity stack projections of GFAP (top) and Iba1 (bottom) staining in pyramidal cell layer-proximal regions of CA1 hippocampus. Cultures were treated with medium alone (left) or NMDAR-Abs (right). Right shows the GFAP+ or Iba1+ pixel positive ratio (top and bottom, respectively). Error bars represent the mean +/- SEM.

Given that immune-mediated action is not likely to play a confounding role on either excitatory or inhibitory signalling, we decided to further investigate the synaptic organisation of principal ion channels regulating the sEPSC and sIPSC events, previously described. To explore this organisation, we employed dissociated cell cultures, as the imaging of individual synapses is complex in intact tissue preparations, and available transfection systems are limited for efficient expression of transgenes in organotypic cultures.

Dissociated hippocampal cultures were prepared from Sprague-Dawley rats, at embryonic day 16 and maintained at 37°C, 5% CO_2. Coverslips were transfected by a standard calcium-phosphate protocol with either a Venus-tagged gephyrin and SEP-tagged gamma 2 subunit of the $GABA_AR$ or dsRed-tagged homer1c and SEP-tagged GluA1 subunit of the AMPAR. This allowed us to visualise the inhibitory and excitatory synapses in transfected cells, co-expressing tagged receptor subunits. At DIV11-12 cultures were incubated with either Control-Ab or NMDAR-Ab at 0.5µg/ml overnight before live staining of surface receptors and fixation. Due to

the incompatible visualisation of the fluorophore combinations (GFP SEP and YFP Venus) we performed live immunolabelling of the cultures, using the extracellular SEP-tag on GABA- and AMPARs to specifically mark surface expressed receptors. Subsequently, cultures were fixed and mounted with a DAPI containing fixative. Intracellularly expressed synaptic markers were not stained for as they could be effectively visualised by excitation of the tagged fluorophores. The images were analysed using a custom build macro which allowed us to quantify the cluster area and density of synaptic marker/scaffolds, homer and gephyrin, the total surface-expressed AMPA and GABAR population, and the synaptic AMPA and GABAR population (identified by colocalisation between the two signals).

We identified, congruent with previous published reports, that NMDAR-Abs do not demonstrate any impact on the cluster size or density of excitatory synaptic scaffolds. We also did not observe any global changes in the surface expression of total (synaptic and extrasynaptic) AMPAR populations, as cluster size and density was unaffected. However, analysis of the synaptic AMPAR population revealed a significant reduction in cluster size ($t(65) = 2.21$, $p = 0.0307$) with no impact on density, suggesting that while AMPAR clusters are not changed in size overall, the extent of overlap with synaptic markers is reduced; indicating a displacement of receptors from the synaptic compartments. Encouragingly, this finding is consistent with the moderate reduction we previously observed in AMPA-mediated sEPSC amplitudes. Further, contrary to our analysis of excitatory synaptic structures, we found that inhibitory synaptic scaffold gephyrin is markedly disrupted by the application of NMDAR-Abs. Demonstrating a significant reduction in size of inhibitory synapses ($t(80) = 4.03$, $p = 0.0001$) and a striking reduction in density ($t(80) = 3.63$, $p = 0.0005$). Interestingly, the total GABAR displayed only a modest disruption in organisation, where we observed a marginally significant and moderate reduction in cluster density ($t(80) = 1.99$, $p = 0.0495$) and no impact on cluster size. However, as expected the synaptic GABAR population was potently impacted, demonstrating a clear reduction in cluster size ($t(80) = 2.34$, $p = 0.0220$) and highly reduced density ($t(80) = 4.88$, $p \leq 0.0001$). Taken together these results recapitulate the findings described in the electrophysiology experiments, as we observe a clear disruption in synaptic GABAR populations, however - contrary to excitatory synapses - this disruption of inhibitory signalling appears to be driven by a gephyrin dependant dysregulation of synaptic scaffolds.

Future experiments will aim to further elucidate the mechanisms by which NMDAR-Abs induce the synaptic loss of non-NMDAR channels. Specifically, we will explore the role of gephyrin phosphorylation on scaffold destabilisation and degradation, utilising current knowledge regarding putative phosphorylation sites S268 and S270, known to regulate gephyrin cluster size and density, respectively. If indeed gephyrin is differentially phosphorylated after exposure to NMDAR-Abs, then we might hope to identify the responsible kinases promoting this phosphorylation and their relationship to NMDAR surface loss. An alternative explanation for the data we have obtained is that surface loss of NMDARs on inhibitory interneurons, alters E/I balance in favour of hyperexcitation, the result of this shift will promote asynchronous GABA release from interneurons, as such a mechanism has been demonstrated to instate homeostasis to hyper-excitable networks. The disruption of AMPAR synaptic localisation is also an intriguing avenue that remains to be further explored. We hypothesis that there may be some compensatory loss of AMPARs from the synapse to maintain a typical NMDAR-AMPAR ratio. It is also plausible that some intracellular mechanisms that are required to allow the internalisation of NMDARs also promotes destabilisation of glutamatergic synaptic proteins. These changes may not be observable at this scale of imaging investigations but it may be possible that super-resolution microscopy techniques (for example uPAINT or dSTORM) will unveil disorganisation of the glutamatergic synaptic scaffolds at the subsynaptic level.

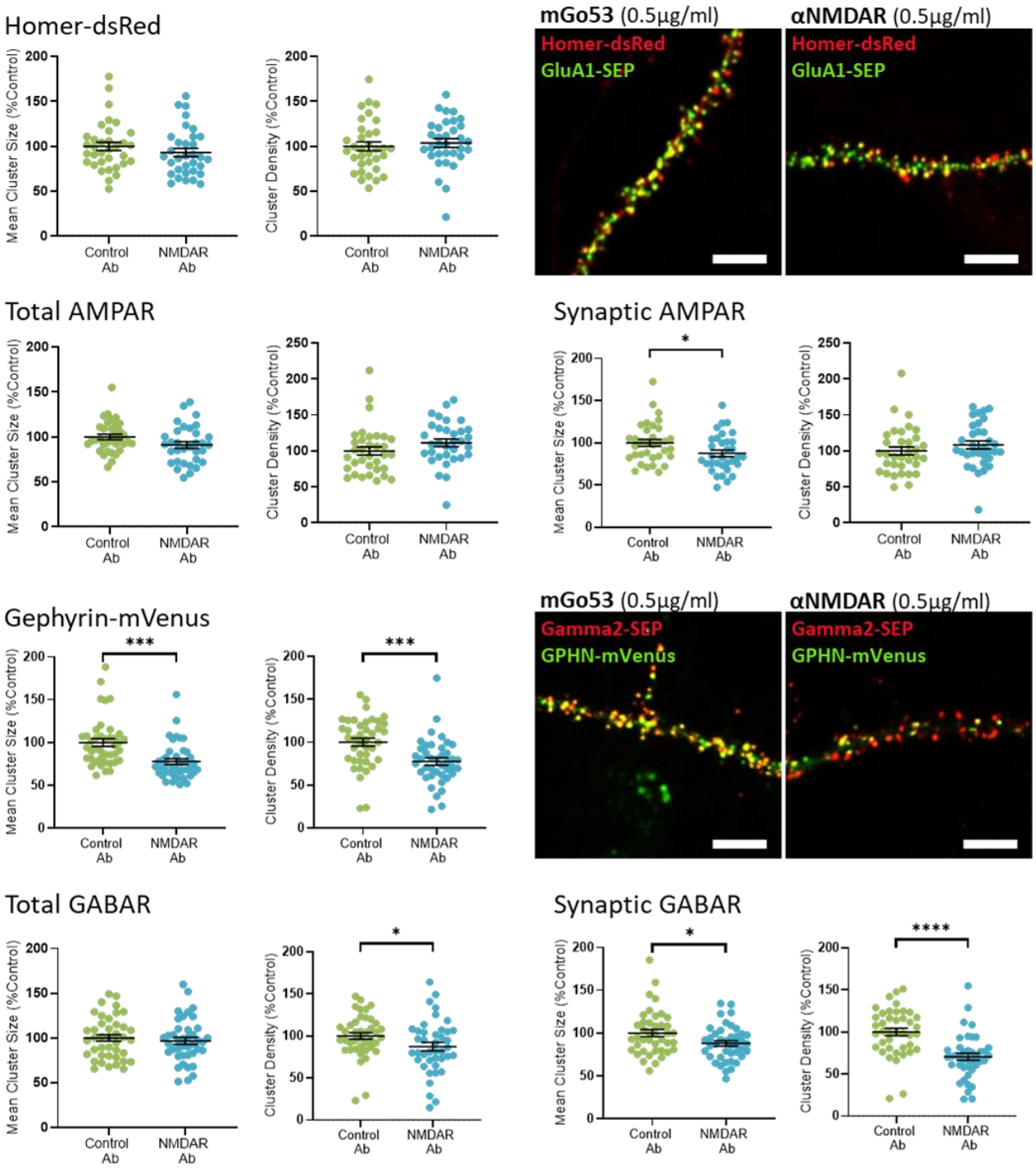


Figure 3. Top panel shows the impacts of NMDAR-Abs at excitatory synapses, with the mean cluster size and mean cluster density of excitatory synaptic scaffolding protein, homer1c, total surface expressed GluA1-containing AMPARs and the colocalised (synaptic) AMPAR population. Error bars represent the mean +/- SEM. Top right immunostaining shows example images of GluA1-AMPAR and Homer1c expression. Lower panel shows these same parameters at inhibitory synapses, with scaffolding protein, gephyrin, total surface expressed gamma2-containing GABAaRs and the colocalised (synaptic) GABAaR population.

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 related to transcriptional silencing of the *Fmr1* gene. This gene encodes for Fragile Mental Retardation Protein (FMRP), an RNA binding protein that regulates the transport and translation of many mRNAs that encode for proteins necessary for a proper synaptic function.

To mimic what happens in this disease, Fmr1 expression was knocked down (KD) using two different sets of short hairpin RNAs (shRNAs) in hippocampal neurons. It was observed that Fmr1 KD was sufficient to increase the synaptic content of GluN2A and GluN2B-containing NMDA receptors, and to impair BDNF-induced synaptic clustering of such receptors.

The efficacy of the above mentioned shRNAs is currently being tested in axons as well, in order to study how the deficiency in FMRP affects presynaptic differentiation. Moreover, a protocol for autaptic cultures has been established, which will allow studying the alterations in the dynamics of synaptic vesicle exocytosis and recycling in Fmr1 KO neurons.

State of the art

Fragile X Syndrome (FXS) is the most common inherited form of intellectual disability (ID), with a prevalence of 1:4000 males and 1:8000 females (Hagerman et al., 2017). It was first described by Martin and Bell in 1943 as an X-linked form of mental retardation (Martin and Bell, 1943), and then related to the presence of a constriction on the long arm of the X chromosome, also known as “Fragile site”, in which *Fmr1* resides (Berger et al., 1985).

This syndrome is characterized by a high incidence of autism. In fact, 90% of FXS patients display some kind of atypical behaviour characteristic of autism spectrum disorder (ASD) (Hernandez et al., 2009). Despite the high variability in symptoms, a group of “core” behaviours has been described, including avoidance of the eye gaze, attention deficit/hyperactivity disorder (ADHD), hand flapping, repetitive behaviours, reduced sociability, anxiety, and speech perseveration. Furthermore, FXS patients may exhibit aggressive behaviours, sleep disturbances, seizures, impaired language and emotional lability (listed in Bagni and Zukin, 2019).

FXS is related to an excessive expansion of a CGG triplet in the 5' untranslated region (UTR) of the *Fmr1* gene, that is indeed located in the abovementioned fragile site (Oberle et al., 1991). Normally, healthy individuals carry between 6 and 54 CGG triplets in the *Fmr1* 5'UTR, granting a correct transcription of the *Fmr1* gene followed by synthesis of the FMRP protein. It may happen that the number of CGG trinucleotide rises from 55 to 200, a condition called premutation (PM). The PM alleles are unstable and the number of repetitions tend to expand upon maternal transmission: more than 200 repetitions are considered to be a full mutation (FM) (Bardoni et al., 2000). The FM triggers DNA hypermethylation and histone deacetylation events, resulting in heterochromatinization and transcriptional silencing of the *Fmr1* locus, with a consequent loss of FMRP (Coffee et al., 2002; Ludwig et al., 2014; Pieretti et al., 1991). Rare *Fmr1* genetic mutations affecting the coding sequence, and thus causing FXS, have also been reported (Sitzmann et al., 2018).

FMRP is a highly conserved, 80 kDa protein that is encoded by the X-located gene *Fmr1*. This gene is diffusively expressed in mice, starting from embryonic day 10 (E10), but after this developmental stage *Fmr1* expression becomes sensitively restricted to few organs, namely the adult brain, ovaries, testes, thymus, oesophagus and spleen. Within the brain, FMRP is enriched in the murine granular layer of the hippocampus and in the cerebellum, while low expression levels are found within the cerebral cortex, thalamus and brainstem. Moreover, FMRP immunoreactivity has been found within the soma, in developing and mature axons and dendrites, and at the base of dendritic spines in cultured hippocampal neurons (Antar et al., 2005, 2006; Parvin et al., 2019).

FMRP is an RNA binding protein (Ashley et al., 1993) that recognizes and binds approximately 4% of the transcripts in the murine brain (Brown et al., 2001). It is canonically considered to be involved in translation, transport and stability of mRNAs (Bagni and Zukin, 2019). Among FMRP targets there are several mRNAs encoding for proteins that are essential for neural development and plasticity, such as Arc, CaMKII, calmodulin and calbindin (Pasciuto and Bagni, 2014). Since the absence of FMRP increases translation, this RNA-binding protein is considered to be a translational repressor (Bhakar et al., 2012). FMRP-mediated inhibition of translation may be mediated by stalling the ribosomes in the elongation phase, or by interaction of the cytosolic complex of FMRP-interacting protein (CYFIP1) with eIF4E, a protein that controls the initial phase of the translation. Beyond its abovementioned functions, it has been shown that FMRP has many other roles, ranging from genome regulation and RNA editing, to the control of the kinetics of cell differentiation and modulation of ion-channels (reviewed in Davis and Broadie, 2017), reflecting the high number of possible FMRP molecular partners.

Considering the broad spectrum of FMRP functions, it is not surprising that FMRP loss represents a key event in the development of FXS symptomatology. Indeed, many synaptic alterations were identified which account for the abovementioned cognitive and behavioural alterations:

- Aberrant dendritic spine morphology and density. Both FXS patients and FXS murine models exhibit an increase in immature dendritic spines at the expenses of mature mushroom spines (Martínez-Cerdeño, 2017; Pan et al., 2010);
- Enhanced metabotropic glutamate receptor (mGluR)-dependent long-term depression (LTD). This form of synaptic plasticity is mediated by AMPA receptor (AMPAr) internalization and requires de novo synthesis of the activity-regulated cytoskeletal protein (Arc) (Huber et al., 2002). Arc mRNA, as well as mGluRs mRNAs, are FMRP targets at synapses, and dysregulation of its activity is implicated in aberrant synaptic plasticity (Park et al., 2008);

- Deregulation of mTOR and MAPK/ERK signalling cascades. These pathways are involved in the regulation of protein synthesis and are hyperactivated in FXS patients, as well as in mouse models of FXS. The ultimate consequence of this hyperactivation is an upregulation of protein synthesis in the dendritic compartment (Darnell and Klann, 2013);
- Disrupted interaction between FMRP and calcium channels: FMRP directly interacts with N-type calcium channels subunits and the absence of this interaction leads to an increase in the synaptic vesicle release probability (Ferron et al., 2014).

Importantly, none of these alterations can account for FXS physiopathology per se. Rather, the different symptoms that characterize FXS are caused by the intricate interplay between signalling cascades and cellular events that are, at least in part, orchestrated by FMRP and by its numerous molecular partners.

Project Goals

This project aims to elucidate the contribution of FMRP to the correct functioning of glutamatergic synapses. Both the presynaptic and postsynaptic compartments will be taken into consideration, using *Fmr1* KD neurons and *Fmr1* KO mice that recapitulate FXS phenotype.

In particular, the objectives of the study are:

1. Evaluate the impact of FMRP absence on the clustering and mobility of NMDA and AMPA receptors at the synapse; investigate if FMRP acts downstream of BDNF-TrkB signalling in mediating receptor clustering.
2. Study of the possible interaction of FMRP with the proteins of the exocytotic machinery and characterize the dynamics of synaptic vesicle exocytosis and recycling in the absence of FMRP.
3. Characterize the effect of FMRP deletion on presynaptic differentiation, as well as on the synaptic proteome and on the presynaptic bouton dynamics.

Presently, there is no cure for FXS, but hopefully unravelling the synaptic defects underlying this neuropsychiatric disorder will help discovering new therapeutic targets or at least ameliorate the course of the disease.

Ongoing work and short-term future steps

To dissect FMRP role in regulating synapse physiology, we used two different sets of shRNAs to knock down (KD) Fmr1 expression in mature hippocampal neurons. We transfected hippocampal neurons with these shRNAs and we analysed FMRP immunoreactivity in dendrites and in somata (Fig. 1 and Fig. 2) to test the efficacy of the KD strategy. Both shRNAs partly decreased FMRP protein levels in the soma and in neurites.

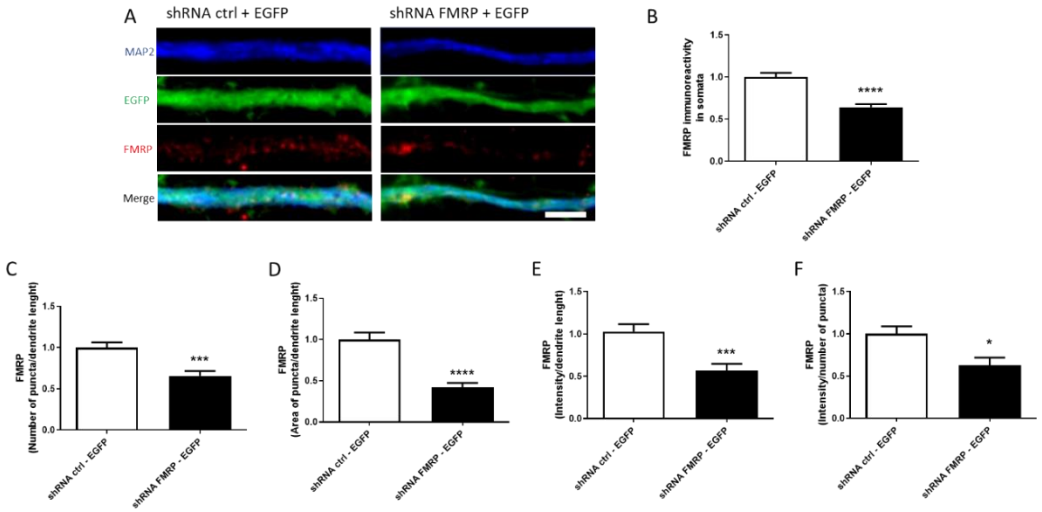


Figure 1. Low density cultured hippocampal neurons (DIV 10) were transfected with a shRNA to knock down *Fmr1* expression or with the relative shRNA control. EGFP has been used as a reporter gene with a ratio of 2µg shRNA: 1µg EGFP in each coverslip. At DIV 13 neurons were fixed and immunostained for MAP2 and FMRP (A). A reduction of FMRP immunoreactivity has been observed in the soma of neurons transfected with the shRNA for FMRP(B), compared to the soma of neurons transfected with the shRNA control (shRNA ctrl + EGFP n=36, shRNA FMRP + EGFP n=32). In the dendritic compartment, the number (C) and area (D) of FMRP puncta per dendritic length, together with the signal intensity per dendritic length (E) and the signal intensity per puncta (F) were analysed. The expression of the shRNA induced a decrease in all the parameters taken into consideration, compared to the control condition (shRNA ctrl + EGFP n=73, shRNA FMRP + EGFP n=37). Three independent experiments using three different preparations have been performed. Data are normalised upon control condition and are plotted as mean ± S.E.M., statistical significance was calculated using an unpaired t-test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001).

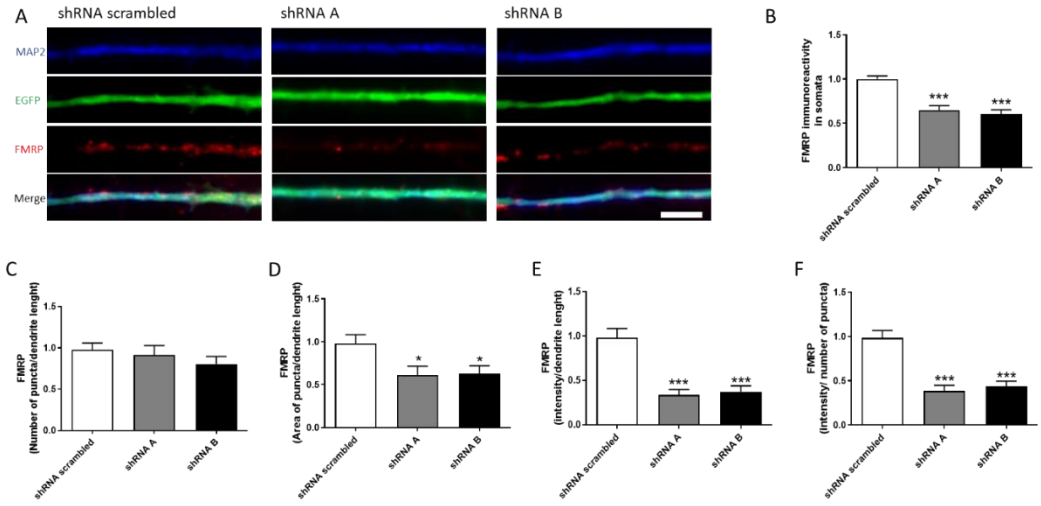


Figure 2. Low density cultured hippocampal neurons (DIV 10) were transfected with one of the shRNA to knock down *Fmr1* expression (here named shRNA A or shRNA B) or with the relative shRNA scrambled, using 1,5µg DNA in each coverslip. EGFP coding sequence was already present in the plasmid. At DIV 13 neurons were fixed and immunostained for MAP2 and FMRP (A). A reduction of FMRP immunoreactivity has been observed in the soma of neurons transfected with both the shRNAs for FMRP (B), compared to the soma of neurons transfected with the shRNA scrambled (shRNA scrambled n=48, shRNA A n=36, shRNA B n=40). In the dendritic compartment, the number (C) and area (D) of FMRP puncta per dendritic length, together with the signal intensity per dendritic length (E) and the signal intensity per puncta (F) were analysed. The expression of the shRNAs A and B induced a decrease in all the parameters taken into consideration, except for the number of puncta per dendrite length, compared to the control condition (shRNA scrambled n=51, shRNA A n=39, shRNA B n=39). Four independent experiments using four different preparations have been performed. Data are normalised upon control condition and are plotted as mean ± S.E.M., statistical significance was calculated using a One-way ANOVA followed by Dunnet's correction for multiple comparisons.

1. Previous studies from our laboratory showed that BDNF induces synaptic accumulation of GluN2A- and GluN2B-containing NMDAR. To study the eventual interplay between FMRP and the neurotrophin BDNF in mediating synaptic receptor clustering, DIV 10 hippocampal neurons were transfected with the two sets of shRNAs and treated with BDNF at DIV13. The receptors associated with the plasma membrane were live-stained with an antibody that recognizes the extracellular domain of each NMDAR subunit. After fixation and permeabilization, neurons were immunostained also for the presynaptic marker vGlut1 (vesicular glutamate transporter type 1) and the postsynaptic marker PSD95. NMDAR clusters that colocalized with PSD95 and vGlut were considered to be synaptic. The number and intensity of GluN2A and GluN2B clusters were analysed.
   Regarding the first set of shRNAs, we observed that FMRP KD was sufficient to impair BDNF-induced synaptic clustering of GluN2A and GluN2B-containing NMDA receptors (Fig. 3 and Fig. 4). Moreover, FMRP KD was sufficient to induce an upregulation of synaptic GluN2B-containing receptors (Fig. 4).

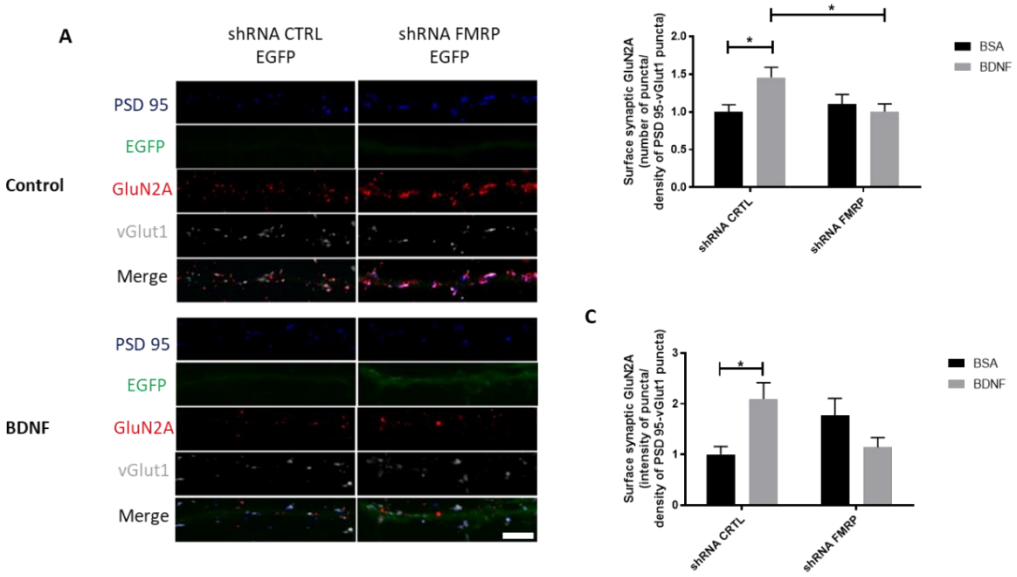


Figure 3. FMRP downregulation impairs the effects of BDNF on the synaptic accumulation of GluN2A-containing NMDA receptors. Cultured hippocampal neurons (DIV 10) were transfected with a shRNA to knock down Fmr1 expression or with the relative shRNA control. EGFP was used as a reporter gene with a ratio of 2 µg shRNA : 1µg EGFP in each coverslip. At DIV 13 neurons were stimulated with BDNF (50 ng/ml) or with BSA 0.1% as a control, for 10 minutes. To visualize receptors present on the cell surface, neurons were live immunostained with an antibody against an extracellular epitope in the GluN2A N-terminus. Following fixation, neurons were immunostained with antibodies for the presynaptic marker vGlut (vesicular glutamate transporter type 1) and the postsynaptic marker PSD 95 (A). GluN2A clusters that colocalized with PSD95 and vGlut were considered to be synaptic. The number (B) and intensity (C) of such clusters was quantified per dendritic length. Four independent experiments were performed, using different preparations. In each experiment 10 to 15 neurons per condition were analyzed. Results are normalised to the control condition (namely neurons transfected with shRNA CTRL and treated with BSA) and expressed as mean ± S.E.M. Statistical significance have been calculated using a 2-way ANOVA with multiple comparisons (*$p < 0.05$). Scale bar = 5 µm. (unpublished observations).

