UNIVERSIDADE D
COIMBRA

Sofia Ferreira Santos

IMPACT OF BIOLOGICAL SEX IN A MOUSE MODEL OF AUTISM SPECTRUM DISORDER:
FOCUS ON ULTRASONIC VOCALIZATION PRODUCTION

Dissertação no âmbito do Mestrado em Investigação Biomédica orientada pelo Professor Doutor Miguel Castelo-Branco e coorientada pela Doutora Joana Gonçalves e apresentada à Faculdade de Medicina da Universidade de Coimbra.

Outubro de 2021

Faculdade de Medicina
da Universidade de Coimbra

IMPACT OF BIOLOGICAL SEX IN A MOUSE MODEL OF AUTISM SPECTRUM DISORDER: FOCUS ON ULTRASONIC VOCALIZATION PRODUCTION

Sofia Ferreira Santos

Dissertação no âmbito do Ramo de Neurobiologia do Mestrado em Investigação Biomédica orientada pelo professor Doutor Miguel Castelo-Branco e coorientada pela Doutora Joana Gonçalves e apresentada à Faculdade de Medicina da Universidade de Coimbra

Outubro de 2021

UNIVERSIDADE D
COIMBRA

This work was supported by grants FLAD Life Sciences Ed 2 2016, Strategic plan UIDP/04950/2020, COMPETE, FCT; ICNAS-P.

Agradecimentos

Primeiramente um agradecimento enorme, possivelmente o maior, aos meus orientadores, Doutor Miguel Castelo-Branco e Doutora Joana Gonçalves. Antes de mais pela oportunidade de mais uma vez fazer parte desta equipa, agora de uma forma mais longa e um nadinha mais séria. Pela incessante ajuda e pelas muitas dúvidas que me tiraram, quaisquer que elas fossem.

Um obrigada muito especial à minha coorientadora Doutora Joana Gonçalves, por me ter dado independência no trabalho, por ter acreditado sempre nas minhas capacidades quando eu própria não o fazia e no fundo por me ter ajudado a crescer, desde a licenciatura até aqui, na pessoa que sou hoje no trabalho. Pelo seu constante apoio e encorajamento que me ajudou tanto nesta fase final.

Um obrigada muito grande também à Daniela Jesus e Rafaela Dias, que me ajudaram muito no meu trabalho e fizeram também com que o que apresento nesta dissertação, possível.

Às minha colegas de laboratório, em particular, à Beatriz Martins e Helena Ferreira. Sem elas tudo teria decerto sido muito mais difícil. Não apenas pelo apoio, mas por me terem vindo fazer companhia a meio do ano e terem tornado o ambiente de trabalho tão melhor. Obrigada, meninas. E um obrigada especial ao/à meu/minha futuro/a pseudo sobrinho/a.

À minha madrinha, colega de licenciatura, colega de mestrado e já agora amiga Carolina Santos! Por me fazer companhia também, tantas vezes em tempos de espera e ser a melhor madrinha de sempre.

À minha colega de licenciatura, mestrado, amiga e parceira em praticamente tudo em Coimbra, Carolina Ferreira, estamos desde o primeiro dia a fazer este percurso juntas e não há ninguém com quem gostaria mais de o fazer.

Para finalizar este leque de pessoas, um obrigado muito especial a uma das melhores amigas, ou a minha siamesa como fomos designadas na licenciatura, Diana Silva. Obrigada por me apoiares e no fundo passares por isto ao mesmo tempo que eu e comigo. Por seres das pessoas que me compreende mais plenamente e por todo o apoio que damos uma à outra.

Por fim às pessoas mais importantes, claro, a minha família.

À minha mãe em especial, mas à minha tia e avó também, podia separá-las, mas não faria sentido pois desempenham o mesmo papel de mãe comigo há pelo menos 23 anos. Por me aturarem e me apoiarem, e aguentarem o meu humor constantemente

inconstante, piadas sem graça ou só o meu desaparecimento por uma qualquer divisão da casa. Também à minha irmã que me defende sempre e desde sempre com unhas e dentes.
Um obrigado muito especial ao meu padrasto, Miguel, que talvez não saiba como me orgulho de ser da família dele, por ser praticamente um pai para nós e agir como tal. E que sei muito bem, e de fonte segura, que sempre me defendeu mesmo quando ninguém mais o fazia.
Ao meu pai que me ensinou o meu lema de vida, talvez um pouco controverso para outros, mas que eu sigo sempre e me guiou muito este ano. Pelas distrações ao longo do ano que também precisei, como sempre o fez.

