UNIVERSIDADE D
COIMBRA

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VISUAL CORTICAL PLASTICITY IN NEUROFIBROMATOSIS TYPE 1

Dissertação no âmbito do Mestrado em Biologia Celular e Molecular, com especialização em Neurobiologia orientada pelo doutor João Martins e pelo doutor João Peça apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra

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Faculdade de Ciências e Tecnologias da Universidade de Coimbra

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Resumo

Os transtornos do espetro do autismo (ASD) são um conjunto de perturbações do neuro desenvolvimento caracterizadas por défices na comunicação social, interesses restritos e comportamentos repetitivos. Os processos de plasticidade sináptica são cruciais para a aprendizagem baseada na experiência e, nestas perturbações, a plasticidade sináptica está desregulada. O córtex visual primário (V1) é uma região atrativa pois desenvolve plasticidade baseada na experiência para um estímulo visual com uma orientação particular. Este tipo de plasticidade é chamada de potenciação de resposta seletiva a um estímulo (SRP).

Esta tese de mestrado pretende dar um particular enfoque ao desenvolvimento de SRP em V1 num modelo animal transgénico de neurofibromatose tipo 1 que apresenta as principais características de ASD. Para isso, utilizámos murganhos *Nf1*$^{+/-}$ e *WT* da mesma ninhada. No nosso protocolo, os animais (*postnatal day* (P) 45) foram habituados com estímulos de inversão de fase com uma orientação particular e ao dia 7 uma orientação nova e familiar foi dada para avaliar a preferência para o estímulo novo. Com outro grupo de animais (P30-45), fez-se a habituação durante seis dias e no dia 7 fez-se registos eletrofisiológicos em V1 usando uma sonda de elétrodos de silicone com 16 canais. Finalmente, fez-se um paradigma de interação social onde se utilizou os cérebros para fazer imunohistoquímica para c-Fos para avaliar quais as regiões mais ativadas. No futuro, estas regiões serão analisadas através de eletrofisiologia em V1 e estimulação visual.

Os nossos resultados demonstraram que a nível comportamental os murganhos sem segregação por genótipo apresentam um interesse superior para a novidade, não havendo diferenças entre *Nf1*$^{+/-}$ e *WT.* Contudo, os registos *in vivo* de eletrofisiologia em V1 não apresentaram diferenças significativas para a latência e magnitude dos potenciais visuais evocados.

Estes resultados sugerem que embora o desenvolvimento de SRP seja aparente a nível comportamental sem diferenças entre genótipos, o nosso paradigma de registo de potenciais evocados em V1 em murganhos anestesiados poderá não ser o ideal para captar os possíveis substratos neuronais deste fenómeno plástico. Além disso, outras regiões que se interconectam com V1 poderão manifestar SRP e estar desreguladas na sua plasticidade. Desta forma, no futuro, seria interessante obter registos em outras regiões que podem estar desreguladas na plasticidade dependente da experiência.

Abstract

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders characterized by deficits in social communication, restrictive interests and repetitive behaviors. Synaptic plasticity processes are vital for learning from experience and it is known that in these disorders, synaptic plasticity is disrupted. The primary visual cortex (V1) is an attractive cortical area since it develops experience-dependent plasticity for visual stimuli with a particular orientation. That type of plasticity is stimulus-selective response potentiation (SRP).

This Master's thesis aims to give a particular focus to SRP development in V1 in a transgenic animal model of neurofibromatosis type 1 (NF1) that resembles the main features of ASD. For that, we used *Nf1*$^{+/-}$ mice and *WT* littermates. In our protocol, animals (P45) were habituated with phase-reversing stimuli with a particular orientation for six days and, on day 7, exposed to novel and familiar orientations to evaluate the preference for novelty. With another group of animals (P30-45), we habituated the animals for 6 days to a particular stimulus, and on day 7 we performed in vivo electrophysiology experiments in V1 using a 16-channel silicon electrode probe. Finally, we performed a social interaction paradigm where we used the animals for c-Fos immunohistochemistry, trying to unveil the most activated brain regions. In future experiments, these later regions will be analyzed with electrophysiology in V1 upon visual stimulation.

Our results showed that at behavioral level, mice with no genotype segregation presented an increased interest for novelty with no differences between *Nf1*$^{+/-}$ mice and *WT* littermates. However, *in vivo* electrophysiology did not present significant differences either for visual-evoked potentials (VEP) magnitude or for latency.

Together these results suggest that although the SRP expression was evident at behavioral level without an effect of genotype, our protocol of evoked potentials recorded in V1 in anaesthetized mice may not be ideal to capture SRP neuronal substrates. Moreover, other regions coupled to V1 might be involved in SRP and disrupted in their plasticity. As a result, in the future, we should obtain more other areas that might be disrupted in this type of experience-dependent plasticity.

Contents

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Abbreviations

ADHD – attention deficit and hyperactive disorder

AMPAR - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ASD – autism spectrum disorders

BLA – basolateral amygdala

BSA – bovine serum albumin

CeA – central amygdala

CP – critical period

E/I – excitation/inhibition

EEG – electroencephalogram

EPM – elevated plus maze

FMRP – fragile X mental retardation protein

GABA - gamma-aminobutyric acid

HCN - hyperpolarization-activated cyclic nucleotide-gated channels

KO – knockout

LTD – long-term depression

LTP – long-term potentiation

MECP2 - methyl-CpG binding protein 2

mGluR – metabotropic glutamate receptor

mPFC – medial prefrontal cortex

MRI – magnetic resonance imaging

mTOR – mammalian target of rapamycin

NAc – nucleus accumbens

NF1 – neurofibromatosis type 1

NLG3 – neurolignin 3

NLGNs – neurolignins

NMDAr – N-metyl-D-aspartate receptor

NRXNs – neurexins

OD – Ocular dominance

OSH – orientation-selective habituation

P – postnatal day

PBS – phosphate buffered saline

PET – positron emission tomography

PN – pyramidal neurons

PNN - perineuronal nets

PSD – post-synaptic density

PTEN – phosphatase and tensin homolog

PV^{+} - parvalbumin positive neurons

RSG – retrosplenial cortex

RT – room temperature

SC – superior colliculus

SHANK - SH3 and multiple ankyrin repeat domains 3

SRP – stimulus- selective response potentiation

SST^+ - somastotin positive neurons

tDCS – transcranial direct current stimulation

TMS – transcranial magnetic stimulation

TSC1 – tuberous complex 1

TSC2 – tuberous complex 2

V1 – primary visual cortex

VEP – visual-evoked potential

Vidget – visually driven behavior in head-fixed mice

VIP^+ - vasopressin positive neurons

VTA – ventral tegmental área

WT – wild-type

Introduction

1) Autism Spectrum disorders

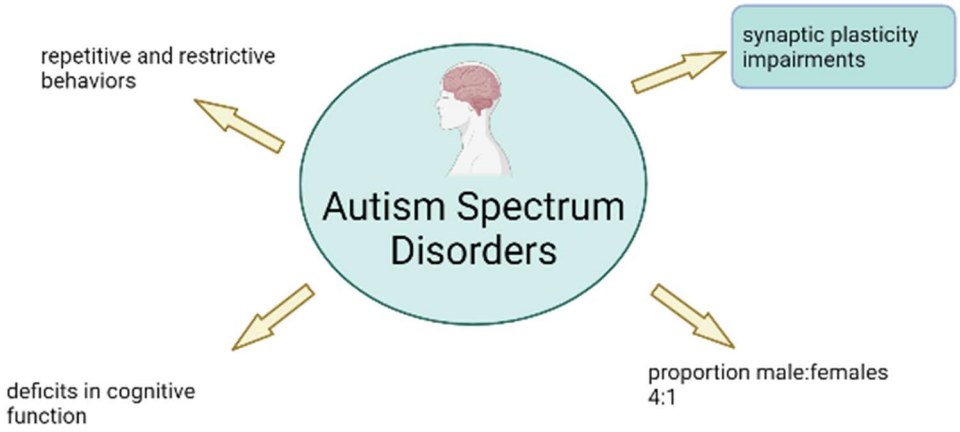

Figure 1 - **Representation of the main features of autism spectrum disorders.**

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Autism Spectrum Disorders (ASD) are a cluster of neurodevelopmental conditions characterized by a specific behavioral phenotype. They are manifested by a deterioration in cognitive function, social interaction, and restricted and repetitive behaviors. It is a condition present worldwide with an incidence of about 1%[1,2]. The proportion between male and females is about 4:1 (Fig.1). The research, usually, puts together ASD and attention deficit hyperactive disorder (ADHD), despite they are sufficiently different to be considered separated[3]. There is an increased interest in the presence of mutations in specific genes, showing that ASD have a genetic risk associated[1]. It was observed that sex chromosomal genes or sex hormones can greatly influence the development of ASD phenotype[4]. As many other disease conditions, ASD depends on the interaction between genetic and environmental factors. A myriad of different biomarkers is being currently developed to have a better and early diagnosis of this condition. This includes the use of cutting-edge technologies such as proteomics, genomics, transcriptomics and

metabolomics[5]. Synaptic plasticity is an essential feature for an appropriate neurodevelopment and ASD patients show impairments in synaptic homeostasis (Fig.1)[6]. As a result, this opens a clear path of research to study if sex variable accounts for synaptic plasticity alterations in ASD. To study this, the visual cortex emerges as an exciting region to start investigating since it is a thoroughly studied region where one can conduct multiple synaptic plasticity experimental paradigms in animal models.

1.1) ASD and the E/I imbalance

The excitation/inhibition balance is defined as the contributions of the excitatory and inhibitory inputs to neural circuits[7].

The theory of E/I imbalance is one explanation given by multiple studies for the neuropathological hallmarks of ASD. These imbalances observed in some ASD models for certain genes associate this imbalance with alterations in GABAergic neurons and to some behavioral deficiencies, particularly, social deficiencies[8]. Alterations in synaptic plasticity are associated/related to alterations in E/I ratio in genetic models of the disorder. Accordingly, it is important to link plasticity with this ratio in models of the disease such as neurofibromatosis type 1 that will be emphasized in the next sections.

2) Synaptic plasticity in ASD

Autism spectrum disorders (ASD) are usually seen as disorders of the synapse called synaptopathies. Indeed, multiple genetic mutations for specific proteins in the pre- and post-synaptic terminals have been shown to be associated with an ASD phenotype. A complete understanding of the interactions between those proteins and the associated signaling cascade was not achieved yet. However, studies using complete or conditional knockout (KO) mice models are revealing the behavior phenotype and neuronal function associated with mutations in those genes (Fig.2).

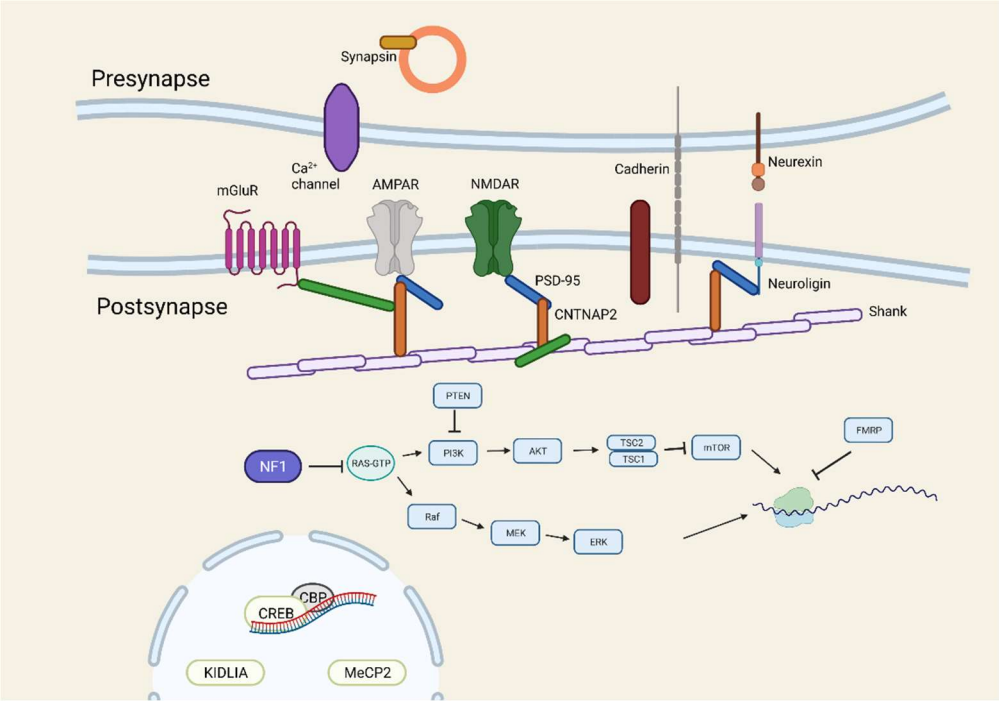

Figure 2 - **Major proteins and signaling pathways involved in the synapse and that are disrupted in ASD**

Synapsin is a protein associated with synaptic vesicles at the pre-synaptic terminal. At the post-synaptic terminal, there are the glutamatergic receptors: mGluR, AMPAR, and NMDAR. The connection between the two terminals is made by the cadherins and by the interaction between neurexins and neuroligins. PSD-95 interacts with CNTNAP2 (contactin-associated protein-like 2 gene) that interacts with Shank proteins at the post-synaptic densities. In the cytosol, we have two crucial pathways: mTOR and Raf/MEK/ERK[18]. NF1 is a protein that inhibits RAS-GTP and consequently the two pathways[55]. PTEN inhibits PI3K and, consequently, mTOR[18]. FMRP is an important protein involved in gene translation[102]. CREB (cAMP response element-binding protein) associated with CBP (CREB binding protein), KIDLIA (protein from KIAA2022), and MeCP2 (methyl CpG binding protein 2) are proteins presented in the nucleus involved in the regulation of gene transcription[9] (Created with biorender.com based on Gilbert,2017[103]).

2.1) *MECP2*

Methyl-CpG binding protein 2 (*MECP2)* mutations are linked to development of Rett syndrome. It is a neurodevelopment disorder characterized by an ASD-like phenotype. The protein encoded by *MECP2* is an epigenetic modulator in the brain involved in many epigenetic mechanisms[9]. In this syndrome, it is observed that sex is an important variable for developing the disorder. Females are more prone to develop Rett syndrome than males, being this mutation a prominent explanation for mental retardation in females[9]. This aspect of the syndrome shows that despite

an increased ratio in ASD for males, we should always consider the genetic factor when we study the epidemiology of the disorder. The reason for that is because this gene is an X-linked gene so, the chromosomic architecture of females is a risk factor for that. Recently, different therapeutical options were tested. One of those options was choline which is a micronutrient found in most of diets and with a role in brain development. The administration of this compound in a mouse model of Rett syndrome showed an intensification in neurons' morphology and dendritic spines as well as a rescue of a normal behavioral phenotype[10]. Another drug used, fluoxetine, proved effective in a heterozygous mouse model of the disease by rescuing the motor deficits observed. The mechanism of action involves stimulation of serotonergic pathways that regulate the motor circuits[11]. In recent times, it was shown that the inhibition of Ras-MAPK signaling, a pathway involved in plasticity, was able to normalize the excessive clustered spine stabilization and rescue the motor defects observed in a mouse model of the disease[12]. When this gene was mutated, alterations in visual cortex circuit maturation were observed. The accelerated maturation of parvalbumin-positive neurons (PV^+) upon vision onset is involved in the early closure of the critical period. These specific alterations in interneurons alter brain connectivity. Also, the binocular function was affected by mutations in *MECP2*[13]. More recently, the same group showed that plasticity development was completely unsettled in the auditory cortices of a Rett syndrome mouse model, conducing to abnormal pup gathering behaviour[14]. It was also demonstrated in other contexts like in a senescence-accelerated model or in stress and depression that *MECP2* is crucial for normal synaptic plasticity[15,16].

All the previously mentioned studies showed that plasticity is impaired in mutations for *MECP2* gene. Also, the proposed therapeutical inhibition of Ras-MAPK signaling, which is implicated in plasticity signaling, may revert the impairment in sensory cortices. Therefore, it is important to study if the different therapeutics already used for Rett syndrome are linked with a normalization of synaptic plasticity in other ASD models.

2.2) mTOR pathway

The mammalian target of rapamycin (mTOR) pathway is one of the most important pathways in the cell involved in cell proliferation and in suppression of autophagy. This pathway is highly studied in cancer research due to its impact on cell proliferation[17]. However, it is also studied in autism research[6]. Indeed, there are multiple proteins involved in this pathway with mutations that mimic autism-like phenotype.

2.2.1) *PTEN*

One of those proteins is the one encoded by *Phosphatase And Tensin Homolog (PTEN)*. In the mTOR pathway, PTEN inhibits PI3K. Dysfunction of PTEN will ultimately, lead to a hyperactivation of the pathway[18]. PTEN has been shown to be important in hippocampal synaptic plasticity independent of neuronal structure[19]. PTEN was shown to be a very important protein in signaling pathways involved in neuronal maturation, particularly, in dendritic maturation[20]. Cortical dysfunction observed in PTEN autism mouse models is a result of the increased expression of small-conductance calcium-activated potassium channels[21]. Mouse models of PTEN loss of function are studied due to their autism-like phenotype. These models show abnormal social interaction and repetitive behavior[22]. Indeed, it was shown in three PTEN mouse models that the normal firing pattern of CA1 neurons is disrupted and that impacts social recognition[23]. As a result, they are good models to study the biological aspects of ASD and to test whether novel therapeutics rescue a normal behavioral phenotype. Targeting mTORC2 complex with an antisense oligonucleotide proved to be a genetic approach with efficacy in improving the behavioral glitches and the neurophysiological impairments observed in models with *PTEN* mutated[24]. Interneuron transplantation proved already that it can be a good therapeutical option in this model by rescuing social behaviors, although this intervention did not restored wild-type circuit states in the prefrontal cortex[25]. This study demonstrated that interneuron manipulation may elicit a therapeutical effect in an autism mouse model marked by excessive synaptic inhibition. In the study previously mentioned, the transplantation was done in the prefrontal cortex of mice. It is not completely understood if interneuron

transplantation to other cortical areas could have the same effect. It is vital to have a more comprehensive picture of the mechanism of action of this therapeutic at a circuit level. An inadequate inhibitory synaptic input towards pyramidal neurons was shown in mice lacking PTEN in PV^+ cells in the visual cortex[26]. Since PTEN is a protein that suppresses mTOR, a proliferation pathway, it could be associated with excessive activity of these PV^+ cells with impairments in plasticity attached. This was shown already for Rett syndrome. Therefore, a question that remains open for this model is to know if it exhibits defects in synaptic plasticity paradigms such as ocular dominance (OD) plasticity.