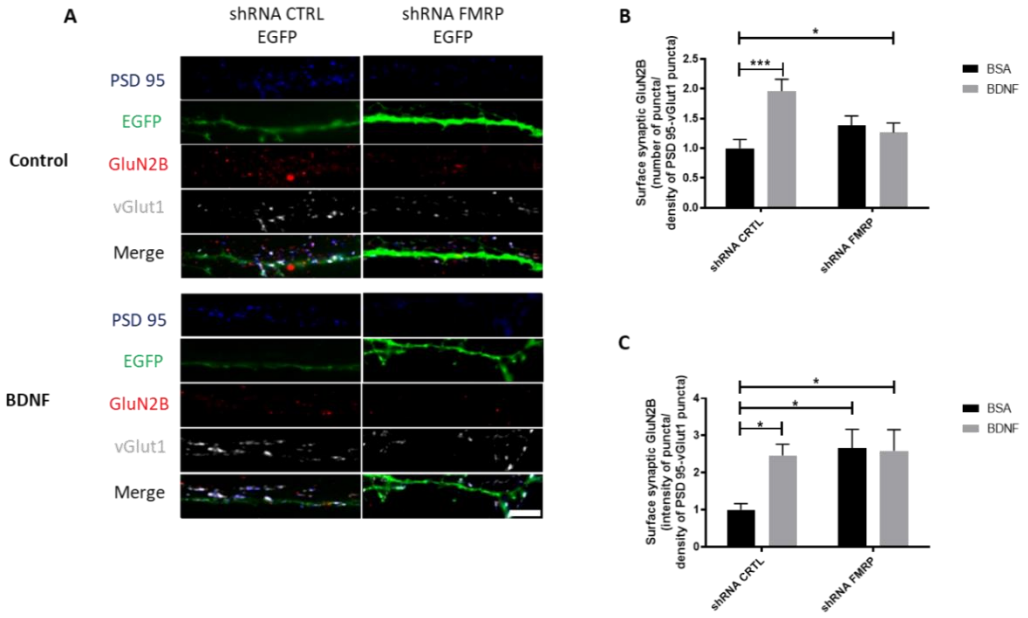


Figure 4. FMRP downregulation upregulates the synaptic expression of GluN2B and impairs the effects of BDNF on the synaptic accumulation of GluN2B-containing NMDA receptors. Cultured hippocampal neurons (DIV 10) were transfected with a shRNA to knock down Fmr1 expression or with the relative shRNA control. EGFP was used as a reporter gene with a ratio of 2 µg shRNA : 1 µg EGFP in each coverslip. At DIV 13 neurons were stimulated with BDNF (50 ng/ml) or with 0.1% BSA as a control, for 30 minutes. To visualize the receptors present on the cell surface, neurons were live immunostained with an antibody against an extracellular epitope in the GluN2B N-terminus. Following fixation, neurons were immunostained with antibodies for the presynaptic marker vGlut (vesicular glutamate transporter type 1) and the postsynaptic marker PSD 95 (A). GluN2B clusters that colocalized with PSD95 and vGlut were considered to be synaptic. The number (B) and intensity (C) of such clusters was quantified. Four independent experiments were performed using distinct preparations. In each experiment 10 to 15 neurons were analyzed per condition. Results are normalised on control condition (namely neurons transfected with shRNA CTRL and treated with BSA) and expressed as mean ± S.E.M.. Statistical significance has been calculated using a 2way ANOVA with multiple comparisons (*p < 0.05, ***p < 0.001). Scale bar = 5 µm. (unpublished observations).

Complementary experiments were performed with a second set of shRNAs and a different scrambled shRNA (shRNA S), to definitely prove the interplay between FMRP and BDNF. In these experiments the effect of BDNF in the synaptic accumulation of NMDA receptors containing GluN2A and GluN2B subunits was not as strong as the effect observed with the previous scrambled plasmid. Moreover, two shRNAs to KD FMRP were used. FMRP KD using shRNA A was sufficient to induce an upregulation of GluN2A and GluN2B synaptic content, and to impair the effects of BDNF treatments. Neurons transfected with shRNA B did not exhibit synaptic accumulation of GluN2A and GluN2B-containing NMDA receptors; on the other hand, we observed that BDNF was able to induce such synaptic clustering (Fig. 5 and Fig. 6). Since these shRNAs target different splice variants of FMR1 mRNA, we hypothesize that the variant targeted by shRNA B is not involved in BDNF-induced clustering of NMDA receptors. Moreover, two more shRNAs belonging to the same set will be tested, to try to correlate what we observe with such different splice variants.

Additional whole-cell voltage-clamp experiments will be performed to determine the effect of FMRP downregulation on BDNF-induced increase in NMDAR-mediated mEPSC.

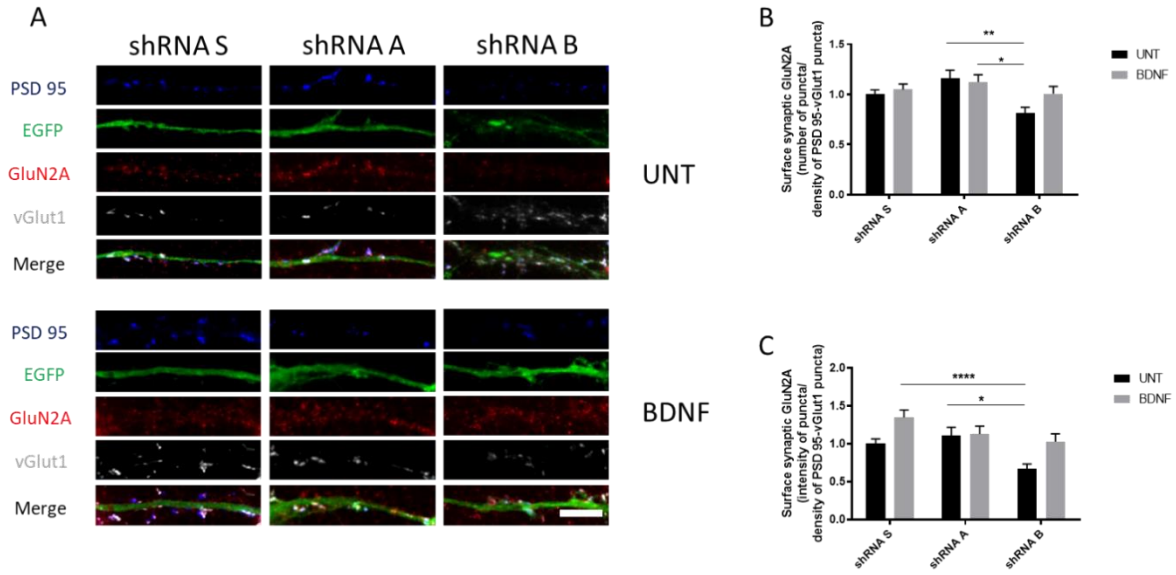


Figure 5. Downregulation of a specific FMRP splice variant impairs the effects of BDNF on the synaptic accumulation of GluN2A-containing NMDA receptors. Cultured hippocampal neurons (DIV 10) were transfected with two different shRNAs, named A and B, to knock down Fmr1 expression or with the relative shRNA control, indicated as S. EGFP coding sequence was included in the vector. 1,5µg of DNA was used in each coverslip. At DIV 13 neurons were stimulated with BDNF (50 ng/ml) or with water as a control, for 10 minutes. To visualize receptors present on the cell surface, neurons were live immunostained with an antibody against an extracellular epitope in the GluN2A N-terminus. Following fixation, neurons were immunostained with antibodies for the presynaptic marker vGlut1 (vesicular glutamate transporter type 1) and the postsynaptic marker PSD 95 (A). GluN2A clusters that colocalized with PSD95 and vGlut1 were considered to be synaptic. The number (B) and intensity (C) of such clusters was quantified per dendritic length. Four independent experiments were performed, using different preparations. In each experiment 10 to 15 neurons per condition were analysed. Results are normalised to the control condition (namely neurons transfected with shRNA S and untreated) and expressed as mean ± S.E.M. Statistical significance have been calculated using a 2-way ANOVA with multiple comparisons (*p < 0.05; **P<0.001; ****P<0.0001). Scale bar = 5 µm. (unpublished observations).

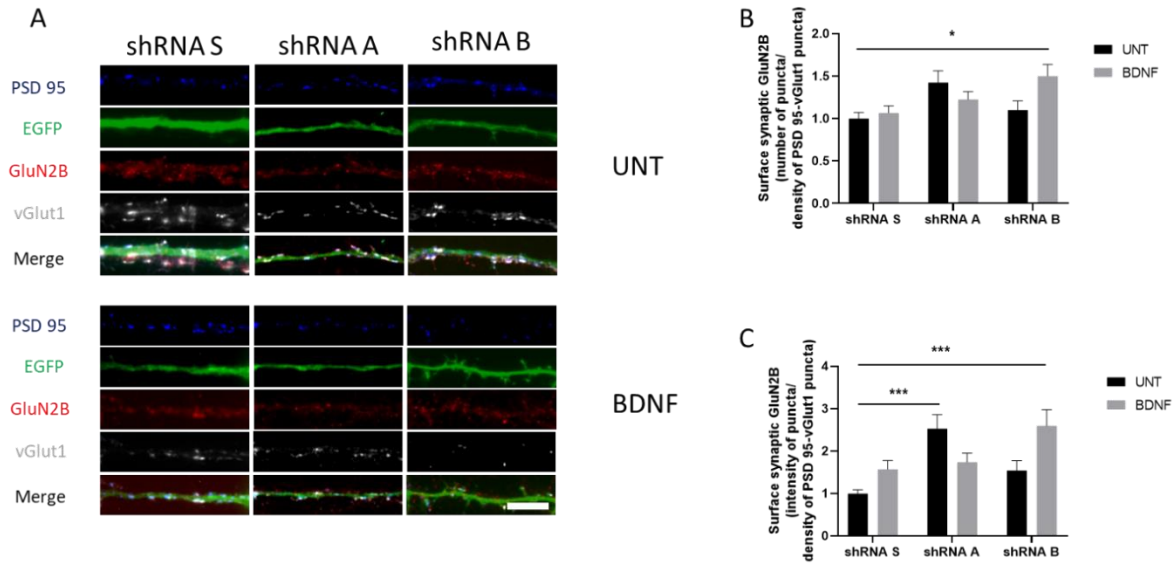


Figure 6. Downregulation of a specific FMRP splice variant upregulates the synaptic expression of GluN2B and impairs the effects of BDNF on the synaptic accumulation of GluN2B-containing NMDA receptors. Cultured hippocampal neurons (DIV 10) were transfected with two different shRNAs, named A and B, to knock down Fmr1 expression or with the relative shRNA control, indicated as S. EGFP coding sequence was included in the vector. 1,5µg of DNA was used in each coverslip. At DIV 13 neurons were stimulated with BDNF (50 ng/ml) or with 0.1% BSA as a control, for 30 minutes. To visualize the receptors present on the cell surface, neurons were live immunostained with an antibody against an extracellular epitope in the GluN2B N-terminus. Following fixation, neurons were immunostained with antibodies for the presynaptic marker vGlut1 (vesicular glutamate transporter type 1) and the postsynaptic marker PSD 95 (A). GluN2B clusters that colocalized with PSD95 and vGlut1 were considered to be synaptic. The number (B) and intensity (C) of such clusters was quantified. Four independent experiments were performed using distinct preparations. In each experiment 10 to 15 neurons were analysed per condition. Results are normalised on control condition (namely neurons transfected with shRNA S and untreated) and expressed as mean ± S.E.M.. Statistical significance has been calculated using a 2way ANOVA with multiple comparisons (*p < 0.05, ***p < 0.001). Scale bar = 5 µm. (unpublished observations).

2. To study the dynamics of synaptic vesicle exocytosis and recycling, the protocol to prepare autaptic cultures from hippocampal neurons have been tested and optimised (Fig. 7). Autaptic cultures are characterised by the presence of single neurons growing in micro-islands with the support of glial cells. In these cultures neurons can make synapses just with themselves, making this a great model to study synaptic connections. Indeed, different groups at CNC will benefit from this model.
   Fmr1 KO animals will be soon present in the CNC animal facility. The next steps will be to prepare autaptic cultures from WT and Fmr1 KO hippocampal neurons to evaluate changes in vesicle pool size and recovery kinetics by performing vesicle pool depletion and recovery protocols.



Figure 7. Representative image of a microisland populated by a glial cell and a single hippocampal neuron. Coverslips were coated with agarose, a biocompatible molecule that does not allow neuronal or glial growth. After that, microislands of poly-D-lysine and collagen have been stamped with a specific tool. Glial cells have been plated 3 days before rat hippocampal neurons. At DIV 14 coverslips have been fixed and immunostained with antibodies against the glial marker GFAP and the neuronal marker MAP2. Nuclei have been stained with Hoechst. Scale bar 50µm.

3. The efficacy of the two sets of shRNAs to KD Fmr1 expression is currently being tested in the axonal compartment as well. After that, hippocampal neurons will be cultured in cloning cylinders to isolate specifically the axonal compartment. At DIV4, neurons will be transfected with the abovementioned shRNAs to KD FMRP and presynaptic proteins, such as vGlut, Synapsin and Bassoon, will be immunostained 3 days later. The goal of these experiments is to investigate the role of FMRP in presynaptic differentiation.
   These experiments will be repeated using hippocampal neurons cultured from WT and Fmr1 KO mice.

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

The current work carried out under ESR8 focuses on understanding the synaptic dynamics of Down Syndrome (DS) cortical neurons by using a new *in vivo* model published by the De Paola Lab in 2018 (Real *et al*., Science). To date, ESR8 has successfully used this *in vivo* model to create new human DS grafts inside the brains of NOD-NSG immunodeficient mice. Briefly, the methodology used is to inject induced-pluripotent stem cell-derived neurons into the primary somatosensory cortex of NOD-NSG mice. A cranial window is then placed over the region and the injected graft cells are imaged over months using 2-photon microscopy. The cells are transduced with lentiviruses prior to injection so that they can be monitored for structural or activity changes respectively. The neurons used are either trisomy 21 (Ts21) cells or isogenic controls (WT) matched to those cells. These grafts differ in their differentiation from previous grafts used in the lab (Real *et al*., 2018) and therefore will provide a new perspective on DS cells in this model type. The De Paola Lab is currently performing a battery of graft characterisation analyses (ESR8, in collaboration with Maria Tortora and Dr. Shabana Khan) which will provide detailed information regarding cell types present in these grafts. Current information indicates that the cells that mature into cortical neurons are mostly deep layer (TBR1+) with a small percentage being upper layer (SATB2+) and a significant number remaining neuronal precursors (Ki67+/Pax6+).

Using the model described above, two main sections of this project have been explored thus far. The first is the understanding of DS synaptic dynamics by evaluating en-passant boutons (EPBs) on axons over time. For this section, a GFP-human Synapsin 1 lentivirus is used to label graft cells for structural imaging. DS axons are compared to WT axons in order to assess bouton turnover, survival rate, density, and size. This information provides us with new knowledge regarding the formation and stability of synapses in DS. Information is collected over 3 timepoints with 1 day between each at both 3 months post-transplantation (mpt) and 5mpt. Current findings indicate no significant difference between DS and WT axons in EPB density, survival or turnover. However, a trend increase in survival rate and decrease in turnover rate are present at 5mpt in Ts21 cells compared to matched WT

controls. This trend follows the findings of the Real *et al*. (2018) paper with the previous cell type. Furthermore, new findings show significant increases in the fluorescent intensity of DS boutons at 5mpt compared to WT boutons at 5mpt and also compared to DS boutons at 3mpt. These findings indicate that DS EPBs are larger in size than WT EPBs at 5mpt and that there is a general increase in EPB size in DS from 3mpt to 5mpt. This implies a potential increase in synaptic strength in DS grafts at 5mpt compared to WT and compared to earlier timepoints. ESR8 hopes to fully understand these findings by completing this dataset shortly, and via the activity and high-resolution microscopy findings to be done in the follow sections.

The second section is the understanding of DS synaptic dynamics by evaluating calcium activity live using t-series imaging on the 2-photon microscope. For this section, a GCaMP6-tdTomato lentivirus is used to label the graft cells for both calcium activity and structural imaging. The goal of this section is to understand the differences between DS and WT spontaneous activity levels in the grafts. These experiments are underway, and will likely lead to future pharmacological experimentation, depending on the final outcomes from these experiments. To date, this section is underway and spontaneous activity has been successfully imaged in both WT and DS grafts. Further data collection and analysis is needed to draw any conclusions, but this data will be available in the near future.

Future experiments include electrophysiological studies (to be undertaken at Lundbeck during secondment), high resolution microscopy studies (to be undertaken at Bordeaux during secondment) and potential pharmacological experiments (depending on the findings from the GCaMP imaging analysis). ESR8 will also complete a 0.5 month secondment at CHUC in Coimbra to learn more about clinical treatment of DS.

This work is incredibly beneficial to the scientific and clinical communities. It will provide data on a new model that is able to be used to study synaptic dynamics of many disorders in a patient-specific way. Furthermore, it will increase the scientific community's understanding of DS and final stage pharmacological experiments may provide potential future drugs options for study and testing in DS. The goal of these future treatments would be to alleviate cognitive decline in DS individuals, who experience a wide range of cognitive and intellectual deficits, including dementia.

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

Previously, our group identified rare mutations in the X-linked gene *GLRA2*, encoding the glycine receptor α2 subunit, in patients with autism spectrum disorder (ASD), implicating altered glycinergic signalling for the first time in ASD (Pilorge et al. 2016, PMID: 26370147). *Glra2* knockout male mice (*Glra2*$^{-/Y}$) exhibited impaired learning and memory in the novel object recognition test, with normal spatial memory, and impaired long-term potentiation in the prefrontal cortex, a region involved in recognition memory and consistently implicated in ASD. These findings unveiled novel roles for the glycine receptor α2 subunit in synaptic plasticity and learning and memory (Pilorge et al. 2016). The aim of my project is to investigate the impact of the loss of *Glra2* on circuit assembly and function in the prefrontal cortex of *Glra2*-deficient mice.

We first **quantified excitatory and inhibitory neuron populations** in the prefrontal cortex using both fluorescent *in situ* hybridisation and immunofluorescence. These experiments showed no difference in density of total excitatory or inhibitory neurons in *Glra2*$^{-/Y}$ mice. However, we observed an increase in the number of somatostatin interneurons (+37%, $p<0.01$, Student's t-test), with no significant differences in the other subpopulations of interneurons studied (parvalbumin, calretinin and cholecystokinin). Immunofluorescence experiments were also performed at postnatal day 14 to rule out a developmental effect, and the results were similar to those in adults. These findings don't confirm the severe loss of cortical projection neurons and inhibitory neurons with microcephaly previously reported by another group in *Glra2*-deficient mice at postnatal days 7 and 14 (Avila et al. 2014, PMID: 24926615; Morelli et al. 2017, PMID: 26891984). While the reasons for this discrepancy are unclear, we are confident that our mice don't have any significant loss of cortical neurons, in agreement with the phenotype of two male ASD patients with *GLRA2* mutations that have normal head circumference, normal brain imaging and only mild or borderline intellectual disability (Pilorge et al. 2016).

In addition, we **quantified excitatory and inhibitory synapses** in the prefrontal cortex. Functional excitatory synapses were identified via immunofluorescent colocalisation of the pre-synaptic and post-synaptic proteins VGlut1 and PSD95, and inhibitory synapses were identified via colocalisation of the

pre- and post-synaptic proteins VIATT and gephyrin. These experiments showed a decrease in the colocalisation of VIATT and gephyrin ($p<0.05$), with no effect on the total density of either marker. By contrast there was no difference in the colocalisation of VGlut1 and PSD95. The decrease in inhibitory synapses with no change in excitatory synapses points to an excitatory/inhibitory imbalance in the prefrontal cortex of *Glra2*-deficient mice, as previously shown for other mouse models of autism.

Analysis of spine density and morphology, and dendritic arborisation in prefrontal cortex pyramidal neurons was carried out by filling the cells with biocytin. Spine density was significantly increased in apical and basal dendrites of *Glra2*-deficient mice compared to wild-type littermates ($p<0.05$ and $p<0.01$, respectively). In terms of the morphology of spines, mushroom or thin spines are defined as mature spines, whereas stubby spines are defined as immature spines. There was a significant increase in mature dendritic spines in mutant mice ($p<0.05$), with no difference in immature spine density. Mature spines vastly outnumbered stubby spines in the sample (roughly 85% of all spines), so this increase in mature spines is likely a reflection of the overall increased spine density observed. In addition, pyramidal neurons in *Glra2*$^{-/Y}$ mice showed increased dendritic arborisation, with increased dendritic length and branching. The number of total and apical branch points was increased by 64% and 83%, respectively ($p>0.01$ and $p>0.001$, respectively). These findings indicate that there is altered connectivity of the pyramidal neurons in the prefrontal cortex of *Glra2*-deficient mice, which is expected to result in perturbations of synaptic function and disruption of normal cortical network dynamics.

Another objective of the project is to **map the brain regions implicated in the novel object recognition deficit observed in *Glra2*-deficient mice, using c-fos**. We observed a decrease in c-fos-positive cells in the infralimbic prefrontal cortex of *Glra2*-deficient mice following novel object recognition, with no significant differences observed in the other regions quantified (prelimbic cortex, perirhinal cortex and entorhinal cortex). In addition, there was no difference in c-fos expression between *Glra2*$^{-/Y}$ and control mice in the basal cohort that did not undergo the novel object recognition phase. Thus, these results suggest that there is hypoactivation of the prefrontal cortex of *Glra2*-deficient mice, which may underlie the cognitive deficit. This experiment is currently being repeated in a larger cohort of mice. We will perform colocalisation immunofluorescence experiments between c-fos and protein markers of different neuronal subtypes, to determine the specific subpopulations that are hypoactivated. In addition, we plan to use fibre photometry in *Glra2*-deficient mice to assess prefrontal cortical activity in freely moving mice. We will also assess functional circuit activity in these mice using electroencephalography and functional magnetic resonance imaging during my secondment in the laboratory of Dr. Peter Kind (University of Edinburgh).

Exploitation of results

I presented a poster with my results in a virtual conference organized by my doctoral school for the PhD students. These results and any subsequent results will be published in a peer-reviewed scientific journal.

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

Many genes implicated in autism spectrum disorder (ASD) and other neurodevelopmental disorders encode proteins involved in synaptic plasticity, especially at glutamatergic synapses. The synaptic dysfunction caused by these genetic defects creates an imbalance between excitatory and inhibitory (E/I) neurotransmission, which is thought to be at the origin of the neurobehavioural deficits observed in patients. Restoring a normal E/I ratio therefore constitutes a promising therapeutic approach to treat different forms of ASD arising from multiple molecular/cellular mechanisms. The endocannabinoid system is a key modulator of neuronal excitability and synaptic plasticity, and regulates the E/I balance. It has already shown promise as therapeutic target in other neurodevelopmental disorders associated with ASD, such as fragile X syndrome and Down syndrome.

The objective of my project is to assess the endocannabinoid system as a therapeutic target in a mouse model of ASD. Specifically, I use a *Shank3*-deficient mouse model. *SHANK3* is one of the genes more commonly mutated in ASD and its loss results in Phelan-McDermid syndrome (PMS), characterized by hypotonia, intellectual disability, severe language impairment, ASD, and motor deficits. *Shank3*-deficient mouse are a well-known model of ASD and exhibit synaptic dysfunction and E/I imbalance. There are several mouse models of PMS with different deletions in *Shank3*. These mice exhibit differing phenotypes, likely due to the fact that the deletions affect different isoforms of *Shank3*. The mouse model we use has a deletion of exons 4-22 and is the first full knockout (KO) model with loss of all isoforms of *Shank3* (Drapeau et al., eNeuro 2018) to better mimic the vast majority of PMS cases, which have deletions affecting all *SHANK3* isoforms. Since PMS patients only have loss of one copy of *SHANK3*, we use heterozygous (Het) mice, but we also include KO mice in our experiments because previous studies have shown that *Shank3* Het mice usually have mild or no behavioural deficits, even if they often show clear deficits in molecular and electrophysiological experiments.

The two main aims of my project are: 1) to assess the consequences of *Shank3* deficiency on the endocannabinoid system; and 2) to assess the effects of administration of endocannabinoid modulators on the phenotype of *Shank3*-deficient mice.

Task 10.1 – Consequences of *Shank3* deficiency on the endocannabinoid system

For the first aim, I collected the brains to evaluate the basal levels of the two main endocannabinoids, anandamide and 2-arachidonoylglycerol, and the two cannabinoid receptors, CB1 and CB2. Endocannabinoid levels will be measured by liquid chromatography-mass spectrometry by our collaborators at the University of Bordeaux. I already learned how to perform Western blots and I tested the antibodies for the receptors; these experiments will be completed in the next few months. The electrophysiology experiments to assess endocannabinoid-dependent synaptic plasticity will be performed in the lab of Dr. João Peça during my secondment in Coimbra.

Task 10.2 – Assess the effects of administration of endocannabinoid modulators on the phenotype of *Shank3*-deficient mice

For the second aim, I performed behavioural experiments to evaluate the effect of rimonabant, an antagonist of the CB1 receptor, in *Shank3*-deficient male and female mice. Rimonabant 1 mg/kg or vehicle were injected intraperitoneally once per day for 7 days in Het, KO and wild-type littermates. Twenty-four hours after the last injection, different groups of mice were tested to evaluate locomotion (actimeter), motor coordination (accelerating rotarod), learning and memory (novel object recognition), and anxiety-like behaviour (dark-light box). KO males exhibited hypolocomotion (both horizontal and vertical activity), which remained unchanged after rimonabant. No significant locomotion deficits were observed in Het males or in females. KO males exhibited a coordination deficit in the rotarod that was rescued by rimonabant. In females, both Het and KO mice had a coordination deficit, which was only partially improved by rimonabant. Learning and memory were tested with two novel object recognition tasks, with novel objects differing from the familiar object either by texture or shape. Rimonabant rescued learning and memory deficits in both Het and KO mice. I am currently evaluating the anxiety-like behaviour in our mice using the dark-light box. Preliminary results indicate that rimonabant has no effects on anxiety-like behaviour in KO males; the experiments in females are ongoing. To better understand the mechanisms underlying the effects of rimonabant on behaviour in *Shank3*-deficient mice, I am setting up a protocol to measure with Western blot the levels of several subunits of glutamate receptors (e.g., mGluR5) and scaffold proteins (e.g., Homer1) in the postsynaptic density of several brain areas. The electrophysiology experiments to analyse the consequences of the administration of rimonabant and other endocannabinoid modulators on synaptic plasticity will be performed during my secondment in Coimbra.

Following the promising results obtained with rimonabant, we tested another drug, AEF0217, a signal specific inhibitor of CB1 receptors developed by the start-up Aelis Farma (Bordeaux). Unlike rimonabant, this compound has no side effects, and is thus suitable for clinical use. I recently completed a pilot test to evaluate the effects of this drug using the same behavioural tests as for rimonabant, after continuous administration of AEF0217 through the drinking water for at least 7 days. The results are promising, and we will soon decide how to continue to study the effects of this drug in our mouse model. If this or other related compounds being developed by Aelis Farma prove

to be effective in rescuing the deficits in *Shank3*-deficient mice, they could be tested in clinical trials in patients with Phelan-McDermid syndrome.

Exploitation of results:

I presented a poster with the results obtained to date in a virtual conference organized by my doctoral school for the PhD students.

The results of my research project will be published in a peer-reviewed journal. The results obtained with the novel compounds developed by Aelis Farma will contribute to the characterization of their effects and will inform future decisions on their potential use in clinical trials in individuals affected with Phelan-McDermid syndrome.

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

Background and rationale for the project

The Thalamic Reticular Nucleus (TRN) is comprised of a thin sheet of GABAergic cells that provide local inhibition to the thalamus and receive glutamatergic inputs from both thalamus and cortex., being a key regulator of E/I balance in the corticothalamic system (Pinault, 2004; Prat et al., 2015; Forgenson and Hugenard 2016). The TRN acts as a gatekeeper for sensory information flowing to the cortex (Halassa et al., 2016), and as pacemaker for sleep spindle generation (Fernandez et al., 2018). Strikingly, sleep spindles, which are known to be a sleep rhythm occurring during NREM sleep involved in the control of sensory gating (Lüthi et al., 2020), have been shown to be markedly reduced in schizophrenic patients and suggested to be an endophenotype for schizophrenia (SCZ) (Manoach et al., 2016; Manoach et al., 2019). Alterations in TRN neurons could decrease the low-threshold bursting that generates spindles and impair the gating of sensory information flow to the cortex. Imbalances in the processing of sensory input may alter both memory and cognition. Thus, treatments that target TRN interneurons are promising therapeutic strategies to restore E/I balance, sleep deficiencies and cognitive disturbances seen in SCZ patients.

Description of the work carried out and results so far

During the first year of the PhD, we studied those ion channels involved in pacing spindle generation in the TRN. Low threshold-Ca2+ channels (CaV3.3) mediate burst firing required for initiating the spindle (Astori et al., 2011), and Small conductance Ca2+-activated K+ channels (SK2) are activated by the Ca2+ ions entering through CaV3.3 channels and induce a burst afterhyperpolarization (AHP) (Cueni et al., 2009; Winner et al., 2012). This AHP is key to ensure that bursting is short and can occur repeatedly. The CACNA1I gene, encoding CaV3.3 channels, has been identified as one of the major risk genes for SCZ (Goshai et al., 2016; Merikanto et al., 2019). Due to the link between SK channels and intracellular Ca2+, we examined effects of allosteric modulation of SK on TRN firing. 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.