Chega finalmente ao fim mais uma etapa. Uma das, mais importantes é certo e que me definirá daqui em diante. A este conjunto de pessoas que de uma maneia ou de outra ajudaram a que este fosse o resultado, muito obrigada.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ADHD - Attention Deficit/Hyperactive Disorder

AdSt – Subjacent Anterodorsal Striatum

AKT – Protein Kinase B

AMPA - α-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid

ANOVA - One-Way Analysis of Variance

ASD – Autism Spectrum Disorder

ASt - Anterodorsal Striatum

dlPAG – Dorsolateral Periaqueductal Gray

dmPAG - Dorsomedial Periaqueductal Gray

DOC - Deoxycholate

EAAT – Excitatory Amino Acid Transporter

EMB – Extreme Male Brain

E/I – Excitation/Inhibition

FPE – Female Protective Effect

GABA – Gamma-Aminobutyric Acid

GAD – Glutamate Decarboxylase

GAP – GTPase Activating Protein

GI – Gastrointestinal

Gln - Glutamine

Glu - Glutamate

GTP – Guanosine Triphosphate

H - Hindbrain

ID – Intellectual Didorder

IFG – Inferior Frontal Gyrus

KO - Knockout

lPAG – Lateral Periaqueductal Gray

LMC – Laryngeal Motor Cortex

M - Midbrain

MAPK – Mitogen-activated Protein Kinase

mGLUr – Metabotropic Glutamate Receptor

mRNA – Messenger Ribonucleic Acid

mTOR – Mammalian Target of Rapamycin

M1 – Primary Motor Cortex

M2 – Premotor Cortex

NDDs – Neurodevelopmental Disorders

NF1 – Neurofibromatosis Type 1

NMDA – N-Methyl D-Aspartate

PAG – Periaqueductal Gray

PDDs – Pervasive Developmental Disorders

PND – Postnatal Day

PVDF – Polyvinylidine Difluoride

Ras – Rat Sarcoma

RRBs – Restricted and Repetitive Behaviours

RT – Room Temperature

SDS – Sodium Dodecyl Sulphate

sEPSCs – Spontaneous Excitatory Post-Synaptic Currents

sIPSCs - Spontaneous Inhibitory Post-Synaptic Currents

T - Thalamus

TBS – Tris Buffered Saline

USVs – Ultrasonic Vocalizations

VGAT – Vesicular Gamma-Aminobutyric Acid Transporter

VGLUT – Vesicular Glutamate Transporter

VL – Ventral Lateral

WT – Wild-Type

LIST OF TABLES

LIST OF FIGURES

RESUMO

O transtorno do espetro do autismo (TEA) é uma doença do neuro desenvolvimento caracterizado por défices em interação e comunicação social, assim como comportamentos repetitivos.

No TEA, há um grande viés masculino no diagnóstico, mas a razão para esta discrepância ainda não está esclarecida.

Compreender as diferenças e a severidade do sintoma de TEA entre machos e fêmeas, é um passo muito importante para descobrir formas de diagnóstico mais eficientes e menos influenciadas pelo sexo.

Esta tese de Mestrado teve o objetivo de caracterizar défices comportamentais diferenciados entre sexos num modelo animal bem estudado de TEA, modelo de ratinho de neurofibromatose tipo 1 (*Nf1*$^{+/-}$). Aqui realizamos um estudo longitudinal do período perinatal até à fase juvenil. Testes de marcos de desenvolvimento foram executados durante a fase perinatal com foco nos sistemas motores e sensoriais. Observámos que os machos *Nf1*$^{+/-}$ mostraram mais défices de desenvolvimento inicial, especificamente em tarefas que envolviam força e coordenação

As produções de vocalizações ultrassónicas também foram gravadas do dia pós-natal 6 ao 16 e foram detetadas muito poucas variações, nomeadamente aos dias 8 e 14. De seguida, os animais juvenis foram sujeitos a um teste social ao dia 25 e aqui as fêmeas *Nf1*$^{+/-}$ mostraram um melhor desempenho social quando comparados aos machos transgénicos.

Durante o teste social, as vocalizações gravadas mostraram que o macho *Nf1*$^{+/-}$ tem preferência por vocalizar num contexto não-social. Ao dia 30, os animais foram sujeitos a um teste de empatia social (com um animal anestesiado) e os machos transgénicos mostraram mais interesse no animal estranho quando comparados com as fêmeas *Nf1*$^{+/-}$. Ao dia 35, os animais desempenharam um teste de exploração de objetos e foi observado que os machos *Nf1*$^{+/-}$ tinham mais comportamento exploratório e maior tendência para repetir sequências quando compar

ados com os animais controlo das suas ninhadas.

Em suma, os resultados apresentados indicam que os machos transgénicos desenvolvem sintomas TEA cedo na sua vida. Estes défices iniciais permanecem até à fase juvenil. De facto, neste modelo mostraram dificuldade em contextos sociais, nomeadamente em interação e comunicação, assim como comportamentos restritos e repetitivos. Todos estes resultados revelam a importância de entender os impactos do

TEA dependentes do sexo, durante o desenvolvimento cerebral, e da descoberta de possíveis biomarcadores precoces para assistir num diagnóstico mais eficiente.

Palavras-chave: Transtorno do espetro do autismo, Neurofibromatose tipo 1, Substância cinzenta periaqueductal, Dimorfismo sexual, Vocalizações ultrassónicas**.**

ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairments in social interaction and communication as well as repetitive behaviour. ASD displays a strong male bias, but the reason for this discrepancy is yet to be clarified. Understanding differences and severity of ASD symptoms between male and females is an important step to find more efficient and less biased ways to diagnose and to discovery new sex-targeted therapies.