2.2.2) *TSC1 and TSC2*

Other mutations affecting the mTOR pathway are the ones related to the tuberous sclerosis complex 2 (TSC2). These types of mutations are linked to dysregulation of some cellular processes such as differentiation, migration and proliferation of neurons as well as behavioral hallmarks typical of autism pathophysiology[27] Both TSC1 and TSC2 are proteins involved in the downregulation of mTOR[28]. Beyond autism and intellectual disability, tuberous sclerosis complex is also associated with increased prevalence of epilepsy[29]. A previous study revealed that TSC2 and FMRP are involved in the management of plasticity, particularly, metabotropic glutamate receptor-dependent long-term depression (mGluR-LTD)[30]. In *Tsc2*$^{+/-}$ mice, an abnormal hippocampal long-term potentiation (LTP) was observed, accompanied by alterations in learning consolidation. The use of an mTOR inhibitor, rapamycin, proved to be effective in rescuing the behavioral problems as well as those associated with plasticity[31]. This paper opened the discussion of whether the use of mTOR pathway as an effective target for other models of ASD with mutations in genes for proteins involved in this pathway. However, there are still points to be uncovered regarding the interactions between those synaptic proteins and all their possible targets. Rheb1 was demonstrated to be involved in memory and behavioral deficits in TSC2 mouse models and synaptic abnormalities. The use of an inhibitor of that protein (lonafarnib) proved effectiveness in improving those abnormalities[32].

2.3) *FMRP*

Another group of important mutations are the one that affect the gene that encodes for fragile X mental retardation protein (FMRP). Those are linked to the development of Fragile X syndrome which increase the risk for ASD. Mutations in *FMR1* gene are coupled to the loss of its product FMRP. It is a protein involved in the translation of mRNAs for proteins essential for neurodevelopment. Defects in neuroplasticity are found in this model[33]. A study demonstrated that the circuit alterations caused by cocaine administration were mediated by FMRP. This was shown in the nucleus accumbens region (NAc)[34]. The evidence that this protein is important for plasticity in this reward circuit shows that perhaps, it could be interesting to study the involvement of this protein in other social/emotional brain areas. There is evidence of altered synaptic plasticity in the hippocampus. In the last years, these were the two regions more actively studied due to their involvement in the behavioral manifestations of ASD. A therapy that was already tested was the administration of minocycline. This type of antibiotic had very interesting results in rescuing cognitive function and intensifying N-methyl—D-aspartate receptor (NMDAR) function in the hippocampal dentate gyrus (DG)[35,36,37].

2.4) Neurexins and Neuroligins

Neurexins (NRXNs) and Neuroligins (NLGNs) are two important types of cell-adhesion proteins at the synapse and that are very important for synaptic function. Neurexins are located at the pre-synaptic terminal and interact at the post-synaptic site with multiple ligands such as neuroligins[38,39]. In mutant mice where neurexin was impaired selectively during late postnatal stages showed impairments in glutamatergic neurotransmission in whole-cell recording experiments in cortical neurons as well as autism-like behavior[40]. Neuroligins are proteins located at the membrane of the post-synaptic terminal. These proteins are important for the formation and maturation of glutamatergic and γ-aminobutyric acid (GABA) synapses[41]. Mutations in neurolignin 3 (NLG3) were found to be paired with modifications in dendritic spine density turnover, and the absence of LTD due to the impaired GluA2 subunit phosphorylation. This leads to a reduction in mGluR-dependent LTD[42]. Due to the role of this group of proteins in

synapse equilibrium when a mutant form of the proteins is present, it is expected an alteration at a circuit level because it will impact synaptic connections between excitatory and inhibitory neurons.

2.5) *SHANK*

There is also another group of scaffolding proteins that are involved in the actin cytoskeleton constitution called SH3 and multiple ankyrin repeat domains (SHANK). These proteins are SHANK1, SHANK2 and SHANK3. Mutations in the genes encoding for these proteins are associated/related to the development of ASD phenotype[43]. Pre-clinical studies using *Shank1* KO mice showed a role of the protein in the regulation of social behavior, more specifically in high-order cognitive functions[44]. Mice lacking the normal protein displayed a shrinkage in dendritic spines, thinner post-synaptic densities (PSDs), and a feeble basal synaptic transmission[45]. In *Shank2* mutant mice it was proved that altered glutamatergic neurotransmission lead to the development of the essential symptoms of ASD[46]. By restoring NMDAR function by specific agonists or indirectly via mGluR5 modulation, the normal phenotype was repaired[47]. Shank2 is important both at a pre and post-synaptic level since it is important for the normal function for social behaviors, and its defects may lead to neuropsychiatric disorders[48]. Shank3 was shown to be a protein with an important function in synapse function. *Shank3* mutant mice were characterized as a model with an autism-like phenotype with manifestations of self-grooming and deficits in social behavior. At a circuit level, impairments in striatal synapses and in cortico-striatal circuits were observed[49,50,51]. All the evidence mentioned showed that Shank proteins are essential to synapse stabilization. If one of these proteins is mutated, defects in synaptic transmission are expected, especially, in glutamatergic synapses. For this reason, we should expect to see defects in LTD or LTP induction when using experimental strategies for their induction. Different strategies were tested for shankopathies. One of them was the administration of agonists for NMDA receptors (NMDAR) or of metabotropic glutamate receptor 5 (mGluR5), an indirect way of activating NMDAR[47,52]. Since mutations in shank proteins affect glutamatergic synapses, the administration of these type of agonists adjusted NMDAR function and rescued the normal behavior phenotype. Another therapeutic option that is being tested in

these diseases is transcranial magnetic stimulation (TMS) which is a non-invasive technique allowing brain stimulation[52]. This approach grants cellular and molecular transformations that promote LTP development and the expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) subunits. This indicates that TMS affects synaptic plasticity. Despite this evidence, a complete understanding of the technique was not achieved yet. Since shank proteins are involved in the mechanisms for LTP development, it should be interesting to conduct studies to evaluate if this technique can elicit effects in shankopathies and in other ASD models with this type of plasticity deficiency[53,54]. In the future, more studies should be undertaken in different brain regions to see if TMS could provide improvements in synaptic plasticity and a normal behavior phenotype in disease conditions marked by a defective plasticity.

3) Neurofibromatosis type 1 (NF1)

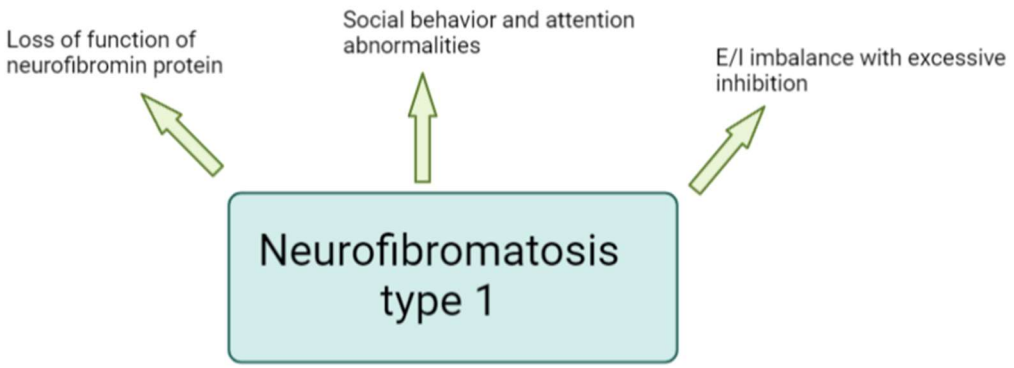

Figure 3 - **Three many features of neurofibromatosis type**

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NF1 is a monogenic disorder, autosomal dominant, that has multiple effects in different organs. This type of mutation is associated with a loss of function of the neurofibromin protein. *NF1* is a

tumor suppressor gene, and the suppression of neurofibromin have impacts in the cell by increasing its growth and by hyper-activating Ras protein. The gene that encodes for this protein is a proto-oncogene. Individuals with this type of mutation expose anomalies in attention and social behavior (Fig.3)[55]. This phenotype was also validated in mouse model studies[56]. In three chamber arena, *Nf1*$^{+/-}$ mice showed lower preference for social interactions and novelty. *Nf1*$^{+/-}$ mice present large volumes for regions involved in social cognition, particularly in prefrontal cortex and *caudate-putamen*. Hippocampal learning and memory deficits are also an hallmark of the mouse model for this disease condition[56]. One clinical manifestation of NF1 is the optic pathway glioma. This is a condition that causes some morbidity in individuals due to the imperfect vision it causes. It was observed that both in humans and in NF1-mutant mice that females present a higher incidence of impaired vision[55]. In this case the mouse model used had a complete ablation of NF1 in astrocytes which facilitates the appearance of optic gliomas. Nevertheless, this is an interesting phenotype observed in NF1 that indicates the sex is a variable with a significant impact on the visual system. As a result, it is a factor that justifies the interest to study sex differences in the visual system of NF1. According to the theory of the excitation/inhibition imbalance, in a mouse model of NF1 (*Nf1*$^{+/-}$), the inhibitory levels are higher in a general perspective[57]. This model presents increased levels of cortical and striatal GABA/glutamate ratios and increased GABA(A) receptor in the hippocampus. Interestingly, in human studies it was shown a decrease in GABA levels in the visual cortex[58]. Despite the contradiction, this is a macroscopic quantification using magnetic resonance spectroscopy and, thus, it doesn't reflect what is happening at a microcircuit level. It could be interesting to observe of how this excitation/inhibition imbalance impacts the visual cortex both in humans and in animal models. Also, NF1 children and adolescents were associated with a potentiation of alpha brain oscillations and aberrant long-latency VEPs. These two factors are reflected in attention deficits and high-order visual processing[59]. These individuals also presented impaired impulse control and a downregulation in electroencephalogram (EEG) signal correlation with early visual processing (parieto-occipital P1)[60]. In both previous two studies, patients with ophthalmological problems (for instance optic gliomas and amblyopia) were excluded. The impairments observed might reflect defects in synaptic plasticity in V1 and are a reason to identify the problems in

plasticity development in that circuit. The visual system is widely recognized as a good model to study the role of inhibition, therefore is very interesting to better characterize this model in NF1.

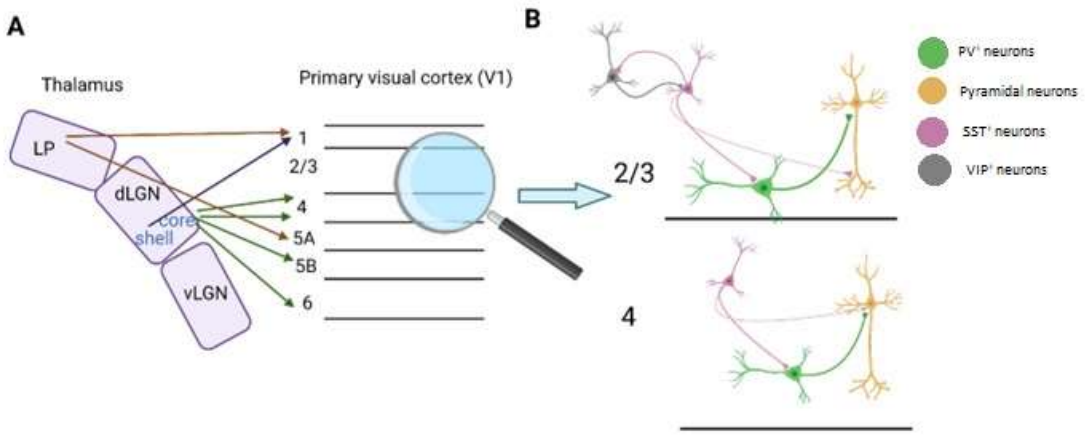


Figure 4 - **Organization of the connections between the thalamus and V1.**

A) The visual thalamus is divided into two regions: dorsal (dLGN) and ventral (vLGN). Only dLGN connects to the V1 through two subregions: core and shell. The connections through the shell are shown in dark purple arrows and through the core are shown in green arrows. Also, there is a high-order region in the thalamus (LP) which the connections to V1 are represented in brown arrows[104]. B) Gross view of the network connectivity in V1.In layer 2/3, VIP+ neurons activate the cortex by inhibiting SST+ neurons. In layer 4, VIP+ neurons are less abundant, but the other connections in the circuit remain the same[63]. (Created with biorender.com)

4) V1 plasticity in the neurodevelopment

The study of plasticity in visual cortex started with Hubel and Wiesel, particularly, the V1[61]. They could observe that there is an innate period of development and another critical period (CP) for experience-dependent plasticity. What happens in these CPs is very important for normal neurodevelopment, that is why this a crucial step that should be studied in neurodevelopmental disorders. In Fig. 4, we have represented the thalamocortical connections. We see the organization of the circuit in layer 4 and layer 2/3, which are the most important ones for

plasticity in V1. The circuit representation shown is the architecture of the organization of neurons by which the connections for experience-dependent plasticity in V1 are developed. There are classes of interneurons present that are of major importance in CP plasticity, which are PV^+ neurons, somatostatin positive neurons (SST^+), and vasopressin intestinal peptide neurons (VIP^+). The strength and organization of these connections will impact the activity of the pyramidal neurons[62,63]. Binocular rivalry is a process where visual perception alternates between the two eyes. For this process to occur in a proper way it needs normal physiology and visual plasticity. It was observed a slower rate of binocular rivalry in the autistic brain due to an alteration in the proportion between excitation and inhibition in the visual cortex[64]. That is another clue that the plasticity in the visual cortex is not happening correctly. This can be an interesting hallmark of autism and may be associated with the severity of autism social behavior symptoms. This suggests different levels of plasticity impairments in ASD. As a result, therapeutics to improve plasticity can have different degrees of efficacy depending on the severity of the disorder. The most used paradigms to study the induction of plasticity in visual cortex are the ones related to ocular dominance (OD) plasticity. This type of experience-dependent plasticity can be induced after monocular deprivation (MD) with NMDAR dependent-plasticity in the non-deprived eye inputs[65]. There is a CP where this type of plasticity is higher during the early periods of development. The differences in the cortical neuronal responses to each of the two eyes are the way we characterize this type of plasticity[66]. In different neurodevelopment disorders, it is expected to see defects in the expression of plasticity, and that can be provoked by the disruption of the CPs. Indeed, for NF1, it was observed that the CP for OD plasticity development was disturbed. The levels of inhibition remained high in the adult visual cortex with no effects in early cortical development[67]. This study suggests that the therapeutic window in NF1 to treat the deficiencies in OD plasticity should be early development. It is important to target therapeutically the increased inhibition observed using strategies that address the GABAergic synapses in V1. A less studied paradigm that has the capacity to evaluate plasticity is SRP[68]. In this type of task, it is observed if mice can discriminate between novel and familiar stimuli consisting of sinusoidal gratings[69]. In previous studies conducted in mice, SRP induction was associated with orientation-selective habituation (OSH) in two types of experimental designs: one with head-fixed mice and in freely behaving mice. It

was observed that a type of behavior called visually driven behavior in head-fixed mice (vidget) required V1. With this behavior one can quantify the familiarity and novelty to stimulus orientation in a SRP paradigm. The activation of NMDARs is important for the development of this type of plasticity in mice, and the genetic ablation or temporary pharmacological blockade of the receptors prevented SRP development[69,70]. Knowing that the inhibition of NMDAR leads to a disruption in the discrimination between novelty and familiarity, a study that follows the one related to the SRP induction with OSH in V1, gave us an insight to explain why the ablation of NMDAR lead to a disruption in familiar vs novelty discrimination. They observed that PV^+ neuron activity in V1 is modulated during SRP in both sexes. Both OD plasticity and SRP required NMDAR activation, however, only SRP recruited the action of PV^+ neurons[71]. Another study evaluated learning-induced changes in stimulus selectivity and interactions in GABAergic interneurons in V1. Notably, it was seen that PV^+ interneurons are as selective as pyramidal cells, and, with learning, they organize stimulus-selective PYR-PV ensembles. This effect was not observed in other class of interneurons such as STT^+ and VIP^+ [72]. A defective in the maturation of fast-spiking PV^+ neurons was observed for neurodevelopmental conditions (autism, schizophrenia, and bipolar disorders). The involvement of this type of interneurons, can be viewed as a common mechanism for neurodevelopmental disorders[73]. A recent study where NF1 was deleted from cortical GABAergic progenitors have shown a specific impact on PV^+ cortical interneurons[74]. The deletion of NF1 leads to Lhx6 transcription factor that is necessary for the maturation of PV^+ interneurons. Therefore, the neurofibromin/Ras/MEK pathway regulates the maturation of PV^+ interneurons. The administration of a MEK inhibitor allowed to rescue the effects of NF1 deletion[74]. These types of neurons are the primary source of synaptic inhibition and are involved in circuit rearrangement in CP plasticity. As a result, it will be interesting to conduct further studies addressing the role of PV^+ cells in the circuitry involving experience-dependent plasticity in V1.

4.1) Sex differences in the visual cortex

In ASD, we observe an increased male: female incidence ratio of the disorder. However, the visual impairments are more extensively described in females in NF1. Females are prone to

impaired visual acuity due to the development of optic pathway gliomas[1]. Despite this oncogenic cause, other causes can be impacting the increased impaired vision acuity in females such as a defective plasticity in the visual cortex. Multiple paradigms can be applied to study plasticity in this brain region. The visual cortex is, in consequence, an interesting brain region to study experience-dependent plasticity. There are sex differences in the visual cortex. Dendritic spine density is lower in females comparing with males. Developmental sex differences are present in the visual and auditory cortices of healthy mice[75]. Due to these differences observed in wild-type mice, it will be important to see if there are sex differences in the visual cortex in neurodevelopmental disorders. Another study demonstrated how the sex variable accounts for the color processing in the brain. Using positron emission tomography (PET) and magnetic resonance imaging (MRI), it was shown that blue color implements left intra-hemispheric connectivity in males and right intra-hemispheric connectivity in females in the healthy brain[76]. This suggests possible differences in the circuit architecture for visual perception.