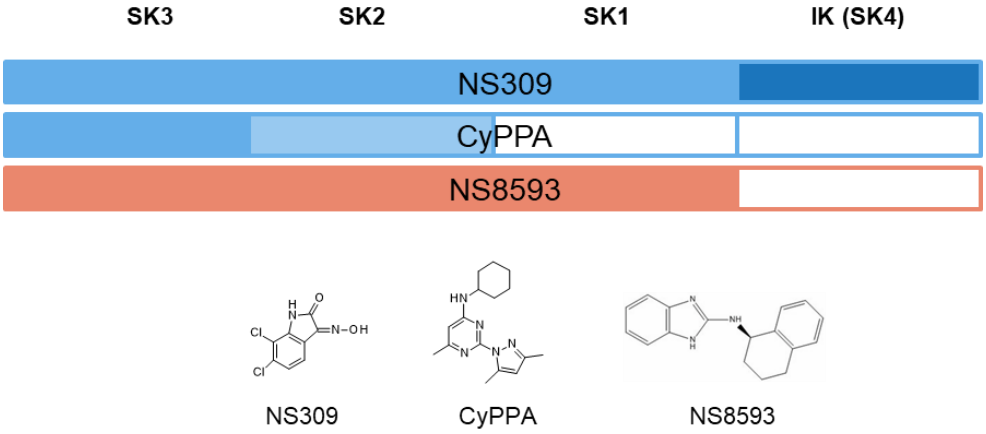


Figure 1. Effect of SK allosteric modulators on the different SK channel subtypes. Two SK PAMs (blue) and one SK NAM (red) are shown. The code reflects the potency on the target; the darker the color more potent on target.

The results showed that NS309 and NS8593 elicited opposite effects on TRN firing as the SK PAM reduced the number of action potentials (APs), whereas, numbers of APs were increased by the SK NAM. No significant effect was observed when applying CyPPA, which acts less potently on SK2 channels (figure 2a). No significant effect was observed in the total number of bursts (Figure 2b). Importantly, both PAM increased the burst AHP and the time between bursts (interburst interval) (figure 3, 4 and 5). NAM NS8593 increased remarkably the number of APs and APs per burst, as well as it reduces the afterburst AHP and interburst interval (figure 3 and 6). When taken together, the results suggest that SK channel allosteric modulators are useful tools for modulating TRN activity. Targeting SK channels might be a promising therapeutic strategy to restore E/I balance, sleep deficiencies and cognitive disturbances seen in SCZ patients.

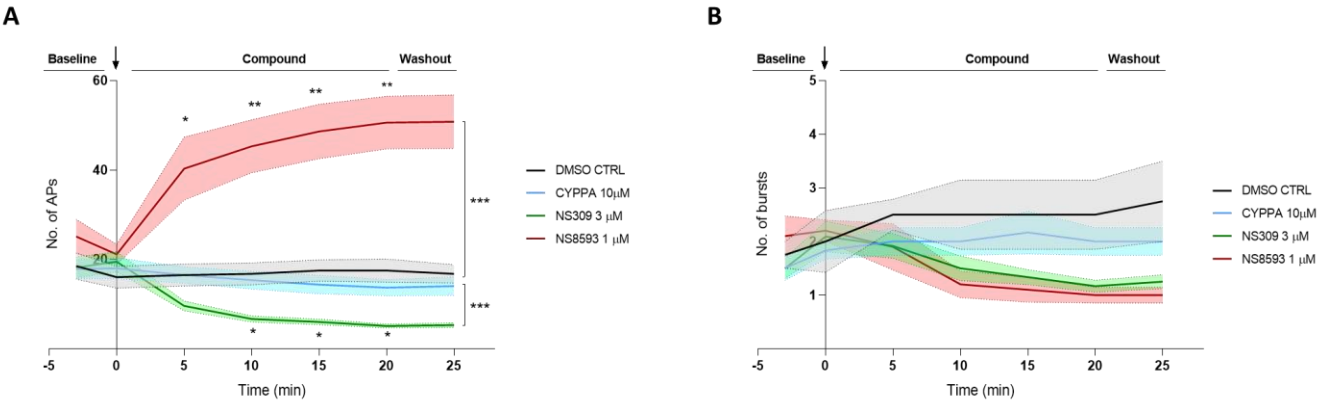


Figure 2. Effect of SK allosteric modulators on TRN firing overtime. A) Number of APs when injecting a 150pA depolarizing current before and after compound administration. In the presence of PAM NS309 3 µM (green), the number of APs elicited is reduced, whereas the opposite effect occurs when applying SK NAM NS8593 1µM (red). No effect on the firing is observed when applying CyPPA 10 µM (blue). B) On the contrary, no effect is observed in the total number of bursts overtime. The statistical analyses performed were repeated measures Two-way ANOVA.

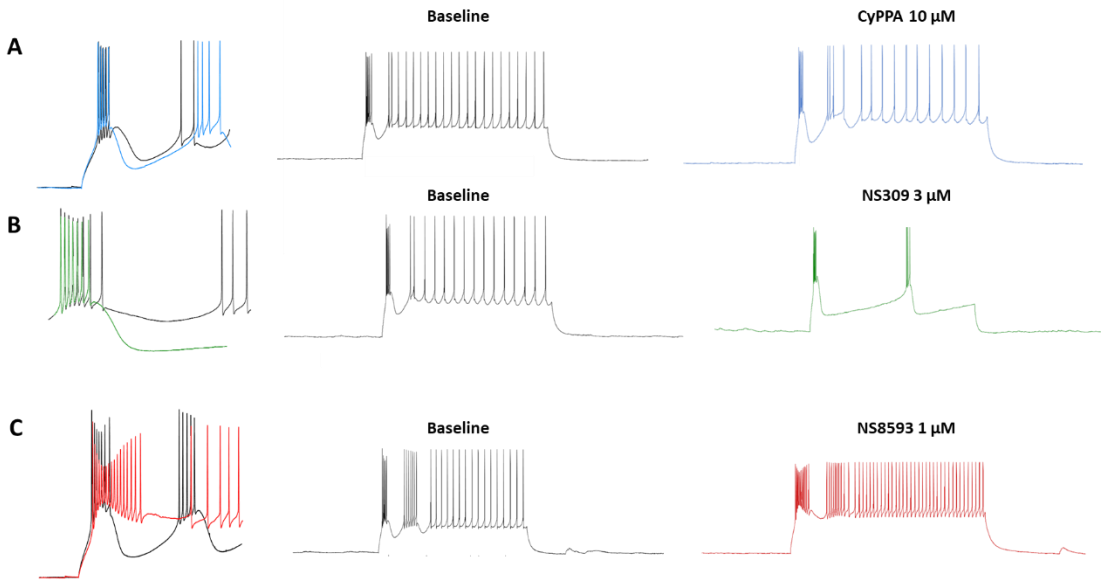


Figure 3. Representative traces of TRN cells firing when injecting a 150 pA depolarizing current step in the presence of CyPPA 10 µM (a), NS309 3 µM (b) or NS8593 1 µM (c). Amplified traces on the left panel show the effect of the different compounds on the afterburst AHP.

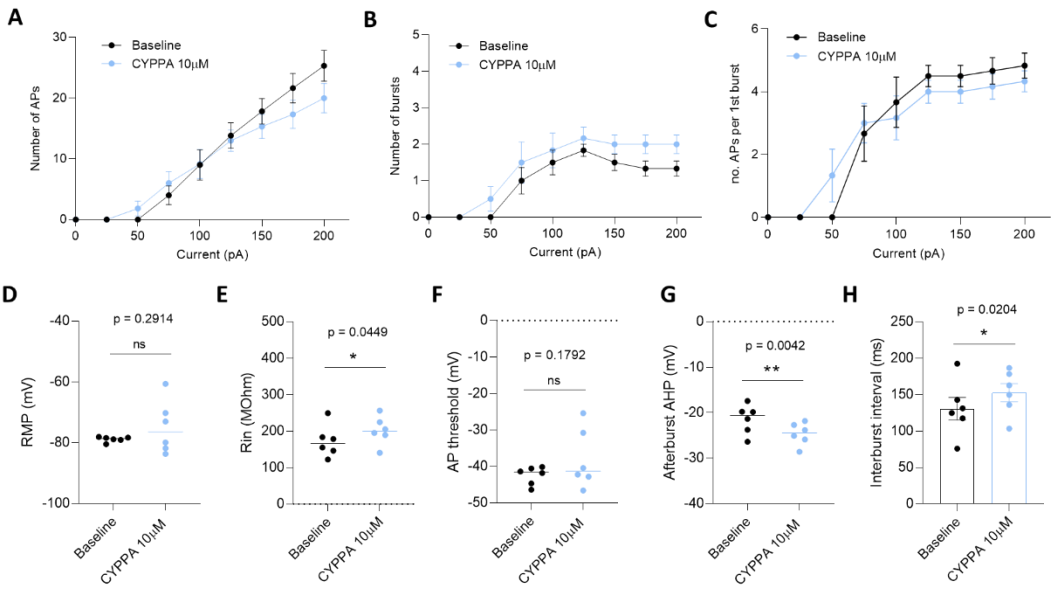


Figure 4. Effect of SK PAM CyPPA 10µM on TRN cells intrinsic properties. After 10 minutes bath-applying CyPPA 10 µM, no effects were observed in the number of APs (a), number of bursts (b) or APs/first burst (c) when comparing with baseline conditions. E) Slight increase is observed in the input resistance, without affecting the RMP (d) or AP threshold (f). Importantly, CyPPA induces a bigger afterburst AHP (g) affecting also the time between bursts (h). The statistical analyses performed were repeated measures Two-way ANOVA (a, b, c) and Student T-test (d, e, f, g, h).

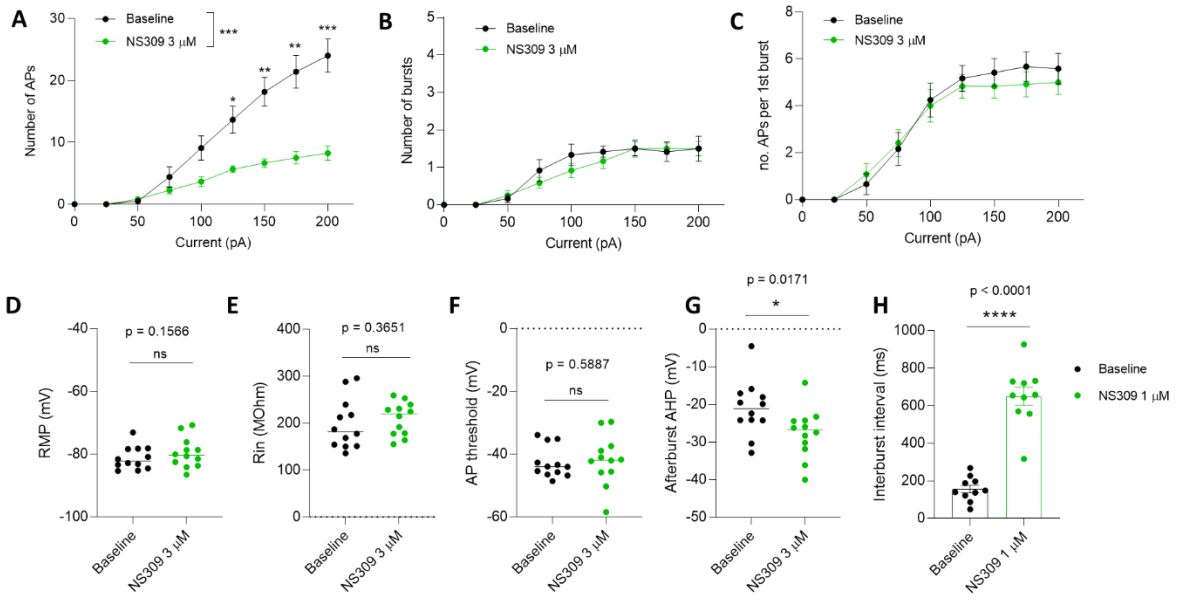


Figure 5. Effect of SK PAM NS309 3µM on TRN cells intrinsic properties. After 10 minutes bath-applying NS309 3µM, a clear reduction in the number of APs was observed (a), with no effect on the number of bursts (b) or APs/first burst (c) when comparing with baseline conditions. No effects were seen on RMP (d), Rin (e), or AP threshold (f). Remarkably, NS309 also increases the afterburst AHP (g) and interburst interval (h). The statistical analyses performed were repeated measures Two-way ANOVA (a, b, c) and Student T-test (d, e, f, g, h).

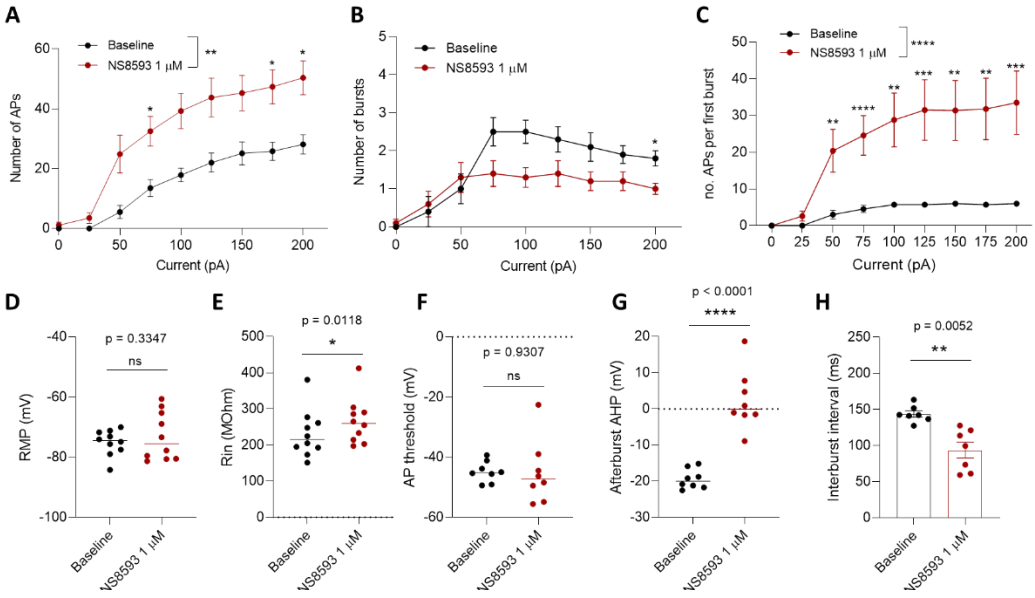


Figure 6. Effect of SK NAM NS8593 1µM on TRN cells intrinsic properties. After 10 minutes bath-applying NS8593 1µM, a significant increase in the number of APs was observed (a), together with a reduction in the number of bursts when applying 200 pA (b) and a remarkable increase in the number of APs during the first burst (c) compared to baseline. No effects were seen on RMP (d), or AP threshold (f), but a higher Rin was noticed. On the contrary to PAMs, NS8593 decrases the afterburst AHP (g) and interval between bursts (h). The statistical analyses performed were repeated measures Two-way ANOVA (a, b, c) and Student T-test (d, e, f, g, h).

Summary of exploitable results

The results obtained so far will be shared with the scientific community in conferences and congresses such as ENCODS 2021 (abstract accepted), and gathered in a future publication (writing in progress).

Next steps on the project

Based on the results obtained during the first year of the PhD, the next step will be to test SK allosteric modulators in an in vivo system. Previous findings by Astori and colleagues showed that SK2 overexpression in mice elevates arousal threshold during NREM sleep (Wimmer et al., 2012), very likely through an increase on sleep spindle density. SK PAMs could therefore have an impact increasing spindle density by enhancing the AHP and ensuring the generation of repetitive, synchronized bursts in the TRN.

In vivo electrophysiology (LFP measurements in thalamus and cortex) will be performed on freely moving rats and will give us information about oscillations (amplitude, power and frequency) in awake and asleep rats. An additional electrode will be placed into muscle tissue to record from electromyographic activity (EMG). Finally, the movement of the animals will also be recorded with an accelerometer. This will allow us to identify different sleep stages (active wake, inactive wake, NREM sleep and REM sleep) in the further polysomnographic recordings. The effect of SK modulators on sleep architecture and sleep spindles (density, duration and amplitude) will be assessed.

Ultimately, modulation of sleep spindles and corticothalamic networks will potentially be studied in a transgenic rat model of the disease in collaboration with Peter Kind (extended stay at UEDIN).

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

The object of study around which my research project revolves is the role of protein synthesis in Autism and Intellectual Disability (ASD/ID). Evidence was gathered in the past 20 years thanks to animal models carrying single genetic mutations which recapitulate specific conditions. Fragile X Syndrome was the first explored of these(Bakker et al., 1994), being the most commonly identified single-gene cause of ASD/ID (Wang, Berry-Kravis, and Hagerman 2010). Mutation in the *FMR1* gene leads to its transcriptional silencing resulting in subsequent loss of the encoded protein Fragile X Mental Retardation Protein (FMRP). These studies revealed insights about dysregulated mechanisms that could lead to the disease, among which the theory that many of the aberrant phenotypes of the disease are due to excessive protein synthesis downstream of group 1 metabotropic glutamate receptors (Gp1 mGluRs) (Bear, Huber, and Warren 2004). The most explored was synaptic long-term depression (LTD) downstream of Gp1 mGluRs which was exaggerated in the *Fmr1*$^{-/y}$ mouse model of Fragile X Syndrome (Huber et al. 2002). In addition to FMRP, many proteins encoded by genes linked to autism were observed to act as negative regulators of synaptic protein synthesis (Khelleher & Bear, 2008). Among these SYNGAP is a GTPase Activating Protein (GAP) encoded by *SYNGAP1* gene that negatively regulates the small G protein Ras and the downstream of ERK1/2 signaling pathway, which limits growth-related processes including protein translation. Mutations in *SYNGAP1* gene are associated with autosomal dominant nonsyndromic ID (NSID) and, in a minority of cases, ASDs (Berryer et al. 2013; Hamdan et al. 2011; Hamdan et al. 2009). A study from Barnes and colleagues demonstrated that excessive hippocampal protein synthesis is a convergent cellular phenotype in *Fmr1*$^{-/y}$ and *Syngap*$^{+/-}$ mouse models of disease. Convergently, hippocampal mGluR-dependent LTD is rendered insensitive to translational inhibitors, further supporting the view that increased translation consequent to loss of SYNGAP and FMRP regulatory roles underlies this LTD phenotype. This study set the basis for my project aimed to identify the specific mRNAs that are mistranslated following disruption of the translation mechanisms in these two models of autism.

1: Identification of differentially translating mRNAs in *Fmr1*$^{-/y}$ *and Syngap*$^{+/-}$ models of disease

Initially I intended to rely on Ribosome Profiling technology to globally monitor mRNA translation in vivo and I started to optimize the protocol in Hek 293 cells. However technical difficulties along with time-related issues and Covid-19 pandemic restrictions, pushed towards integration of TRAP-sequencing approach instead to be used for the exploration of the differentially translated transcripts in specific regions of the brain.

Cell-type specific mRNA purification by Translating Ribosome Affinity Purification (TRAP) allows to target translated mRNA content of specific cell population by affinity tag such as by enhanced green fluorescent protein (EGFP) tagging of ribosomal subunit protein L10a (Heiman et al. 2014). A reliant TRAP-pipeline was

optimized in Osterweil team and revealed unknown dysregulated mechanisms and possibly therapeutic targets in *Fmr1*$^{-/y}$ models of disease (Thomson et al. 2017). In addition, a preliminary dataset was obtained from hippocampal CA1 region of *Syngap*$^{+/-}$ mouse models and I focused on the analytical processing of this dataset to conduct differential expression analysis. More specifically I carried out the analysis in different steps:

- Characterization of the *Syngap*$^{+/-}$ CA1 dataset

  A brief introduction must be made on the way the data was collected. 4 pairs from WT and *Syngap*$^{+/-}$ littermate mice underwent isolation of specific ribosome bound fractions using anti-GFP coated beads targeting specific CA1 region fraction. Unbound fraction was also isolated to represent total RNA fraction.
  mRNA fractions were amplified and sequenced through Illumina HiSeq, generating Paired ends reads in fastq-format files.
  Then these data underwent pre-processing steps to obtain the raw count files on which I performed DESeq analysis to generate a table of differentially expressed genes.
  Significant genes can be filtered by applying specific cut-offs, I specifically selected pvalue <0.01. 248 genes were found differentially downregulated vs 218 upregulated in the translatome (CA1 TRAP) fraction, while 248 downregulated vs 132 upregulated genes characterized the total RNA fraction (Figure 1). In order to identify the functions of these differentially expressed genes, these lists underwent enrichment analysis. KEGG pathway analysis in Enrichr tool was specifically used. However, any significant gene terms were detected considering a cut-off pvalue of <0.01, apart from downregulated terms in the Input fractions in the category of Carbohydrate digestion and absorption and Proximal tubule bicarbonate reclamation (Figure 2 A, bottom right). In addition, I performed rank-based enrichment analysis (GSEA) where gene set statistics are calculated for each gene set ranked upon differential expression analysis of ribosome bound or total RNA fractions. This analysis revealed terms such as transcription to be upregulated in CA1 TRAP fraction and terms such as metabolism, translation and ribosome to be upregulated in total RNA fraction (Figure 2B top).
  Instead, synaptic activity, G protein coupled receptors terms were downregulated in CA1 TRAP fractions while synaptic function was downregulated in total RNA fraction (Figure 2B, bottom).

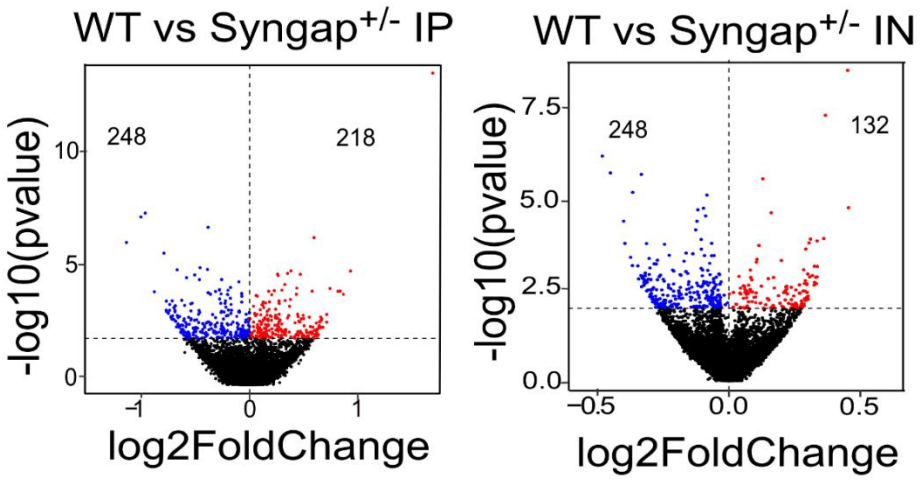
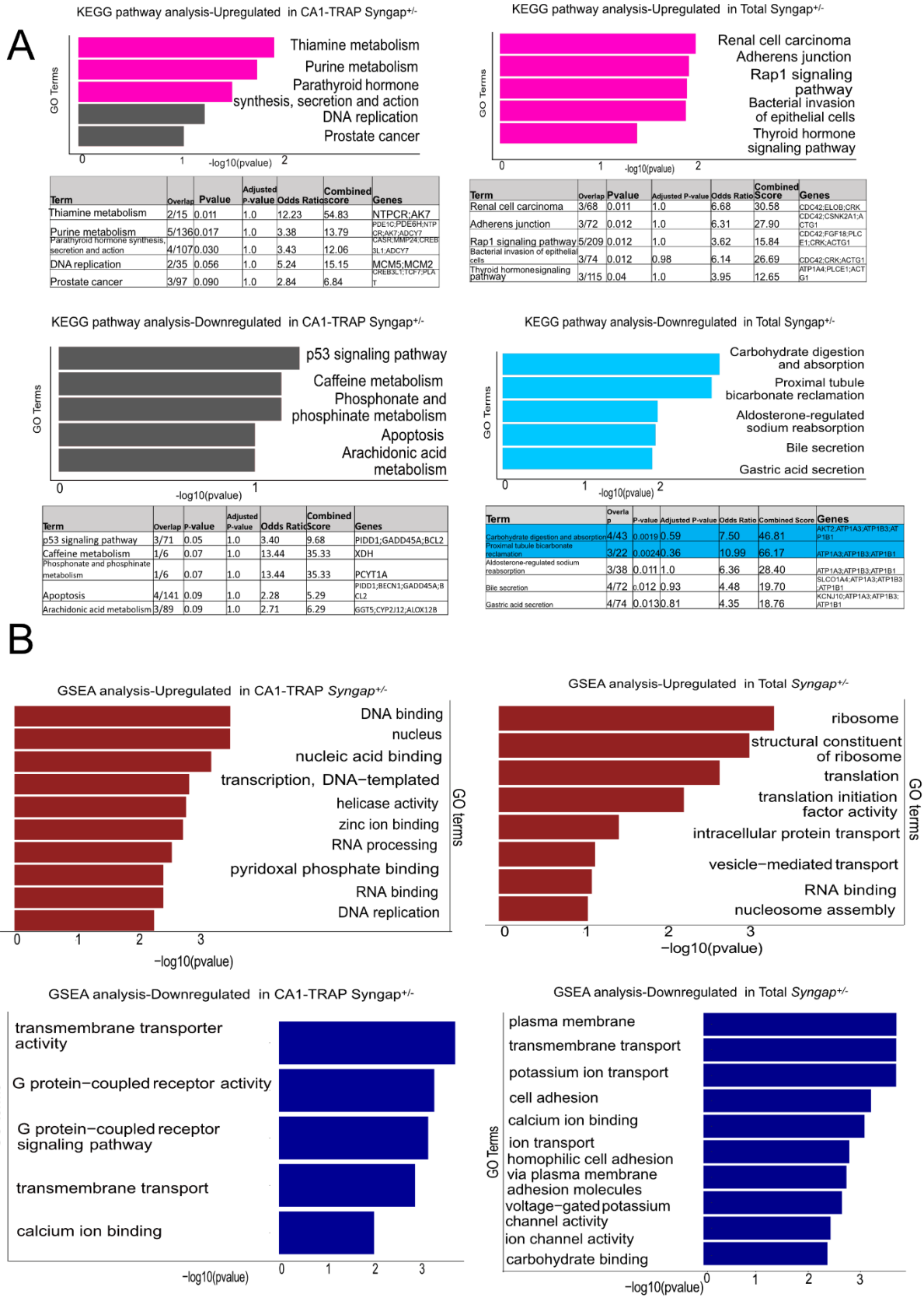


Figure 1. Volcano plots of *Syngap*$^{+/-}$ CA1 dataset showing difference between WT and *Syngap*$^{+/-}$ pairs in the CA1 TRAP (IP) and Total RNA (IN) Fractions. Blue defines downregulated genes and red defines upregulated genes. Plots show log2FoldChanges vs -log10 (pvalue set at cutoff of <0.01).