This Master Thesis aimed to characterize differential behavioural impairments between male and female in a well-stablished animal model of ASD, neurofibromatosis type 1 (*Nf1*$^{+/-}$) mouse. Here, we performed a longitudinal study from perinatal period to juvenile phase. Developmental milestones were performed during the perinatal period, focusing on motor and sensory systems. We observed that *Nf1*$^{+/-}$ males were more susceptible to early developmental delays, specifically in tasks involving strength and coordination. Maternal-induced separation ultrasonic vocalizations (USVs) were also recorded between postnatal day (PND) 6 and 16 and few changes were detected, namely at PND8 and PND14. Next, juvenile mice were subjected to a social play test at PND25 and here *Nf1*$^{+/-}$ females showed a better social performance when compared to transgenic males. During social test, USVs were recorded showing that *Nf1*$^{+/-}$ males show a preference of vocalization in a non-social context. At PND30 mice were subjected to a social empathy test (with an anesthetized animal) and transgenic males display more interest in the stranger animal when compared to female *Nf1*$^{+/-}$. At PND35, the animals performed the novel object exploration test and we observed that *Nf1*$^{+/-}$ males had more exploratory behaviour and had more tendency to repeat sequencies when compared to their wild-type (WT) littermates.

Overall, our data demonstrated that *Nf1*$^{+/-}$ males showed ASD symptoms early-on in life. These initial impairments are also observed later in the juvenile phase. Indeed, they demonstrated difficulty in social contexts, namely in interaction and communication, and repetitive/hyperactive behaviour. All these results highlight the importance of understanding sex-dependent impacts of ASD during brain development and of discovering possible early biomarkers to help in a more efficient diagnosis.

Key-words: Autism spectrum disorder, Neurofibromatosis type 1, Periaqueductal gray, Sex dimorphism, Ultrasonic vocalizations.

Chapter 1 **| STATE OF THE ART**

1.1 Neurodevelopmental disorders (NDDs)

Neurodevelopmental disorders (NDDs) are a set of disorders with impairments in growth and development of the brain and/or central nervous system. These impairments appear in the developmental phase of the brain, tend to show well before children even enter school and may affect learning, executive function, social abilities and/or cognition (DSM-5, APA). Moreover, there is a tendency for these symptoms to occur simultaneously. In fact, no symptom exclusively belongs to one single disorder (DSM-5, APA). The impairments manifested in these disorders vary from very specific to global limitations of cognitive and executive functions, social skills, or intelligence (DSM-5, APA).

According to the *Diagnostic and Statistical Manual of Mental Disorders, 5th edition* (DSM-5), NDDs include: intellectual disability (ID); autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), motor disorders, specific learning disorder, and communication disorder (Table 1).

Table 1: DSM-5 Neurodevelopmental Disorders Classification and Features.

Disorder	Characteristics
Intellectual Disorder (ID)	Deficits in adaptative and intellectual functioning with same level peers
Autism Spectrum Disorder (ASD)	Impairments in social interaction and communication and repetitive behaviours
Attention Deficit/Hyperactive Disorder (ADHD)	Persistent problems, lack of attention, excessive energy and impulsivity interfering with functioning
Motor Disorders	Involuntary repetitive movements associated with affliction (i.e., tics)
Specific Learning Disorder	Persistent deficits in reading, writing and mathematical reasoning
Communication Disorder	Difficulty with the practical aspects of language

However, various authors do not favour this grouping due to definition of NDD as a disruption to brain development. They claim that it is more correct to include some rare genetic syndromes, such as cerebral palsy, congenital neural anomalies, schizophrenia

and even epilepsy (BOZZI et al. 2012; OWEN et al., 2011), considering the broad heterogeneity of neurodevelopmental impairments (THAPAR et al., 2015). Some of these disorders stabilize over time, such as autism spectrum disorder, attention deficit/hyperactivity disorder, intellectual disability, learning and communication disorders, whereas other tend to follow a remitting and relapsing pattern, such as tic disorders and schizophrenia (THAPAR et al., 2015).

Diagnosis of NDDs is extremely complex but one common feature is the fact the males tend to have a higher rate of formalized diagnosis when compared to females. In fact, ASD presenting a male bias of 3:1, ID presenting a ratio of 1.6:1 for mild cases and 1.2:1 for severe and ADHD having a 2:1 ratio (LOOMES et al., 2017; MORRIS-ROSENDAHL et al., 2020).

The major difficulty to diagnose children with NDDs relies in the common symptoms of the disorders. So, it becomes importance to find distinguishing symptoms/features between NDDs, not only will this help in diagnosis, but also found effective therapeutic approaches. Currently, the diagnostic of NDDs, especially ASD and ADHD, is based on symptomatic criteria, namely DMS-5 in the United States and ICD-11 outside of United States (DOERNBERG et al., 2016). This diagnosis system consists of using particular features in each individual disorder. Accordingly, the clinicians search for specific characteristics in their patients to help them formalize diagnosis. For example, Tourette's syndrome, a tic disorder is diagnosed when the individual has at least one year of prevalent vocal and motor tics, while specific learning disorder is diagnosed when the individual display impairments in the ability to perceive and process information in an accurate way (DSM-5, APA). The use of well characterized features is important since it can help enrich the diagnosis with extra information. Furthermore, there are specifiers "associated with a known medical or genetic condition or environmental factors" (DSM-5, APA). These allow to identify factors that may underlie aetiology of the disorder, such as, genetic conditions, medical conditions, or environmental factors (DSM-5, APA).