5) Plasticity in brain circuits involved in sociability

There are different brain regions that interconnect within themselves to produce sociability. One of them is ventral tegmental area (VTA) which is involved in the reward system with its reward processing and exploration to novel stimuli. It was demonstrated that dopamine (DA) neurons of VTA increase their activity during an interaction with an unfamiliar conspecific and thus is very important for social novelty[77]. A recent study presented an interaction between the superior colliculus (SC) and the VTA that is important for social behavior induction. They showed that SC-VTA pathway is critically involved in social novelty towards an unfamiliar conspecific whereas medial prefrontal cortex (mPFC)-VTA is relevant for the maintenance of the social interest. SC neurons project towards DA and GABA neurons of VTA[78]. This can be impaired in ASD, and particularly, NF1 where GABA levels abnormalities are a hallmark. NAc is another very important region for regulating social behaviors. It is a region that interplay with other brain regions and it is dysregulated in neuropsychiatric disorders[79]. The hippocampus is another region with particular interest in social memory. There is evidence for the involvement of CA1 where CA2 make projections and its relevance for the constitution of social engrams. The

storage of social information in CA2, however, is less elucidated. Recently, it was described the importance of CA2 in the encoding of contextual changes between a novel and a familiar. In a mouse model that mimics schizophrenia, CA2-dependent/related/mediated social memory was impaired and the blockade of TREK-1 K^+ current rescued the normal phenotype of firing of CA2 pyramidal neurons (PNs)[80]. The pathway between the lateral entorhinal cortex and the CA2 has a role in the storage of social information in CA2[81]. The prelimbic cortex and the cingulate cortex are part of the called mPFC and, in rodents is involved in the adaptation of the animals to changing environments. The connections between BLA and mPFC subregions are crucial for social perception and social-decision making[82]. Central amygdala is important for emotion discrimination via oxytocin. It is important to discriminate fear and relief with unfamiliar conspecifics[83]. Orbitofrontal cortex is important to an animal quantify the reward of an action and decide to go or not go in a social context[84]. In addition, retrosplenial cortex is important in social contexts since it is a hub for a network of brain regions that interconnect for episodic memory and planning for the future[85].

6) Objectives

The defective synaptic plasticity development is a specific hallmark of neurodevelopmental disorders. With a mouse model of NF1, we analyzed the expression of synaptic plasticity. The visual system, as part of the sensorial system is an interesting circuit to study. The SRP is a specific type of plasticity that is developed in the V1 and can be induced by habituation to a specific stimulus with a particular orientation in a mouse model. Thus, we induced this type of potentiation in our model and evaluated it either at behavior level and with *in vivo* electrophysiology recordings in V1. Specifically, we wanted to discriminate the layers of the cortex most affected and hypothesize which population of neurons are more important in that process. From the literature, we can hypothesize PV^+ neurons are critically involved in this potentiation. We want, in the future, to associate this type of plasticity with a social context. To identify brain regions mostly involved in social processing in our mouse model, we conducted an exploratory analysis using social interaction and quantification of c-Fos^+ neurons as a proxy of neural activation.

Materials and Methods

1) Animals

During the experiments performed, all the legislation was carefully followed (both the EU directive 2010/63/EU and Decreto-Lei 13/2013). The project was licensed by the national authority for animal experimentation (DGAV – Direção-Geral de alimentação e veterinária) and monitored by the commission for experimentation and animal welfare (ORBEA – Órgão responsável pelo bem-estar animal) of ICNAS – Instituto de Ciências Nucleares aplicadas à saúde. All of efforts were implemented to minimize the number of animals and their suffering. The animals were housed in groups of 2-5 individuals on a 12h light/dark cycle with access to food and water ad libitum. The transgenic mouse models used were NF1 and TSC2. For all experiments mice were obtained by crossing *Nf1*$^{+/-}$ mice (C57BL/6N background) with 129/Sv mice or crossing *TSC2*$^{+/-}$ mice (C57BL/6N background) with C57BL/6J mice. Age and number of animals is indicated for each experiment.

2) Genotyping

To distinguish the newborn mice between the transgenic mice and the wild-type (*WT*) littermates a portion of 3-4 mm of tail of mice until 14 days after birth was obtained for DNA extraction and genotyping.

The first step of the protocol was DNA extraction. In an Eppendorf with the biological sample, 487.5µL of lysis solution (10mM Tris (Sigma Aldrich), 5mM EDTA (Panreac), 200mM NaCl (Panreac), 0.3% SDS (Bio-Rad), pH8) and 12.5µL of proteinase K (0.025% final solution, Thermo Fisher Scientific) and leaved in a warped plate for 4 h at 55ºC. After this step, a first centrifugation was performed at 13400g for 15 min at 4ºC (centrifuge from Eppendorf). Supernatant was removed and put in a new Eppendorf, and 1mL ethanol 100% (iced) (Sigma Aldrich) was added. Then, a second centrifugation (1500g 5min at 4ºC) was performed with the supernatant discarded. After the third centrifugation (1500g 15 min 4ºC), the supernatant

was discarded, and the ethanol let to evaporate overnight by leaving the tubes undisturbed at room temperature. The DNA is resuspended in 40 µL of Tris-EDTA (TE) buffer (10mM Tris (Sigma Aldrich), 1mM EDTA (Panreac), pH 8,0) and leaved in a warmed plate for 10 min at 70ºC.

Before DNA amplification, DNA was quantified at nanodrop (dsDNA) (Thermo Fisher Scientific). DNA was then amplified with three selected primers for NF1 (NF1-P132: 5' TTC AAT ACC TGC CCA AGG 3'; NF1-P133: 5' ATT CGC CAA TGA CAA GAC 3', NF1-P134: 5' GGT ATT GAA TTG AAG CAC 3') and three selected primers for TSC2: P605: 5' CAA ACC CAC CTC CTC AAG CTT C 3'; P606: 5' AAT GCG GCC TCA ACA ATC G 3' and P607 5' AGA CTG CCT TGG GAA AAG CG 3'. The program of amplification included 34 cycles (2' 94ºC, 45'' 94ºC, 1' 56ºC, 1' 72ºC, 10' 72ºC) for NF1 and 35 cycles (3'94ºC, 45''94ºC, 1'64ºC, 1'72ºC, 10'72ºC) for TSC2.

The Polymerase chain reaction (PCR) product was used in an agarose gel (2% for NF1 and 3% for TSC2, Thermo Fisher Scientific) and the electrophoresis run at 120 V during 20 min with the electrophoresis system fully covered with Tris-acetate-EDTA (TAE) 1x buffer (40mM Tris (Sigma Aldrich) 5mM EDTA (Panreac), 5.71% acetic acid (Sigma Aldrich)). The bands of PCR products were acquired in a transilluminator (Vilber Lourmat) (under UV light). For NF1, the WT product was 230bp and the NF1 mutant product 350bp. For TSC2, the WT product was 86bp and NF1 mutant product is 105bp.

3) Estrous cycle

The evaluation of vaginal smears is extremely important when conducting research using females. The estrous cycle can have an impact in behavioral paradigms and, thus is important to be determined[86]. The estrous cycle is divided in four stages: proestrus, estrus, metestrus, and diestrus[86]. In last day of each behavioral paradigm, vaginal swabs were collected using a cotton swab dipped in water and placed in glass slide and let air drying. The slides were stained with cresyl violet for 1min and then gently washed with tap water. The slides were observed in the morphometric microscope (Axio Lab A1, Zeiss) using a 10x objective. After that, the images collected were analyzed and the different cell populations quantified.

4) Behavioral paradigm

4.1) Behavioral SRP

14 *Nf1*$^{+/-}$ animals (9 males and 5 females) and 11 *WT* littermates (7 males and 4 females) were used. 2 females and 5 males *Nf1*$^{+/-}$ and 2 males *WT* were removed because they present either discrepant values or very few exploration in the test. 6 *Tsc2*$^{+/-}$ (5 males and 1 female) animals and 14 *WT* littermates (11 males and 3 females) were used.

Mice aged 45 post-natal days (P45) were used. Experiments were performed during night phase to take advantage of mouse increased exploratory activity. Before this behavioral paradigm, animals' cage is placed in the room test 30-60min.

Before the initiation of the training sessions, the animal was allowed to freely explore the testing arena (40x40x20cm) for 2 days over 30 min per session in which both ASUS monitors were placed 20cm from the center of the arena with a mean luminance 34cd/m^2 presenting full fields of gray. This behavioral paradigm had a period of training for 6 days. During each day of the training period, the mouse experienced two free exploration sessions separated by 1h (Fig.5). After that, the animal returned to its home cage. Each session consisted of 5 min of full-field gray on both monitors which was followed by a presentation of a visual stimulus on only one side of the arena with the side the stimulus counterbalanced from day to day. The stimulus which the animal is experiencing consisted in a 100% contrast, sinusoidal grating that phase-reversed at a frequency of 2Hz with a spatial frequency of 0.05 cycles per degree (cpd). The stimulus was initiated automatically. Visual stimuli were presented in five blocks of 100-phase-reversals per block with 30s of gray-screen stimulus during the interblock interval. On day 7, the animal experienced four training sessions: two sessions with a stimulus with the familiar orientation (the same as in training) and two sessions with a stimulus with a novel orientation that consists in a rotation of 90º to the familiar orientation stimulus (Fig. 6).

Figure 5 - **SRP behavioral test.**

Representation of a mouse performing the test with the two monitors that present the visual stimuli with the location counterbalanced from day to day.

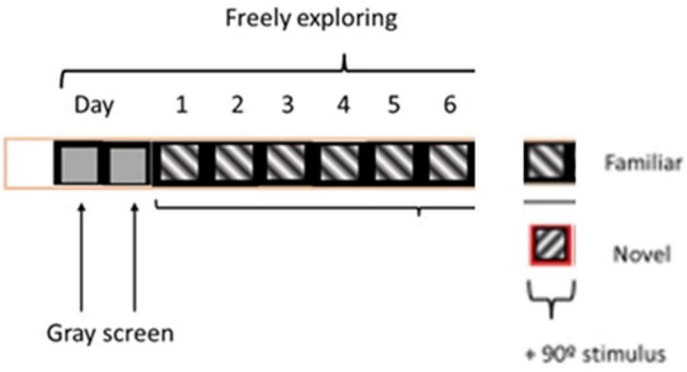


Figure 6 – **SRP protocol**

Mice are habituated during 6 days with a particular orientation. At day 7 with the anesthetized animal with the probe implanted the familiar orientation is presented and a familiar stimulus that is generated by adding 90º to the familiar stimulus (Adapted from Cooke et al, 2015)

4.1.1) SRP behavior quantification

We used an open-source *software* (*ezTrack*)[87]. The time in which the animal explored in each side of the arena was quantified.

Firstly, we cropped the image to define the arena zone. Then, we created a reference frame by generating an average frame of an ensemble of frames the animal was moving. After that, we divided the arena in two halves (left and right) to draw our regions of interest. We defined the dimensions of the arena and started tracking location which was saved in a .csv file. A video of the tracking was generated to confirm the animals were tracked properly. After that, the preference of the animal for the stimulus was calculated using the following formula: $Preference\ index = \frac{Stimulus - Grey}{Stimulus + Grey}$.

5) *In vivo* electrophysiology measurements in V1

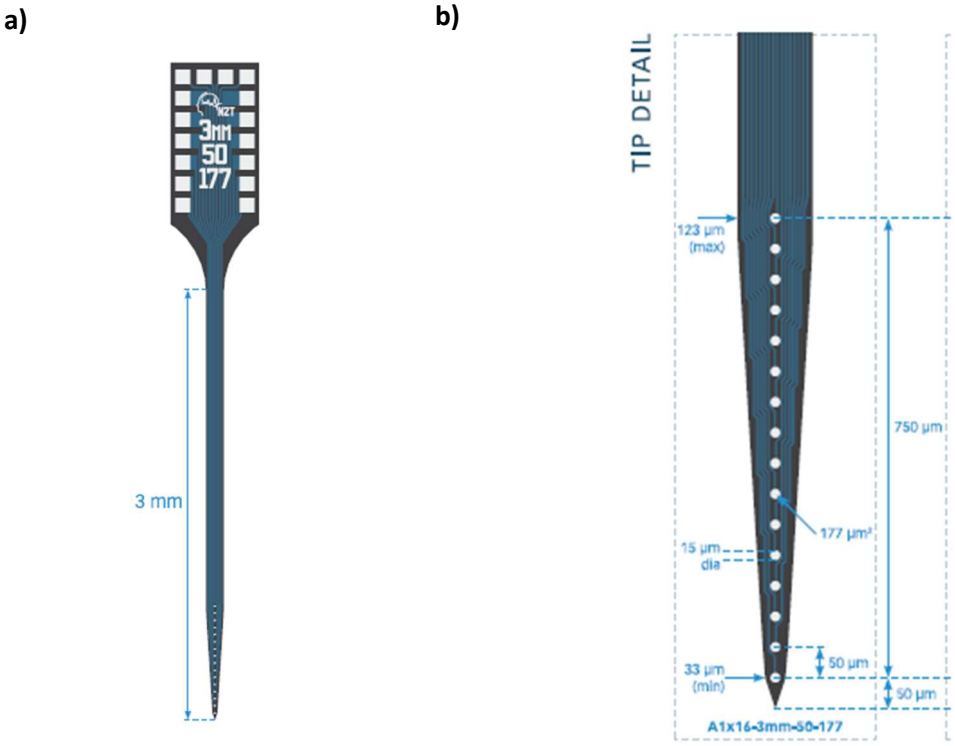


Figure 7 - **16-electrode channel probe**

a) General image of the Neuronexus probe (ref. A1x16-5mm-50-177) with 5mm length. b) Close-up for the 16 recording sites. (Retrieved from Neuronexus catalogue).

Animals aged between postnatal day 30 and 45 were used to perform the *in vivo* electrophysiology experiments. Before the visual stimulation protocol, the animals were handled one week before. In the first two days, mice were placed in the arena with gray stimulus for habituation to the arena during 20min. A single DELL monitor was placed with 30 cm distance from the arena. For 6 days, the animal is placed in an arena (40x40x20cm divided in the center with a PVC plate) to be stimulated with a visual stimulus with a particular orientation – familiar. Visual stimulus was generated by custom script using PsychoToolbox (http://psychtoolbox.org) run by Matlab (Mathworks). The animal was placed only in one half of the arena. The stimulus consisted in a 100% contrast, sinusoidal grating that phase-reverse at a frequency of 2Hz with a spatial frequency of 0.05 cycles per º. The stimulus was initiated manually, only when the animal was looking to the monitor. Mean luminance was 34cd/m^2. Visual stimuli were presented in five blocks of 100-phase-reversals per block with 30s of gray-screen stimulus during the interblock interval. On day 9 of the habituation protocol the animal was anesthetized by an intraperitoneally (IP) injection of urethane (1.5g/kg urethane in saline; Sigma). Xylazine was also administered IP (5mg/kg; Dechra). Additionally, atropine (0,1mg/kg; Labesfal) and dexamethasone (2mg/kg; Campifarma) were also administered subcutaneously. When, the animal was deeply anesthetized, was placed in the stereotaxic apparatus to do the craniotomy surgery following the coordinates of the binocular primary visual cortex (+0.5 anterior to lambda, 2.8ML right hemisphere, angle 10º). A small cranial window (~1x1mm) was drilled using a 0.5 burr, dura removed, and a 16-channel electrode silicone probe (Neuronexus, ref. A1x16-5mm-50-177) (Fig. 7) was placed. For reference, other a small burr hole (~0.5mm) was drilled to insert 50µm nichrome wire (80% Ni, 20% Cr, Science Products) in left prefrontal area. The ground electrode consisted in a stainless-steel needle inserted in the nuchal muscle of the animal. With the electrophysiology apparatus assembled, the recordings were obtained using MultiChannel Experimenter software (MultiChannel Systems) at 20kHz sampling rate, with online monitoring

with specific filters to local field potentials (1-300Hz) and spiking activity (>300Hz). The recordings were obtained while a visual stimulation was presented with blocks of the familiar stimulus and blocks of novel stimulus (rotating 45 or 90º), using the same DELL monitor as during training/habituation, 30cm from animal eyes, covering 73º -horizontal and 45º-vertical of the visual field. Mean luminance was 34 cd/m^2. The orientations used were -30º, 15º and 60º (considering 0º the vertical orientations). It was also tested other orientations for visual stimulus with -30º and 60º with different spatial frequencies (0.01, 0.03, 0.1, 0.3, 1cpd). At the end of the experiment, a green ink coated 30G needle was inserted in the recording site using the same depth as the silicon probe. The brains were perfused and sliced in a cryostat (Leica). These slices are then colored with cresyl violet staining and observed in the morphometric microscopy (Axio Lab A1, Zeiss) to confirm the coordinates of the silicon probe.

5.1) VEP magnitude quantification

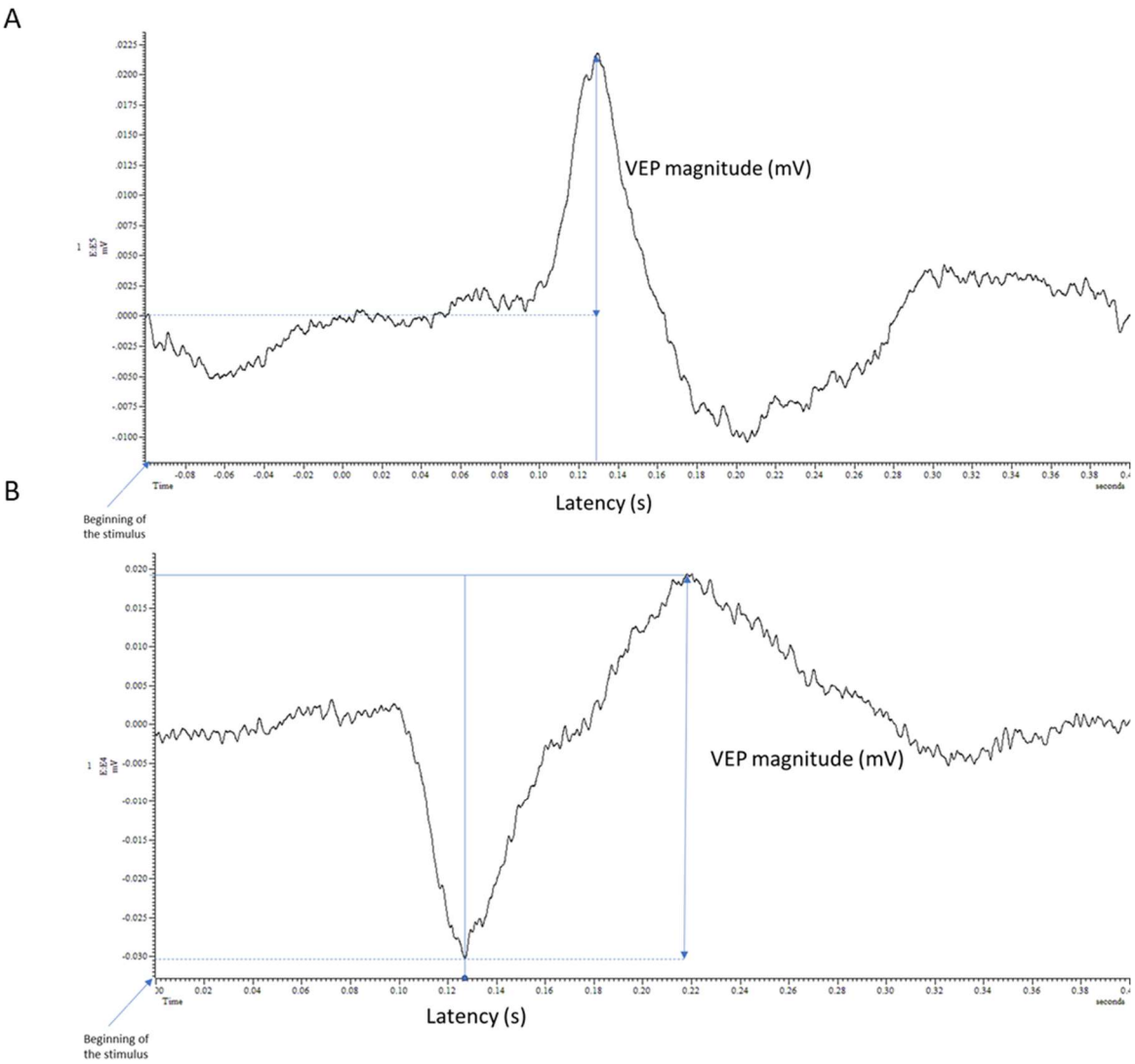


Figure 8 – VEP quantification

A – Quantification in L1 of the cortex

B – Quantification in L2/3, L5, L5 and L6

Raw data was filtered with a high pass butterworth order 2 filter with a 5Hz cutoff and a low pass butterworth order 2 filter with a 200Hz cutoff. This was performed with MultiChannel Analyzer software (Multi Channel Systems). When files presented a very intense noise in the

Raw data was filtered with a high pass butterworth order 2 filter with a 5Hz cutoff and a low pass butterworth order 2 filter with a 200Hz cutoff. This was performed with MultiChannel Analyzer software (Multi Channel Systems). When files presented a very intense noise in the signal, a Notch filter with 50Hz cutoff was applied. Then, the file was converted to .smr using Multi Channel DataManager (Multi Channel Systems) for Spike 2 software analysis (Cambridge Electronic Design).