Term	Overlap	Pvalue	Adjusted P-value	Odds Ratio	Combined Score	Genes
Thiamine metabolism	2/15	0.011	1.0	12.23	54.83	NTPCR;AK7
Purine metabolism	5/136	0.017	1.0	3.38	13.79	PDE1C;PDE9H;NTPCR;AK7;ADCY7
Parathyroid hormone synthesis, secretion and action	4/107	0.030	1.0	3.43	12.06	CASR;MMP24;CREB3L1;ADCY7
DNA replication	2/35	0.056	1.0	5.24	15.15	MCM5;MCM2
Prostate cancer	3/97	0.090	1.0	2.84	6.84	CREB3L1;TCF7;PLAT

Term	Overlap	Pvalue	Adjusted P-value	Odds Ratio	Combined Score	Genes
Renal cell carcinoma	3/68	0.011	1.0	6.68	30.58	CDC42;ELOB;CRK
Adherens junction	3/72	0.012	1.0	6.31	27.90	CDC42;CSNK2A1;ACTG1
Rap1 signaling pathway	5/209	0.012	1.0	3.62	15.84	CDC42;FGF18;PLCE1;CRK;ACTG1
Bacterial invasion of epithelial cells	3/74	0.012	0.98	6.14	26.69	CDC42;CRK;ACTG1
Thyroid hormonesignaling pathway	3/115	0.04	1.0	3.95	12.65	ATP1A4;PLCE1;ACTG1

Term	Overlap	P-value	Adjusted P-value	Odds Ratio	Combined Score	Genes
p53 signaling pathway	3/71	0.05	1.0	3.40	9.68	PIDD1;GADD45A;BCL2
Caffeine metabolism	1/6	0.07	1.0	13.44	35.33	XDH
Phosphonate and phosphinate metabolism	1/6	0.07	1.0	13.44	35.33	PCYT1A
Apoptosis	4/141	0.09	1.0	2.28	5.29	PIDD1;BECN1;GADD45A;BCL2
Arachidonic acid metabolism	3/89	0.09	1.0	2.71	6.29	GGT5;CYP2J12;ALOX12B

Term	Overlap	P-value	Adjusted P-value	Odds Ratio	Combined Score	Genes
Carbohydrate digestion and absorption	4/43	0.0019	0.59	7.50	46.81	AKT2;ATP1A3;ATP1B3;ATP1B1
Proximal tubule bicarbonate reclamation	3/22	0.0024	0.36	10.99	66.17	ATP1A3;ATP1B3;ATP1B1
Aldosterone-regulated sodium reabsorption	3/38	0.011	1.0	6.36	28.40	ATP1A3;ATP1B3;ATP1B1
Bile secretion	4/72	0.012	0.93	4.48	19.70	SLCO1A4;ATP1A3;ATP1B3;ATP1B1
Gastric acid secretion	4/74	0.013	0.81	4.35	18.76	KCNJ10;ATP1A3;ATP1B3;ATP1B1

Figure 2. A) GO enrichment was performed by submitting the lists of downregulated or upregulated genes with cutoff pvalue<0.01. KEGG Pathway analysis is shown in the bar graph and also in the format of table reporting statistics and single genes represented in that terms. Really different categories appear significant for pvalue <0.05 and two only for pvalue<0.01 in the Total fraction. B) GSEA analysis was performed using PIANO package in R. Rank-based analysis of total CA1-TRAP and Total fractions resulted in genes being grouped in GO terms for which statistics is calculated in order to attribute directions towards downregulation or upregulation. Translation, ribosome-related terms were upregulated in the Total fraction, while transcription-related terms were upregulated in the CA1-TRAP fraction. Commonly downregulated transport-related terms were found in both Total and CA1-TRAP fractions. Bar graphs represent GO terms significantly changed based on cutoff of p<0.01.

- Comparison between *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ datasets

  After characterization of *Syngap*$^{+/-}$ dataset, a comparative analysis was carried out with the previously published *Fmr1*$^{-/y}$ dataset. Gene set and correlation analysis revealed a significant inverse correlation between the two datasets (Figure 3). At the single gene level, genes with opposite changes were found to be implicated in autism and synaptic plasticity and could represent potential targets for subsequent validation.
  In addition, FMRP targets expression (FMRP is estimated to bind over 800 mRNA targets and repress the highest part (Darnel et al., 2011)) in *Syngap*$^{+/-}$ CA1 TRAP and total RNA fractions revealed a significant shift toward upregulation in the CA1 TRAP fraction of *Syngap*$^{+/-}$ dataset opposite to the downregulation observed in *Fmr1*$^{-/y}$ dataset (Figure 4A).
  The Input fraction instead showed a shift towards downregulation in both datasets. The same was observed when analyzing the SFARI target expression in both datasets (these are genes implicated with autism risk collected in the SFARI database) (Figure 4B).

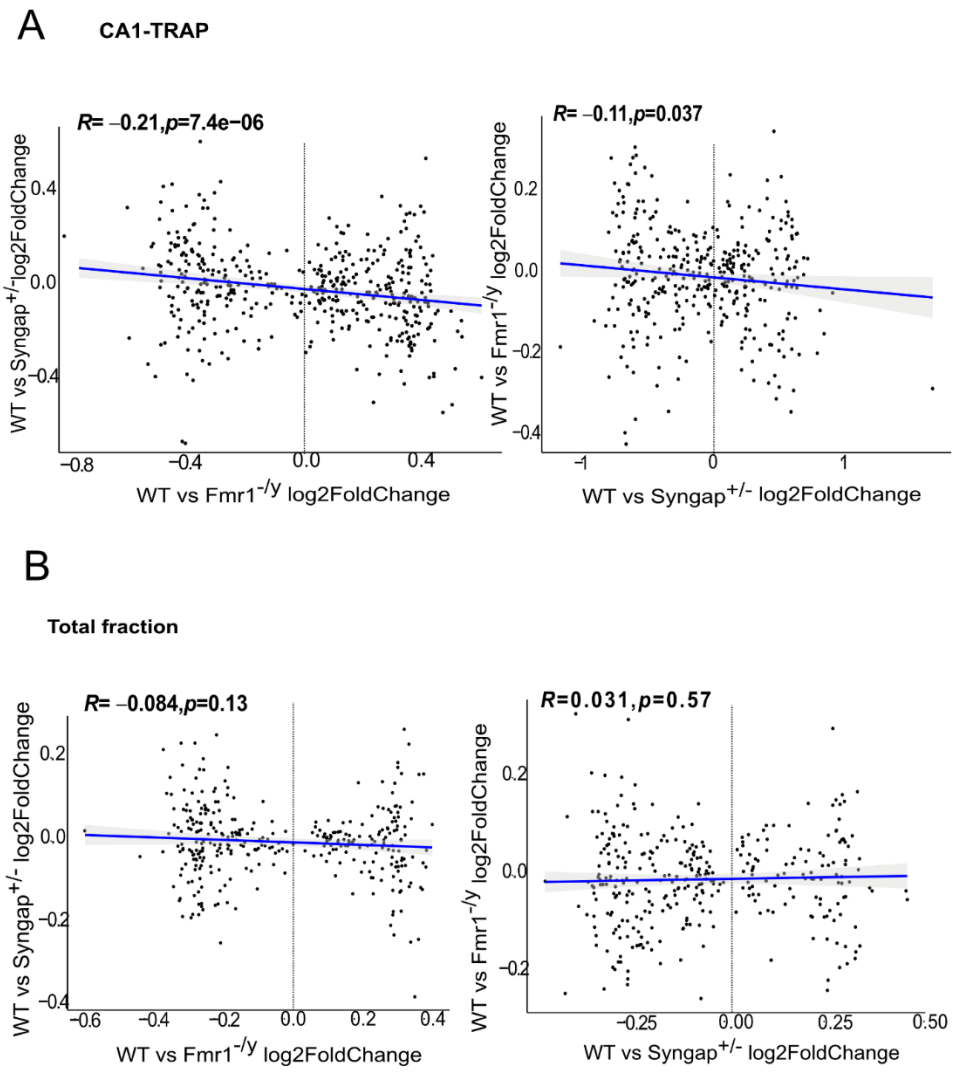


Figure 3. A) Correlation analysis between WT/*Syngap*$^{+/-}$ CA1 fraction and WT/*Fmr1*$^{-/y}$ CA1 fraction (left y vs x axis) and between WT/*Fmr1*$^{-/y}$ CA1 dataset and WT/*Syngap*$^{+/-}$ CA1 (right y vs x axis) show significant negative correlation. B) Correlation analysis between WT/*Syngap*$^{+/-}$ Total fraction and WT/*Fmr1*$^{-/y}$ Total fraction dataset (left y vs x axis) and between WT/*Fmr1*$^{-/y}$ Total dataset and WT/*Syngap*$^{+/-}$ Total (right y vs x axis) show any significant positive or negative correlation.

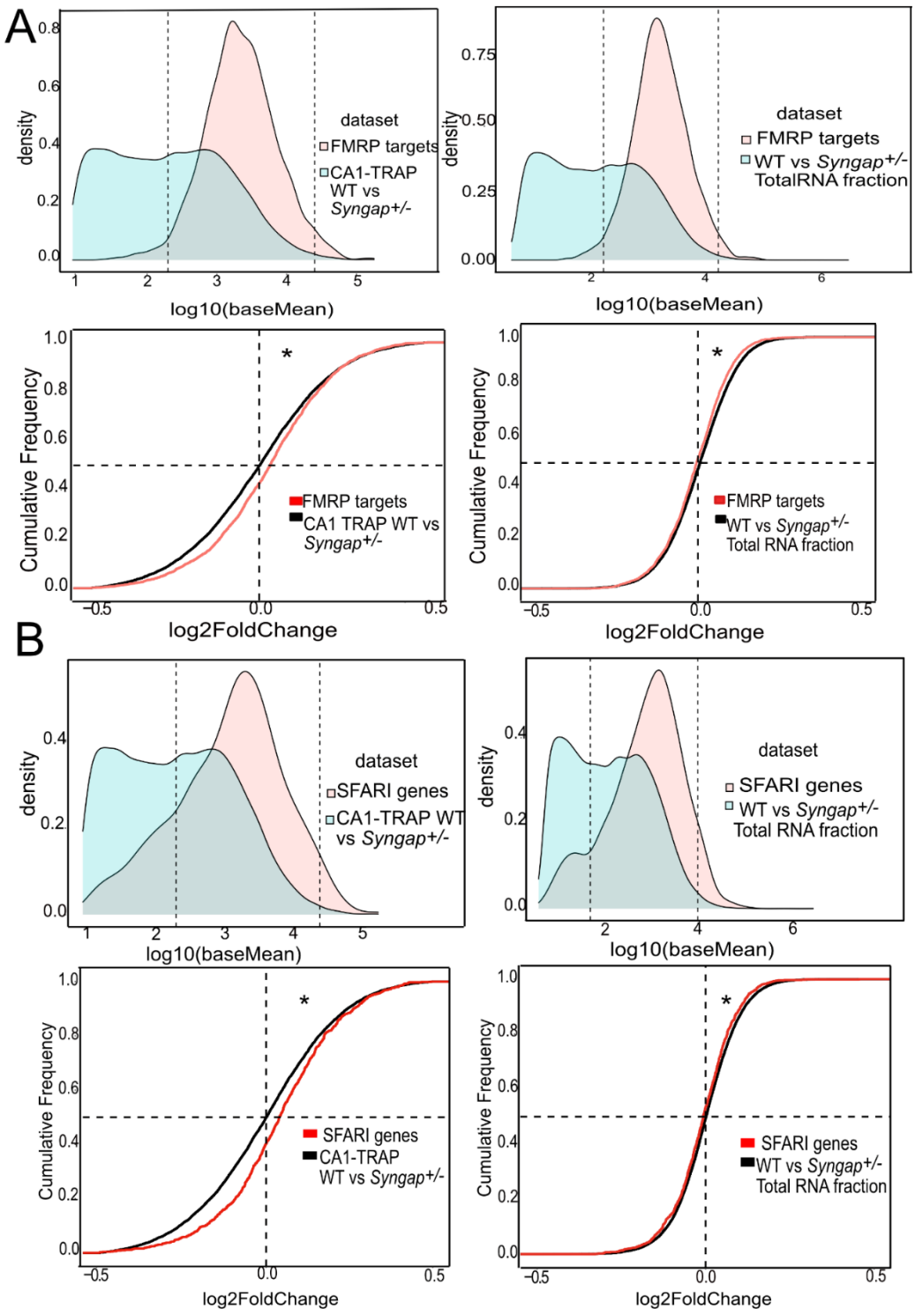


Figure 4. A) FMRP targets were compared to differentially expressed (total) genes with the same level of abundance (normalized count between $10^{2.2}$ and $10^{4.2}$ for *Syngap*$^{+/-}$ CA1-TRAP and $10^{2.3}$ and $10^{4.4}$ for *Syngap*$^{+/-}$ Total fraction). A cumulative distribution of FMRP targets shows a significant shift toward upregulation when compared to the distribution of total differentially expressed genes with the same level of abundance in *Syngap*$^{+/-}$ CA1-TRAP and towards downregulation in *Syngap*$^{+/-}$ Input fraction (K-S test, D = 0.081643, p-value = 2.773e-12; D = 0.040703, p-value = 0.00229). B) SFARI targets were compared to differentially expressed (total) genes with the same level of abundance (normalized count between $10^{1.7}$ and $10^{4.4}$ for *Syngap*$^{+/-}$ CA1-TRAP and $10^{2.3}$ and $10^{4.4}$ for *Syngap*$^{+/-}$ Total fraction). Cumulative distribution of SFARI targets shows a significant shift toward upregulation when compared to the distribution of total differentially expressed genes with the same level of abundance in *Syngap*$^{+/-}$ CA1-TRAP and a shift towards downregulation in *Syngap*$^{+/-}$ Input fraction (K-S test, D= 0.12727, p-value = 3.218e-11 ; D = 0.053061, p-value = 2.778e-05).

- Validation of differentially regulated genes of the *Syngap*$^{+/-}$ dataset

  Syngap$^{+/-}$ dataset underwent further validation in order to confirm what the analysis pipeline detected. More specifically, TRAP-sequencing was associated with qPCR analysis to validate specific targets. These were picked from the downregulated and upregulated lists of genes. The cutoff was always set at $p<0.01$ and genes were selected also based on the abundance level (mean Count) and on the log2fold change ($\geq\pm0.5$) in order to select for the most differentially regulated. More specifically Nf1 (neurofibromin 1), Xpo5(exportin5) and Paf1 (RNA polymerase II complex component) were selected from the upregulated genes and Cplx1(complexin 1), Lynx1 (Ly6/ neurotoxin 1), Nnat (neuronatin), Pianp(PILR alpha associated neural protein) were detected from the downregulated. Some of these genes such as Nf1, Nnat, Pianp are autism-risk associated genes as well (Mbarek et al., 1999; Oyang et al., 2011; Winkler et al., 2020). Primers were designed with NCBI Primer Tool. 5 WT/*Syngap*$^{+/-}$ pairs were TRAP sequenced and extracted RNA was used to generate cDNA in order to validate these genes through means of qPCR validation. Statistical analysis (t-test) did not show significant upregulation or downregulation for any of the targets. Plots of single genes mean counts showing expression of the selected genes in the 4 pairs included in the original sequencing experiment show variability across samples as well (Figure 5). This could possibly prevent from getting significant validation. In this optic, future analysis may include targets that are significantly different at more stringent cutoff levels such as $padj<0.1$ or include more samples. This is a first step towards validation that is then followed by exploring the functional role looking at the effect exerted by the specific targets on LTD measurements for example, being this phenotype exaggerated in these models of disease (Barnes et al., 2015).
  This could give new insights in the mechanisms that are disrupted in *Syngap*$^{+/-}$ models of disease and the current state of the art would be enriched by the comparison with evidence gathered in Fragile X syndrome, defining possible mechanisms that could converge and/or diverge in these conditions. This also gives a powerful means in order to identify possible targets that could be amenable to treatment strategies to a larger slice of the Autism related disorders' pie.

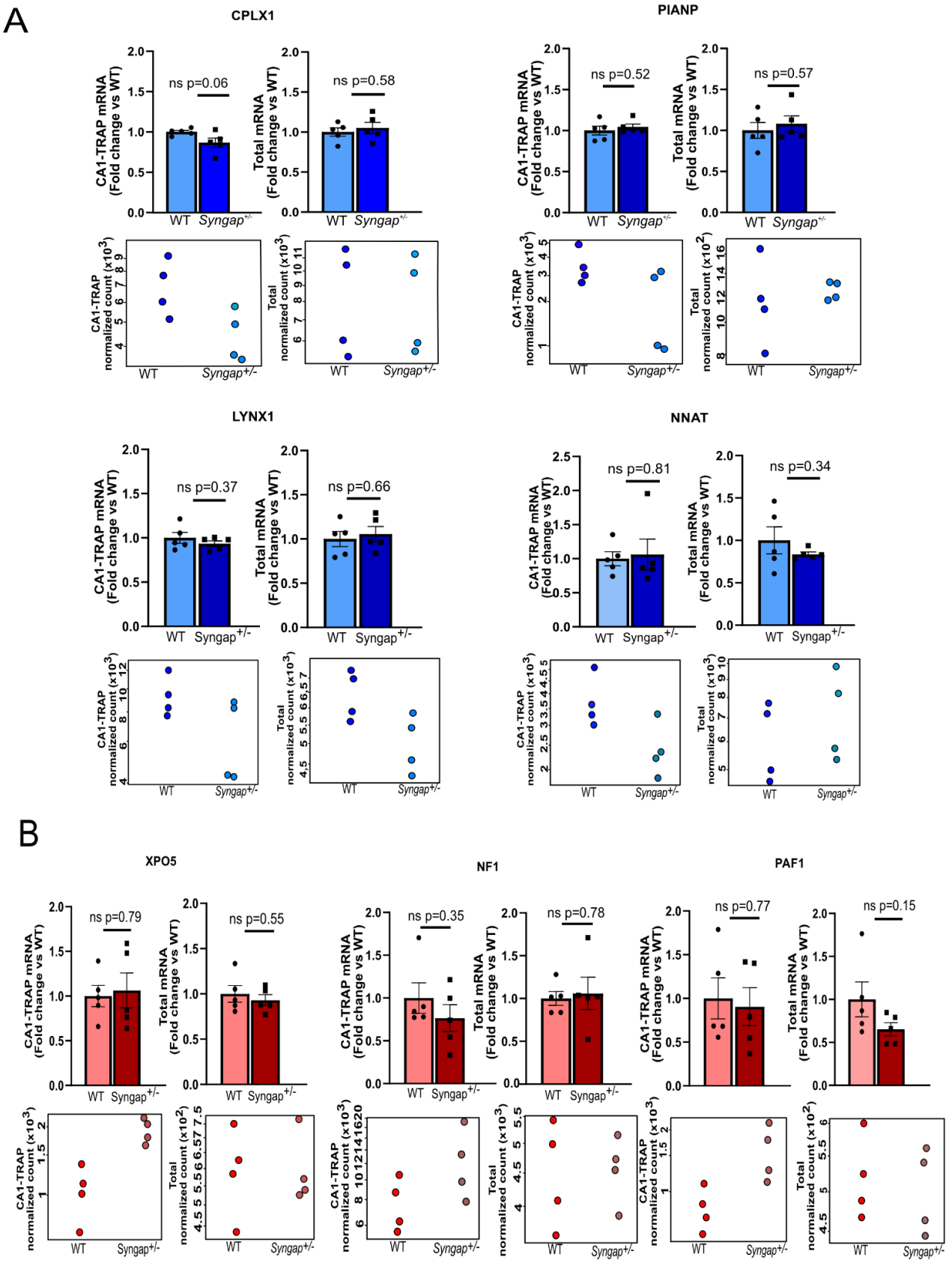


Figure 5. qPCR validation of selected targets from WT/Syngap$^{+/-}$ CA1 dataset. A) Targets in blue were selected from list of downregulated with cutoff at pvalue<0.01. They were ordered from highest to lowest for meanCounts and log2FoldChange. None of the above show significant changes (Mean,±SD; t-test). In addition, plot of normalized counts is shown for each target derived from DESeq analysis, marking high variability B) same as above for upregulated targets in red.

2: Determine differential translation mechanisms underlying learning in these models of disease

In the experiments described above CA1 hippocampal region was targeted since it is the structure of the brain critical for learning and memory (Bird and Burgess 2008; Jarrard 1993) and relies specifically on de novo protein synthesis(Fischer et al. 2004), processes both disrupted in these disorders. Impaired spatial memory and context recognition are observed in *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ models of disease (Bostrom et al. 2016; Clement et al. 2012; Komiyama et al. 2002) as exemplificative of disrupted hippocampal function. However, cued and contextual fear conditioning tasks and social behavioural tasks performed in these models revealed additional deficits in learned fear and alteration in social learning (Guo et al. 2009; Liu and Smith 2009) suggesting functional impairments in other structural regions of the brain involved in the processing of information related to fear and emotion (Amygdala) and regulation of social behaviour (Prefrontal Cortex) found both disrupted in ASD (Amaral and Corbett 2003; Bicks et al. 2015; Schumann et al. 2004).

Evidence of the impact of differential translation during learning in *Fmr1*$^{-/y}$ and *Syngap*$^{+/-}$ models of disease is still poor as well as its contextualization across diverse brain regions thought to be involved in this complex process. This can help create a more comprehensive picture of what goes unfunctional in these circuits. In addition to CA1 region I will explore the amygdala brain region, more precisely Basolateral Amygdala since it is highly involved in the association of fear memories. Moreover, Fragile X Syndrome and Syngap1 hapolinsufficiency disorders presents with phenotypes such as anxiety, exaggerated fear and gaze avoidance that are specific for amygdala function. LTP was also found deficient in BLA principal neurons from *Fmr1*$^{-/y}$ mouse model (Suvrathan et al., 2010). The molecular characteristics that determine amygdala functions are still unexplored and possibly this will result in unravelling the molecular mechanisms of function of this brain region as well as analyzing its contribution in autism-like disorders.

In order to study this brain region, I will use a Rasgrp1 PO1 BAC-Cre (MGI:5051790) mouse line, which exhibits robust expression in this area as validated by Immunofluorescence. This line has been crossed with a TRAP Ribotag line already established in the lab. The latter carries a ribosomal protein gene (Rpl22) with a floxed C-terminal exon followed by an identical exon tagged with hemagglutinin (HA). When RiboTag is crossed to a mouse expressing Rasgrp1 driven Cre recombinase, expression of the epitope-tagged protein is activated in the BLA (Figure 6, A).

In the establishment of this line, however I targeted some recombination occurring from the Cre line into the Ribotag leading to ectopic expression in other non-specific brain regions and this was spotted by means of Immunofluorescence and Genotyping (Figure 6, B). Subsequently, this delayed the crossing with *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ mouse lines. The main experiments will consist of TRAP sequencing from BLA specific population and follow a path similar to that used for the CA1 dataset in terms of analysis and validation. However, Rasgrp-CRE$^{+/-}$/Ribotag$^{+/-}$ mice were perfused to look at the expression of HA antibody in the BLA region. In addition, Rspo2+ neuronal population was targeted to be present in 82.5% of HA+ cells. (Figure 6, D). Further targets thought to mark BLA population will be tested, including markers of excitatory neurons (the major population in the BLA).

In addition to a first naïve interrogation of protein translation in these models of disease, TRAP-seq will then be applied to underpin the molecular mechanisms underlying protein synthesis in the context of learning and memory and this will take advantage of the specific behavioural paradigm of fear conditioning.

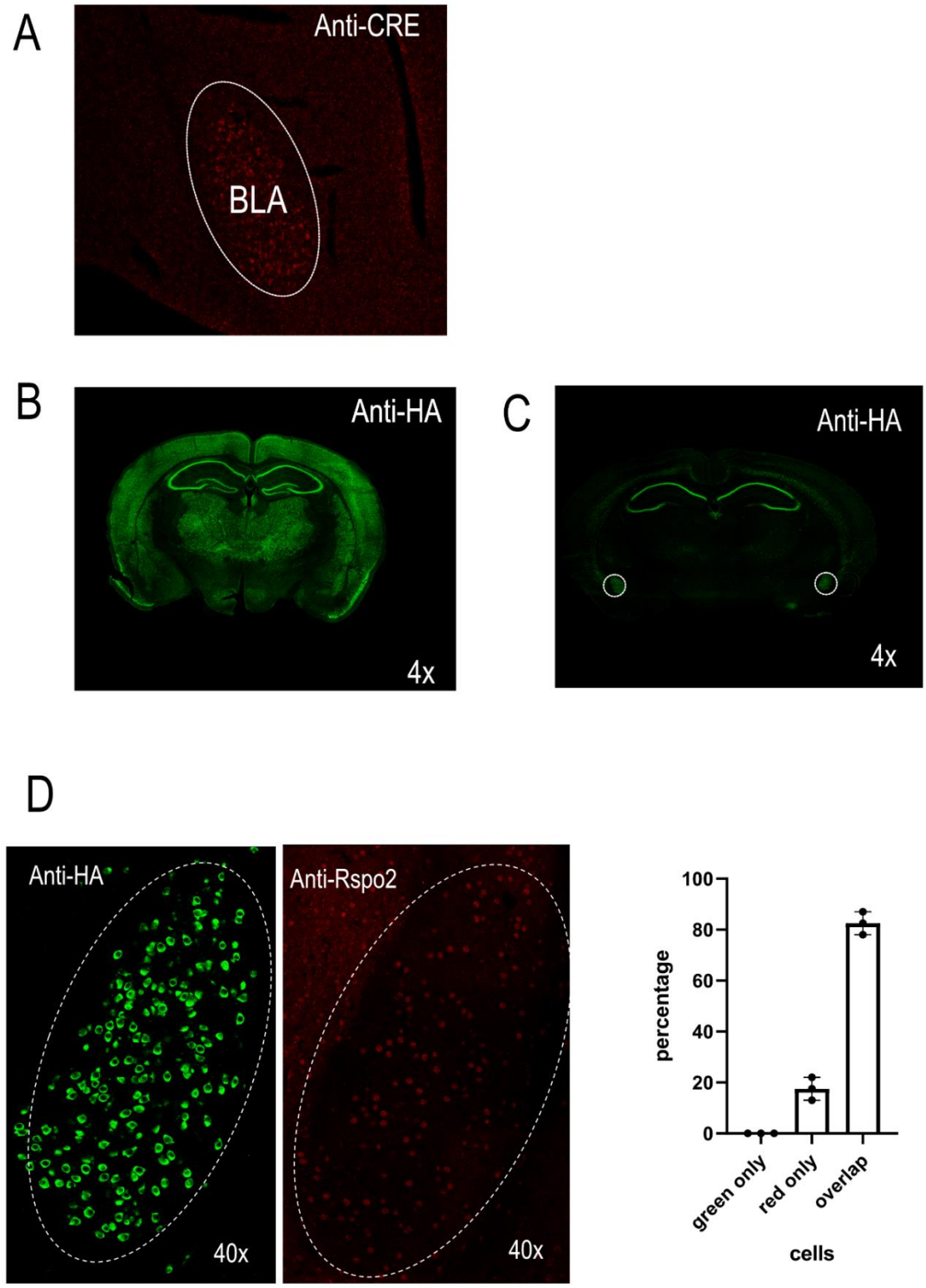


Figure 6. A) Rasgrp-CRE line was first validated staining Rasgrp-CRE mouse brains with Anti-CRE(1:100) antibody which showed high expression in the BLA region of Amygdala. Subsequently Rasgrp-CRE mice were crossed with Ribotag mice and the progeny was marked with HA-antibody which should be specific for BLA neurons. Figure B shows a recombination example with ectopic expression of HA while figure C shows how the expression should look like with BLA regions highlighted in white circles. Figure D shows quantification of Rspo2+ cells (Anti-Rspo2 antibody 1:500) and colocalization with BLA-specfic HA+ neurons (Anti-HA antibody 1:500) across multiple sections (2 sections per mouse were considered as replicate) taken from three mice . Cell counter plugin in Image J was used to count HA+cells(green) excluding out of focus cells (well-defined perinuclear stain was considered as criterium to distinguish between in focus and out of focus cells) and Rspo2+cells (red). The graph represents the percentage of Green only cells(HA+) , Red only (Rspo2+) cells and Overlapping cells(Rspo2+ and HA+). 82.5% of Rspo2+ cells colocalize with HA+ cells, while 17.5 % are Rspo2+ cells only.

Progress and expected results

Together these experiments aim to identify a population of commonly mistranslating mRNAs in the *Fmr1-/y* and *Syngap+/-* models and possibly help to identify targeted therapeutics. An opposite outcome such as identification of non-overlapping targets will still be key to delineate further hypothesis, such as shared dysregulation in translation not necessarily leading to pathology through a specific set of transcripts. Moreover, looking at misregulated targets individually for each disorder, may still point out at interesting targets.

Exploration of different brain regions could also identify diverse pools of mistranslated genes which could define a specific area to be more or less impacted by the changes in protein synthesis.

In addition, the impact of exaggerated protein translation would be interrogated also in relationship to different circuits during fear learning, and help identify the disrupted mechanisms that define pathogenicity in these two models of disease. More generally, it will also convey important and novel information regarding the processing of fearful memory.

Importantly, regardless of outcome, this study will create cell type-specific TRAPseq datasets from both *Fmr1-/y* and *Syngap+/-* mouse models and represent a shareable resource.