Authors have shown the importance of evaluating the patient in a more thorough way, rather than only applying core symptoms for a primary diagnosis. They state it is important to understand the patient itself, analyse the characteristic features and common symptoms with other neurodevelopmental disorders, but also assess additional deficits present and emotional features (THAPAR et al., 2015). This would translate to richer diagnosis, but also, and possibly more important, a better understanding of the individual and its necessities. For clinicians this could be of great help in understanding different therapeutic strategies. In fact, the more information on the individual, the better may be its follow-up. Therapeutics for children with these disorders are extremely

complex and tend to be multidisciplinary treatments, requiring specialists in child psychiatry, psychology, and speech therapy for example (THAPAR et al., 2015).
There are no concrete treatments for NDD, there are some therapeutic approaches to control some symptoms for example hyperactivity and attention, and some to alleviate other signs, such as tics and anxiety, depending on the disorder (VASA R.A. et al., 2016). NDDs, being extremely complex disorders, with needs ranging from the health care field to the educational field, representing a hurdle for NDDs patients but also for to their families and caretakers. Children with NDD have the need for many different services throughout their lives, which represents different services and costs through time (THAPAR et al., 2015; LAMSAL et al., 2017). The importance of the study of these disorders becomes evident when we scrutinize their real impact in society.

1.2 Autism spectrum disorder (ASD)

Autism spectrum disorder (ASD) is a pervasive developmental disorder affecting 1 in every 160 children worldwide (ELSABBAGH et al., 2012). In DSM-5, this group is characterized as a joining of some disorders that had been separated in previous editions, namely autistic disorder, Asperger's disorder, childhood disintegrative disorder and pervasive developmental disorders (PDDs). The range and quantity of information concerning these disorder's bases and origins are amongst the most complex in their group, namely psychological and psychiatric disorders (V. M. DURAND, APA, 2014).
It is known that ASD has a genetic component and high heritability which explains recurrence risk in families as well as associated characteristics (HALLMAYER et al., 2011; DE RUBEIS et al., 2015). However, ASD's overall complexity extends to the genetic influence, with many studies concluded that there is a strong genetic foundation where both gene-gene interaction and gene-environmental factors play an important role (LI et al., 2012). In fact, the increase in genetic studies of ASD helped to elucidate the role of a set genes responsible for ASD etiology in some cases, counting on copy number variations (CNVs) studies and Mendelian mapping, for example (LI et al., 2012; GESCHWIND, 2011; MURDOCH et al., 2013).
There is a strong and consistent male bias concerning ASD with a ratio of 3 males to 1 female (LOOMES et al., 2017). Several hypotheses have been postulated to explain this discrepancy based in genetic, epigenetic, and environmental factors, which seems could have an important influence on ASD heterogeneity (PERSICO et al., 2006).

These disorders are characterized by three main core traits: impairments in social interaction and communication – both verbal and nonverbal communication is affected, with problems ranging from speech delays, poor diction, monotony in speech, poor eye contact, difficulty in evaluating facial expressions and gestures and difficulty in understanding the other end of the conversation overall - and restricted/repetitive behaviours – ranging from physical behaviours such as hand flapping, to the use of objects and to echolalia, i.e. repetitive speech (DMS-5, APA; CAMPISI et al., 2018). People with ASD have also been found to, along with core symptoms, present comorbid intellectual disability, anxiety or depression (CAMPISI et al., 2018).

According to the DSM5, ASD diagnostic criteria are based on evaluation of main core symptoms of ASD impairments in social interaction and communication **(A)** and repetitive behaviours **(B)** by focusing on:

A1 – Social initiation and response – evaluates social approach, ability of back-and-forth communication skills, sharing of emotions and interests and ability of social context initiation;

A2 – Nonverbal communication - evaluates use and ability to understand eye contact, posture and gestures, the use of normal speech characteristics (such as volume and pitch) and coordination between verbal and nonverbal communication;

A3 – Social awareness and relationships - evaluates behaviour adjustment, imaginative play, ability to make friends and take interest in others;

B1 – Atypical speech and movements - evaluates stereotyped and/or repetitive speech, use of objects and movements;

B2 – Resistance to change - evaluates ability to follow a routine, resistance to change, inability to understand humour or irony in speech (rigid thinking);

B3 – Repetitive use of objects or topics - evaluates obsessions, few but extremely intense interests, obsession with objects, numbers, letters, symbols, topics, and unusual fears or attachments;

B4 – Abnormal sensory behaviours - evaluates pain tolerance and unusual interest in sensory characteristics of the involving environment (sound, visual, sell taste, for example).