The VEPs were obtained by waveform average with a 0.5s width, 0.1s offset and using with the trigger being the digital event. We quantified VEP magnitude and latency for each file (Fig. 8). For layer 1 VEP quantification is shown in fig.8-A for deeper layers is shown in fig.8B. Then, we calculated amplitude and latency ratios by dividing the values for familiar and novel stimuli.

6) Brainwide c-Fos mapping

6.1) Social Interaction

This behavioral paradigm aimed to expose the experimental animals to three types of social relevant paradigms: novel, familiar and object, using *Nf1*$^{+/-}$ and *WT* littermates mice aged P90-P120. In the novel paradigm, unfamiliar animals age-, sex-, and genotype-matched were allowed to freely interact. In the familiar paradigm, two littermates were allowed to freely interact. In the object paradigm the experimental animal was exposed to an object (black and silver VGA/DVI adaptor). A total of 56 animals were used (28 *Nf1*$^{+/-}$ (14 males and 14 females and 28 *WT* littermates (11 males and 17 females). Prior to the behavioral test, the animals were socially isolated for 24 hours. The test started with a square arena (40x40x20cm) with a partition in the middle of it that separates two sides: one animal or object on each side during a period of 3 min for habituation after which the partition was removed and the animals allowed to freely explore or interact for 10 min (Fig.9).

a b

Figure 9 – **Social behavioral test**

a) Mouse interacting with an object b) Two familiar mice interacting

The videos were analyzed manually. The time of social interaction or object exploration was quantified.

6.2) Social interaction-dependent c-Fos: Immunohistochemistry

The animals that performed the social or object behavioral paradigm were sacrificed 90 min after the social or the object interaction. Before perfusion, the animals were anesthetized with ketamine (100mg/kg; Dechra) and xylazine (10mg/kg; Dechra). The animals were transcardially perfused with PBS 1x (NaCl 137mM (Panreac), KCl 2.7mM (Panreac), Na_2HPO_4 10mM (LabKem), KH_2PO_4 (Sigma Aldrich) 1.8mM, pH 7.4) followed and 4% paraformaldehyde (PFA, Sigma Aldrich). The day after the brains were immersed in 20 % sucrose (Sigma Aldrich), and after sunk stored at -80ºC. From the initial set of animals 19 animals were used (6 *Nf1*$^{+/-}$ (2 males and 4 females) and 13 *WT* littermates (4 males and 9 females).

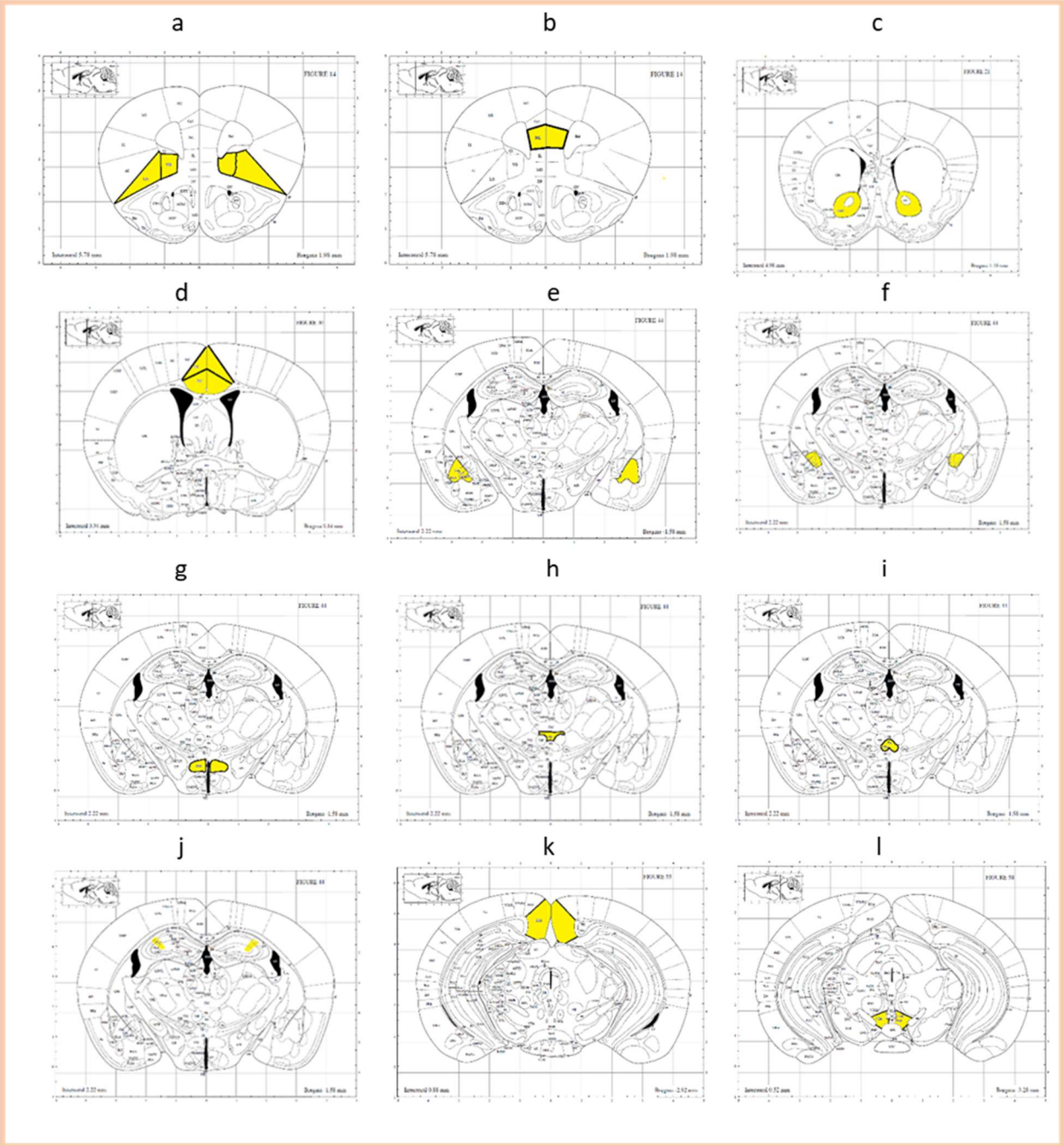


Figure 10 - **Brain slices selected for selected brain regions**

based on the “Mouse brain atlas in stereotaxic coordinates” from George Paxinos and Keith B. J. Franklin. a) orbitofrontal cortex; b) prelimbic cortex; c) nucleus Accumbens; d) cingulate cortex; e) basolateral amygdala; f) central amygdala; g) dorsomedial hypothalamic nucleus; h) rhomboid thalamic nucleus; i) reuniens thalamic nucleus; j) CA2; k) Retrosplenial cortex; l) VTA

The brains were sliced in a cryostat (Leica) with a slice thickness of 40 µm and placed in a 24 multi-well plate with phosphate buffered saline (PBS) 1x. Using a mouse brain atlas "The mouse brain in stereotaxic coordinates" from George Paxinos and Keith B. J. Franklin, certain slices (14, 21, 30, 36, 44, 55, and 58) were selected to undergo the free-floating immunohistochemistry protocol in different regions of the mouse brain (Fig. 10). They were selected based on their relevance in a social context. VTA and NAc are involved in the reward system[77,79]. Prelimbic and cingulate cortices are important for the animals adapt their behavior to environments that are continuously changing. These regions link with BLA for social perception[82]. CeA is important for emotion discrimination by oxytocin release[83]. CA2 is important for encoding social memories and orbitofrontal cortex is important to process if a social interaction is worth it or not[81,84]. RSG being an hub of many regions is important for episodic memory in a social context[85]. In addition to the most important areas, we also quantified reuniens and rhomboid thalamic nucleus involved in aspects of behavior related to anxiety, stress and photoperiod and the dorsomedial hypothalamic nucleus involved in the circadian rhythms[88,89]. Firstly, the slices were rinsed with PBS 1x 2 times for 5min at room temperature (RT). After that, slices were incubated with a block solution PBS supplemented with 1% bovine serum albumin (BSA) (Merck) 6% Goat*Serum (Merck) 0.25% Triton X-100 (Sigma Aldrich) for 30 min at RT. The sections were then incubated with primary antibody anti-c-Fos (Cell Signaling, ref. c-Fos (9F6) Rabbit mAb #2250, 1:3000 dilution) in PBS supplemented with 1% BSA 1% Goat*Serum 0.25% Triton X-100 (Sigma Aldrich) during 48h at 4ºC. After the incubation with the primary antibody, the slices were washed with PBS 1x 6 times for 10min at RT with agitation. The secondary antibody, Alexa Fluor 488 (ThermoFisher Scientific, LTI A11008, 1:500 dilution) in PBS 1% BSA (Merck) 1% Goat*Serum (Merck) 0.25% Triton X-100 was applied in a 24 multi-well plate covered with aluminum foil for 1h at RT with agitation. The following step were three washes with PBS 1x for 10min at RT with agitation. The sections were incubated with DAPI (Invitrogen, ref. #D1306, 1:5000 dilution) in PBS 1x at RT with agitation to mark DNA in cells. The sections were then washed in PBS 1x 3 times for 5 min at RT with agitation. The sections were mounted in glass slides with Dako Fluorescence Mounting Medium (ref. S3023) and covered with glass coverslips. After Dako is solidified, the glass slides were sealed with nail polish. The slides were then observed in a

fluorescence microscope using 10x objective (AXIO Observer Z7, Zeiss) with tiles acquisition to obtain an image of the full section.

6.2.1) Fluorescence analysis

In order perform the fluorescence analysis, two softwares were used: Fiji imageJ and Adobe Photoshop 2022. The brain slice images were obtained in a .czi format and opened in Fiji Image J to select the green channel with c-Fos$^+$ cells and the image saved in a .jpeg format. After that, the image was opened in Adobe Photoshop 2022 where the green channel image was overlapped with a corresponding image of "Mouse brain atlas in stereotaxic coordinates" from George Paxinos and Keith B. J. Franklin (Fig.11A-C). With the two images overlapped, the selected region of interest (ROI) was drawn in the green channel and saved in a .png format. This image was, finally, opened in Fiji Image J where c-Fos$^+$ cells were manually counted, and the ROI area quantified. These data are presented in cell density in a ratio of c-Fos$^+$ cells with the ROI area in µm^2.

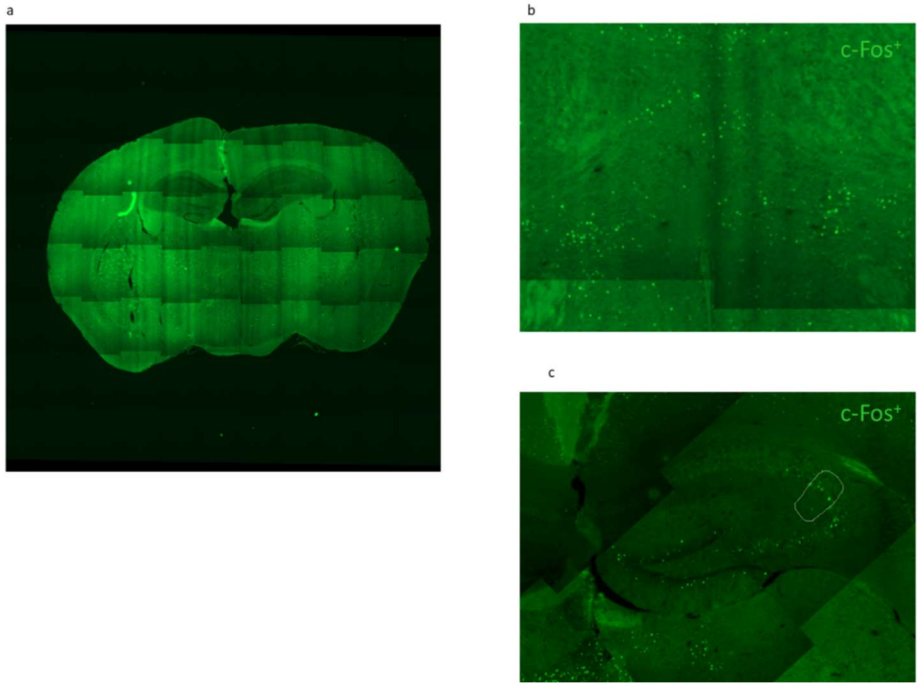


Figure 11 - Representative image of c-Fos immunohistochemistry

a) Image of an image with tiles acquisition from slice 44 of the atlas.
b) C-Fos marking
c) CA2 region of interest with c-Fos$^+$ cells

7) Statistical analysis

To do the statistical analysis Microsoft Office Excel and GraphPad Prism 8 software. All statistical tests are indicated in the legend of the figures.

Results

To study visual cortical plasticity in our NF1 mouse model we divide this section in three topics: 1) Behavioral SRP – where we compare the preference index of the *Nf1*$^{+/-}$ mice for novel vs. familiar stimuli with *WT* littermates. We also have data from another ASD-like behavior model to compare (*Tsc2*$^{+/-}$ mice); 2) *In vivo* electrophysiology in V1 when we segregate VEPs for different layers and compare between two conditions (*Nf1*$^{+/-}$ mice and *WT* littermates) if there are significant differences between the responses to familiar and novel stimuli; 3) Brain wide c-Fos mapping when we performed a social behavior paradigm and evaluate the most important brain regions for social behavior by analyzing c-Fos activation.

1) Behavioral SRP

1.1) Familiar vs. Novel stimuli

In this behavioral paradigm we want to analyze if there is a potentiation in a form of SRP which promotes increased interest for a novel stimulus. In Fig. 12, we present the results for two different groups of animals NF1&WT (composed by *Nf1*$^{+/-}$ mice and *WT* littermates) and TSC2&WT (composed by *TSC2*$^{+/-}$ animals and *WT* littermates). With these results, we want to analyze for each genetic background if the protocol was successful in the promotion for interest for novelty in both backgrounds. As we can observe in Fig. 12A1 and 12A2, novelty increased preference of mice for that stimulus ($p=0.0011$ and $p<0.0001$, respectively). However, for the exploration time, no significant differences were observed (Fig. 12B1-B2). In the figure we can observe that TSC2&WT animals present a more pronounced preference index compared with NF1&WT animals. The time of exploration is also higher for TSC2&WT animals mice compared with NF1&WT animals. The genetic background maybe influencing these results since there are some differences in some phenotypes. C57BL/6J have lower locomotion activity in an open-field arena compared with 129S1/SvImJ but increased rearing movements. Also, C57BL/6J have a lower anxiety phenotype compared with 129S1/SvImJ measured in elevated plus-maze test. These can contribute to a general lower social approach observed in these 129S1/SvImJ[90].

Therefore, the greater performance in the protocol of TSC2&WT animals might be explained with the genetic background more receptive for novelty.

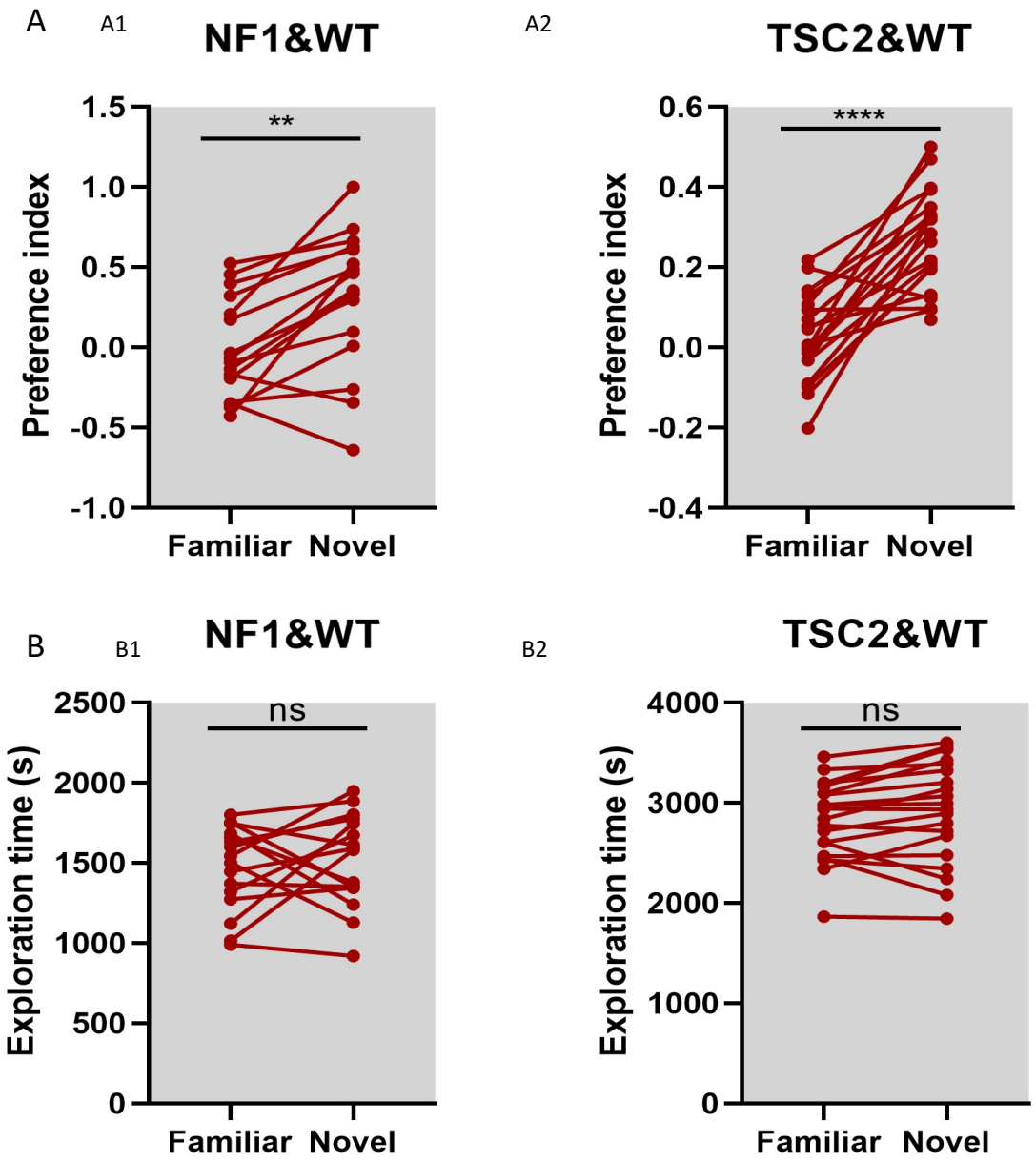


Figure 12 - **SRP expression in the two genetic backgrounds used**

A- Preference index for the visual stimulus presented (novel or familiar). Normality was tested in all groups.