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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 Thalamic Reticular Nucleus (TRN) is a shell of GABAergic neurons surrounding the thalamus, modulating sensory processing, attention, thalamocortical sleep rhythms, and sleep-dependent memory consolidation[1]. It is also recognized as a functional hub for thalamocortical network dysfunction in neurodevelopmental disorders, such as schizophrenia and autism[2]. Evidence from the literature indicates that TRN neurons express the Corticotropin-Releasing Hormone Receptor 1 (CRHR1)[3] that binds Corticotropin-Releasing Hormone (CRH), a key modulator of the stress response. However, whether and how stress-induced CRH release affects TRN function has not been explored.

The project aims to investigate the following objectives in mice:

1. Quantification of the expression of CRHR1 in TRN neurons.

2. Characterization of the effects of CRH on TRN neurons excitability.

3. Identification of the source of endogenous CRH projections to the TRN.

4. Investigation of the impact of CRH on TRN-related sleep rhythms.

5. Characterization of the effect of CHR on TNR gene expression.

Results obtained for objectives 1-2-3:

1. Quantification of the expression of CRHR1 in TRN neurons.

Highly specific quantitative *in situ* hybridization of mRNA confirmed the expression of CRHR1 in TRN neurons (1.61×10^{-3} a.u.)(fig.1D,F). Additionally, the second CRH receptor, CRHR2, was also found to have a low-density expression (0.31×10^{-3} a.u.)(fig.1E,F) in these neurons. Sex differences in the levels of CRH receptor expression will be further investigated as well as cell-type specificity in different TRN neuronal subpopulations (parvalbumin vs somatostatin-expressing neurons) along with its correlation with trait anxiety.

2. Characterization of the effects of CRH on TRN neurons excitability.

To assess the impact of CRH on the TRN neuron firing modalities, that are relevant for sleep rhythmogenesis (low-threshold Ca^{2+} bursts vs. tonic firing – fig.2A), *ex-vivo* patch-clamp recordings were performed in acute slices. Bath application of CRH (500nM) decreased the number of bursts by 22.3±8.4% (p=.0211, n=10) and increased tonic firing frequency by 11.5±4% (p=.0002, n=10)(fig.2B). The CRHR1 antagonist (NBI39565, 3µM) completely abolished the CRH induced modality shift (bursts increased by 10.6±7.2% and tonic firing decreased by 5.5±5.9%, n=8)(fig.2C). This suggests that a CRHR1-mediated intracellular signaling cascade affects intrinsic ionic mechanisms that govern TRN excitability. As TRN repetitive bursting is generated by the interplay between low-threshold voltage-activated calcium channels (T-channels) and small conductance calcium-activated potassium channel 2 (SK2 channels), the effects of CRH were investigated on composite T-currents and SK2-currents. Preliminary data suggest CRH preferentially impacts the SK2 component, the major contributor to the burst afterhyperpolarization (AHP) that allows repetitive bursting, and not the T-currents. Isolated calcium recordings using cesium to block potassium conductances confirmed that CRH has no significant effect on T-currents elicited by somatic depolarization. Further experiments will be conducted to better understand how the CRH receptors contribute to modulating the ionic mechanisms responsible for the AHP, in order to conclude how CRH shifts the TRN's firing modality.

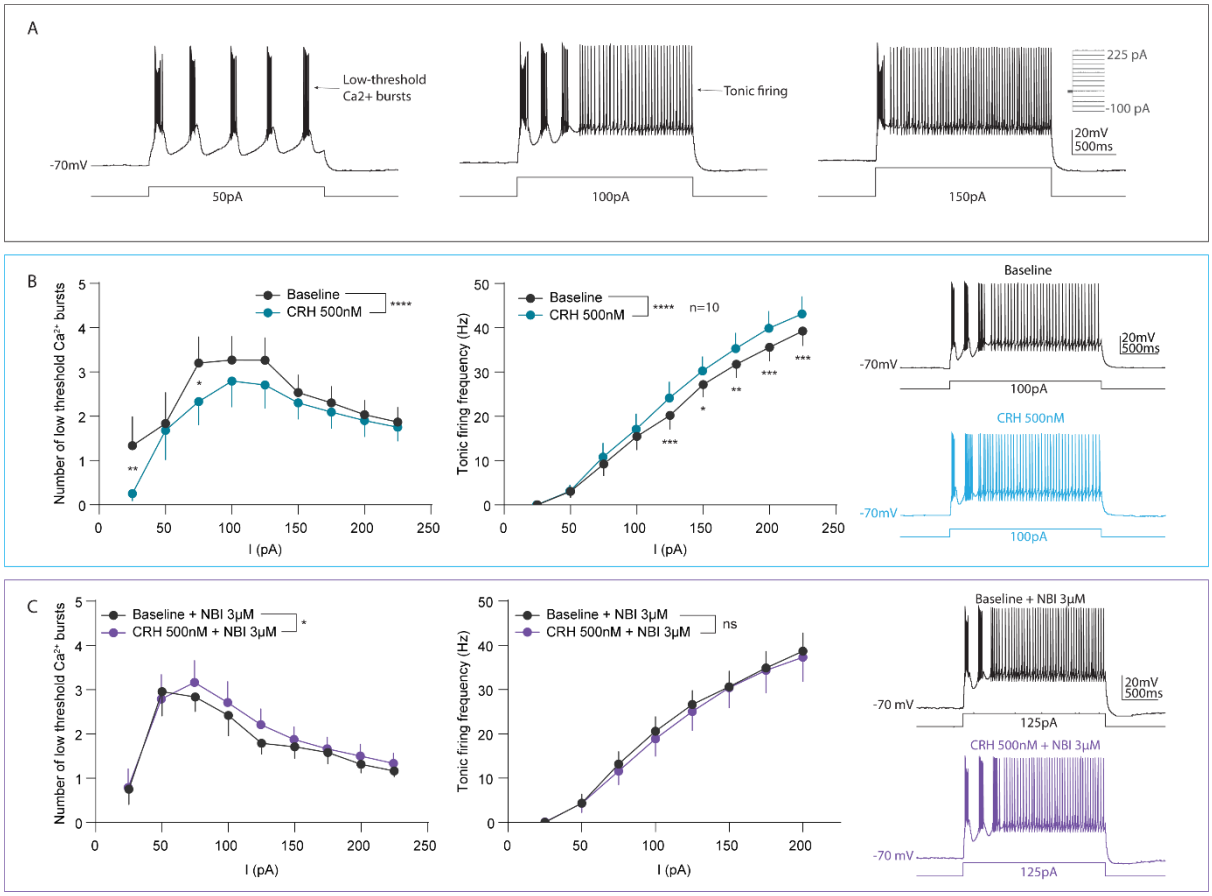


Figure 2 – CRH shifts the TRN neurons firing modality. (A) Example traces of current-clamp recordings showing low-threshold bursting activity at low depolarizations followed by the appearance of tonic firing with increased current injection. (B) Significant decrease in the number of bursts ****p<0.0001; post hoc Sidak: 75pA *p=.0211; 25pA **p=.0016; n =10. Significant increase in tonic firing frequency with CRH 500nM ****p<0.0001; post hoc Sidak: 225pA ***p=.0009; 200pA ***p=.0002; 175pA **p=.0034; 150pA *p=.0158; 125pA ***p=.0008; n=10. Example traces (right side) comparing the effect of CRH in the same cell before and after CRH application. (C) CRHR1 inhibition abolishes the effect of CRH, the number of bursts tend to increase *p=0.0109, n=8, while tonic firing is unaffected, ns. Example traces (right side) in the same cell exposed to CRH 500nM in the presence of the selective CRHR1 antagonist NBI39565. All error bars shown are S.E.M.. All statistical tests are 2way RM ANOVA.

Transgenic mice, expressing ChannelRhodopsin2 (ChR2) and tdTomato in all CRH producing neurons (Ai27DxCRH_IRES_Cre), were used to investigate the effect of endogenous CRH release on TRN excitability. Using *ex-vivo* patch-clamp recordings, a train stimulation of 20Hz with blue light (470nM) that activates ChR2 in axon terminals contacting TRN neurons, mimicked the effect of CRH bath application on the TRN firing modalities. The number of bursts decreased by 22.2±6.7% (p=.01, n=8) and tonic firing increased by 21.5±8.9% (p=.04, n=6). Preliminary data with the CRHR1 antagonist suggest that this effect is mediated by endogenous CRH, released at TRN synaptic terminals. Future experiments will be done to further confirm receptor specificity. The optogenetic protocol tested here *ex-vivo* will be used *in-vivo* during polysomnographic recordings to investigate the effect of endogenous CRH release on sleep-rhythms and arousal threshold (Objective 4).

3. Identification of the source of endogenous CRH projections to the TRN.

In order to identify the sources of endogenous CRH projections to the TRN, transgenic mice expressing Cre in all CRH producing neurons (CRH_IRES_Cre) were stereotaxically injected with a Cre-dependent retrograde Adeno-Associated Virus (AAV). The CRH sources to the TRN appear to be heterogenous, with different cortical and subcortical areas, contacting different regions of the TRN. The middle and posterior regions of the TRN (that have been shown to contribute most to sleep spindle rhythmogenesis[4]) receive CRH inputs from layer 6 of cortical areas: primary sensory cortex (S1), primary and secondary visual cortices (V1, V2), entorhinal cortex (LEnt, MEnt) and ectorhinal cortex (Ect)(fig.3B-C). They also receive subcortical CRH inputs from thalamic nuclei, the ventral-lateral geniculate nucleus (VLG) and the medial geniculate nucleus (MGM)(fig.3D-E) as well as extra-thalamic such as the zona incerta (ZI) and the baso-lateral amygdala (BLA)(fig.3F-G). No projections from the paraventricular nucleus of the hypothalamus (PVN) were found (fig.3H), suggesting the TRN is modulated by a central CRH system and not the hypothalamic-pituitary-adrenal (HPA) axis. Further confirmation of these regions is in process as well as uncovering new potential sources of CRH to the TRN. The final aim is to manipulate the relevant CRH sources to the TRN using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)(Objective 4).

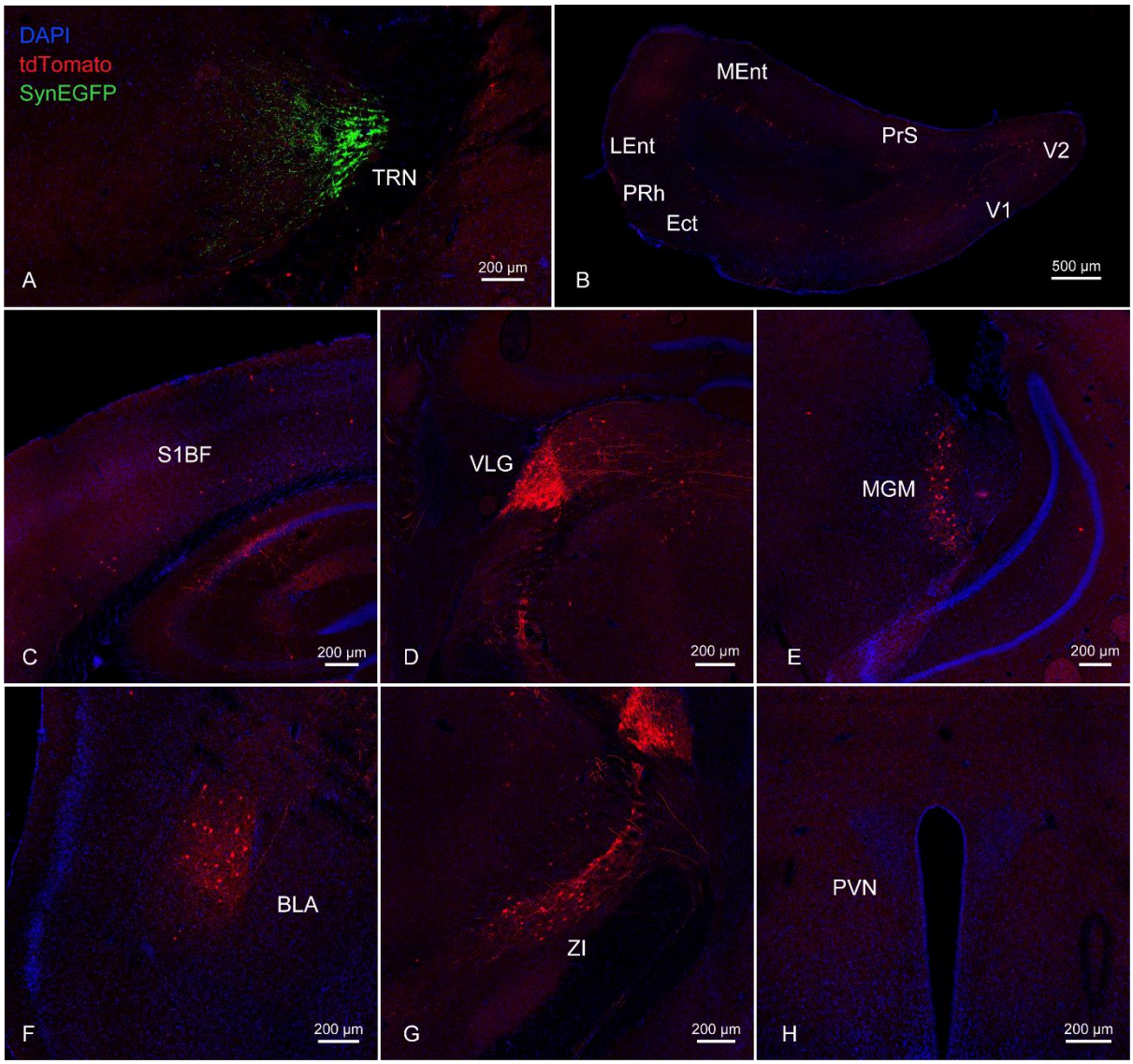


Figure 3 – Source of endogenous CRH projections to the TRN. Confocal images showing nuclei stained with DAPI (blue), CRH projections to the TRN virally-labelled with tdTomato (red) and synapsin EGFP (green) indicates the injection site. (**A**) Injection site targeting the somatosensory and posterior regions of the TRN. (**B, C**) Cortical CRH projections to the TRN. (**D,E**) Thalamic CRH projections to the TRN. (**F,G**) Other subcortical CRH projections to the TRN. (**H**) The PVN showing no CRH projections to the TRN.

Outlook for objectives 4-5:

4. Investigation of the impact of CRH on TRN-related sleep rhythms.

Sleep disturbances are a common occurrence in most psychiatric disorders, and in humans stress is known to induce sleep dysregulation and insomnia[5], that can predispose, contribute to and possibly cause symptoms associated with neuropsychiatric illnesses[6]. Normalizing sleep patterns is thought to have a beneficial impact on the trajectory of symptomatology and aid in the treatment of such disorders[7]. During sleep, the TRN's bursting activity is a critical component that engages the thalamo-cortical loop, which results in oscillations within the sigma power band (10-15Hz), known as sleep spindles[8]. The density of sleep spindles is affected in neurodevelopmental disorders[9], however how or if stress modulates sleep spindles is unknown.

This objective aims to investigate the impact of stress through CRH on sleep rhythms, particularly on the TRN-related sleep spindles, using *in-vivo* polysomnographic recordings combined with optogenetic manipulation of CRH afferents to the TRN. The CRH-induced decrease in the number of bursts found *ex-vivo* indicates that the CRH system impinges on the generating mechanism of sleep spindles[10], thus supporting the relevance of this aim. Furthermore, in case CRH is found to affect sleep spindles *in-vivo*, its impact on sleep spindle-related functions, such as sleep-dependent memory consolidation, will be assessed using specific behavioural paradigms (e.g.: for contextual memory).

Additionally, as part of the secondment at Lundbeck, the sources of endogenous CRH projections to the TRN, found in objective 3, will be manipulated chemogenetically with DREADDs in order to further inspect the potential effect of CRH on sleep spindles. Single unit recordings from the TRN combined with local field potential (LFP) recordings from cortical areas will be used to evaluate the impact of manipulating different CRH projections to the TRN during sleep and wakefulness. Furthermore, pharmacological manipulation of SK channels will be possible at Lundbeck to assess their contribution.

CRH is also known as a regulator of arousal[11,12], therefore, using *in-vivo* fiber-photometry with the aid of a CRH sensor (GRAB-CRH), potential endogenous CRH release will be assessed in response to environmental disturbances (e.g.: loud noise) during sleep to investigate its impact on arousal threshold.

5. Characterization of the effect of CRH on TRN gene expression.

During all stages of the *in-vivo* experiments, the control animals will be compared to the experimentally manipulated animals in terms of gene expression in the TRN (e.g.: Cacna1l, Kcnn2, Grm3, Ptchd1, Crhr1, Crhr2). Genes such as the Cacna1i (Cav3.3) or Kcnn2 (SK2) are involved in the ionic mechanisms responsible for the bursting of the TRN neurons but also represent risk genes for schizophrenia and autism[2]. Screening for genes across experimental manipulations that induce either acute (e.g. optogenetic stimulation) or prolonged stress (e.g. DREADDs activation) will allow us to evaluate if CRH induces changes in their expression that may be linked to individual vulnerability to stress or lead to increased risk to develop psychiatric disorders.

In summary, CRH shifts the TRN's firing modality, decreasing the number of bursts, that are the generating mechanism of sleep spindles within the thalamo-cortical loop. The *ex-vivo* data support the future aim of the project to study the effect of CRH *in-vivo* on sleep rhythms. Relevant CRH afferents to the TRN originating in cortical (S1, Ect, Ent) and subcortical areas (VLG, BLA, ZI) will be manipulated using DREADDs to understand their contribution to TRN function. Screening of genes related to stress and psychiatric disorders will help to uncover potential links between individual vulnerability to stress, and stress-induced sleep disturbances.

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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

Summary description of the project and objectives

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[1,2]. *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[1]. Alterations in social behaviour, especially social interaction and communication impairments, are one of the major traits in *SYNGAP1* haploinsufficiency and in general in ASDs[1,4]. 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[5–7]. However, ethologically, male mice social interactions are limited and predominantly antagonistic since they live in single-male territorial structures [8]. In contrast, rats live in complex hierarchical colonies with more dynamic dominance structures and exhibit complex social behaviours such as social play[9]. 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[3]. 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[3,10]. The calcium dependent lipid binding or C2 domain is essential for the normal SynGAP Rap-GTPase activity in addition to mediating calcium binding[11]. 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[12,13].

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[1]. 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 [14]. 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[15]. However, little is known about the specific circuit deficits causing social interaction alterations in *SYNGAP1* haploinsufficiency.

The main goals of this project are:

- To characterize the impairments in social interactions in both, *Syngap1* $^{+/\Delta GAP}$ and *Syngap1* $^{+/-}$ rat models
- To define the brain regions and circuit deficits linked to social alterations by using *in vivo* electrophysiology

Description of the work performed since the beginning of the project

The work carried out so far has mainly focused on the first aim, studying alteration in social interactions in two novel rat models of *SYNGAP1* haploinsufficiency using the following approaches. The experiments have been conducted on young male adults to detect the alterations after the developing period. Once social alterations are detected and time-permitting I aim to also look at female subjects. Moreover, I intend to study younger stages in development in order to find out critical periods[16,17].

1) <u>Study of social interactions on a naturalistic environment: The Habitat</u>

The Habitat idea was initially perceived through a collaboration with Dr Oliver Hardt. It consists of 16 modules interconnected by tubes, ramps, ropes, where a group up to 54 rats can freely explore and interact to each other. This habitat allows a more natural and enriched behaviour in addition to the possibility of free social interactions with multiple individuals free of any experimenter intervention. It aims to simulate an ethologically relevant habitat consisting of a burrow system where rats live in colonies of more than 150 individuals[9]. For the experiments described in Results section, rats were habituated to the habitat by cage and for 2 consecutive days, 30 min every cage. All cages were then introduced together for 3 consecutive days for 4 hours each day.

Social recognition in rats plays an important role for mate choice and maintaining social hierarchy and social dominance[9,18,19]. Characterizing social dominance in *Syngap1* models might contribute to a better understanding of their social alterations. I analysed the scoring of tube encounters in a cohort

of 8 Wildtype, 8 *Syngap1*$^{+/\Delta GAP}$ and 8 *Syngap1*$^{+/-}$ rats to address dominance behaviours[18] in the context of The Habitat.

I have also been developing methods for scoring behaviours with a positive valence such as allogrooming and self-grooming. Allogrooming is the grooming of one animal by another by licking or gently nibbling one part of the conspecific body. Alternatively, it may be considered an altruistic behaviour, it may enhance sociability and its reciprocity[20]. Regarding self-grooming, it may be a valuable measure of repetitive behaviours[21]. Since the scoring is still in process, I am unable to show the results of this study at the date of this report but will be updated on following reports.

2) Study of social interaction, habituation, and social discrimination to a conspecific by dyads: The habituation-dishabituation test

When assessing how rats habituate to a conspecific *(defined as a rat from the same strain)* and discriminate a novel stimulus rat from a familiar one, a less complex more controlled approach than the habitat is required. I have implemented a new protocol of habituation-dishabituation to a conspecific based on previous approaches by Bariselli et al., 2018 and Ferguson, Young, & Insel, 2002. This paradigm allows me to study how *Syngap1*$^{+/-}$ and *Syngap1*$^{+/\Delta GAP}$ rats habituate and discriminate between conspecifics, how conspecifics habituate and behave to *Syngap1*$^{+/-}$ and *Syngap1*$^{+/\Delta GAP}$ rats and the potential differences between pairs of different genotypes when interacting. The protocol design, process of optimization of this social task and the obtained results are discussed in *Results* section.

3) Study of olfactory function: Detection, habituation, and discrimination to non-social and social odours

For most non-primate mammals, olfaction is the dominant sense used to discriminate between conspecifics[24] . The olfactory system plays an important role in conspecific recognition by detecting each subjects' unique secreted chemical signals, such as pheromones[25]. In order to determine if the social deficits observed in the *Syngap1* rats might be due to alterations in olfaction, I designed and implemented a behavioural protocol to test if *Syngap1*$^{+/-}$ and *Syngap1*$^{+/\Delta GAP}$ rats are able to detect, habituate and discriminate between different social and non-social odours. To test whether they could discriminate between odours of two different individuals, a second test was designed using urine as social odorant of a single individual. Urine (from male conspecifics) was selected as an odorant since it is one of the substances where chemo signals are secreted[26], its accessibility. Moreover, studies in rodents showed they could discriminate the urine of two different individuals[27].

Theses protocols were designed as a control for the habituation-dishabituation test (see above). As so, every cohort used for the habituation dishabituation test was also used for the olfactory tests. The protocol design and consequent implementations and the results obtained from each of them are discussed in *Results*.

Description of the main results achieved so far

1) Assessing tube encounters in the Habitat

Tube encounters were defined as the encounter of two rats face to face in a tube. The 'winner' is the rat which pushed the other one out of the tube and is considered more dominant. The preliminary results suggest that *Syngap1*$^{+/-}$ rats tend to have more tube encounters than wildtypes (Figure 1C and Figure 1E). Moreover, on the first day in the habitat, the most frequent types of tube encounters occur

between *Syngap1*$^{+/-}$ rats with wildtypes or *Syngap1*$^{+/-}$ with *Syngap1*$^{+/\Delta GAP}$ rats (Figure 1D). Comparing the total number of wins over a different genotype, the data suggests that wildtype might win more encounters than either of the mutants.

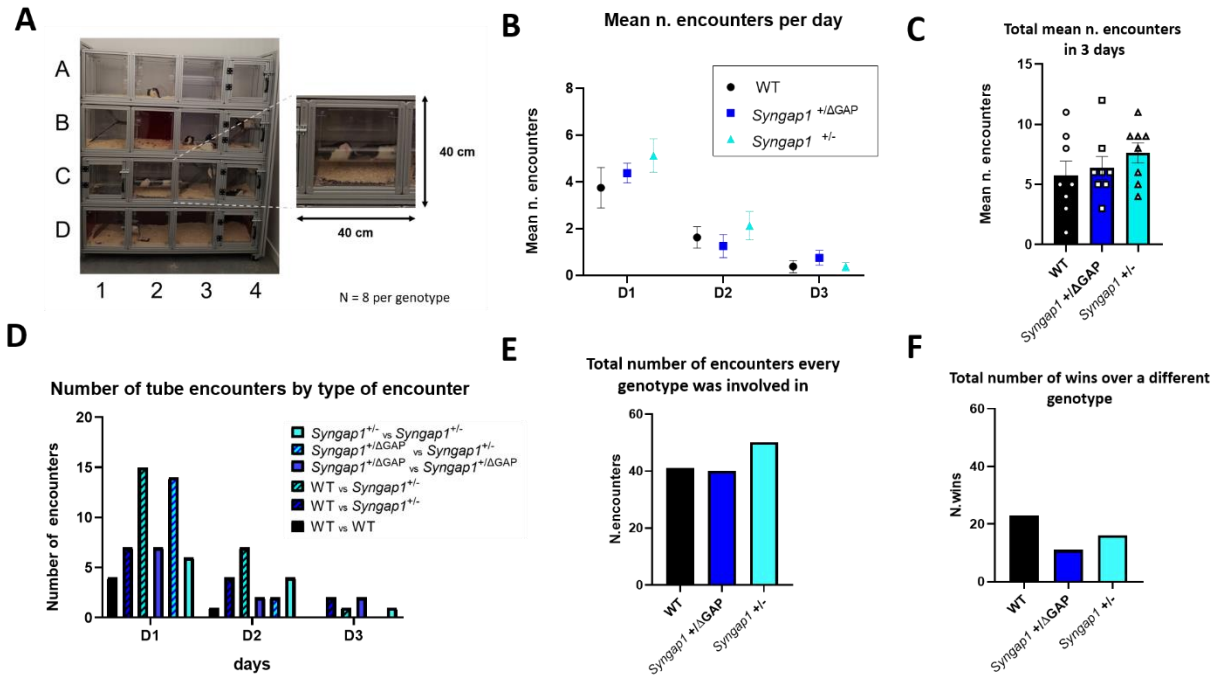


Figure 1. Representation of tube encounters in *Syngap1*$^{+/\Delta GAP}$ and *Syngap1*$^{+/-}$ animals. A) Picture showing the layout of the Habitat. There are 4 floors (A-B) and four columns (1-4) making a total of 16 modules. The tube encounters occur in long tubes connecting two different modules as shown in the inset. **B)** Mean number of encounters ± standard error occurred on every experimental day. The mean decreases with days on three conditions. **C)** Mean number of encounters ± standard error in total of the three days. **D)** Total number of encounters by genotypic pair in the tube. **E)** Total number of encounters every genotype was involved in. **F)** Total number of wins of each genotype over a different one. N= 8 wildtype, 8 *Syngap1*$^{+/\Delta GAP}$ and 8 *Syngap1*$^{+/-}$ rats.

2) <u>Habituation-dishabituation to a conspecific</u>

Briefly, the initial version protocol consisted of two days of handling and one day of habituation to the arena (two exposures to the arena of 5 min each and placed in a black box between exposures) prior to the experimental day. The experiment consisted of introducing the subject rat for 10 min to the arena, after which a new and non-littermate stimulus rat (Stimulus 1) was introduced in the arena for 5 min. This step is repeated presenting the same initial conspecific (Stimulus 1) 3 more times to a total of 4 presentations. At the end of the 4 presentations Stimulus rat 1 is considered a familiar social stimulus. Following this, the subject rat is exposed to a different conspecific (Stimulus 2), new to the subject and un-related to both the first stimulus and the subject rat. Between exposures to conspecifics, both subject and stimulus rats were individually placed in a black box for 5 min. This version of the protocol was piloted using a wildtype cohort to test its efficacy. Wildtype rats show a gradual decrease in time spent on social exploration from exposures 1 to 4 and an increase in time spent in social exploration when exposed to the new conspecific (Stimulus 2/ exposure 5).