It is also important to notice that the symptoms observed impair everyday life, with onset at early childhood. Moreover, ASD can be divided in three different severity levels (Figure 1).

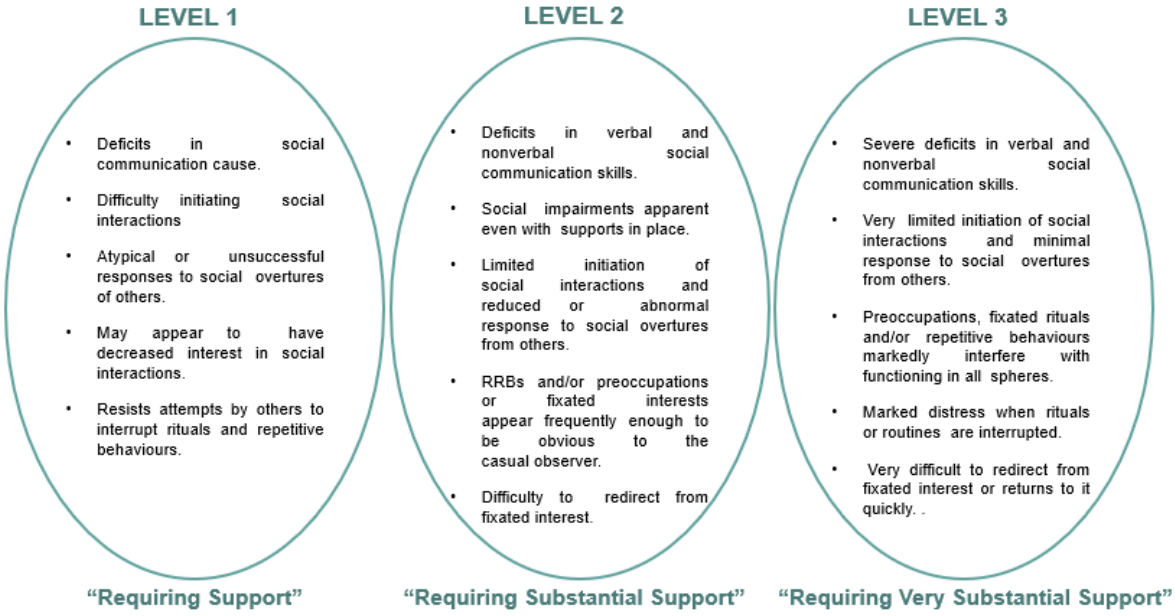

Figure 1. Graphic scheme of ASD levels of severity and their characteristics. Adapted from DSM-5.

Similar to NDDs, there is no pharmacological treatment for the core manifestations of ASD. The most effective treatments available today are applied behavioural analysis, namely occupational therapy, speech therapy, physical therapy (DURAND, 2014). As mentioned previously, several authors have described genes that seem to be implicated in ASD development (KAZDOBA et al., 2015, LI et al., 2012; GESCHWIND, 2011; MURDOCH et al., 2013). Consequently, many individuals with ASD display comorbid disorders such as intellectual disability, epilepsy, anxiety disorders, mood disorders, ADHD, gastrointestinal problems (GI), eating problems, sleep disorders (Figure 2) (DURAND, 2014). On the other hand, there are monogenic disorders comorbid to ASD, such as neurofibromatosis type 1 (NF1), which identified comorbidities have great interest in the research field of NDD (GARG et al., 2013; WALSH et al., 2013; PLASSCHAERT et al., 2015; GARG et al., 2015). Importantly, animal models of several monogenic diseases, inclusive NF1, have been used as preclinical tools to study aetiological mechanisms in ASD (MOY et al., 2006).

Figure 2. ASD symptomatology. Scheme of ASD core symptoms - Social interaction, communication impairments and repetitive behaviours and their association with other conditions. Adapted from KLINGER et al., 2014.

1.3 Neurofibromatosis type 1 (NF1)

Neurofibromatosis type 1 (NF1), a common autosomal dominant disorder (NORTH et al., 2000; GARG et al., 2015), is caused by a mutation on gene *Nf1*. About fifty percent of individuals with NF1 show clear inheritance patterns, whereas the rest come from a random mutation of the *Nf1* tumour suppressor gene (GARG et al., 2013; GUTMANN et al., 1991).

This gene is located on the long arm of human chromosome 17 (17q11.2.) and its encoded protein neurofibromin (Figure 3) (ANDERSON et al., 2015; GARG et al., 2013; GUTMANN et al., 1991).

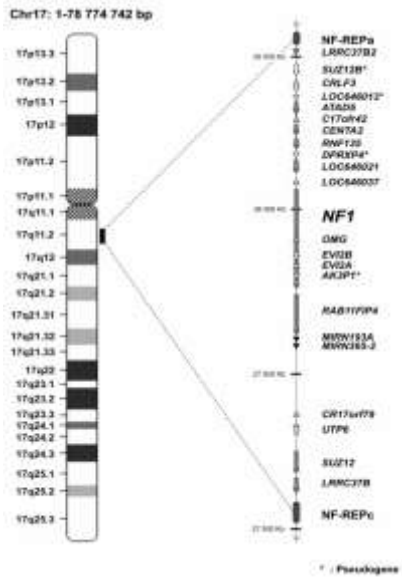

Figure 3. NF1 mutation site. Representation of NF1's mutation site in the tumour suppressor gene, on the long arm of the human chromosome 17(17q11.2). Adapted from PASMANT et al., 2011.