A1) Two-tailed paired t-test (n=16 cell pairs, p=0.0011)

A2) Two-tailed paired t-test (n=19 cell pairs, p <0.0001)

B- Total exploration time (s) the animal explored the stimulus presented (novel or familiar). Normality was tested for all groups

B1) Two-tailed paired t-test (n=16, p=0.05180)

B2) Two-tailed paired t-test (n=20, p=0.0816)

1.2) Familiar vs. Novel with genotype segregation

After, analyzing if the different genetic backgrounds had a good performance for that protocol, we then analyzed between transgenic mice and *WT* littermates the differences. For the preference index we didn´t find any significant differences Δ (N-F) (calculated by subtracting the value of the index for familiar to novel stimulus) between *WT* and $Nf1^{+/-}$ mice and *WT* and $Tsc2^{+/-}$ mice. $Nf1^{+/-}$ mice present a slightly decrease in Δ (N-F) compared with *WT* littermates (Fig.13A1). We also didn't find any significant differences for the total exploration time (presented in N/F ratios) (Fig.13B1-2). We also performed sex segregation (Supplementary data Fig. 25).

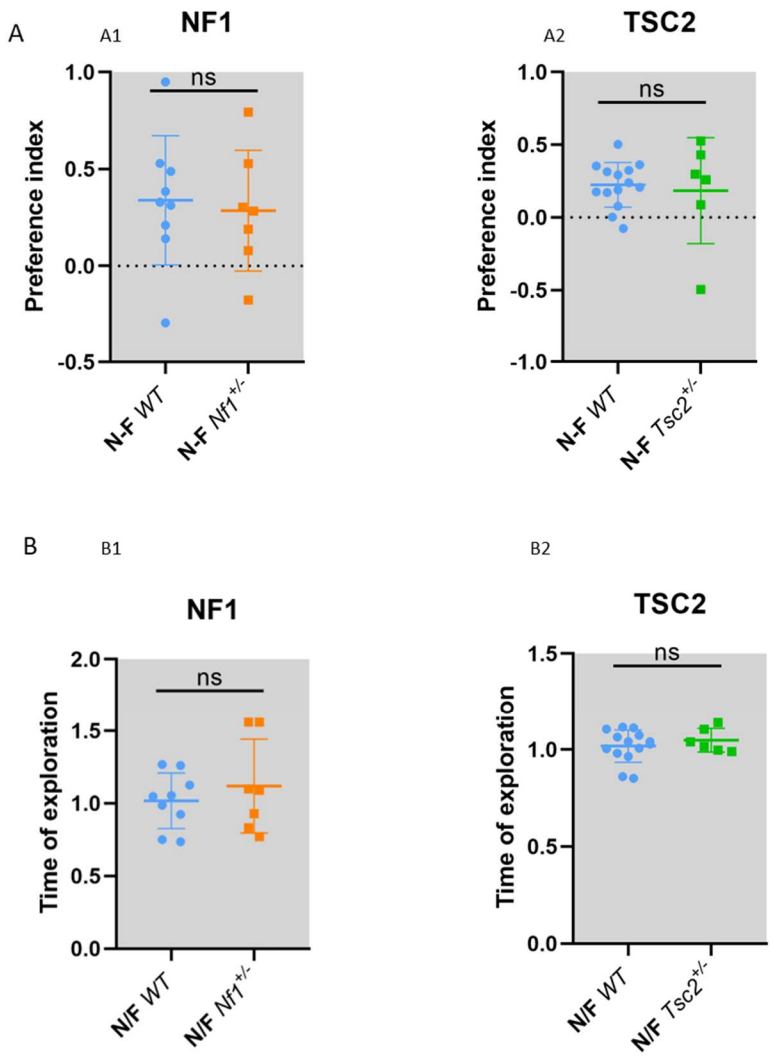


Figure 13 - **SRP development in two genetic models of ASD: *Nf1*$^{+/-}$ and *Tsc2*$^{+/-}$**

A) Preference index for the visual stimulus presented in Δ (familiar-novel stimuli) for *Nf1*$^{+/-}$ mice and *WT* littermates and for *Tsc2*$^{+/-}$ mice and *WT* littermates. Normality was tested for all groups.

A1) Unpaired t-test (n=16, p=0.7482)

A2) Unpaired t-test (n=20, p=0.6127)

B) Total exploration time (s) the animal explored presented in novel/familiar stimuli ratios for *Nf1*$^{+/-}$ mice and *WT* littermates and for *Tsc2*$^{+/-}$ and *WT* littermates. Normality was tested for all groups.

B1) Unpaired t-test (n=16, 0.4409)

B2) Unpaired t-test (n=20, p=0.4406)

2) *In vivo* electrophysiology in V1

2.1) Cortical layer division per channel (representative image) and electrode site position

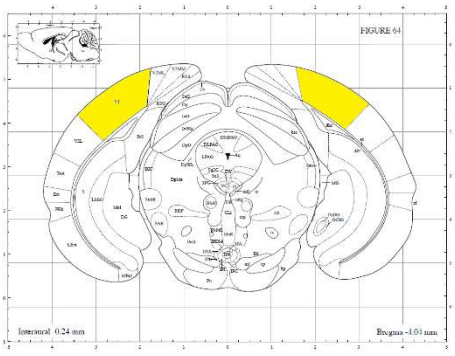
After evaluating if in the behavioral experiment, *Nf1*$^{+/-}$ mice and *WT* littermates discriminate between a novel and a familiar stimulus, we wanted to perform *in vivo* electrophysiology experiments in V1. Our objective was to evaluate for the different V1 cortical layers if potentiation was occurring. To do that, we had to insert a 16-channel electrode silicone probe that was described in Methods section.

In fig. 14A we have represented a representative image of averages VEP responses for the 16 channels and the assignment of each channel to a different cortical layer based on VEP shape[91] (Fig.14D). The probe was inserted in right binocular V1 (Fig.14B). After, electrophysiology recording, brains with the insertion site labeled with a green ink were sliced to confirm in a morphometric microscopy if the probe was successfully implemented in V1 (Fig.14C).

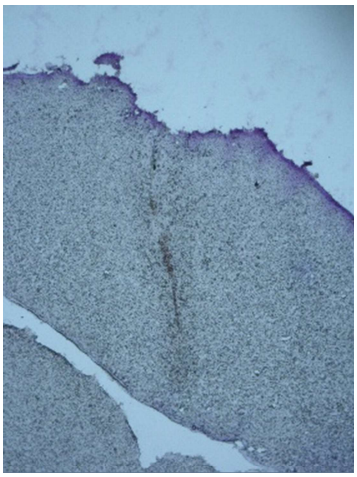
A

L1
L2/3
L4
L5
L6

B



C



D

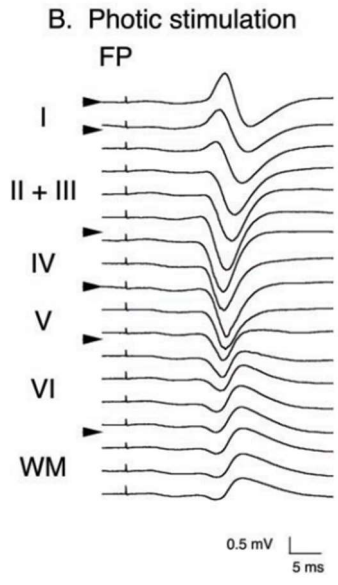

B. Photic stimulation
FP
I
II + III
IV
V
VI
WM
0.5 mV
5 ms

Figure 14 - ***In vivo* electrophysiology in V1**

A – 16-channel electrode probe signals. Channel 1 corresponds to layer 1, channel 5 to layer 2/3, channel 1 to layer 4, channel 4 to layer 5 and channel 10 to layer 6.

B – Figure 59 of mouse brain atlas "The mouse brain in stereotaxic coordinates" from George Paxinos and Keith B. J. Franklin with V1 region colored in yellow.

C- Representative image of a cresyl-violet stained brain slice with the electrode insertion site.

D – Field potentials in the primary visual cortex of adult rats for different cortical layer where we based our division of VEPs in different cortical layers (retrieved from Heynen et al, 2021)

2.2) Amplitude ratio for different cortical layers

Different metrics were analyzed in electrophysiology data. One of them was the amplitude of the VEP (described in Methods section). When, comparing only *WT* animals, we did not observe any significant differences for an increase in VEP magnitude for the familiar stimulus vs. novel 1 (60º or -30º depending on the familiar stimulus) or vs. novel2 (15º) (Fig. 15A-E).

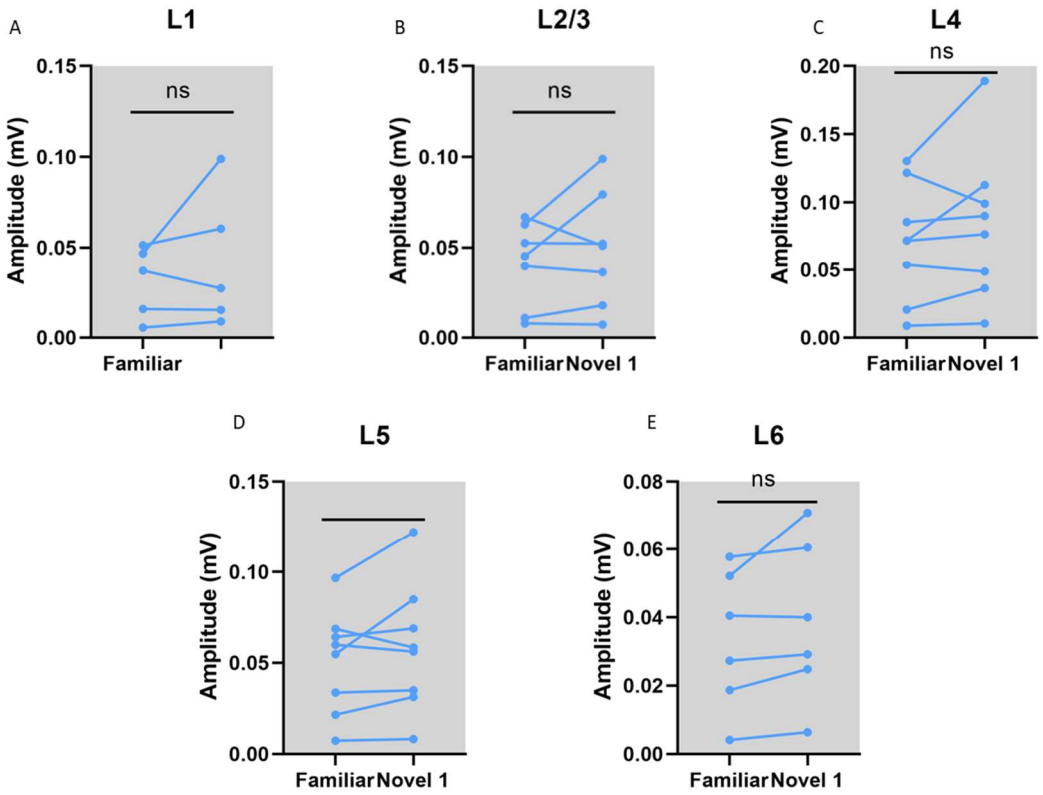


Figure 15 - **VEP magnitudes (Familiar/Novel1) in different cortical layers.**

Two novel orientations tested: N1 (-30º or 60 depending on the familiar stimulus given) and N2 (15º). Normality was tested for all groups.

A – L1 – Two-tailed paired t-test (p=0.3700, n=5 cell pairs)

B- L2/3 – Two-tailed paired t-test (p=0.3154, n=7 cell pairs)

C- L4 – Two-tailed paired t-test (p=0.2215, n=8 cell pairs)

D- L5 – Two-tailed paired t-test (p=0.1819, n=8 cell pairs)

E- L6 – Two-tailed paired t-test (p=0.1250, n=6 cell pairs)

2.3) Amplitude ratio for different cortical layers with genotype segregation

We wanted to discriminate for the different genotypes if potentiation was different. Therefore, we measured VEP magnitude calculated ratios VEP ratios (A ratio) to four conditions (*WT* F/N1, *WT* F/N2, *Nf1*$^{+/-}$ F/N1 and *Nf1*$^{+/-}$ F/N2). We also did segregation for sex (Supplementary Data Fig. 26-27).

We did not observe any significant differences for the 5 cortical layers (Fig 16A-E and Fig.17A-E)). Despite that, we could find a slightly increase in the amplitude ratio in *Nf1*$^{+/-}$ mice in L1 and L2/3 for novel 1 (p=0.0585, Fig. 16B)).

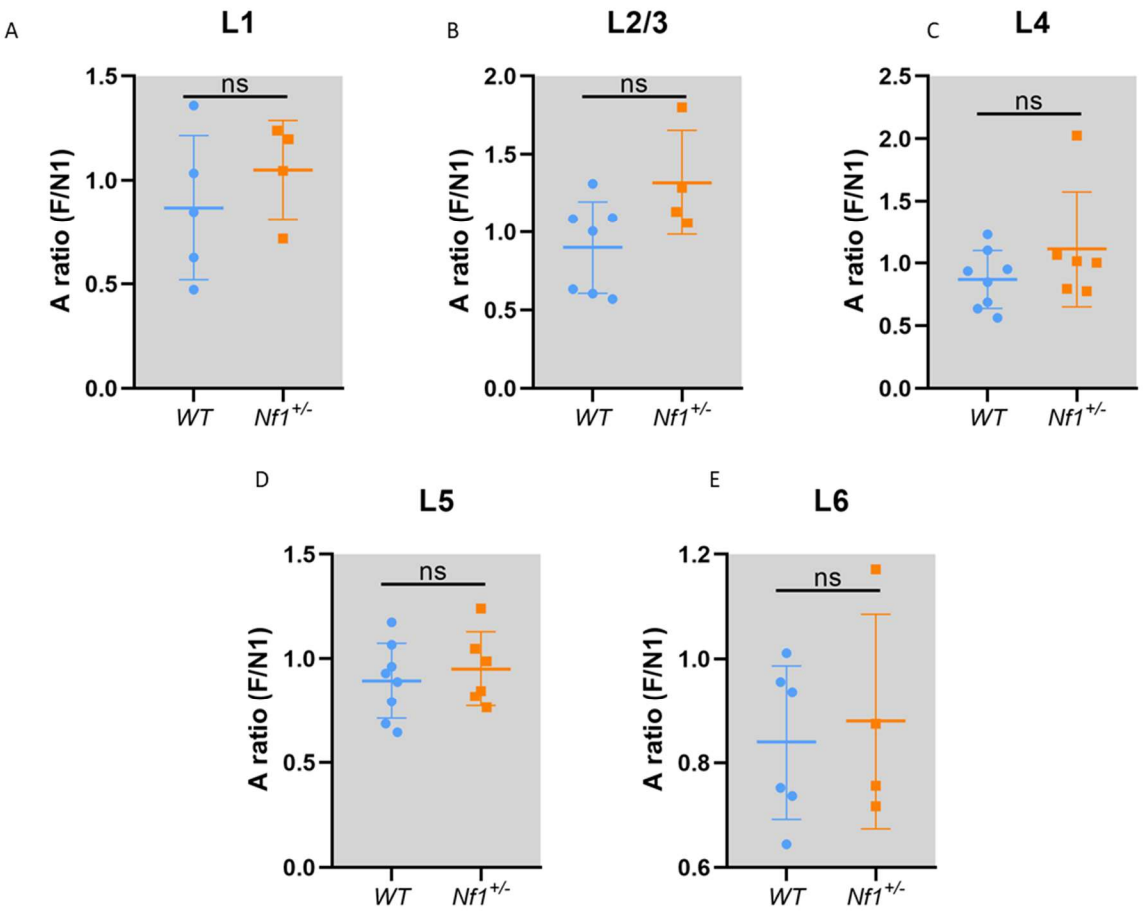


Figure 16 - **Amplitude ratio for VEP magnitudes (Familiar/Novel1) in different cortical layers for *Nf1*$^{+/-}$ and *WT* mice.**

Normality was tested for all groups.