The first cohort of *Syngap1*$^{+/-}$ and *Syngap1*$^{+/\Delta GAP}$ rats (3 wildtype 3 *Syngap1*$^{+/\Delta GAP}$ and 3 *Syngap1*$^{+/-}$ subjects) and their respective stimuli rats (all wildtype and un-related) were subjected to the initial

protocol described as previously described. Although many social behaviours were scored, such as sniffing, following, avoiding, self-grooming, allogrooming, approaching and huddling. However, the most frequent in this paradigm was the total time sniffing the conspecific (social sniffing). In Figure 2A the sniffing time to the conspecific is plotted for both, the subject rats (3 wildtypes, 3 *Syngap1*$^{+/\Delta GAP}$ and 3 *Syngap1*$^{+/-}$) and for their respective stimuli rats (all wildtypes and non-related). In this preliminary data, we observe as both, wildtype subjects and their Stimuli 1 rats decrease their social sniffing decreases from exposure 1 to 4 (wildtype subjects mean sniffing on e1 of 124.99s ± 22.65 SEM and on e4 23.28s ± 6.9 SEM; Stimuli 1 mean sniffing on e1 of 75.84s ± 47.07 SEM and on e4 12.42s ± 5.51 SEM). Therefore, wildtype subjects and their Stimuli rats habituate to each other. Wildtype subjects also present an increase of sniffing when Stimulus 2 rat (new conspecific) is presented on exposure 5 (wildtype subjects mean sniffing on e5 of 73.03 ± 10.22SEM) and the Stimuli 2 rats also present an elevated time of sniffing (Stimuli 2 rats mean sniffing on e5 of 80.85s ± 2.9SEM). This result suggests wildtype subject increase their sniffing exploration of their conspecific when is a new one, and they discriminate it is a new one. Also, their Stimuli 2 rats recognize the subject rats as new as well.

Regarding *Syngap1*$^{+/\Delta GAP}$ subjects, the data suggest they habituate to the conspecific among exposures 1-4 since there is a gradual decrease on social sniffing among exposures (*Syngap1*$^{+/\Delta GAP}$ subjects social sniffing mean on e1 of 77.35s ± 20.33 SEM and on e4 of 22.79s ± 14.19 SEM). Moreover, their Stimuli 1 rats also habituate to them (Stimuli 1 rats social sniffing mean on e1 of 75.02s ± 3.4SEM and on e4 of 15.83s ± 4.8 SEM). When the new conspecific is presented on exposure 5 (Stimulus 2 rat), *Syngap1*$^{+/\Delta GAP}$ sniffing time increases (social sniffing mean on e5 of 38.67s ± 18.74 SEM). The mean value of social sniffing towards Stimuli 2 rats is 0.5 times the mean value observed in wildtype subject for exposure 5. Additionally, the mean value of social sniffing of Stimulus 2 rats presented to *Syngap1*$^{+/\Delta GAP}$ subjects appears to be 2.1 times higher than *Syngap1*$^{+/\Delta GAP}$ mean sniffing to them (Stimulus 2 rat sniffing mean, on e5 of 82.4s ± 21.60 SEM). In conclusion, although *Syngap1*$^{+/\Delta GAG}$ habituate to their conspecific, their increase interest on a new conspecific seems to be smaller than wildtype subjects and their new presented conspecific (Stimuli 2 rats).

Regarding *Syngap1*$^{+/-}$ subjects, a similar tendency than in *Syngap1*$^{+/\Delta GAP}$ subjects is observed (*Syngap1*$^{+/-}$ subjects mean sniffing values on e1: 71.16s ± 4.7 SEM; e4: 12.42 ± 5.5 SEM; e5:36.71s ± 23.96; Stimuli 1 rats mean sniffing values on e1: 79.73s ± 4.4 SEM; e4:25.63 ± 3.1 SEM and Stimuli 2 rats mean sniffing values on e5: 80.41s ± 5.7SEM). The mean sniffing time of *Syngap1*$^{+/-}$ subjects on exposure 5 was 0.5 times compared to wildtype subjects. The mean sniffing time of Stimulus 2 rats on e5 was 2.1 times compared to the *Syngap1*$^{+/-}$ subjects they interacted with. Altogether, both *Syngap1*$^{+/\Delta GAP}$ and *Syngap1*$^{+/-}$ subjects although they increase their social sniffing to a new conspecific, their interest appears to be half the mean value compared to wildtype subjects or the Stimuli 2 rats they interact with. However, more animals are needed to gain statistical significance.

Since *Syngap1*$^{+/\Delta GAP}$ rats have shown to display absence seizures during period of quite wakefulneess[14], the time spent in the black box between social exposures could be triggering seizures on the rat models and consequently, affect their performance on the test. To study if a prolonged time in the black box could alter their social performance the protocol was modified by adding new step on the experimental day. The subject rats spend 20 min in the black box before being introduced to the arena.

I conducted this protocol on a second cohort composed of 4 subject rats (2 wildtypes, 1 *Syngap1*$^{+/\Delta GAP}$ and 1 *Syngap1*$^{+/-}$ subjects) and their respective stimuli rats (all wildtype and un-related). The results

showed on Figure 2B reproduce a similar tendency to the one observed in previous cohorts under the initial version of the protocol (Figure 2A).

The data on travelled distance during the day of habituation to the arena (day prior to the experiment) and wall climbing (not shown) suggested that rats were not completely habituated to the arena by the day of the experiment. The lack of habituation to the arena could be interfering with their exploration because they would still be exploring the arena (context novelty) while also interacting new conspecifics (social novelty). Therefore, I modified the protocol such that each experimental rat was subjected to 4 days of habituation to the arena instead of 1, prior to the start of the experiment. I have recently run two cohorts consisting of 14 animals split among the three genotypes. However, since this is an ongoing experiment for which I am unable to show the results at the date of this report. They will be updated in the following reports.

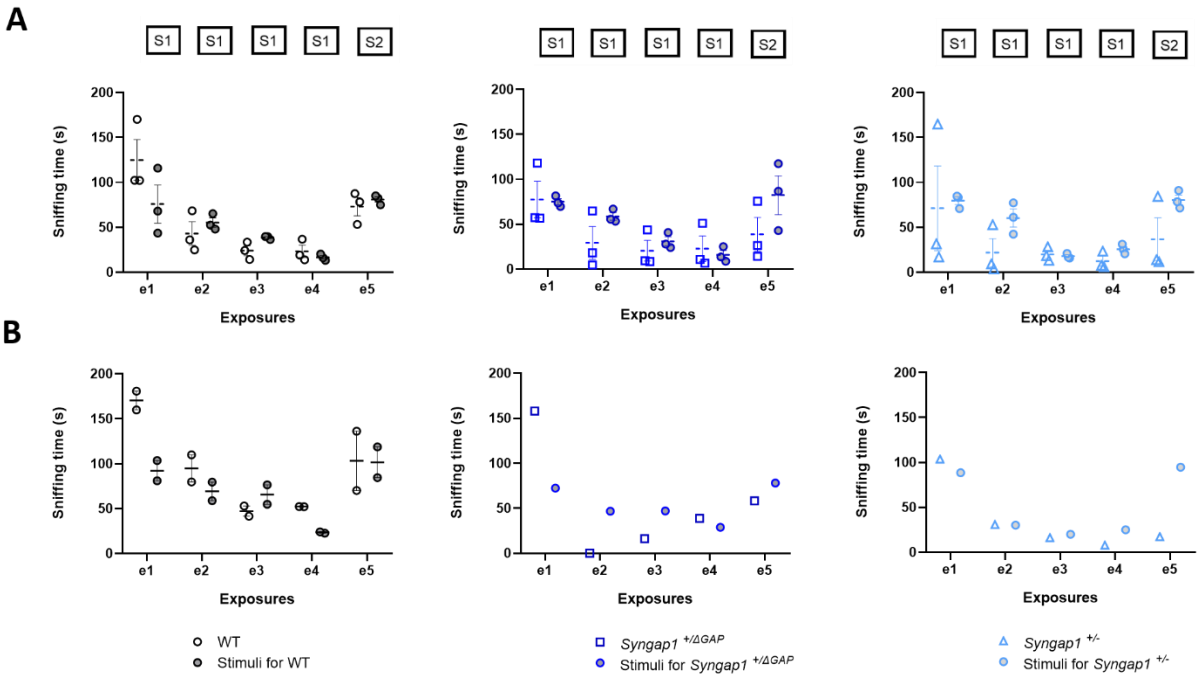


Figure 2. Sniffing time of a conspecific in *Syngap1*$^{+/\Delta GAP}$ and *Syngap1*$^{+/-}$ subjects and their stimuli rats on the habituation dishabituation test. A) Mean sniffing time in seconds on the first version of the habituation dishabituation protocol. N= 3 wildtype, 3 *Syngap1*+/ΔGAP and 3 *Syngap1*+/- rats for subjects. **B)** Mean sniffing time in seconds on the second version of the habituation dishabituation protocol. N= 2 wildtype, 1 *Syngap1*$^{+/\Delta GAP}$ and 1 *Syngap1*$^{+/-}$ rats for subjects. S1 and S2 means Stimulus 1 and Stimulus 2 respectively. e1-e5 means exposure 1-5. Stimuli rats are shown as circles (since they are wildtype rats) and on the contour colour of the subject's genotype they were matched with. Mean ± standard error shown.

3) Study of olfactory performance

3.1) Non-social odours

After different designs tested on pilot experiments on wildtype rats (data and protocol not shown in this report) the first protocol applied on a *Syngap1* cohort was as follows: two days of handling, one day of habituation to the arena (two exposures of 10 min each), one day of habituation to the arena with a pot (3 exposures of 5 min each and placed in a black box between exposures) and experimental day. Sand mixed with spices (5g dried oregano and cinnamon powder per kg of sand) was used as non-

social odorants. This mix was poured inside a pot covered with a lid containing holes. The experimental rat was introduced on the arena containing the pot for 5 min. The rats were introduced to the pots in the following sequence: one exposure to plain sand, four exposures to the first odour and one exposure to the second odour. The odours were counter-balanced, such that the first odour was oregano for half of the cohort and cinnamon for the other half. Between exposures, rats were kept in a black box for 5 min. The results on the first *Syngap1* cohort (3 wildtype, 3 *Syngap1*$^{+/\Delta GAP}$ and 3 *Syngap1*$^{+/-}$subjects) following this protocol are shown in Figure 3A. The preliminary data suggests no increase in sniffing time between unscented sand and the first odour in wildtype subjects or any of the *Syngap1* models (Mean sniffing time for wildtype subjects on plain sand: 63.71s ± 9.7 SEM and on first exposure to first odour: 41.2s ± 5.7 SEM. Mean sniffing time for *Syngap1*$^{+/\Delta GAP}$ subjects on plain sand: 18.4s ± 7.7SEM and on first exposure to first odour: 19.1s ± 6.7 SEM. Mean sniffing time for *Syngap1*$^{+/-}$ subjects on plain sand: 20.6s ± 1.8 SEM and on first exposure to first odour: 23.06s ± 1.9 SEM)

The lack of an increase in sniffing time when wildtype rats are presented the first odour suggests that the test needs is not working to show olfaction skills to detect an odour. However, in wildtype rats an increase of 3.3 times in the sniffing mean time was observed when the second odour was presented compared to the fourth exposure of first odour (Mean sniffing time for wildtype subjects on fourth exposure to first odour: 9.2s ± 4.6SEM and when second odour presented: 30.4s ± 7.6 SEM). For the same comparison, an increase of 1.4 times in the mean sniffing time was observed for *Syngap1*$^{+/\Delta GAP}$ subjects (Mean sniffing time for *Syngap1*$^{+/\Delta GAP}$ subjects on fourth exposure to first odour: 18.4s ± 6.2 SEM and when second odour presented: 25.8s ± 5.5 SEM). There was no increase observed for the second odour on *Syngap1*$^{+/-}$ subjects. Since wildtype rats showed increased to the second odour but failed to detect the first odour, the problem to detect the odour could be the lack of enough habituation to the pot.

Consequently, for the following cohort (2 wildtypes, 1 *Syngap1*$^{+/\Delta GAP}$ and 1 *Syngap1*$^{+/-}$) I increased the habituation day to 4 days. Animals were habituated both, to the arena and to the pot containing plain sand simultaneously (3 exposures to the arena with the pot of 5 min each every day). Additionally, on the experimental day 3 exposures to plain sand instead of only one were conducted. Results of this experiment are shown on Figure 3B.

The main result of this pilot experiment is the optimization of the detection and habituation of this test. Wildtype rats present an increase of 4.3 times between the mean sniffing time on the third exposure to plain sand and the first odour (Mean sniffing time for wildtype subjects on third exposure to plain sand: 10.1s ± 0.7 SEM, and for first exposure to first odour: 43.5s ± 20.8 SEM). They also gradually habituate to the first odour (mean sniffing time for wildtype subjects on fourth exposure to first odour: 1.1s ± 0.6 SEM). However, the discrimination phase robustness is still to be confirmed since 1 of the 2 wildtype subjects of this cohort didn't present an increase in sniffing. On the one hand, it could be an outlier since on the previous version of the protocol, the discrimination seemed to work for wildtype subjects. However, since the protocol is now longer in time, that could affect the interest of the subjects on the task. More experiments have been conducted to increase the N and gain statistical significance.

Regarding the performance of *Syngap1* rats under this protocol. The only *Syngap1*$^{+/\Delta GAP}$ subject did not show a substantial increase of sniffing when presented the first odour (Sniffing time for *Syngap1*$^{+/\Delta GAP}$ subject on third exposure to plain sand: 5.1s and when first presented the first odour: 6.13). Since it is only animal, a higher N is needed to confirm or discard a possible impairment in odour detection. The only *Syngap1*$^{+/-}$ subject showed an increase of 10.76s when presented the first odour (Sniffing time for *Syngap1*$^{+/-}$subject on third exposure to plain sand: 2.6s and when first presented the first

odour: 12.8). When looking at their ability to discriminate a second odour, *Syngap1*$^{+/\Delta GAP}$ subject sniffing time when presented the second odour increased 2.9 times and for *Syngap1*$^{+/-}$ subject 1.78 times. The variation in response in wildtype for the discrimination phase and the small N of this pilot experiment difficult state any clear conclusions.

Altogether, the current version of the protocol appears to succeed in show detection and habituation to odours. An increase in N will give insight into a possible impairment in odour detection for *Syngap1*$^{+/\Delta GAP}$ subject and the robustness of the discrimination phase of this test.

I have recently run two cohorts consisting of 14 animals split among the three genotypes. However, since this is an ongoing experiment for which I am unable to show the results at the date of this report. They will be updated in the following reports.

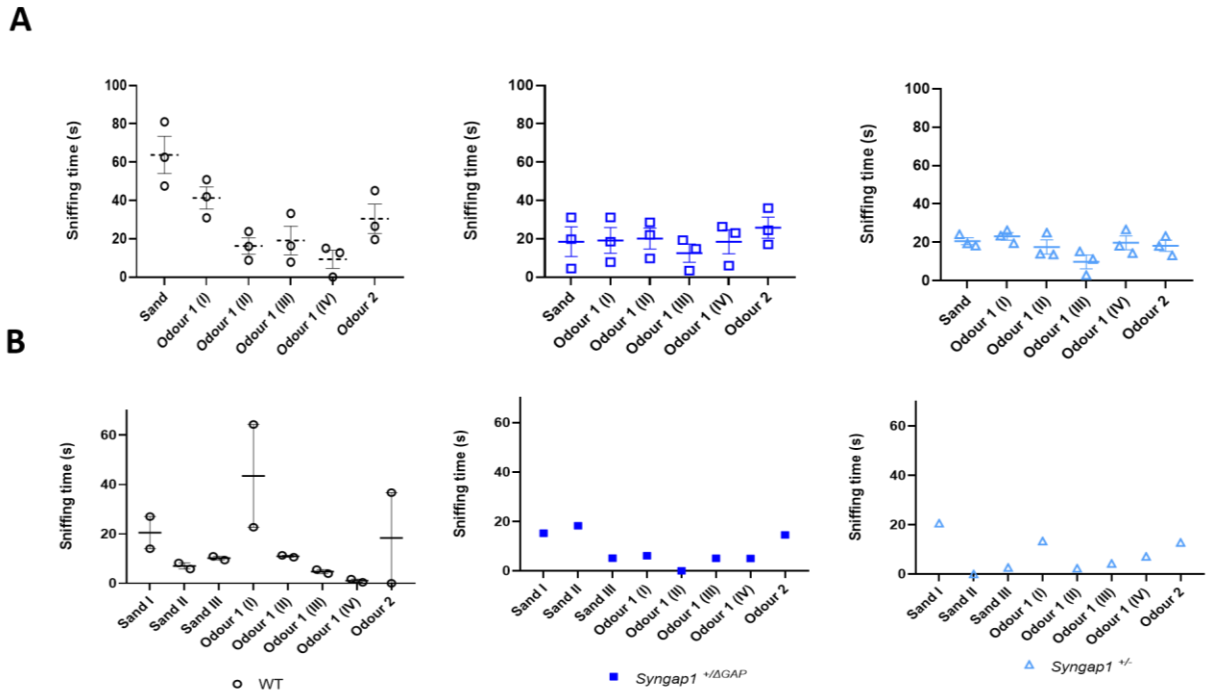


Figure 3. Sniffing time in *Syngap1*$^{+/\Delta GAP}$ and *Syngap1*$^{+/-}$ rats on the detection, habituation, and discrimination of non-social odour test. A) Mean sniffing time in seconds on the first version of the protocol. N= 3 wildtype, 3 *Syngap1*$^{+/\Delta GAP}$ and 3 *Syngap1*$^{+/-}$ rats for subjects. **B)** Mean sniffing time in seconds on the second version of the protocol. N= 2 wildtype, 1 *Syngap1*$^{+/\Delta GAP}$ and 1 *Syngap1*$^{+/-}$ rats. Mean ± standard error shown.

3.2) Social odours

The aim of this paradigm is to test if animals are able to differentiate between social odours from two different conspecifics, as in the habituation-dishabituation test, they are presented with two different stimulus rats.

The initial protocol applied on a *Syngap1* cohort consisted of introducing the experimental rat to a cage containing a plastic tube with a cotton bud. The plastic tube had an open-end that served as sniffing hole. The rat was exposed first to a cotton bud soaked in distilled water. Following which, it was exposed to a cotton bud soaked in urine of subject 'A'. Similar to the habituation-dishabituation test and the non-social odour discrimination task, this step was repeated 4 times. Then, the subject rat was exposed to a cotton bud soaked in the urine of a novel rat, 'B'. Each exposure was 5 min long and the results for this cohort are shown on Figure 4A.

Wildtype subjects show an increase of sniffing when presented the first urine (Mean sniffing in wildtype subjects on water exposure: 5.9s ± 2.1 SEM and on first urine: 19.8s ± 7.3 SEM). This preliminary result supports the use of urine as a social odorant since rats are able to detect urine odour.

Regarding the performance of *Syngap1* subjects on the detection phase, *Syngap1*$^{+/\Delta GAP}$ subjects also present an increase in sniffing when presented the first urine (Mean sniffing in *Syngap1*$^{+/\Delta GAP}$ subjects on water exposure: 5.4s ± 1.1 SEM and on first urine: 13.9s ± 20.08 SEM). *Syngap1*$^{+/-}$ subjects also present an increase on average in sniffing when presented the first urine (Mean sniffing in *Syngap1*$^{+/-}$ subjects on water exposure: 5.8s ± 1.7 SEM and on first urine: 12.86s ± 6.9 SEM).

Regarding the discrimination between two urine of different subjects, on wildtype subjects (2 of 3) subjects increase their sniffing time being the mean sniffing time when presented urine from subject 'B' 18.07s ± 8.9 SEM among the three subjects while on the previous exposure (fourth exposure to the urine 1 from subject 'A') was 0.25s ± 0.25 SEM. This result suggest that rats are able to discriminate urine from two different subjects and supports the use of urine as a social odorant of individuals/conspecifics.

Regarding the performance of *Syngap1* subjects on the discrimination phase, *Syngap1*$^{+/\Delta GAP}$ subjects also present an increase in sniffing when presented the urine of subject 'B' (Mean sniffing in *Syngap1*$^{+/\Delta GAP}$ subjects on fourth exposure to urine 1-subject 'A': 0s ± 0 SEM and on urine 2 from subject 'B': 12.04s ± 7.2 SEM). *Syngap1*$^{+/-}$ subjects increase in sniffing when presented the urine from subject 'B' is on average of only 1.7s (Mean sniffing in *Syngap1*$^{+/\Delta GAP}$ subjects on fourth exposure to urine 1-subject 'A': 1.5s ± 1.5 SEM and on urine 2 from subject 'B':3.2s ± 1.79 SEM).

Altogether, this first study supports the use of urine as a social odorant from two different conspecifics. Both *Syngap1*$^{+/\Delta GAP}$ and *Syngap1*$^{+/-}$ subjects seem to be able to detect the odour of urine.

Syngap1$^{+/\Delta GAP}$ subjects also seem to discriminate between the two subjects' urine but it remains unclear *Syngap1*$^{+/-}$ subjects are able to discriminate two subjects' urine. If it was confirmed with a higher N size that *Syngap1*$^{+/-}$ subjects are unable to discriminate two social odours, that could be linked to their decrease sniffing exploration of a new conspecific on the habituation dishabituation test.

In order to match the non-social odour test and serve as a control of the habituation-dishabituation test the protocol went through several modifications. First, instead of a cage, an arena was used as the context, instead of a plastic tube, a pot filled with sand was used (similarly to the non-social odour test). Secondly, instead of a cotton bud, a filter paper was used since it offered a higher surface to be used on a pot. Lastly, the habituation period to the arena and pot was set to 4 days (equal to the non-social odour test) and on the experimental day three exposures to the plain pot were prior to the first urine. The plain pot contains sand and on top of it, a filter paper with 500uL of distilled water. The exposures on the experimental day are as follows: 3 exposures to the pot with filter paper and 500uL distilled water, 4 exposures to the pot with a filter paper with 500uL of urine from subject 'A' and one last exposure to the pot with a filter paper with 500uL of urine from subject 'B'. Results of this study on a cohort of *Syngap1* rats (2 wildtypes, 1 *Syngap1*$^{+/\Delta GAP}$ and 1 *Syngap1*$^{+/-}$) are shown in Figure 4B.

Wildtype subjects show an increase of sniffing when presented the first time the urine 1 from subject 'A' (Mean sniffing in wildtype subjects on third exposure to water: 4.7s ± 0.9 SEM and on first urine: 37.4s ± 20.08 SEM). The only *Syngap1*$^{+/\Delta GAP}$ also shows an increase for urine 1 (sniffing time on third

exposure to water: 3.06s and sniffing time to first exposure to urine1: 31.16s). The only *Syngap1*$^{+/-}$ also shows an increase for urine 1 (sniffing time on third exposure to water: 1.3s and sniffing time to first exposure to urine1: 9.06s). However, their increase in sniffing time seems to be 4.21 times smaller compared to the mean presented in wildtypes. As we are describing the data of a single animal, it is not enough to extract any conclusions.

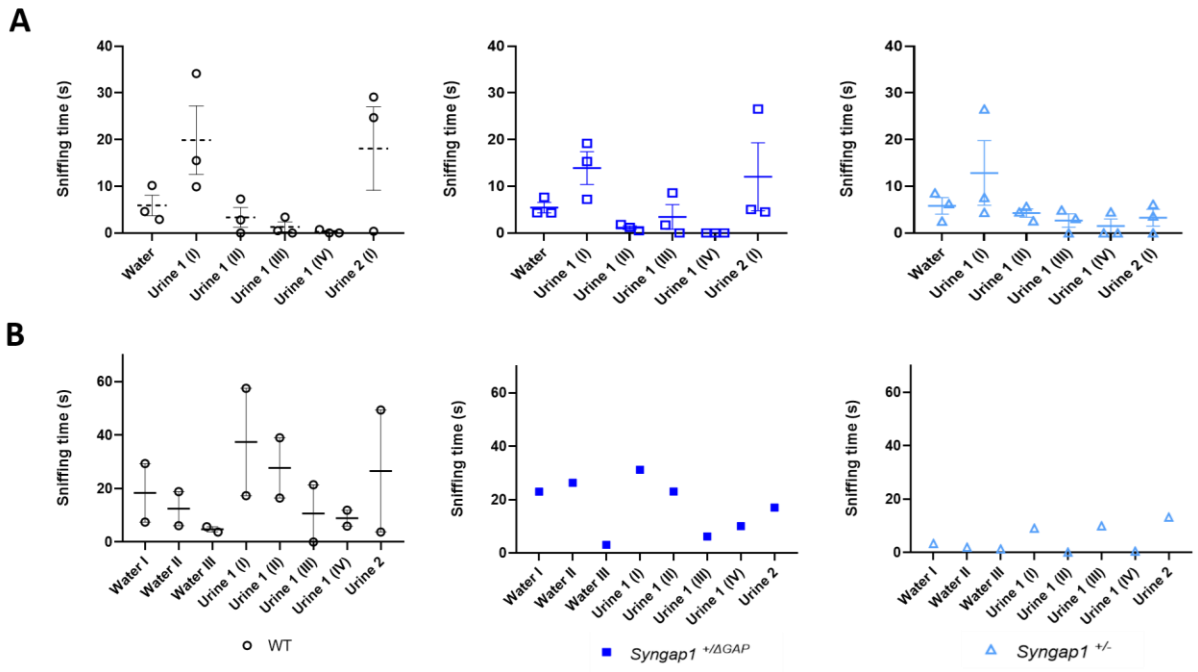


Figure 4. Sniffing time in *Syngap1*$^{+/\Delta GAP}$ and *Syngap1*$^{+/-}$ rats on the detection, habituation, and discrimination of social odour test. A) Mean sniffing time in seconds on the first version of the protocol. N= 3 wildtype, 3 *Syngap1*$^{+/\Delta GAP}$ and 3 *Syngap1*$^{+/-}$ rats for subjects. **B)** Mean sniffing time in seconds on the second version of the protocol. N= 2 wildtype, 1 *Syngap1*$^{+/\Delta GAP}$ and 1 *Syngap1*$^{+/-}$. Mean ± standard error shown.

While the overall profile is similar to the data shown in Figure 4A, the cohort size is too small to draw any conclusions. I have recently run two cohorts consisting of 14 animals split among the three genotypes. However, since this is an ongoing experiment for which I am unable to show the results at the date of this report. They will be updated in the following reports.

Exploitable results

The COVID-19 pandemic and subsequent severe lockdown in Scotland has interrupted my experimental work for a minimum of 6 months. Despite the strong impact of COVID-19 pandemic on this project, I have succeeded in implementing 3 different behavioural protocols to assess social and olfactive alterations in two novel rat models of *SYNGAP1* haploinsufficiency, as well as measuring alteration in social behaviours in a naturalistic 'Habitat' environment.

The most important results shown in this report are:

- *Syngap1*$^{+/-}$ rats appear to participate in more tube encounters and *Syngap1*$^{+/\Delta GAP}$ and *Syngap1*$^{+/-}$ win fewer encounters when compared to wildtype rats

Replication of this experiment are needed to confirm these results. However, there are still questions that can be addressed with this data such as looking at the flexibility on dominance between days, the percentage of winning by genotypic pairs and the influence of encounters with litter and cage mates which can give insight in the creation of hierarchy with unfamiliar conspecifics

- On the habituation dishabituation test, *Syngap1*$^{+/\Delta GAP}$ and *Syngap1*$^{+/-}$ rats subject sniffing of new conspecific appears to be less than the stimuli 2 rat sniffing on them. Moreover, *Syngap1*$^{+/-}$ subjects interest on a new conspecific is less than wildtype subjects.

Preliminary results show that *Syngap1*$^{+/-}$ rats present less social interaction with a new conspecific in the short term. This data also allows us to characterize the temporal distribution of sniffing and how this is modulated upon every exposure, thus monitoring the dynamic changes during habituation to a conspecific and upon the introduction of a novel stimulus rat. The different sequential dynamic in social interaction between each rat of the pair/dyad is also planned to be further examined. This could give us information such as latency to start an interaction, of the interactions are usually reciprocated and if not, what other behaviours are being done while the conspecific is interacting with them. This analysis is planned to be done using the Analysis tool of Observer XT together with analysis scripts to be made on MATLAB. These details could give further insight if social behaviours are active, reactive or passive. Finally, behavioural differences classified by genotypic pair can be analysed. Finally, it is possible that social interactions are influenced by the motor performance and/or hyperactivity of *Syngap1* subjects[1,14]. The analysis of possible differences in movement and approaches during the social interaction could solve this question. Therefore, I plan to analyse the tracking of the videos by using DeepLabCut tool[28,29].