Neurofibromin has a molecular weight of 220–250 kDa and is abundantly expressed in neurons, oligodendrocytes, and Schwann cells (ANDERSON et al., 2015). This protein is a negative regulator of Rat Sarcoma protein (Ras) pathway (JETT et al., 2010). Neurofibromin due to the similarity with GAP (GTPase Activating Protein) family of proteins, can activate signalling cascade by turning guanosine diphosphate (GDP) into guanosine triphosphate (GTP) (BUCHBERG et al., 1990; XU et al., 1990; BALLESTRER et al., 1990; MARTIN et al., 1990). Since this protein is a negative regulator of a Ras intracellular signalling pathway, is extremely important in cell division, differentiation, proliferation, growth, and apoptosis (Figure 4) (PYLAYEVA-GUPTA et al., 2011; GARG et al., 2013). In NF1, the loss of neurofibromin function leads to an up-regulation of Ras/MAPK signaling and, consequently, its downstream pathways MAPK/ERK and AKT/mTOR (BOLLAG et al., 1996; JESSEN et al., 2013). Studies from Alcino Silva lab have suggested that hyperactivation of Ras activity leads to an increase of gamma-aminobutyric acid (GABA) neurotransmission observed by individuals with NF1 (COSTA et al., 2002; MAINBERGER et al., 2013).

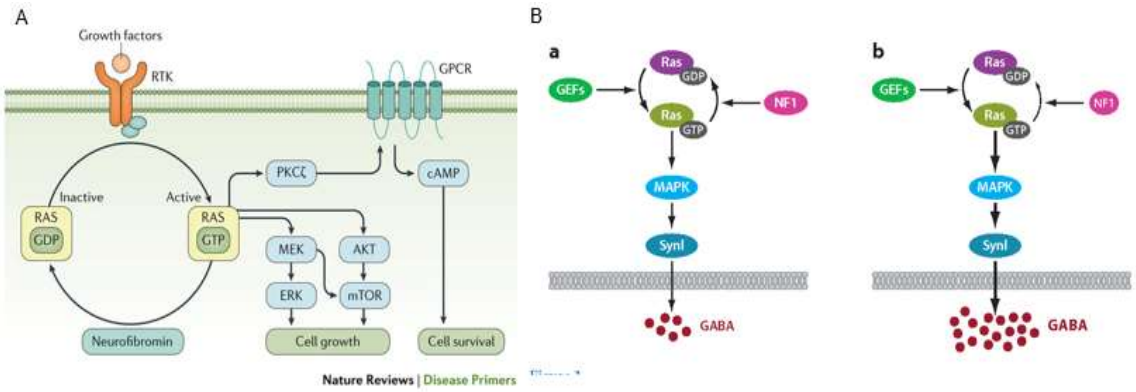

Figure 4. Molecular function of neurofibromin. A – Schematic representation of both pathways affected in the NF1 disorder, Ras/MAPK and AKT/mTOR, as well as their regulation by neurofibromin. Lower levels of neurofibromin leads to an upregulation o both pathways affected and consequence increased levels of their products, that enhances the effects of these pathways in the system by increasing, for example, cell growth and proliferation. Adapted from GUTMANN et al., 2017. **B** – Schematic representation of the role of *Nf1* gene mutation in neurotransmission. The lack of neurofibromin function leads to an upregulation of the Ras/MAPK pathways, leading to higher levels of neurotransmitter gamma-aminobutyric acid (GABA) and consequent increase in inhibitory neurotransmission. Adapted from SHILYANSKY et al., 2010.

The diagnosis of NF1 is based on the presence some clinical features summarised in Table 2, accordingly to Anderson and colleagues (2015). Moreover, since NF1 is a genetic disorder, it is possible to do prenatal diagnosis if it is known one of the parents carries this disease. However, it is not possible to determine its severity (VERLINSKY et al., 2002).

As above-mentioned, NF1 children are susceptible to have learning disabilities together with attention deficits, motor delays and impaired social functioning (MAUTNER et al., 2002; PRIDE et al., 2012; WESSEL et al., 2013; LEHTONEN et al., 2013). Since some of these features are core symptoms of ASD, it has been considered that NF1 has a high comorbid with ASD. In fact, many studies were dedicated to understanding and characterize of the emergence ASD symptoms in NF1 children (WALSH et al., 2012).

Table 2: Symptomatology of Neurofibromatosis type 1 and corresponding ages of onset.