A – Unpaired t-test (n=9, p=0.4026)

B – Unpaired t-test (n=11, p=0.0585)

C – Mann-Whitney test (n=14, p=0.3450)

D – Unpaired t-test (n=14, p=0.5678)

E – Unpaired (n=10, p=0.7254)

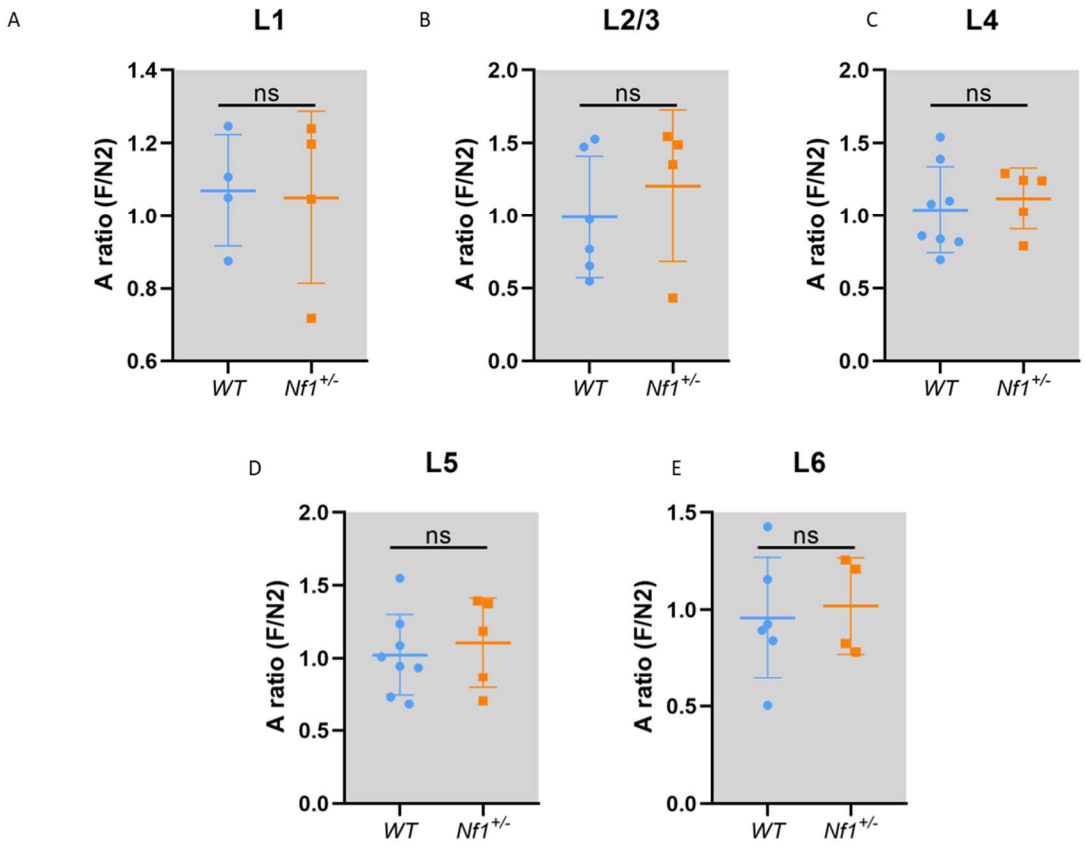


Figure 17 - **Amplitude ratio for VEP magnitudes (Familiar/Novel2) in different cortical layers for *Nf1*$^{+/-}$ and *WT* mice.**

Normality was tested for all groups.

A – Unpaired t-test (n=8, p=0.8958)

B – Mann-Whitney test (n=13, p=0.6095)

C – Unpaired t-test (n=13, p=0.6200)

D – Unpaired t-test (n=13, p=0.6236)

E – Unpaired t-test (n=10, p=0.7529)

2.4) Latency for different cortical layers

Latency to the appearance of VEP is another metric usually quantified in electrophysiology data. We calculated latency ratios (L ratio) in similar way we did for amplitudes and again, we did not find any significant differences between the different cortical layers and the genotypes (Fig.18A-E and Fig.19A-E). We also did segregation for sex (Supplementary data Fig.28-29).

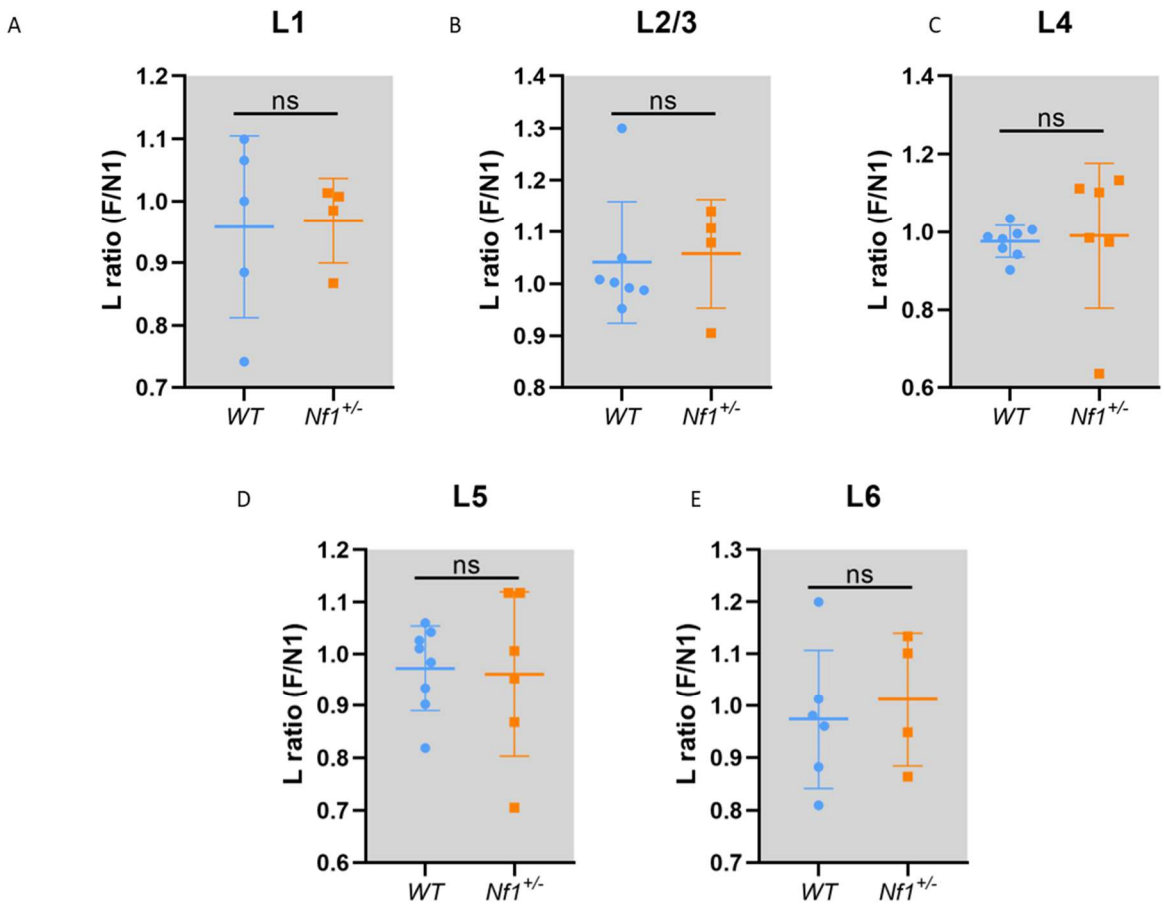


Figure 18 - **Latency ratio for VEP magnitudes (Familiar/Novel1) in different cortical layers for *Nf1*$^{+/-}$ and *WT* mice.**

A – Unpaired t-test (n=9, p=0.9041)

B – Mann-Whitney test (n=13, p=0.5273)

C – Mann-Whitney test (n=15, p=0.4136)

D – Unpaired t-test (n=14, p=0.8680)

E – Unpaired t-test (n=10, p=0.6659)

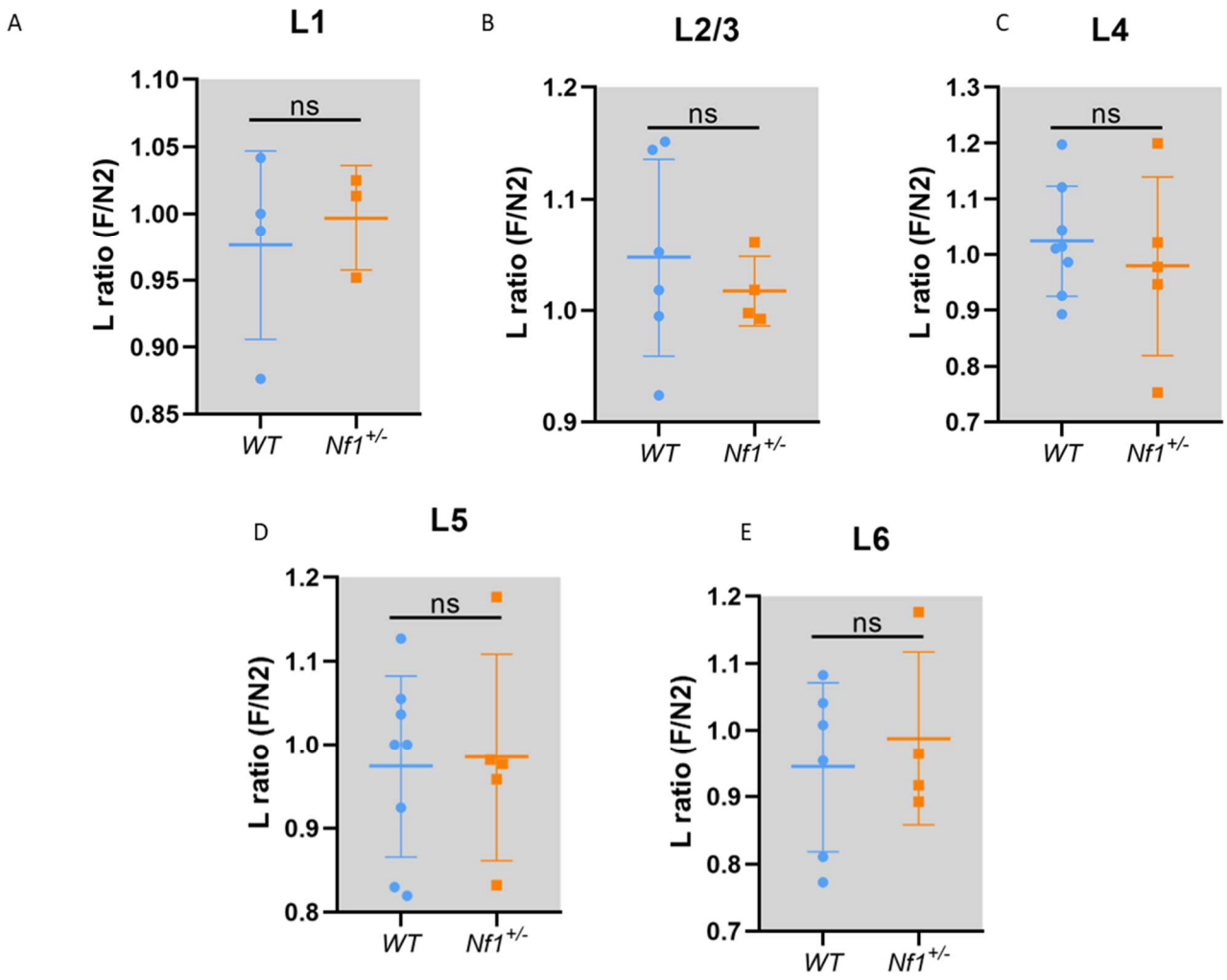


Figure 19 - **Latency ratio for VEP magnitudes (Familiar/Novel2) in different cortical layers for *Nf1*$^{+/-}$ and *WT* mice**

Normality was tested for all groups.

A – Unpaired t-test (n=7, p=0.6746)

B – Unpaired t-test (n=10, p=0.5358)

C – Unpaired t-test (n=13, p=0.5440)

D – Unpaired t-test (n=13, p=0.8709)

3) Brain wide c-Fos mapping

3.1) Social interaction-dependent c-Fos

The last group of results aimed to open news paths of this line of research since we want in the future to couple V1 electrophysiology data with a more social relevant stimulus. Because of that, we performed a behavioral paradigm where animals interacted with a familiar or novel conspecific or with an object. As expected, a social interaction significantly increased interaction time comparing with an object (Fig. 20A) ($p=0.0002$ and $p<0.0001$, respectively), although we difference was observed between familiar or novel interactions.

However, when we segregate data between genotype, we did not find any significant differences between *Nf1*$^{+/-}$ mice and *WT* littermates (Fig.20B). We also segregated for sex (Supplementary data Fig. 31).

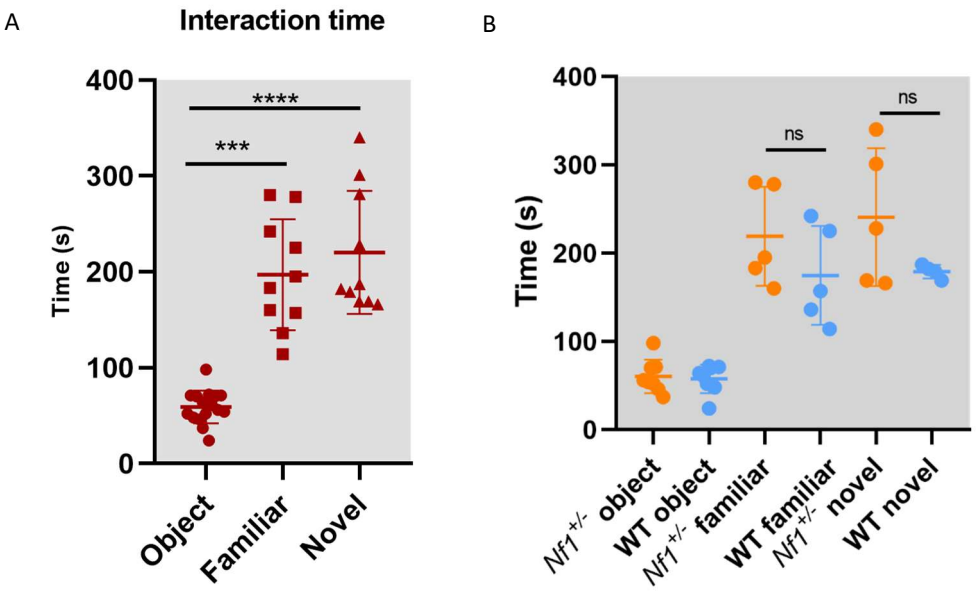


Figure 20 - **Social interaction time.**

Normality was tested for all groups.

A – One-way ANOVA (n=35, p<0.0001)

B – Unpaired t-test (*Nf1*$^{+/-}$ vs *WT* familiar) (n=10, p=0.2456)

Unpaired t-test (*Nf1*$^{+/-}$ vs *WT* novel) (n=9, p=0.1652)

3.2) c-Fos immunohistochemistry

Some of the brains of the animals that performed the behavioral paradigm were used to performed free-floating immunohistochemistry to quantify c-Fos^{+} cells in different brain regions. Our aim was to identify the most promising regions that encode familiarity or novelty to a social stimulus. We did not find any significant differences for the regions analyzed for the three conditions (familiar, novel and object) (Fig.21).

Our objective was to see if there was any significant difference for the three conditions. This is an ongoing exploratory analysis where samples were processed randomly and blinded to the experimental group. Therefore, when don't have a sufficient n to segregate for genotype or sex to do statistical analysis. Nevertheless, we segregated for genotype (Supplementary Data Fig. 32). We also quantified other regions (Supplementary data Fig.31).

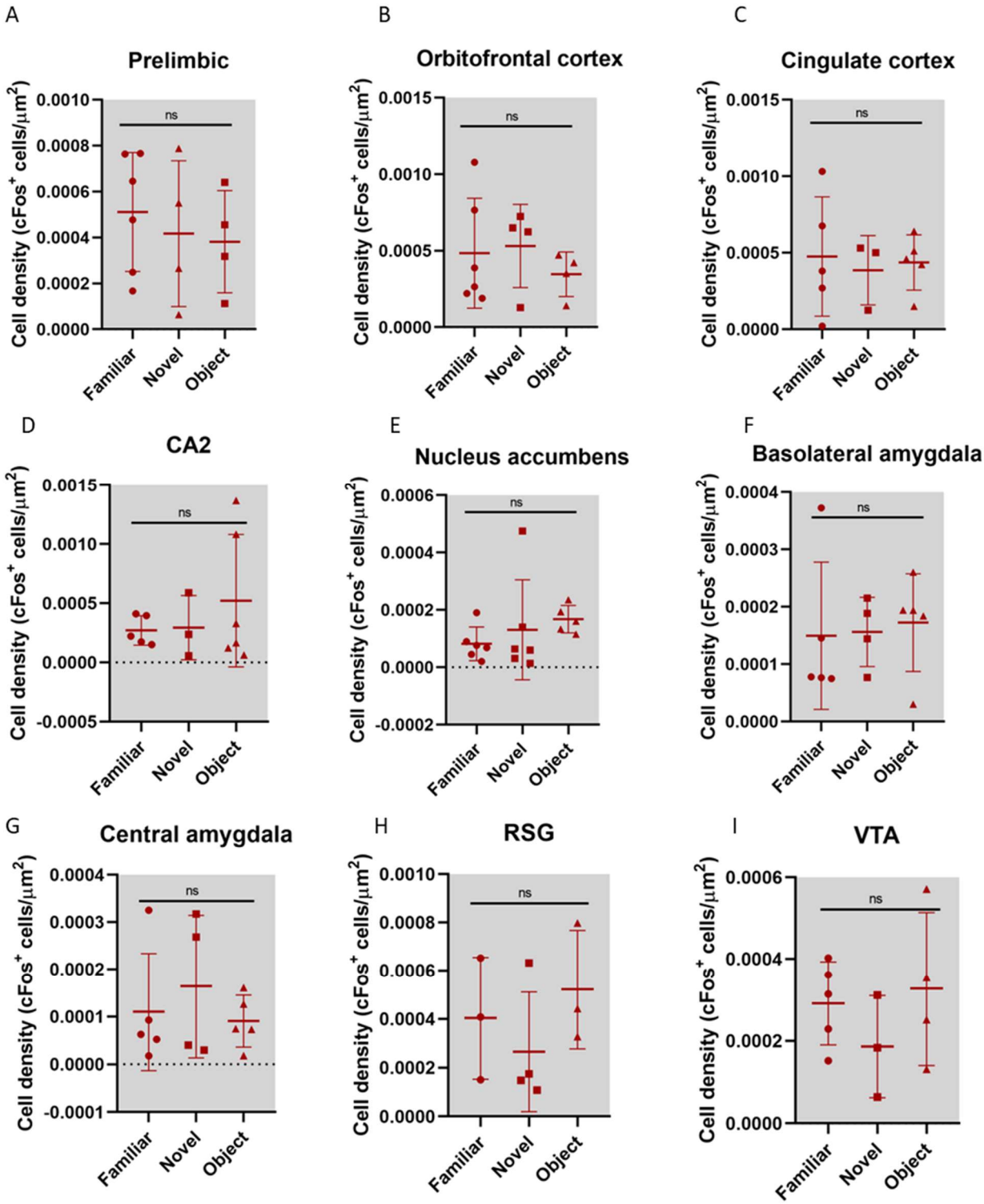


Figure 21 - **c-Fos$^+$ cells for different regions in three different groups: novel, familiar and object.**

A) Prelimbic cortex – One-way ANOVA (n=14, p=0.7337); B) Orbitofrontal cortex – One-Way ANOVA (n=14, p=0.6552); C) Cingulate cortex – One-way ANOVA (n=13, p=0.9140); D) CA2 – One- way ANOVA (n=12, p=0.5524); E) Nucleus Accumbens – Kruskal-Wallis test (n=17, p=0.1076); F) Basolateral amygdala – Kruskal-Wallis test (n=14, p=0.6837); G) Central Amygdala – Kruskal-Wallis test (n=14, p=0.9463); H) RSG – Kruskal-Wallis test (n=10, p=0.3138); H) VTA – One-way ANOVA (n=12, p=0.4344)

Discussion

In this section the most important results will be discussed, dividing it in three parts: 1) Behavioral SRP; 2) *In vivo* electrophysiology in V1; 3) Brain wide c-Fos mapping.