- *Syngap1*$^{+/\Delta GAP}$ rats appear to be able to distinguish between urine from different subjects. The ability of *Syngap1*$^{+/-}$ rats to detect, habituate and discriminate to social odours is yet to be determined.

Syngap1$^{+/-}$ subjects ability to discriminate two odours has not be confirmed yet. The lack of a clear sniffing response on average could mean an impairment in their olfaction function. However, replication of these experiments will increase the N size to gain statistical significance.

One confounding factor that could explain the low sniffing time in the *Syngap1*$^{+/-}$ subjects is overall reduced exploration time[14].In order to eliminate this, I intend to normalize the sniffing time of the odours to their sniffing time on first exposure of every odour.

Finally, the first results on the analysis of allogrooming and self-grooming are still under analysis.

Altogether, I am now in position to replicate my preliminary finding and understand which part of social alterations are truly altered in our models of *SYNGAP1* haploinsufficiency. Moreover, thanks to the use of these two rat models, I will be able to discriminate if there are any differences exclusively linked to a heterozygous disfunction in the C2/GAP domain of *Syngap* gene.

Once this first part of the project is complete, I will follow-up these results by determining which specific neural circuits are responsible for each of the observed behavioural alterations.

The circuits to be studied will depend on the exact behaviour and its alterations. For example, a potential impairment in social and olfaction discrimination could be linked to alterations in medial amygdala (MeA)[30,31]; alterations in social motivation could be caused by impairments in ventral tegmental area (VTA) and nucleus accumbens (NAc) circuitry[32,33], deficits in social memory are highly

determined by alterations in ventral hippocampus and CA2 region of the hippocampus and prefrontal cortex (mPFC) [34–36] and social dominance can be reinforced by manipulating the thalamo-mPFC circuit[37].This second part of the project could help with the discovery of potential therapeutic targets.

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Annex 2 – Articles published in *Biologi per la Scienza* (Biologists for Science)

Gli organoidi di cervello - L'emancipazione etica degli studi sullo sviluppo cerebrale umano

by Giuseppe Cammarata

Fino a pochi anni fa, la ricerca sui disturbi dello sviluppo cerebrale umano era molto limitata. Il principale ostacolo era di tipo etico, in quanto l'accessibilità a tessuti cerebrali umani vitali sulla quale effettuare esperimenti era scarsa. La controversia legata all'utilizzo di cellule umane derivanti da embrioni o da prelievo di cellule da tessuti vitali umani era la principale responsabile dell'esigua accessibilità a queste importanti risorse di studio.

La soluzione a questo problema è arrivata solo pochi anni fa grazie alla scoperta della riprogrammazione cellulare. In biologia, la riprogrammazione è un processo che agisce su una cellula somatica, cioè già differenziata e specializzata per svolgere una funzione precisa (gli epatociti nel fegato, i neuroni nel cervello ecc.), portandola a uno stato di pluripotenza. Queste cellule pluripotenti, definite cellule staminali indotte, possono differenziarsi in molti tipi cellulari diversi, diventando epatociti, neuroni, cellule della pelle. Grazie all'esposizione a fattori di trascrizione definiti "Yamanaka" siamo adesso in grado di riprogrammare cellule somatiche e riportarle allo stadio staminale con la quale è possibile produrre una gran varietà di cellule specializzate importanti non solo per lo sviluppo cerebrale, ma anche per altre malattie. L'aspetto più rivoluzionario e vantaggioso è che queste cellule somatiche possono essere ottenute facilmente dalla pelle oppure tramite un prelievo di sangue da soggetti adulti, sostituendo la necessità di prelievi invasivi o dell'utilizzo di cellule embrionali.

L'uso delle cellule staminali umane *in vitro* nella ricerca biomedica ha portato a nuove innovazioni nella biologia dei disturbi neurodegenerativi e dello sviluppo neurologico. Lo studio su neuroni derivati da cellule staminali indotte, prelevate dai pazienti, ha permesso di approfondire nuovi aspetti dei disturbi dello sviluppo neurale umano, come ad esempio il disturbo dello spettro autistico. In particolare, lo studio su colture 2D (il classico "vetrino" su cui crescono le cellule in un unico strato piatto) ha portato a nuove informazioni su come i neuroni umani si sviluppano e maturano fino a raggiungere funzionalità che somigliano a quelle trovate *in vivo*. Nonostante la grande utilità delle colture 2D che hanno avuto nello studio delle malattie del neuro sviluppo, queste tecniche sono limitate nel riprodurre la architettura cellulare di un cervello umano in via di sviluppo.

Ed è qui che entrano in gioco gli organoidi cerebrali. Questi, infatti, sono una tecnica alternativa alle colture 2D che puntano al superamento dei limiti sopracitati, con la creazione di complesse colture tridimensionali. Il metodo di coltura 3D si basa sull'intrinseca abilità delle cellule staminali di auto organizzarsi durante la differenziazione in neuroni o altri tipi di cellule specializzate. Questa differenziazione però non avviene su una superficie, come nelle colture 2D, ma in un ambiente che ne consente il libero sviluppo in tutte le direzioni, con il supporto di una matrice extracellulare. Questi aggregati cellulari che galleggiano nel mezzo di coltura sono pertanto in grado di riprodurre alcune delle caratteristiche strutturali specifiche dello sviluppo della corteccia umana: la separazione in distinti strati corticali con un'alta varietà di tipi cellulari; la migrazione neuronale fondamentale per la formazione degli strati corticali; e soprattutto la maturazione funzionale dei neuroni.

Gli organoidi cerebrali umani sono un modello in vitro che presenta diversi vantaggi, ma anche alcune limitazioni. Per certi aspetti e certi tipi di ricerche, gli organoidi potrebbero essere considerati una più che valida alternativa alla sperimentazione animale. Per cominciare, uno degli svantaggi dei modelli

animali è il divario presente tra gli animali utilizzati e noi esseri umani. Questa differenza viene poi particolarmente accentuata quando si parla di studi sulle malattie del neuro sviluppo umano: lo sviluppo di strutture cerebrali, i meccanismi legati alla maturazione del cervello e i comportamenti che ne sono il loro diretto risultato possono rivelarsi più complessi o completamente differenti se comparati a quelli degli animali modello. Bisogna quindi considerare con cautela i risultati riguardanti le malattie del neuro sviluppo umano ottenute lavorando su modelli animali. Con l'utilizzo degli organoidi cerebrali come modelli umani *in vitro* si potrebbe coprire quel vuoto presente tra i modelli animali e gli umani. Certo, sono presenti alcune limitazioni importanti, come l'impossibilità di condurre studi comportamentali sugli organoidi.
Per concludere, gli organoidi cerebrali sono colture in grado di riprodurre i primi stadi di sviluppo dello sviluppo neuronale. Con il tempo e con il perfezionamento di questa tecnica saremo in grado di dare un grandissimo contributo nello studio dei meccanismi che portano alla maturazione di un cervello umano, e questo ci aiuterà a capire come controllare e trattare le malattie del neuro sviluppo. E poi diciamocelo: sono veramente fotogenici.

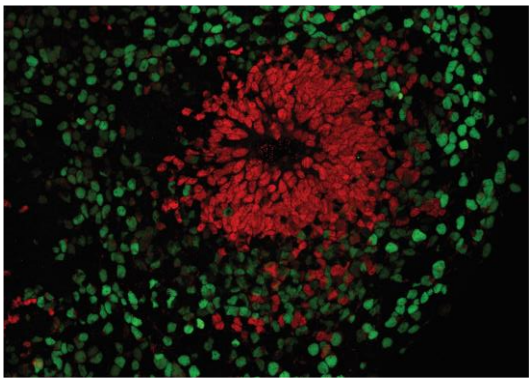


Figura 1. Nella figura si può apprezzare la zona simil ventricolare in rosso (positiva a SOX2 marker per i progenitori neuronali che andranno a formare le diverse stratificazioni), e i primi strati profondi in verde (positiva a CTIP2 marker per neuroni degli strati profondi). Dalla zona simil ventricolare i progenitori si moltiplicano e le cellule che si specializzano in neuroni migrano verso l'esterno formando i diversi strati della piastra corticale.

Ma la libertà di scelta esiste? By Elisa Corti



Ciao, sono Elisa e quando mi è stato chiesto di preparare un pezzo su un argomento che reputavo interessante, io ho scelto di acconsentire. Forse.

Ognuno di noi si trova quotidianamente di fronte a delle scelte: cosa indossare, cosa mangiare per colazione, se rispondere a una telefonata o se andare in palestra. Tutti abbiamo, almeno fino a un certo punto, il controllo delle nostre azioni e siamo in grado di scegliere di prendere dall'armadio la maglietta blu, di mettere la moka sul fornello, di alzare la cornetta e di riposarci sul divano dopo una giornata stancante. Le nostre vite sono piene di decisioni che crediamo di prendere in autonomia. Ma è davvero così?

Da sempre i filosofi si interrogano sull'esistenza e sul significato del libero arbitrio. La maggioranza delle visioni convergono su uno scenario in cui gli uomini sono liberi: liberi di scegliere per esempio cosa è bene e cosa è male, cosa è importante e cosa no, per cosa lottare e per cosa arrendersi.

Il *Bereitschaftspotential*, o potenziale di prontezza

Nel 1964 però, due ricercatori tedeschi aprirono il vaso di Pandora. Il lavoro di Hans Helmut Kornhuber e Lüder Deecke originava dalla loro critica alla visione, imperante a quell'epoca, del cervello come una macchina che produce pensieri e azioni sempre e solo in risposta agli stimoli in arrivo dal mondo esterno. Per soddisfare il loro desiderio di capire come il cervello sia in grado di generare un'azione in assenza di stimoli esterni, Kornhuber e Deecke invitarono una dozzina di persone a sedersi a un tavolo e chiesero loro di eseguire un'azione molto semplice: flettere l'indice della mano ogni qualvolta lo volessero. L'attività cerebrale dei soggetti era costantemente monitorata tramite una tecnica chiamata elettroencefalogramma (EEG), che consiste nell'utilizzo di una cuffia sulla quale sono montati vari elettrodi. Ogni elettrodo è in grado di registrare l'attività elettrica dei neuroni sottostanti l'elettrodo stesso, che viene descritta tramite tracce, linee spezzate che ricordano vagamente i grafici dell'andamento dei mercati finanziari. Proprio osservando queste tracce, i due si accorsero che mezzo secondo prima della flessione del dito si osservava un particolare pattern nell'attività cerebrale dei soggetti (un aumento seguito da un brusco decremento) che i due scienziati chiamarono *Bereitschaftspotential*, o potenziale di prontezza. Il *Bereitschaftspotential* scoperto da Kornhuber e Deecke rappresentava ciò che anticipa il compimento di un'azione, il primo segno che indicava che la decisione di flettere il dito era stata presa. In altre parole, osservando la presenza del potenziale di prontezza, gli scienziati potevano affermare che mezzo secondo dopo il soggetto avrebbe flesso il dito.

È il cervello a scegliere per noi?

Due anni dopo, il ricercatore Benjamin Libet cercò di capire cosa succedesse nel mezzo secondo che separa la comparsa del potenziale di prontezza e il compimento dell'azione (la flessione del dito). Egli ripetè lo studio di Kornhuber e Deecke, chiedendo ai partecipanti di comunicare il momento in cui la decisione di flettere l'indice veniva presa. Come atteso, il *Bereitschaftspotential* comparve 500 millisecondi prima della flessione del dito. Il problema è che i soggetti riportarono di aver deciso di flettere il dito solamente 150 millisecondi prima di muoverlo effettivamente. C'era dunque un divario di 350 millisecondi tra il momento in cui nel cervello compariva il segno che l'azione stava per essere compiuta (il potenziale di prontezza) ed il momento in cui i soggetti riportavano di aver effettivamente preso quella decisione. Era come se il cervello avesse autonomamente deciso di iniziare un'azione ancor prima che la persona ne diventasse cosciente.

Gli sviluppi moderni

Un drastico cambio di paradigma si ebbe nel 2010 grazie al ricercatore Aaron Schurger. Egli stava studiando tramite EEG le fluttuazioni spontanee dell'attività cerebrale a riposo, che derivano solamente dal funzionamento di miliardi di neuroni interconnessi tra loro. Schurger ebbe l'idea di combinare le tracce ottenute dai singoli elettrodi, per avere un'idea del livello di attività generale del cervello e ottenne così qualcosa che assomigliava molto al *Bereitschaftspotential*. In questo caso nessuna azione stava per essere compiuta, quindi egli giunse alla conclusione che il potenziale di prontezza non ha uno scopo preciso (quello di iniziare un'azione), ma è qualcosa di correlato alle fluttuazioni spontanee dell'attività cerebrale.

Il nuovo modello di Schurger

Due anni dopo, Aaron Schurger, Jacobo Sitt e Stanislas Dehane, proposero una teoria: quando dobbiamo scegliere tra due opzioni, per esempio A e B, ci sono gruppi di neuroni che hanno il compito di valutare i pro e i contro di ogni opzione, e fanno ciò rispettivamente aumentando o diminuendo la loro attività. Se l'opzione A presenta più contro che pro, i neuroni coinvolti non saranno molto "entusiasti" e mostreranno un livello minore di attività. Viceversa se l'opzione B è quella vincente, i nostri neuroni saranno più "entusiasti", cioè più attivi. Quando l'attività di questo gruppo di neuroni cresce a tal punto da superare un determinato valore soglia, noi siamo pronti a scegliere B e a non scegliere A. Però c'è un però. Negli esperimenti di Kornhuber, Deecke e Libet non c'erano opzioni tra cui scegliere ma un'azione spontanea da compiere. In questo caso non ci sono gruppi di neuroni che devono valutare qualcosa, quindi come è possibile raggiungere e superare il valore soglia? Ecco che ritornano in gioco le fluttuazioni dell'attività cerebrale a riposo: quando casualmente l'attività dei neuroni è più alta, essa è più vicina al valore soglia. In questa circostanza sarebbe quindi più probabile per i soggetti decidere di flettere il dito. Ciò non significa, come sosteneva Libet, che il cervello decide per noi, piuttosto che queste fluttuazioni spontanee permettono di facilitare o meno il raggiungimento del valore soglia, quindi di prendere o meno una decisione in assenza di fattori esterni su cui basarci.

Un esperimento più rassicurante

Recentemente, Schurger ha ripetuto l'esperimento di Libet, includendo nello studio anche persone che non avrebbero dovuto muovere il dito. Se Libet aveva ragione, cioè se il cervello decide autonomamente quando flettere il dito, l'attività cerebrale dei soggetti che dovevano muovere il dito avrebbe dovuto differire da quella dei soggetti che dovevano stare fermi circa mezzo secondo prima della flessione dell'indice: nel primo caso si sarebbe osservato il *Bereitschaftspotential*, nel secondo caso no. Ciò non è accaduto. Anzi. Differenze tra l'attività cerebrale dei due gruppi di soggetti sono emerse solamente 150 millisecondi prima del movimento, cioè nel momento in cui i soggetti

dichiaravano di essere coscienti dell'urgenza di muovere il dito. Quindi la presa di coscienza della decisione coincideva con la presa della decisione stessa.

Non è detto che Schurger abbia ragione, ulteriori studi servono per chiarire la questione e per capire se effettivamente siamo in grado di prendere decisioni, di modificare l'influenza esercitata dal mondo esterno, o se semplicemente il nostro prendere decisioni sia il risultato di una soglia oltrepassata in seguito all'azione che il mondo esterno esercita su di noi.

Ciao, sono Elisa e non so più se ho scelto di scrivere questo articolo o no.

Annex 3 – 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 4 – Ethical report by External Ethical Advisor

Grant Agreement number: **813986**

Acronym: **Syn2Psy**

Title: **Synaptic Dysfunction in Neuropsychiatric Disorders**

Deliverable date: 26.04.2021

Document Title	Ethical Report
Document Author:	External Advisor
Approving Body:	External Advisor
Date:	26.04.2021
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 SETS OUT ETHICAL CONSIDERATIONS RAISED BY THE syn2psy PROJECT, AND IDENTIFIES AREAS IN WHICH ATTENTION SHOULD BE FOCUSED.

1.2 PROJECT GOALS

The large public health burden and individual suffering associated with mental disorders speaks to the need to study their biological underpinning. Genetic research, animal models of disease and human neuroimaging studies have provided convergent evidence to common pathways implicated in their aetiology, namely pathways that regulate synapse development and plasticity, which result in abnormal structural and functional connectivity in the brain of neuropsychiatric patients. The time has come to integrate studies across different levels, leading to the identification of signatures of illness associated to specific risk pathways, which can drive the development of novel therapies.

The objective of Syn2Psy is to provide high-level Ph.D. training for 14 early stage researchers (ESRs) to answerthree critical questions:

1) What are the synaptic defects at the basis of neuropsychiatric disorders?

2) Which are the neuronal circuits that are disrupted, and may be targets for therapies?
3) How and when can we modulate the course of disease?

Syn2Psy combines 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 provide them with the transferable skills necessary for thriving careers in a flourishing area. Industrial partners Lundbeck, Eurotrials and ZEISS provide experience on drug, clinical trials and technology development, the Coimbra University Hospital will expose the students to clinical research, while the clinic for child development PIN and the Marionet theatre company offer training in societal awareness and outreach. The excellent basic scientific knowledge and diverse skills acquired by ESRs will enhance their employment prospects in both the academic and non-academic sectors, and their scientific contributions will

inform novel therapeutic approaches for alleviating this leading cause of disability worldwide.

The 14 studies under development can be summarized as follows (currently in the following development phase):

ESR1

*To establish the role of Stargazin in psychiatric disorders (**Task 1.1**), **ESR1**, **Orsolya Antal**, under the supervision of Ana Luísa Carvalho at CNC, has generated two knock-in mouse models of neuropsychiatric disorders, each carrying a disease-associated variant of the AMPAR-auxiliary protein Stargazin, encoded by the CACNG2 gene. ESR1 is currently performing behavioural and morphological analysis in these animals, which will be complemented by further functional characterisation, all focusing on alterations in synaptic function in the prefrontal cortex and its behavioural consequences. Furthermore, since exposure to chronic stress is a major risk factor for neuropsychiatric disorders, ESR1 aims to assess its impact on these structural, functional and behavioural phenotypes in the disease-associated models.*

ESR2

*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, has conducted genetic manipulations in mice to induce loss of function of Mitofusin 2 (Mfn2) in the Nucleus Accumbens (NAc) and has assessed the impact thereof on motivated behaviour. Downregulation of Mnf2 in medium spiny neurons expressing the dopamine receptor 1 (D1-MSNs) induced an impairment in effort-related behavior, as shown by the adoption of a passive coping behavior in the forced swim test, indicative of depression-like behavior. ESR2 performed cFos mapping of neuronal activation during the behavioral task, which revealed decreased engagement of NAc neurons. Based on these results, it was decided to first study the cellular and synaptic consequences of Mfn2 downregulation in accumbal cells, before exploring the impact in NAc downstream regions. ESR2 performed electrophysiological recordings in mice with Mfn2 downregulation in D1-MSNs, which indicate aberrant intrinsic excitability, altered excitatory synaptic inputs and reduced dendritic complexity in D1-MSNs. ESR2 will extend this analysis by exploring the reciprocal inhibitory connections between D1- and D2-MSNs. Ribosome-associated mRNA (Ribotag) and mitochondrial isolation (Mitotag) approaches were set up to perform an unbiased cell-type and brain region specific RNA sequencing, that will allow the identification of the molecular mechanisms responsible for the dendritic and synaptic defects induced by Mfn2 deregulation.*

ESR3

*To produce human-derived neurons and brain organoids (**Task 1.3**), ESR3, Giuseppe Cammarata, supervised by João Peça, chose a contingency plan that involved the reprogramming of dental stem cells instead of direct differentiation into neurons. Human Induced Pluripotent Stem Cells (HiPSC)-derived human brain organoids have been shown to be more stable and consistent in the generation of neurons when compared to the direct differentiation of dental stem cells. Stem cells from patients and control subjects have been collected. ESR3 has contributed to the reprogramming of 3 cell lines into hiPSC from the ProTeAN biobank. These lines will be characterized to test their pluripotency capability and, if they show positive outcomes, they will be used as a source for the development of 3D brain organoids. ESR3 is currently testing valproic acid (VPA) exposure on human brain organoids derived from hiPSCs of the control pool and is testing the neurophysiological, morphological and network activity properties of both control and VPA-treated organoids. Preliminary data suggest that exposure to VPA leads to the development of hyperactive network. These results may contain hints of a possible impact of VPA on the inhibitory system formed in the organoids treated with this drug, and more investigations are needed to assess what is the cause of this excitatory/inhibitory imbalance.*

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 it was decided that new models should be developed: human pluripotent cells (hPS) and Shank3 knock-out (ko) mouse. This task is in stand-by since former ESR4, Diogo Soares resigned in October 2020, until the official start of the new recruited ESR4, Veronica Villeri, in May 2021. During this waiting time, the ko Shank3 mouse model was established and hPS was implemented on another pathological model.*

ESR6

*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 studying the impact of patient-derived anti-NMDA receptor autoantibodies (NMDAR-Abs) on GABAA receptor-mediated inhibitory signalling and on AMPA receptor-mediated hippocampal excitatory signalling and observed a disruption of both networks.*

To investigate the structure and global regulation of excitatory and inhibitory synapses after exposure to NMDAR-Abs, ESR6 transfected fluorescently-tagged synaptic scaffolding proteins of excitatory and inhibitory synapses: Homer1c and Gephyrin, respectively. ESR6 observed reduced density and size of inhibitory synaptic clusters, but no effect on the excitatory synapses was apparent. Similarly, ESR6

investigated the synaptic organisation of GABAARs and AMPARs, and observed discrepant disturbance of inhibitory and excitatory synaptic organisation. Furthermore, ESR6 analysed preparations exposed to NMDAR-Ab for indicators of activation of the immune system, which did not reveal an activation of astrocytic or microglial cell populations.

ESR7

*To examine alterations of the synaptic properties in Fragile X Syndrome (**Task 2.4**), a condition related to the transcriptional silencing of the Fmr1 gene, **ESR7, Elisa Corti**, supervised by Carlos Duarte, Ramiro Almeida and Paulo Pinheiro at CNC, has mimicked what happens in this disease, by knocking down (KD) Fmr1 expression using two different sets of shRNAs in hippocampal neurons. ESR7 observed that Fmr1 KD was sufficient to increase the synaptic content of GluN2A and GluN2B- containing NMDA receptors, and to impair BDNF-induced synaptic clustering of such receptors. ESR7 is currently testing the efficacy of the above mentioned shRNAs in axons as well, in order to study presynaptic differentiation. Moreover, ESR7 has established a protocol for autaptic cultures, which will be useful to study the dynamics of synaptic vesicle exocytosis and recycling in Fmr1 KO neurons.*

ESR8

*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, has performed experiments to dissect the mechanisms of the increased synaptic density in DS. ESR8 performed surgeries and 2-photon imaging for the evaluation of synaptic bouton density, survival, turnover, and size. The dataset is close to completion with the current DS clone set and shows a trend increase in survival and decrease in turnover rate in trisomic cells. Additionally, a significant increase in bouton size has been found in trisomic cells compared to isogenic controls, indicating potentially increased strength of synapses. This dataset will be built on with the findings resulting from dissecting the mechanisms of the functional network defects in DS. Surgeries for this dataset have recently be optimized for GCaMP 2-photon activity imaging of somas. Imaging is ongoing in a large group of animals at 1 month, 3 months and 7-8 months post-transplantation. Analysis is currently underway to determine if the data fits with the hypothesis, but imaging of activity (both low-level and across multiple somas) has been successfully performed. It is expected that electrophysiological data and super-resolution data from future secondments will further expand on this dataset and its findings.*

ESR9

*To understand the role of glycinergic signalling in ASD (**Task 2.6**), **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. Mutations in the gene GLRA2, encoding*

the glycine receptor α2 subunit, have been reported in patients with autism. Glra2 knockout mice exhibit deficits in learning and memory in the novel object recognition test and impaired long-term potentiation in the PFC. First, the cellular correlates of PFC synaptic dysfunction in Glra2-deficient mice were explored. ESR9 quantified excitatory and inhibitory neuron populations in the PFC using fluorescent in situ hybridisation and immunofluorescence. Although there were no differences in the density of total excitatory or inhibitory neurons in Glra2−/Y mice, ESR9 observed an increase in the number of somatostatin interneurons, with no significant differences in the other subpopulations of interneurons studied (parvalbumin, calretinin and cholecystokinin). Immunofluorescence experiments were also performed at postnatal day 14 to rule out a developmental effect, and the results were similar to those obtained in adults. Quantification of excitatory and inhibitory synapses in the PFC via immunofluorescent co-localisation of pre-synaptic and post-synaptic proteins showed a decrease in inhibitory synapses with no change in excitatory synapses, pointing to an excitatory/inhibitory imbalance in the PFC of Glra2−/Y mice. Analysis of spine density and dendritic arborisation in PFC pyramidal neurons after biocytin injection revealed increased spine density and dendritic arborisation, with increased dendritic length and branching. These findings indicate that there is altered connectivity of the pyramidal neurons in the PFC of Glra2-deficient mice, which is expected to result in perturbations of synaptic function and disruption of normal cortical network dynamics. In addition, ESR9 mapped the brain regions implicated in the novel object recognition deficit observed in Glra2-deficient mice, using c-fos. ESR9 observed a decrease in c-fos- positive cells in the infralimbic PFC of mutant mice following novel object recognition, with no significant differences in the other regions or under basal conditions. These results suggest that there is hypoactivation of the PFC of Glra2−/Y mice, which may underlie the cognitive deficit. This experiment is currently being repeated in a larger cohort of mice.

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 (**Task 3.1**), **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. Endocannabinoid levels will be measured by our collaborators at the University of Bordeaux. ESR10 learned how to perform Western blots and tested the antibodies for the receptors; these experiments will be completed in the next few months. To study the effects of administration of endocannabinoid modulators on the phenotype of Shank3-deficient mice, ESR10 performed behavioural experiments to evaluate the effect of the CB1 receptor antagonist rimonabant (1 mg/kg/day i.p. for 7 days) in male and female heterozygous (Het), knock-out (KO) and wild-type littermates. After the last injection, mice were tested to evaluate locomotion, motor coordination, learning and memory, and anxiety-like behaviour. KO males exhibited hypolocomotion, which remained unchanged after rimonabant. No significant locomotion deficits were observed in Het males or in females. KO males exhibited a coordination deficit in the rotarod that was rescued by rimonabant. In females, both Het and KO mice had a coordination deficit, which was only*

partially improved by rimonabant. In two novel object recognition tasks, with novel objects differing from the familiar object either by texture or shape, rimonabant rescued deficits in both Het and KO mice. ESR10 is currently finishing the evaluation of anxiety-like behaviour in the dark-light box. In addition, ESR10 recently completed a pilot test to evaluate the effects of another drug, AEF0217 (Aelis Farma), a signal specific inhibitor of CB1 receptors, which unlike rimonabant, has no side effects, and is thus suitable for clinical use. AEF0217 was administered continuously through the drinking water for at least 7 days; the results are promising, and we will soon decide how to continue to study the effects of this drug in our mouse model.

ESR11

*To investigate the excitation/inhibition imbalance hypothesis of neuropsychiatric disorders in the corticothalamic system (**Task 3.2**), **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. Positive and negative modulators of these channels elicited opposite effects on TRN firing, after burst afterhyperpolarization and time between bursts. These results allowed the project to move forward to the next step. Experiments to test the impact of pharmacological modulation of TRN neurons in thalamocortical rhythms during sleep and wakefulness in freely moving rats, are now ongoing.*

ESR12

*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, started by characterizing Syngap+/- dataset precedently pre-processed in the lab. DESeq analysis revealed misregulated targets which were compared with the previously published Fmr1-/y CA1 dataset leading to the following observations: Syngap+/- CA1-TRAP revealed 218 upregulated transcripts and 248 downregulated transcripts; gene set analysis showed upregulation of terms for metabolic and RNA processing, downregulation of terms for GPCRs and serotonergic synapses; comparison of Fmr1-/y and Syngap+/- GO terms revealed common upregulation in rRNA processing, but opposite changes in several other*

gene sets. Interestingly, changes in Fmr1-/y CA1-TRAP were negatively correlated with changes in Syngap+/- CA1-TRAP. FMRP targets and SFARI transcripts, which are downregulated in Fmr1-/y CA1- TRAP, were upregulated in Syngap+/- CA1-TRAP. In addition to this dataset obtained for CA1 region, ESR12 started to originate a new TRAP line specific for amygdala BLA region of Syngap+/- and Fmr1- /y mouse models of disease. Through Immunofluorescence and Genotyping, recombination was spotted, which delayed the establishment of the line. However, amygdala BLA-specific TRAP line obtained through Ribotag technology, was validated through Immunofluorescence with HA antibody that showed BLA-

specific staining. In addition, Rspo2+ population described to be BLA specific was showed to be present in the BLA region and colocalization with HA staining was present for 82.5% of the cells.