Symptoms	Age
Café-au-lait macules with diameters greater than 5mm in a prepubertal patient and greater than 15mm in a post pubertal patient	Birth (increase in number over the first 1-2 years of life)
Two or more neurofibromas or one plexiform neurofibroma	Peripubertal years
Skinfold (axiliary or inguinal) freckling	Early childhood (5-8 years)
Optic pathway tumour	Before 7 years
Two or more iris hamartomas	30%-50%% until 6 years of age 92% until adulthood
Characteristic bony lesion	Early infancy
First-degree relative with neurofibromatosis type 1	-

Until now many authors have been dedicated to understanding NF1 and trying to find efficient treatments. For that, animal models, namely the *Nf1*$^{+/-}$ mouse, were amply used. Transgenic mice present a pattern of cellular and molecular change very similar to the human phenotype, which means that it is a good model to study disorders. There are many well trustworthy and well characterized mouse models of NF1 inserted in one of

two major groups, namely: mouse models of cell growth and differentiation abnormalities and mouse models of learning disabilities (COSTA et al., 2003).

The similarities between human individuals with NF1 and NF1 mouse models was huge (COSTA et al., 2003). In fact, humans and mice with NF1 share susceptibility to develop tumours, skin pigmentation problems, brain abnormal function, learning deficits (CICHOWSKI et al., 1999; VOGEL et al., 1999; ATIT et al., 1999; NORDLUND et al., 1995; COSTA et al., 2001; JACKS et al., 1994). Important, NF1 phenotypes are observed for heterozygous individuals, and the presence of a homozygous mutation is lethal to both humans and animals.

1.4 Sex Dimorphism in ASD and NF1

As mentioned previously, there is a consistent male bias present in ASD with a 3:1 male to female ratio (LOOMES et al., 2017). Sex dimorphism is of growing interest in the autism research field, the mechanism(s) that underlies in this difference are still unclear. However, recently, several reports described several relevant behavioural and neurological differences between males and females.

Regarding behaviour, it is known that girls with ASD show better ability to mental processes related with goal-oriented behaviours (BÖLTE et al., 2011) as well as concerning visual perception (CARTER et al., 2007). Girls also have less social and communicative impairments (STACY et al., 2014), less repetitive behaviours (SUPEKAR et al., 2015; LEEKAM et al., 2007), and are more able to maintain reciprocal conversation. In addition, girls have enhanced attention to faces and are able to execute gaze patterns, helping them in social contexts (HARROP et al., 2018). On the other hand, boys have higher scores in language and motor skills (CARTER et al., 2007).

Behavioural features of ASD girls represent a huge challenge to diagnosis, due to a mechanism of “camouflaging”-type behaviour (DEAN et al., 2017; HULL et al., 2017; BARGIELLA et al., 2016). In fact, it has been shown that even when ASD symptoms are demonstrated in both sexes, girls find it harder to get an official diagnosis (GIARELLI et al., 2010; WILSON et al., 2016). The complicated diagnosis of females may be due to the possible conceptualization of symptoms as male-like in the diagnosis of ASD, which may translate into a lower sensitivity of professionals to sex-dependent symptoms, causing female symptoms to be overlooked and/or misdiagnosed (HARROP et al., 2019; LAI et al., 2015).

Sexual dimorphism in ASD is not only observed in behaviour, but also in brain pathophysiology. Indeed, males show cortico-cerebellar hypoconnectivity, while females show signs of brain damage (according to the Rutte and Lockyer, 1967 criteria) and lower intelligence quotient (IQ) (SMITH et al., 2019; TSAI et al., 1981; LORD et al., 1982). Moreover, males have shown enlarged cerebellum, hippocampus, and amygdala (CHEN et al., 2017; SPARKS et al., 2002), as well as increased microglial activation mainly in the cerebellum (SUZUKI et al., 2013).

Nowadays, two major theories explain the ASD male bias. One of them is the Extreme Male Brain theory (EMB) that supports the idea of a hypermasculinization of the brain (BARON-COHEN et al., 2002; BARON-COHEN et al., 2005; MAY et al., 2019; TAN et al., 2017) (Figure 5). This hypermasculinization is stated to then lead to a more systematizing brain, prioritizing the ability to foresee and react according to behavioural systems rules which need to be deduced rules. On the other hand, female brain gives emphasis to the ability to understand and react according to others mental state (Baron-Cohen et al., 2005). EMB is linked to a potential variation in foetal testosterone exposure levels, that has been shown in ASD individuals (BARON-COHEN et al., 2015; GEIER et al., 2012). Another theory is the Female Protective Effect (FPE), opposing itself to the EMB theory, it assumes that females have specific factors preventing them from developing ASD, accordingly to the idea that a greater genetic/mutational load is necessary for them to reach a diagnosis (Figure 5) (JAQUEMONT et a., 2014). The FPE is explained by two models: the greater variability model that says males have higher incidence but lower severity (WING et al., 1981); and the liability-threshold model that states that females when reaching threshold for ASD diagnosis present a higher mutational load when compared to males (TSAI et al., 1981).

Another possible explanation for the sex differences observed in ASD focuses on the differences in sex chromosomes, where the Y chromosome may present itself as a risk, and/or the extra X chromosome that of women protects them in some way (SKUSE, 2000). In fact, males with Y aneuploidies, such as XYY and XXYY, were shown to be 20 times more likely to receive a diagnosis (Figure 5) (ROSS et al., 2012).