1) Behavioral SRP

Visual stimulus habituation which is not associated with reward or punishment is vital to distinguish a novel and a familiar stimulus in the environment. Using specific visual stimuli with a particular orientation is used to study the development of plasticity in V1 in a form of SRP. The animal is habituated for several days to those stimuli. Over time the preference of the animal to the stimulus decreases while VEP amplitude increases. In last day of test it is expected that the animal spends more time exploring the novel stimulus comparing with familiar stimulus (Fig.22)[69]. Before taking any conclusions from familiar vs. novelty discrimination in SRP, we should evaluate visual acuity in transgenic models. Data from our lab had already confirmed that visual acuity was not impaired in *Nf1*$^{+/-}$ and *TSC2*$^{+/-}$ models measured by optomotor response (Fig. 23A-B) Visual acuity was quantified by increasing spatial frequency of grating presented to the animal until optomotor response disappears[92]. In figure 12A1-A2 we observed that the genetic background was not influencing the discrimination familiar vs. novel since novel stimulus increased the preference for novelty. For TSC2&WT animals and for NF1&WT animals it was observed SRP induction, shown by an increased preference for novelty. This suggests that SRP induction was occurring properly in the behavioral phenotype but with no effect at a genotype level. TSC2&WT animals presented a higher preference for novelty. Total exploration was not altered between familiar and novel for both animal groups with TSC2&WT animals showing increased total exploration time compared with NF1&WT animals. This is in line with what is known about the genetic backgrounds. TSC2&WT animals are hybrids BL/6NxBL/6J whereas NF1&WT animals are hybrids BL/6Nx129. 129S1/SvImJ mice present a lower performance in social tasks which is in part explained to the general low levels of locomotion activity measured in an open-field arena and the high levels of anxiety measured in EPM. Compared with C57BL/6J, 129S1/SvImJ mice present higher locomotion activity and fine movements but lower rearing movements. However, they present lower % entries in the open arms of EPM[90]. These features

of 129S1/SvImJ strain combined may contribute to some hesitancy to explore the stimulus and a consequent lower interest registered for novelty in SRP protocol.

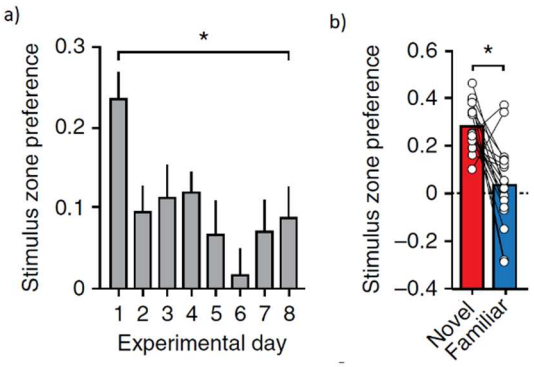


Figure 22 - **SRP behavior**

A) Day after day animals reduce stimulus preference B) On day 9 animals prefer the novel stimulus comparing to the familiar stimulus (Retrieved from Cooke et al, 2015)

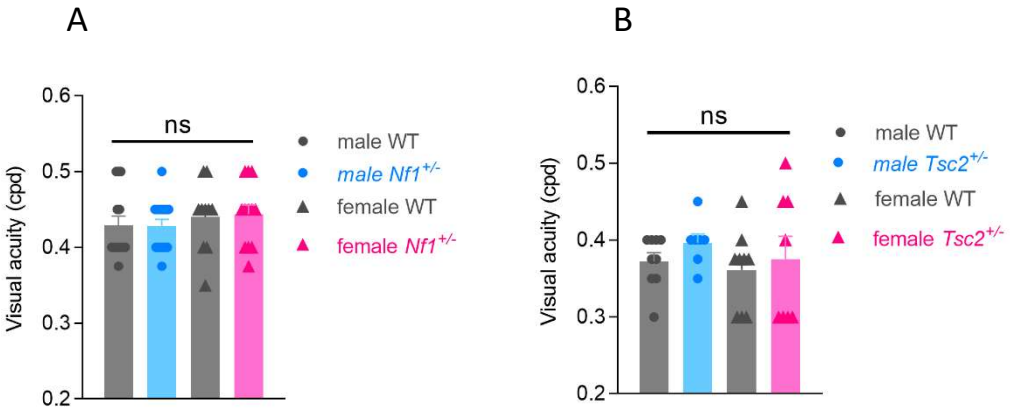


Figure 23 - **Optomotor response in in a visual acuity test.**

A) *Nf1*$^{+/-}$ mice and *WT* littermates with sex segregation. B) *TSC2*$^{+/-}$ mice and *WT* littermates with sex segregation. (unpublished data).

2) *In vivo* electrophysiology in V1

Animals that underwent an SRP protocol are expected to develop potentiation in V1. That potentiation can be analyzed in VEP magnitude since it increases gradually during the habituation days (Fig.24A). On the last day of the experiment, it is expected to see that a novel stimulus has a smaller VEP magnitude when compared with the familiar (Fig.24B).

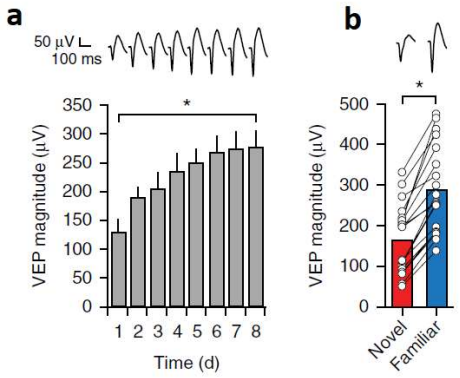


Figure 24 - **SRP in vivo electrophysiology in V1**.

a) Day after day animals increase VEP magnitude for the familiar stimulus b) On day 9 animals VEP magnitude is higher in the familiar stimulus than in the novel one. (Retrieved from Cooke et al, 2015)

We did not observe any potentiation of VEP amplitude for familiar stimulus. In addition, no significant differences in VEP magnitude measured in amplitude ratios were observed between *Nf1*$^{+/-}$ mice and *WT* littermates. In behavioral results we observed increased novelty preference which suggested that the protocol was eliciting SRP properly. However, the results for VEP magnitude are conflicting with behavioral results. Unlike other forms of plasticity, SRP requires PV^{+} neurons for its induction[74]. These neurons are important in V1 for the formation of PYR-PV ensembles for stimulus selectivity[74]. We hypothesize that this transgenic model of ASD had a defective SRP development since this model had deficient maturation of cortical PV^{+} interneurons[74]. However, that was not observed for VEP magnitude in our experiments. PV^{+} interneurons can be more impactful in other sensorial areas that link with V1 to form the

habituation to visual stimuli. That is why we performed c-Fos immunohistochemistry in the last set of results. Another factor that we could take in consideration to explain these results are sex differences. Developmental differences are reported in auditory and visual cortices. Females presented less DSDs, particularly in layer 4[75]. If we increase the n of the experiments, we could find statistical differences in VEP magnitude for females *Nf1*$^{+/-}$ vs *WT* littermates. With our protocol we didn't find SRP development in VEP magnitude. We could hypothesize that perhaps the use of anesthetized animals in day 7 of the protocol could not be the best option. Cooke et al in their protocol implant the electrode before the habituation days. Since the animals are allowed to fully recover 24h from the surgery, the implantation of the probe should be more efficient than with our protocol. On the day of electrophysiology, they didn't anesthetize the animals but only restrained mice with head fixed[69,93]. With this protocol visual perception should be more effective in awake mouse. We already observed that, in this genetic background, SRP development was not so evident. VEP responses, should, therefore less salient and, consequently, difficult to identify. Also, our technique of probe insertion should be more accurate in the future, for example, using micromanipulators to allow a slow insertion in the cortex[94].

3) Brain-wide c-Fos mapping

3.1) Social interaction-dependent c-Fos

To investigate which regions were mostly activated in a social context, we performed a behavioral test in which we compared the performance of *Nf1*$^{+/-}$ mouse with their *WT* littermates. In the first graph, when we only compare the three conditions (object, novel, and familiar interaction), we clearly see that mice show a clear preference for the social context comparing with the object, though they show similar interaction times for familiar and novel subject. These results could be influenced by some constraints such as the limited n of the experiments, the age of the animals that was between P90 and P120, the availability of animals for the experiments and the access to the animals' facility. The advanced age of the animals used could not be appropriate, since in their ages, mice present lower interest for social interaction. It should be more appropriate to use animals in adolescence phase[95]. Other variable that could

be influencing is the environment of the behavioral room that is novel for both animals. Perhaps, it should be interesting to increase the time the animals are habituated to the room to eliminate this confounding effect. When we segregate for genotype, we did not see any significant differences between *WT* and *Nf1*$^{+/-}$ animals. A decreased interest for a novel interaction was hypothesized for *Nf1*$^{+/-}$ mice a priori since this model presents learning abnormalities and memory deficits[96].

3.2) c-Fos activation

We wanted to conduct immunohistochemistry c-Fos mapping because we wanted to evaluate what were the regions that were mostly activated by a social context both in health and in a disease condition.

In our results, we did not observe any significant differences between the three conditions (novel, object and familiar) for the different brain regions. We mainly focused our attention to regions that constitute the social brain. In rats it was demonstrated that NAc plays an important role in regulating helping behavior in individuals of the same strain, suggesting that reward and motivation are vital for this type of behavior. In our results we couldn't see tendency for an increased c-Fos activation in familiar condition. Other region, OFC plays an important role in signaling reward expectations. It is important to quantify how important a specific reward can be which is important in situations where it is important to decide if a reward is worth it or not to go[84]. This region can be linked to SRP since it can be important to the animal decide to choose or not a novel or a familiar orientation. In adult mice, by analyzing c-Fos activation patterns in adult mice interacting with a familiar or novel juvenile, it was demonstrated that long-term social integration requires olfactory circuits that interconnected with medial amygdala. They used a behavioral paradigm where they used control adult mice placed in an empty cage, a novel condition where mice were exposed to an unknown juvenile and perfused afterwards and a familiar condition where mice were exposed with an unknown juvenile, have 24h for memory consolidation and another interaction with the same juvenile before perfusion. The information is also integrated in the hippocampus which add to that information non-social cues. The mPFC, particularly, the prelimbic areas are crucial for a better transduction of the signal, allowing a

better recognition of a familiar conspecific[97]. In our results, we could see a slight increase in c-Fos activation in mPFC regions (prelimbic and cingulate cortices) for the familiar compared with novel interaction. This is in line with the observed recruitment of these areas to long-term social memory. We couldn't see the same tendency for CA2 and for amygdala (BLA and CeA). Therefore, we could quantify c-Fos expression for medial amygdala to see the tendency.

There are some limitations to take conclusions from these results because this is an ongoing exploratory work in which the animals are being processed blinded to the experimental groups. With an increased n to make statistical analysis by sex and genotype, we could identify a region particularly interesting to obtain with *in vivo* electrophysiology recordings and correlate with SRP.

Conclusions and future perspectives

Neurodevelopmental disorders are known for the presence of impairments in synaptic plasticity development, particularly in the sensorial areas of the cortex. Here, we these results, we could give some insights using a genetic mouse model of NF1 about what was happening in the visual cortex. This gave us an idea about how the specific circuitry of the V1 could be impaired in a neurodevelopmental model. The results of the electrophysiology with extracellular recordings gave us an idea about what was generically happening with the potentiation in that circuit. However, these results cannot answer more concrete questions about the effect in specific population of inhibitory and excitatory neurons in V1. Therefore, using other techniques such as optogenetic and chemogenetic tools we can activate different populations of neurons and evaluate if we that activation we can reverse the impairments in SRP. Chemogenetic activation of PV^{+} neurons in the adult visual cortex was already tested, with verified impairments in OD plasticity[98]. Other techniques like patch clamp electrophysiology should also be considered to give us more answers at a cellular level[99].

Our protocol used a habituation paradigm that consisted in the habituation of the animal for several days with a visual stimulus with a particular orientation. This gives answers about the efficacy of the animal to store that information but gives very little information about social recognition. The idea in the future is to associate that visual information with a more social relevant information such as social interaction in an unfamiliar conspecific. For that, it is important to continue to evaluate c-Fos activation, trying to unveil a possible disruption region in $Nf1^{+/-}$ that can be linked to SRP.

Finally, we can also try to explore other sensorial areas of the cortex which can have links with social behaviors. The olfactory system is another very interesting topic to explore where we can test different odors associated with different aspects of social interactions (an odor of a mating partner, an odor of a predator, an odor of a sibling). The importance of this system for the generation of long-term social memory is described[97].

If we characterize in detail the key cortical areas most affected in this neurodevelopmental genetic model, we can define a precise therapeutical strategy to the most affected circuits. These strategies can be either pharmacologic or non-pharmacologic. These include the use

of drugs like fluoxetine which was shown to open the critical period of plasticity in the visual cortex of mice[100] or the use of anodal transcranial current stimulation which was shown to promote hippocampal LTP and the epigenetic regulation of brain-derived neurotrophic factor in mice[101].

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Supplementary data

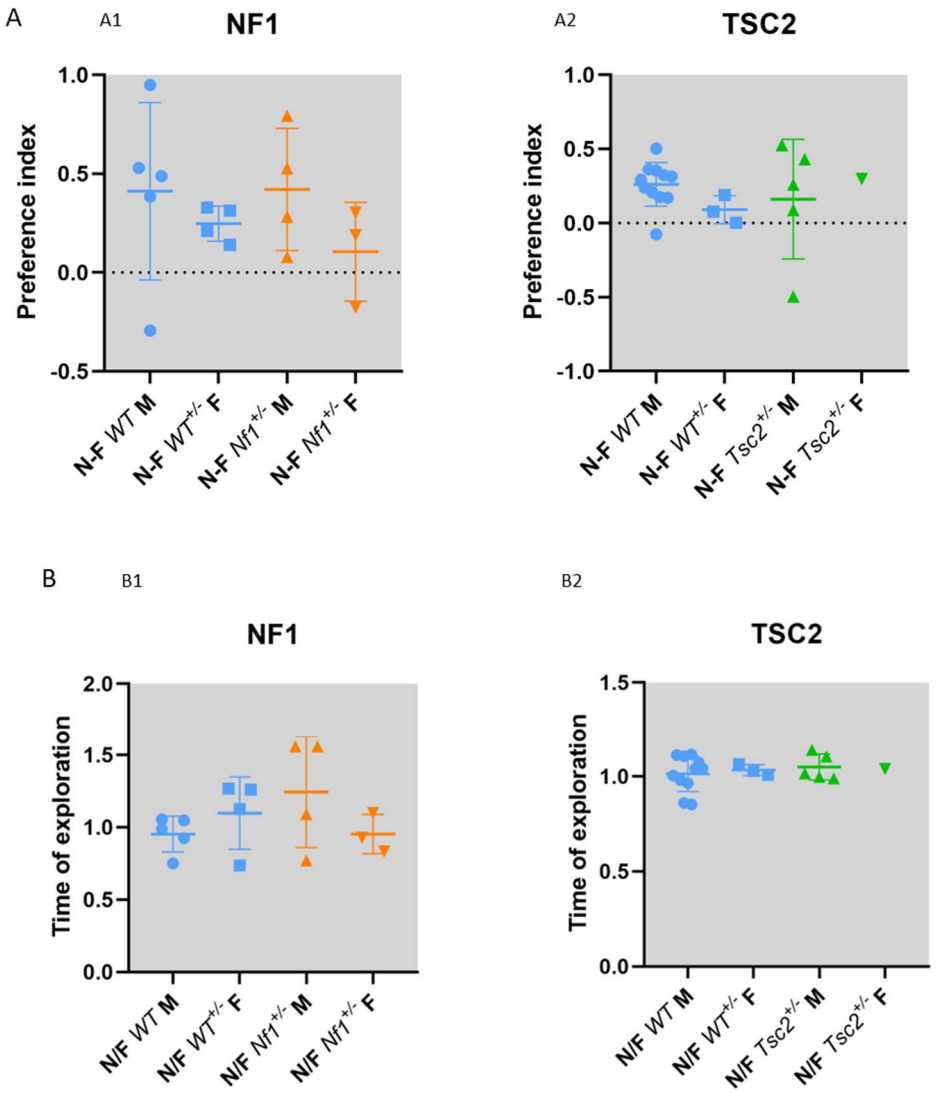

Figure 25 - **SRP development in two genetic models of ASD: *Nf1*$^{+/-}$ and *Tsc2*$^{+/-}$ with sex segregation.**

A1) Preference index in *Nf1*$^{+/-}$ and *WT* littermates expressed in Δ(N-F); A2) Preference index in *Tsc2*$^{+/-}$ and *WT* littermates expressed in Δ(N-F); B1) Total exploration in *Nf1*$^{+/-}$ mice and *WT* littermates expressed in novel/familiar ratio; B2) Total exploration in *Tsc2*$^{+/-}$ mice and *WT* littermates expressed in novel/familiar ratio.