ESR13

*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, conducted Electrophysiological experiments in neurons of the thalamic reticular nucleus (TRN) from mouse slices. Exposure to the corticotropin-releasing hormone (CRH), either bath-applied or released by photoactivation of CRH-releasing fibers, was found to alter TRN cell excitability by decreasing low-threshold bursting via activation of the CRH receptor 1. The expression of this receptor in TRN neurons was confirmed by specific quantitative in situ hybridization of mRNA (RNAscope). ESR13 is further dissecting the ionic mechanisms involved in burst reduction, which will indicate molecular targets to be tested in experiments in vivo. By performing circuit mapping through viral infections, ESR13 has identified several extra-hypothalamic populations of CRH-releasing cells projecting to the TRN. These data provide the first demonstration that the stress-related CRH system affects TRN function. Based on progress achieved so far, ESR13 will soon set up polysomnographic recordings in freely-behaving mice and examine the effects of CRH on sleep rhythms through chemo- or optogenetic manipulations of TRN-projecting CRH-releasing cells. ESR13 has started pilot analyses of gene expression in TRN samples collected from mice exposed to early life stress. The results will guide further experiments in which the expression of genes relevant for psychopathology will be assessed after chronic exposure to CRH or stress.*

ESR14

*To study the circuit deficits associated with ASD/ID (**Task 3.5**), **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, ESR14 has achieved to implement new protocols and methods to study robust social phenotypes. ESR14 has also obtained results that show a potential altered phenotype in social interaction with a new conspecific in the short-term in Syngap1+/ΔGAP and Syngap1+/- rats as well as potential alterations in social dominance. Furthermore, since these results are shown in young adults, ESR14 is in position (time-permitting) to characterize younger ages and define critical periods when these phenotypes are manifested. Once these potential phenotypes are confirmed, ESR14 will work on the second task of the objectives as described in the Individual Research Project. To study if cellular phenotype can be prevented, ESR14 first aims to define the specific brain circuits mediating the described social alterations.*

All partners/researchers within the Syn2Psy Team are aware of the relevant ethical principles in scientific research, and thereby they have 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 is to inform better therapeutic strategies, configures a new mindset for the research community devoted to the investigation of neuropsychiatric disorders, and urges the creation of innovative advanced training programs to adequately prepare the next generation of researchers in the field.

A. Participants

It is 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 will not interfere with the medical care received. There will be no payment of travels or pecuniary counterparts for this participation. The participation in the study and sample donation are voluntary and revocable and don´t cause damage to the participants, who at any moment, by their free will, or by representation by their legal representative, have the right to remove their data, the sample and interrupt the collaboration, if they think is convenient, without the need to justify and not being discriminated against. In case the participant or donor (or his legal representative) wants to remove his personal data and/or the sample or interrupt the collaboration, he should describe this by writing. Under such situation the dada will be erased, and the sample will be immediately destroyed.

Informed consent is a key principle of ethical research, ensuring that research participants are adequately informed of the risks of taking part in studies, that their participation is voluntary, and that the information about them gathered remains under their control. The two key principles of informed consent, can be defined as:

- Principle 1: Research participants must be informed fully about the purpose, methods and intended possible uses of the research, what their participation in the research entails and what risks, if any, are involved.

- Principle 2: Research participants must participate in a voluntary way, free from any coercion.

We analyze how **Syn2Psy** project data processing activities comply with the two key principles separately for the involved partners.

The partners in the project, except for CNC, do 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 (hence Principles 1 and 2) 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 does not collect data directly from users, but only indirectly though the user-facing partners of the project. We foresee such collection is lawful under the existing Privacy Statements of the partners.

All the data collected is 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 requires strong data minimization and purpose limitation.

B. Data Protection

The partners in the project, except for CNC, do 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, collect human adult stem cells (from teeth) and fibroblasts (from gingival biopsy, associated with the tooth extraction) from patients

and healthy controls. The rationale to collect dental stem cells is two-fold: a) these cells originate from the ectoderm and neural crest and therefore share a similar lineage to human neurons that may facilitate downstream differentiation protocols; b) tissue collection occurs from exfoliated teeth or is performed as part of prescribed dental treatment, no patient or controls are recruits with the intent of performing biopsies (i.e. tooth collection for this project is secondary to standard dental treatment and will only be performed if deemed necessary by the patient/caregiver).

Only in this part, the project involves 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 will be 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).

It is important to mention that the Syn2Psy consortium does not change the nature of existing data management procedures (collection, storage, protection, retention, destruction, and confirmation) for CNC and neither poses a hindrance in terms of the ability to comply with current and future regulations in data management.

The only partner engaging in data collection for research is CNC. All research proposals involving living human participants and the collection and/or study of data derived from living human participants undertaken by CNC staff or students on the CNC premises and/or by CNC staff or students elsewhere requires ethical approval to ensure that the research conforms with general ethical principles and standards.

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 are aware of them. The Syn2Psy project involves a very low risk of occurrence of incidental findings since the participants are clinical subjects with known

diagnosis, or lack thereof and the analytical component of the research work does not lend itself to incidental findings (e.g. there is no foreseen wideband DNA sequencing or similar methodology). Regardless of the above, since the original project approval granted from Ethics Committee at the University Hospital of Coimbra does not foreseen disclosure, nor not the non-disclosure of incidental findings, any such discovery with clinical relevance will have to first submitted for Ethical Board review board on a one-to-one cases.

D. Animal experiments

Several strains of transgenic mice and rats are being used in Syn2Psy. Some experiments include 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 is 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 is pseudonymized. The confidentiality of participants information is protected and the nature of the proposed studies does 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. As ethics advisor to the project, I am satisfied that the research in the project has been designed with full consideration for ethical concerns, and that the appropriate measures are 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 is pseudonymized. The confidentiality of participant information will be protected and the nature of the proposed studies does not give rise to stigmatization for those bearing the diseases under study. Personal data of participants, including medical or health information collected or created as part of the study (such as medical records or test results) will not be required for this project after its collection. Animal experiments are compliant with the European and national laws for animal experimentation and the principle of 3Rs is well addressed. The partners had obtained the relevant authorizations for animal experiments.

The Syn2Psy project is unusual, in some senses. The nature of the project does raise some broader ethical considerations beyond the basic compliance with data protection.

As ethical advisor I am satisfied that consideration of these questions have been fundamentally incorporated into the research of the project.

Assinado com Assinatura Digital Qualificada por: CARLA LILIANA FERREIRA BARBOSA BAPTISTA
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Carla Barbosa
External Advisor

Annex 5 – Data Management Plan – v2

Grant Agreement number: **813986**

Acronym: **Syn2Psy**

Title: **Synaptic Dysfunction in Neuropsychiatric Disorders**

Data Management Plan - v2

WP6 – D6.9 – D43

Date: 30-04-2021

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1. Document information and Summary

Deliverable Number:	D6.9 update
Deliverable Title:	Data Management Plan
Work Package:	WP6 - Management
Lead Beneficiary:	CNC
Type:	ORDP: Open Research Data Pilot
Dissemination Level:	Confidential, only for members of the consortium
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Author	Margarida Figueiredo and Raquel Rodrigues
Reviewed and Authorized	Ana Luísa Carvalho (coordinator)

Summary

This document describes the second version (v2) of the data management plan (DMP), based on the initial DMP, submitted as a deliverable in December 2019, for the Syn2Psy ITN. The DMP was prepared taking into account the EC Open Research Data Pilot. The data generated during the Syn2Psy project is intended to follow the FAIR principles for data storage and sharing (findable, accessible, interoperable and reusable).

Syn2Psy ITN aims at training a new generation of researchers to unravel the cellular and molecular basis underlying neuropsychiatric disorders (like autism, schizophrenia, intellectual disability) with the goal of contributing to the development of better therapies. A strong focus of this network is in describing the mechanisms by which mutations on synaptic protein-encoding genes cause defects in neuronal networks and in behaviour, since recent studies describe that synaptic pathology is observed in many neuropsychiatric disorders (altered synapse morphology and density, aberrant synapse signalling and synaptic plasticity). The Syn2Psy ITN combines human geneticists with laboratories developing new animal models and with a pharmaceutical company and research groups testing new hypotheses and compounds. The main goals are to: i) generate novel models of disease, including human-derived neurons and brain organoids; ii) test the promising focal point of excitatory/inhibitory imbalance in neuropsychiatric disorders; and iii) explore disease modulatory elements (both positive and negative).

The Syn2Psy research carried out by the ESRs will generate data from studies on: novel animal models created to mimic specific human mutations, human-derived neurons, brain organoids and IPSCs derived from patients as models of neurodevelopmental disorders. The data will include videos of behavioural studies, super-resolution microscopy images and synaptic and mesoscopic imaging, fiber photometry data, optogenetic data and electrophysiology data, among others.

This initial DMP is a living document and its contents will be updated to the end of the project.

2. Data collection and documentation

2.1 Description of the data collected, observed, generated or reused

General data collected and generated:

- Animal data from mice (*Mus musculus*) and rats Wistar Han and Long Evans (*Ratur norvegicus*), including new animal strains (100-150 rats/mice per year for each organization involved).
- Data from Human cells/tissues: iPSCs; dental stem cells extracted from donated teeth from study participants (patients with autism and healthy individuals).
- Viral vectors (Adeno-Associated Virus, lentivirus, etc) and plasmid vectors.

Administrative data (provided as ethics deliverables - due date, February 2020):

- Ethics approvals and certifications for animal experimentation: institution licences (ethics approval for the animal house including authorizations from breeders, suppliers, users), project licences (ethics approval for each project); personal licences (ethics certifications for ESRs - FELASA B minimum).
- Use of Human cells / tissues in research: ethics approvals for the use of cells from patients and informed consent forms; material transfer agreements - MTA (for cells obtained from other laboratories/institutions); details on cell/tissue types and details on biobanks and commercial providers.
- Documentation on health and safety procedures from local/national guidelines/legislation, GMO authorizations, BSL2 measures (security level 2 for work with virus).

Animal husbandry and experimental design data:

- Dark/light cycle, husbandry statistics, animal provenance, mutants.
- Blinding, attrition, randomization, allocation concealment, power analysis, termination criteria, genotyping after animal delivery, genotyping before and after experiment, etc.
- All animal research data will be managed using electronic spreadsheets, animal facility management software **PyRAT®** , **Anibio®, A-Tune** and **Tick@Lab**, and animal behaviour detection and analyses software **Ethovision ®**, **Observer ®**, **Freezeframe**, and Macros designed using **Matlab®**.

Animal physiology and behavioural data:

Behavioural data from living animals and data from *ex vivo* analyses (e.g. brain tissues) will be collected according to standard procedures, and all biological samples will be prepared according to standard published protocols that are well recognized in the field. Data from video tracking of animals' behaviour are expected to reach a total size between 3 TB and 5 TB over the course of each project. The remaining data, together with other derived data (measurements and quantifications), are not expected to exceed 2TB, per project.

Data and Human cells from patients:

For ESR3 project (involving the extraction of dental stem cells from teeth donated by the study participants) we will collect the following data from patients: demographic Data - age, gender; Clinical Data – Diagnosis and Clinical History. The experimental data will be generated from the cell cultures established from the donated tissues (teeth) and from material extracted from them, such as mRNA. The data is expected to have a size < 1 TB. Our data might be useful for other researchers working in similar areas who wish to perform meta-analyses or for comparison of results. Publicly available databases will be re-used for data comparison, such as GTex database and the Allen brain atlas.

Specific types of data generated:

(a) Video files monitoring animal's behaviour during specific tasks
(b) Quantitative analysis data of genes or proteins from brain tissue
(c) Quantitative analysis data of hormones from blood samples
(d) Quantitative analysis data on brain metabolites with magnetic resonance spectroscopy
(e) Polysomnographic Electroencephalogram/ Electromyography (EEG/EMG) recordings
(f) Field and whole-cell electrophysiological data on neuronal cell activity *ex vivo* and *in vivo*.
(g) RNA expression data, including data from ribosome profiling analysis (RNAseq), Drop-seq and qRT-PCR
(h) Protein expression data
(i) Flow cytometry data
(j) Immunocytochemistry data
(k) Whole-cell patch clamp data/field recordings
(l) Two-photon microscopy data
(m) Data obtained by super-resolution imaging methods (PALM, 2-color 3D Storm, UPaint)
(n) Data from mesoscopic optical voltage imaging
(o) Data from real-time quantum dot tracking of single receptors
(p) Data from monosynaptic retrograde tracing
(q) Data from magnetic resonance diffusion tensor imaging (MRI-DTI)
(r) Demographic Data - age, gender and Clinical Data - Diagnosis, Clinical History (ESR3 – using autism patients dental stem cells)
(s) Neuronal morphometric analyses - Sholl
(t) Neuronal activity measurements (GCaMP)
(u) Ultrasonic Vocalizations (USVs) recordings

Syn2Psy data files:

When possible data will be saved as commonly used file types, e.g. .**tiff**, **.jpeg, .xlxs, .png, .txt** and **.xlm**. For video tracking, files will be saved as .**avi**, **.mp4** and **.raw**. Below are examples of file types that might be generated from specific software and techniques:

.lsm and **.czi** files(**ZEISS** microscope format) - confocal data;

.lif, **.lifext** (**Leica SP8** microscope format) - confocal data;

.tiff files (**ImageJ** and **Prairie View**) - *in vivo* 2-photon imaging, macros, analysis & processing of images; **.vsi** - scanned slides;

.sds - gene expression;

.prm - chromatography primary files for HPLC;

.pvfs (**Sirenia**® software) - *EEG*/EMG;

.dat, **.hdf5** (**Stimfit** software) and **.pClamp** - electrophysiology;

.png, .txt, .xlm (**Neuroexplorer** and **Openphys** software) - *in vivo* electrophysiology recordings;

.pxp or **.ibw** (**WaveMetrics Igor Pro**® Software) - to analyze electrophysiology data or exported as **.csv;**

.xlxs, **.png** or .**xlm** (**GraphPad-Prism** Software) - to make graphics of electrophysiology data;

.ab1, **.seq,**.**fastq** - sequencing data;

.gel (**Typhoon** software) - laser scanner data;

.ims (**IMARIS** software files) - Sholl and spine analyses;

.m (acquired data from **Matlab** custom software scripts) – animal behaviour;

.imu - accelerometer data;

.gpio and **.isxd** (**Inscopix** software) - DAQ and for ancillary data respectively;

.wav and .**aiff** (**Audacity** and **Raven Lite** software) - USVs;

.xlsx - measurements of behavioural parameters (for long-term preservation: **.csv**);

.ffii (**Freezeframe** Software) - behaviour data.

2.2. Documentation and metadata provided

Nomenclature:

Datasets will be stored with criteria that will ensure accessibility to all current and future members of the research group and that will facilitate future consultation and reuse of the data. Files will be named according to a pre-agreed convention that will include identification of Syn2Psy, researcher name, date and the type of data, e.g. **Syn2Psy_ResearcherName_date_DataType**'.

We will provide keywords in the published scientific articles. We will follow the Human Genome Organisation naming conventions, defined by the HUGO Gene Nomenclature Committee (HGNC).

Unique identifiers:

Animals used in each dataset will be identified by the **unique code** assigned by the animal facility software **PyRAT®, Anibio®, A-Tune** and **Tick@Lab** or blindly by the animal facility personnel**,** allowing access to genetic background and pedigree.

For iPSC cells derived from patients by collaborators, the collaborators coding system for the cells will be used (designated by clone number and cell type [disomic or trisomic]). All other identifier information pertaining to patients will be kept blinded from the researcher.

For ESR3 project (involving the extraction of dental stem cells from donated teeth of the study participants): each patient sample is assigned a Project ID number unique to each patient, which will be provided in a pseudonymized form along with relevant information (age, gender, diagnosis).

Syn2Psy Publications, underlying data, and corresponding metadata/bibliographic metadata will be assigned a **DOI (Digital Object Identifier)**. All Syn2Psy researchers will have an **ORCID** number (non-proprietary alphanumeric code to uniquely identify scientific and other academic authors and contributors).

Metadata

Each dataset will be accompanied by a **README.txt** file which will describe the directory hierarchy, the purpose, origin, description, time reference, creator, access conditions and terms of use of a data collection and providing details on the experimental procedures.

The final datasets, as deposited in the chosen data repositories, will be accompanied by the relevant metadata documentation that will be compliant with DataCite Schema standards. This will ensure that the produced data can be analysed together with other data types during the project and following project completion.

Bibliographic metadata, which identify publications deposited in open access repositories, will include: [EU_Horizon2020_MSCAITN_Syn2Psy_GA813986_PublicationDate_LenghtEmbargoPeriod_DOI].

Documentation procedures:

Along with the stored data, we will annotate what has been done in detail: the conditions of the analytical procedures; sample type, size, date of analysis, fixed and variable modification done during the sample preparation, method used for sample preparation, units of measurements, version of software used, the date on which each modifications were made, the type of modification or data processing, if any.

3. Ethics and security issues

3.1. Handling of ethical issues

All research work proposed in Syn2Psy will be conducted in accordance with the relevant national rules of the country where the research will be carried out. Whenever necessary, authorization will be requested by the competent research ethics committee or the medical ethical committee of the country where the work is conducted. The proposed work has implications on two different levels: the use of (i) laboratory animals and (ii) human-derived samples, from healthy volunteers and patients with *Shank3* mutations.

Animal Experimentation:

All Syn2Psy research groups have adhered to the European Commission guidelines for the care and use of laboratory animals, which have been acquired and transformed into operative rules by the national (Ministry of Health or Ministry of Agriculture) and local institutions (Health Services, Universities and their operative branches, the Departments and Institutes), and monitored by local Ethical Committees.

Research involving animals - small rodents, in the case of the present project - will be performed in compliance with Directive 2010/63/EU (revising Directive 86/609/EEC), which is transposed into the national law of all partners involved.

The **Syn2Psy** project will comply with the *Amsterdam protocol on animal protection and welfare*, which is characterized by the need to **Replace** animals whenever possible, to **Reduce** the number of animals used in experimental research, and to **Refine** procedures to minimize suffering (3Rs approach). The following principles will be implemented: **Reduction:** we will make all efforts to *reduce* the number of animals used in experiments to the minimum that is required for obtaining meaningful results. **Replacement:** A major effort will be made to keep animal experimentation to the minimum required and to *replace* the use of live animals by non-animal alternatives. **Refinement:** Mice (wild type and genetically modified) will be housed in certified animal facilities at all participating institutions and all effort will be made to minimise animal suffering and distress during handling and experimentation.

In order to ensure the best practice in experimental research with animals, all **ESRs** will be required to **successfully conclude a FELASA course - Category B, or equivalent** (for persons carrying out animal experiments).

Human cells from patients

For ESR3 project (involving the extraction of dental stem cells from teeth donated by the study participants): Only the clinical staff has direct access to patients and all medical information. The clinicians explain the overall goals of the project, recruit participants, store informed consents, extract biological samples and perform pseudonymization. The research team is given accessed to limited data from the participants, which is mostly restricted to age, gender, clinical condition and relevant biological samples. The pseudonymized personal data of the patients (e.g demographics and clinical data) is kept alongside participants' IDs and other relevant information related to the biological sample (e.g. passage number, cell types, etc.). Only the healthcare team at the Pediatric Hospital will have access to the key that can identify the participants. No identifiable personal data will be made available to the research team or for publication. The biobank responsible for storing the samples manages its own license for use of samples for research and is under the purview of the Portuguese Ministry of Health. The informed consent given to participants clearly states that generated data can be shared within the research community and states the above-mentioned safeguards regarding personal data protection. Regarding long-term preservation of data and biological samples, the Portuguese Legislation indicates that research study participants cannot require erasure or destruction of samples.

3.2. Management of data access and security

All computers in the laboratory are only accessible through a session opened by users. User accounts (user_ID) and passwords are managed by the Institution's IT Departments. Access to the various servers in the laboratories is assigned to users according to their needs and controlled by the PI.

Human cells from patients (ESR3)

For ESR3 project (involving the extraction of dental stem cells from teeth donated by the study participants): the best security practices will be followed in order to ensure the **confidentiality**, **authenticity** and **integrity** of the information. The participants' pseudonymised personal data (e.g. demographics and clinical data) is kept in a password-protected platform- *S.Clinico* - that is maintained by the Portuguese Government through the Ministry of Health, and is accessible only by clinicians at the Paediatric Hospital. Project-specific information is transferred to *CliniData*, a platform associated with the Paediatric Hospital Biobank, where the participant ID/codes is deposited. Researcher staff only has access to pseudonymised data in the *CliniData* platform.

The research team does not have access to the key that can identify the participants. The healthcare team (primary caregivers) at the Pediatric Hospital retains all patient data and clinical history and deliver the relevant information (age, gender, diagnosis) to the research team in an associated, pseudonymized form, alongside an Project-specific ID number unique to each patient.

Research data will be archived in a durable form for a period of 10 years with the research team in password-protected offline storage. The team has implemented a Standard Operating Procedure (SOP) (in Annex 1) to cover handling of samples and assure the maintenance of patient confidentiality. This outlines the controls and checkpoints that are in place when processing different types of confidential information and biological samples.

Syn2Psy Intellectual Property (IPR)

The rights of beneficiaries and partner organisations to use the knowledge produced within Syn2Psy are subjected to IPR clauses in the **consortium agreement** that was signed by all parties. The Syn2Psy consortium will examine and operate any potential IPR issues as they happen.

4. Data storage and preservation

4.1. Data storage and back-up during the research

Animal husbandry data from **PyRAT®**, **A-TUNE**, **Anibio®** and **Tick@lab** software will be stored at the animal husbandry units and exported and stored at the institutional servers.

Data is collected in original notebooks and hard- copies of quantification will be stored in the PI's laboratory or office. Additional electronic data will be stored on the individual experimenter's computer and backed up on external disks / Lab Server only accessible from the research group. Additionally, the laboratory will make use of the PI's lab server space at institution's storage facility for a second repository of data storage (when available). Data will be preserved for 10 years on the Institution's servers.

4.2. Depositing data in repositories

For long-term data preservation, Syn2Psy research results in the form of peer reviewed final manuscripts and in the form of underlying data (data required to validate the results described in the scientific publication and corresponding metadata) will be immediately deposited in a digital **data repository** conforming with the FAIR data principles (e.g. Zenodo), at the latest on the publication day. Bibliographic metadata, which identifies the publications, will also be deposited. Manuscripts ready for peer-review (preprint) might in some instances be released as open-access preprint on **BioRxiv**.

Datasets will be given a Digital Object Identifier -**DOI**- and associated metadata. The **DOI** corresponding to the datasets in the repository will be included in the article's reference list, allowing identification and access of any dataset in a publication.

DOIs will also be linked to appropriate records in the Institution's publication repository, to enhance the dataset's visibility. Metadata about datasets will be publicly searchable and discoverable and will indicate how and on what terms the datasets can be accessed. Iinformation about datasets will be displayed on the laboratorys' webpage, on researcher profile pages, as well as on researchers' **ORCID_iDs**, which will increase the visibility of the datasets.

After depositing the data, open access to the publications will be ensured.

Unpublished high-quality final data generated during this project will be saved for reuse in the research groups' future projects. We will make some data available for use by researchers in future collaborations if our lab no longer uses it. Bad quality data will be permanently discarded at the end of the project. Where possible, we will store files in open archival formats e.g. **.docx** files converted to **.pdf-A** or simple **.txt** files encoded in **UTF-8**, **.xlsx** files converted to **.csv**, **.LSM** and **ImageJ** files to **.tiff**. In case this is not possible, we will include information on the software used and its version number.

Coding data and custom software will be shared by deposition on appropriate online repositories (e.g. direct link to lab server, Bitbucket, GitHub, and ModelDB), and custom design files will also be made available (e.g. www.thingiverse.com and GitHub).

5. Public data sharing

5.1. Providing Open Access to data

Syn2Psy will, as far as possible, enable the generated data to be openly accessible, free of charge, allowing the users to mine, exploit, reproduce and disseminate it (unless specified in this document or in future Syn2Psy data management plans).

Syn2Psy will provide **open access** to published data, and corresponding bibliographic metadata, via self-archiving (in **Zenodo** and Institution's data repositories) no longer than 6 months after the publisher's publication date (green open access) or immediately upon publication (gold open access – via open access journals or by paying additional fees in hybrid journals). Syn2Psy will aim at establishing CC BY license with the publishers regarding the researchers/authors copyrights over their work. The EC Open Access Publishing Agreement: V1.0 – 20.03.2017 might be used, when necessary, to ensure that the publisher's embargo period for public dissemination is not longer than 6 months.

Underlying data (needed to validate results presented in scientific publications), and corresponding metadata, will also be openly accessible, through deposition in **Zenodo**.

5.2. Data sharing constraints

Syn2Psy will, in general, have no data sharing constraints, except when using data from patients due to the need to protect personal data and assure their confidentiality and security (see below). All chosen digital repositories are/will be conformed to the FAIR Data Principles (e.g. **Zenodo**).

Data from patients:

For ESR3 project (involving the collection of human dental stem cells from autist patients), to assure participant confidentiality, the following data will not be publicly available: Demographic Data, Clinical History. **Neuromorpho.org** will be the data repository used for morphometric data. Since the data will be stored in a public repository and used for scientific purposes, the identity of those accessing the data will, therefore, not be ascertained. Our data will be uploaded without any identifiers, tags or metadata, regarding participant identity. The available data produced in the project will be interoperable, allowing data exchange and re-use between researchers, institutions, organizations and countries. The data will be licensed through creative commons attribution and non-commercial licenses. Data will be available upon publication through scientific articles, which will include the appropriate link to find the relevant datasets. The shared data will be re-usable, since it will be deposited in public repositories and, upon request, given to the leaders of the project. The data will remain re-usable during the lifetime of the public repositories and offline back-ups will be kept for a period of 10 years. Data quality assurance will be described in the methods section of the associated scientific articles.

6. Allocation of resources

The costs of making the Syn2Psy data FAIR are related to the time spend in these activities and are estimated to be about 1 week/year for each partner. There is a continuous need to update and maintain informatics hardware associated with the maintenance of data storage. Those costs are funded by project funds.
Every Syn2Psy researcher is responsible for the management of the data that he/she will collect/generate. The project manager will overview the implementation of this data management plan.

For the costs related to preservation of digital data, Syn2Psy will use the free data repository **Zenodo**.

Preservation of biological samples will be the responsibility of the partners that generated them. Important samples can be transferred to third-party repositories that do not charge for archiving (e.g. Adgene).

Annex 1

Standard Operating Procedure (SOP) for ESR3 Project (involving the collection of human dental stem cells from patients with autism)

Below we describe the procedures, since sample collection until data preservation:

1) Participant personal and clinical data is collected by clinicians and stored in S.Clinico Platform.

2) Informed consent form is given and signed during medical consultation with the medical staff.

3) In case of deciduous teeth, parents take a tooth kit home, which includes a tube with medium to preserve the dental cells and instructions to have the sample kit delivered to the Paediatrics Hospital.

4) In case of third molars, a surgical excision is scheduled.

5) Both the tube and consent form are coded by attaching a tag with an ID code.

6) Once the tooth is shed or collected, it is placed inside the coded tube and brought to the Paediatric Hospital by the parents or clinical staff.

7) The coded tube is then picked up by the research team and sent to the lab at CNC, University of Coimbra.

8) The sample is cultured and expanded by cell culture procedures.

9) Frozen sample is then sent to the Biobank at the Paediatric Hospital for storage, where the identification of participants is stored in their platform CliniData.

10) Pseudonymized data generated in the lab will be stored in password-protected drives.