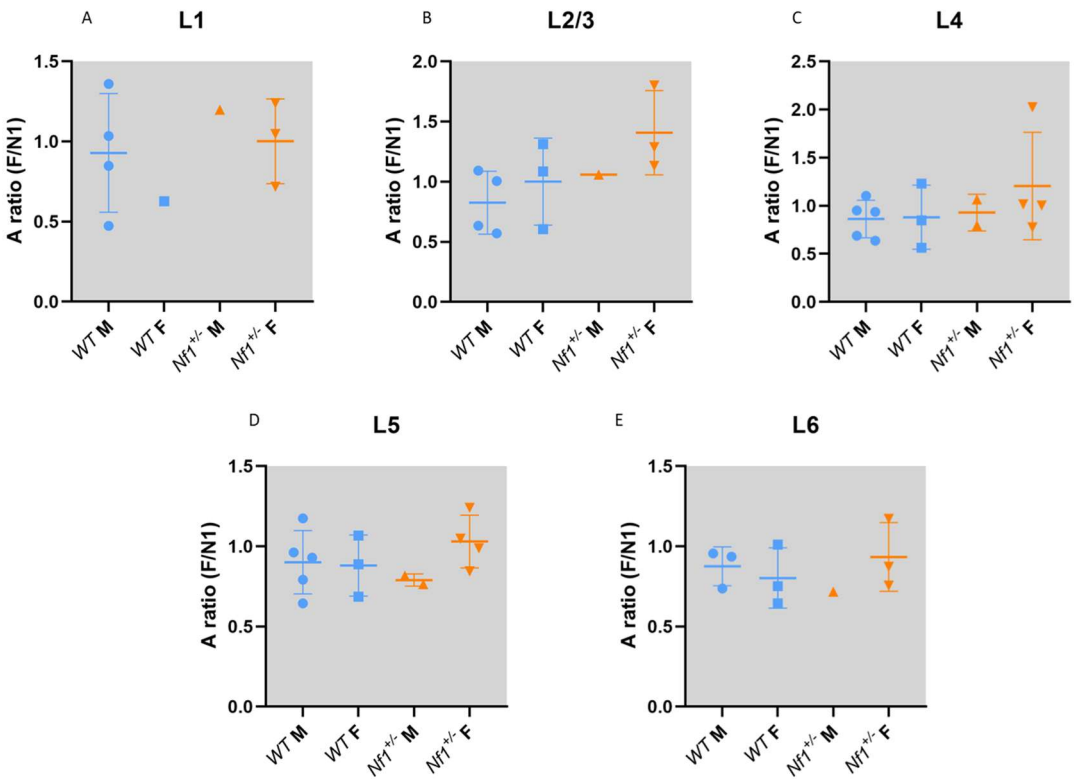

Figure 26 - **Amplitude ratio for VEP magnitudes (Familiar/Novel1) in different cortical layers for *Nf1*$^{+/-}$ and *WT* mice with sex segregation.** A) L1, B) L2/3, C) L4, D) L5, E) L6

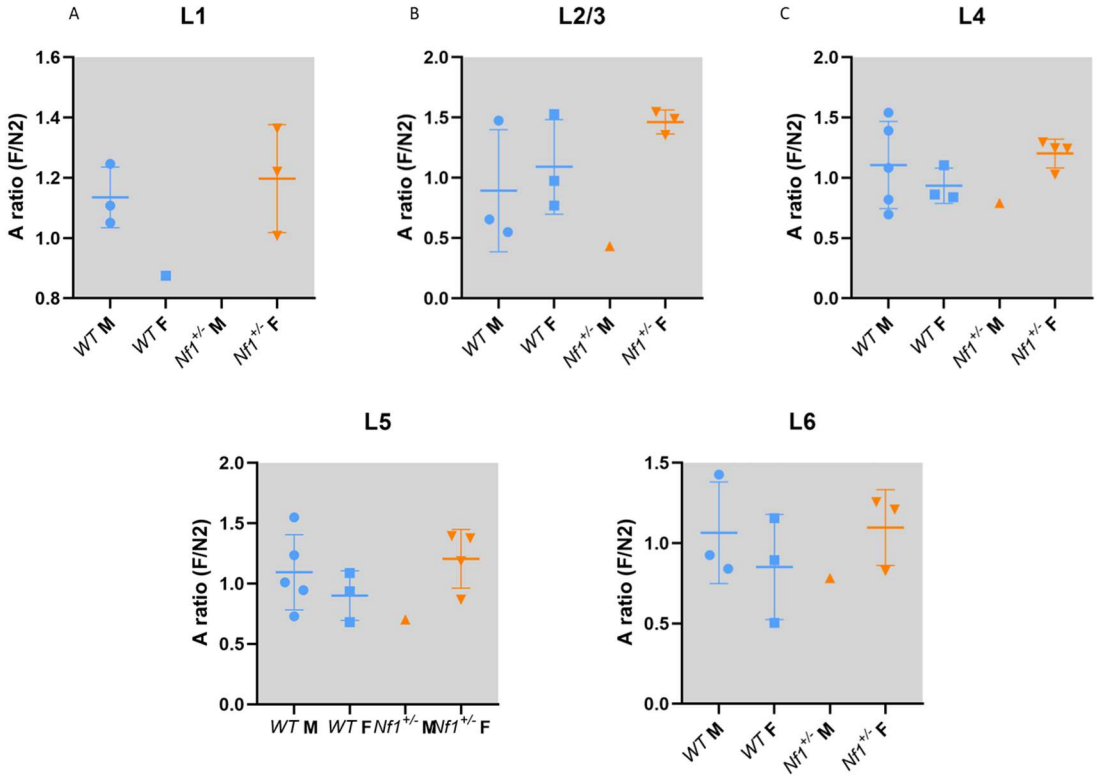

Figure 27 - **Amplitude ratio for VEP magnitudes (Familiar/Novel2) in different cortical layers for *Nf1*$^{+/-}$ and *WT* mice with sex segregation.** A) L1; B) L2/3; C) L4; D) L5, E) L6

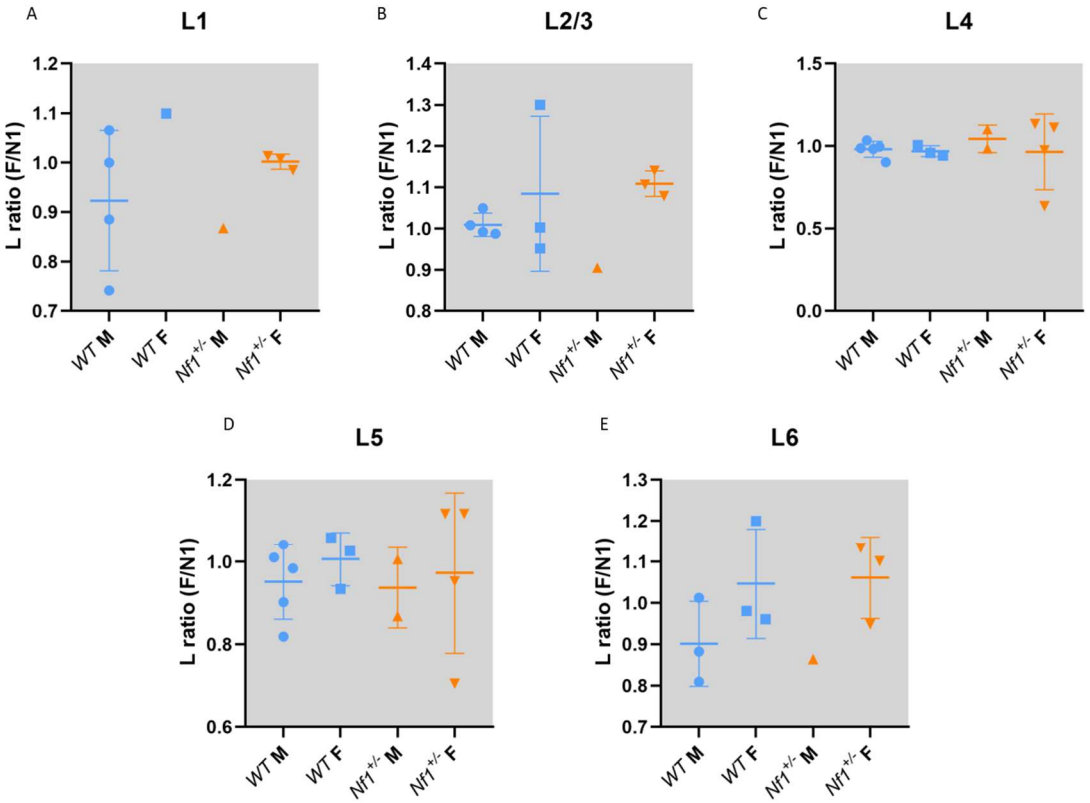

Figure 28 - **Latency ratio for VEP magnitudes (Familiar/Novel1) in different cortical layers for *Nf1*$^{+/-}$ and *WT* mice with sex segregation.** A) L1; B) L2/3; C) L4; D) L5; E) L6

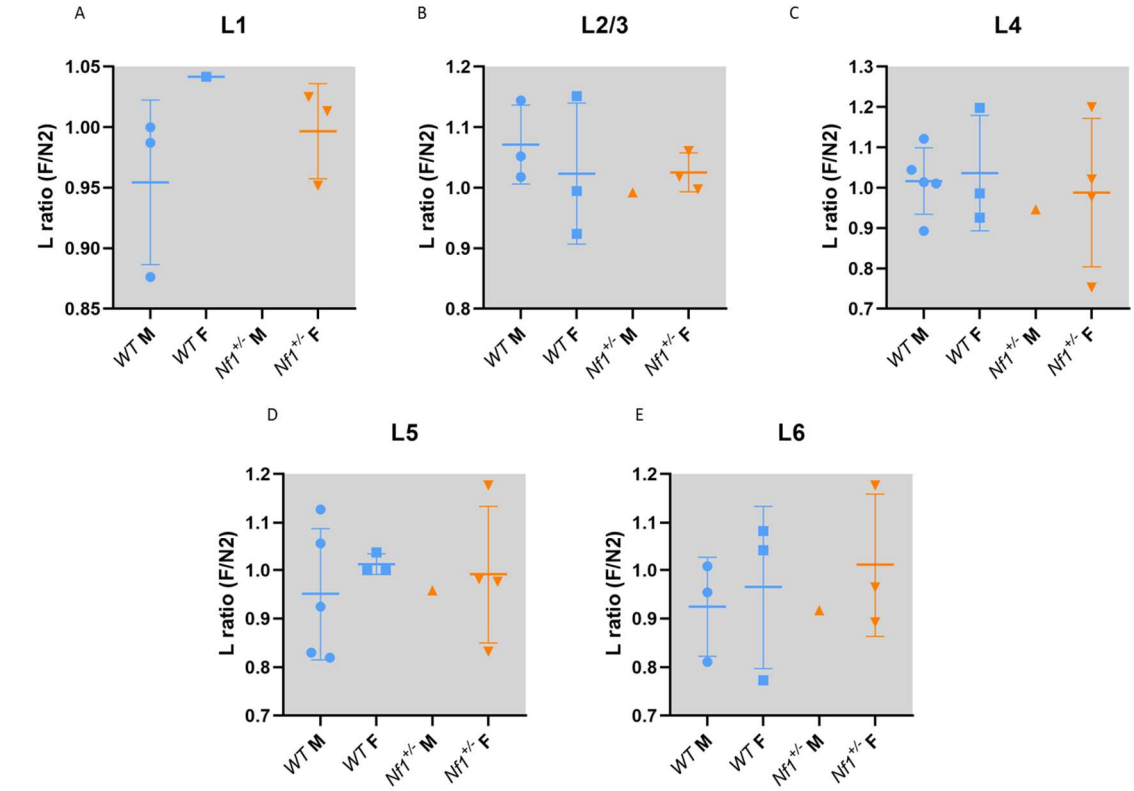

Figure 29 - **Latency ratio for VEP magnitudes (Familiar/Novel2) in different cortical layers for *Nf1*$^{+/-}$ and *WT* mice with sex segregation.** A) L1; B) L2/3; C) L4; D) L5; E) L6

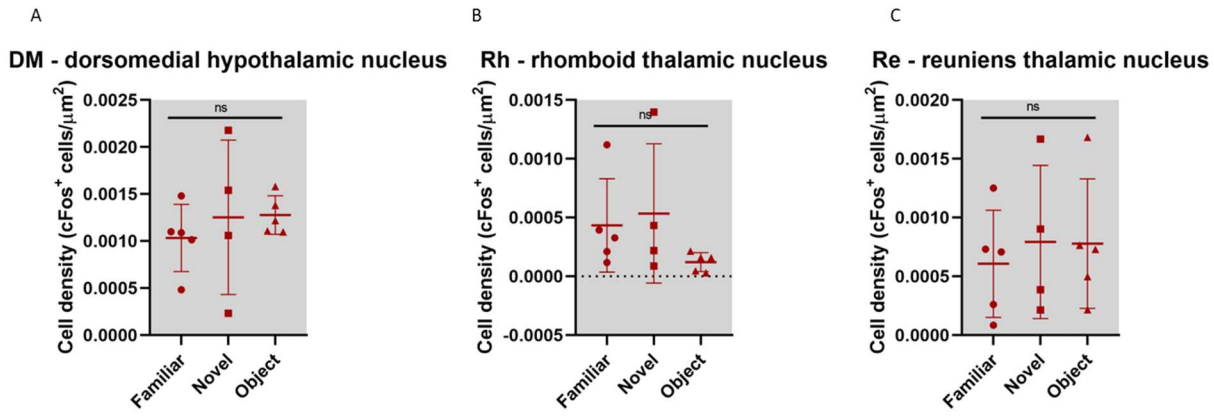

Figure 30 - **c-Fos⁺ cells for different regions in three different groups: novel, familiar and object**

A – One-way ANOVA (n=14, p=0.7052)

B – One-way ANOVA (n=14, p=0.3385)

C - One-way ANOVA (n=14, p=0.8470)

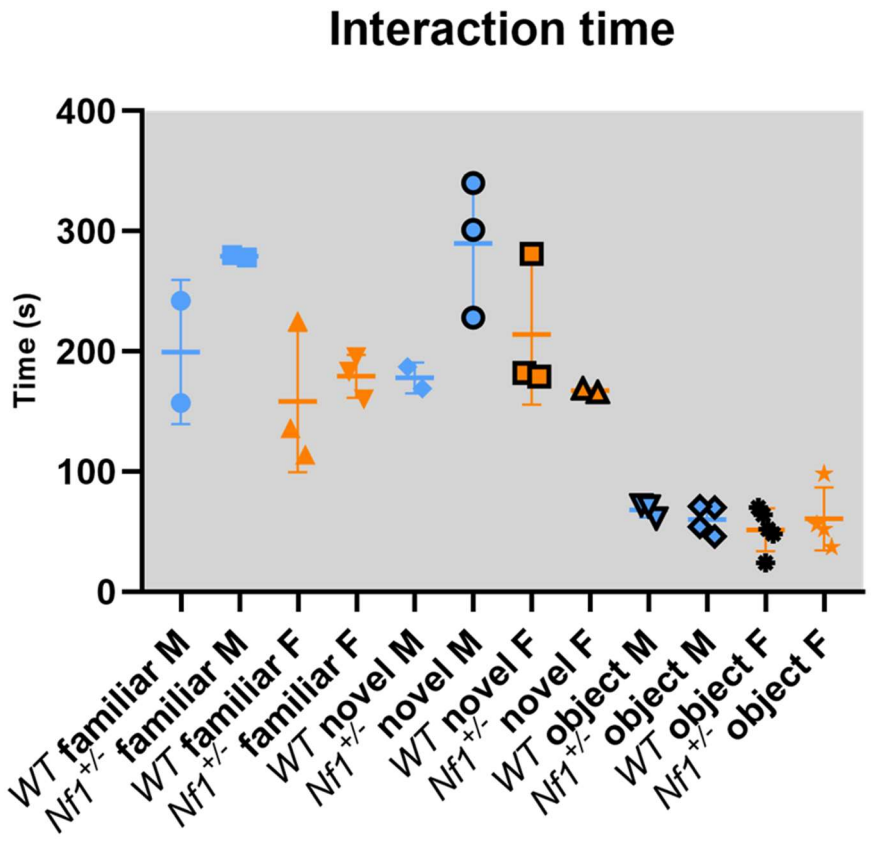

Figure 31 - **Social interaction-dependent c-Fos with sex segregation.**

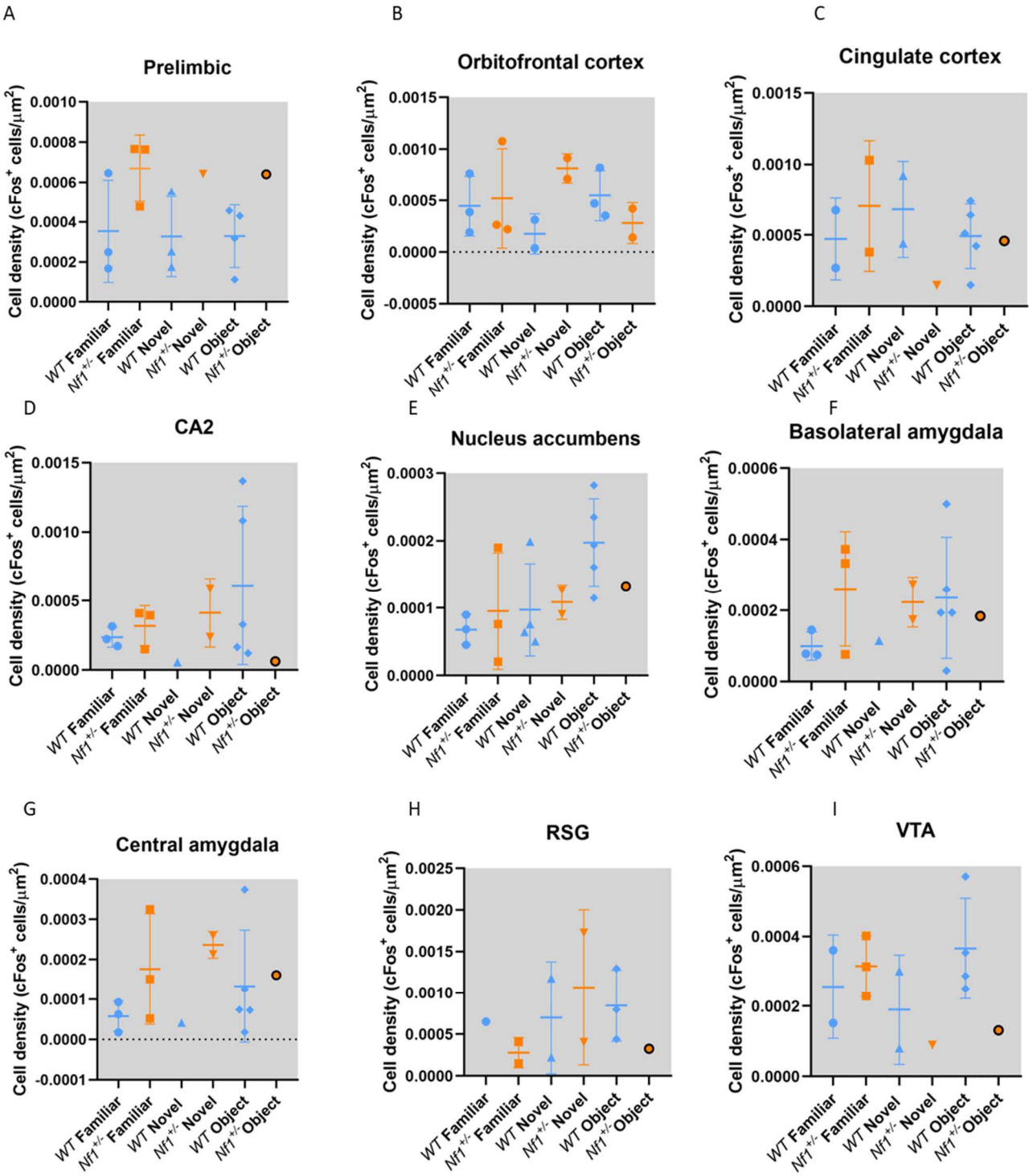

Figure 32 - **c-Fos⁺ cells for different regions in three different groups: novel, familiar and object with genotype segregation**

A) Prelimbic cortex B) Orbitofrontal cortex); C) Cingulate cortex; D) CA2; E) Nucleus Accumbens); F) Basolateral amygdala; G) Central Amygdala; H) RSG; I) VTA