Concerning NF1, it has also been described that sex is determinant in the development of the disease, in spite of the number of affected male and females being similar. Diggs-Andrews and colleagues (2014) demonstrated, both in humans and mice, that females have tendency to develop NF1-associated optic gliomas, while males have showed more

propensity to academic underachievement (COUDÉ et al., 2006; DIGGS-ANDREWS et al., 2014).

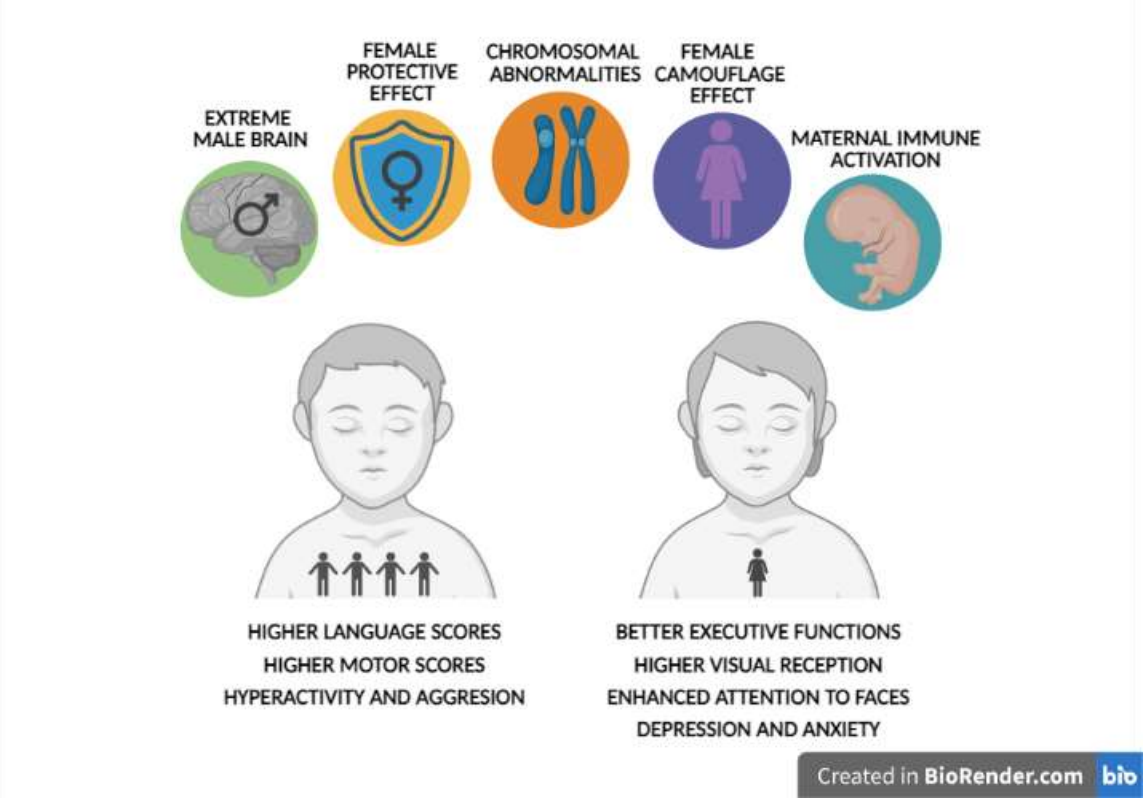

Figure 5. Theories for sex dimorphism in ASD. Representation of the currently studied theories that explain sex bias in ASD: extreme male brain theory, female protective effect theory, chromosomal abnormalities, female camouflage and maternal immune activation. All these theories have been suggested throughout the years to possibly explain male and female diagnostic discrepancy, of 3:1 boy to girl, and underlie sex-dependent behavioural responses. Adapted from SANTOS et al., in preparation.

1.5 Ultrasonic vocalizations (USVs)

Rodent communication is essential for animals' life since it is used to share several messages for socialization or threat situations. Vocalization production involves a neural network within the forebrain and brainstem (JÜRGENS, 2002, 2009; SIMONYAN, 2014). Rodents emit ultrasonic vocalizations (USVs), typically from 30kHz to 90kHz with an average of 80ms during diverse social contexts (CARUSO et al., 2020). Specifically, pups' USVs are induced by maternal separation, while production of USV in juvenile and adult mice is stimulated by social interaction (HECKMAN et al., 2016), new or stressful environments and mating (CHABOUT et al., 2012). For these reasons, the study of USVs

is extreme importance to assess emotional ability and development (CARUSOS et al., 2020).

The vocal repertoire is influenced by several factors, namely environment, genetic background, and development of the animal (HECKMAN et al., 2016; PELEH et al., 2019). Moreover,the vocal repertoire is composed by various USV call, where each of them has particular characteristics, such as start and end frequency, principal frequency, slope and peak frequency, duration, amplitude and waveform, which allow its classification into different categories. Several authors have been proposed classification systems based different syllable types of USV, whose temporal sequencing includes the utterance of repeated phrases (HOLY & GUO, 2005). One of the most important and accepted classification system was proposed by SCATTONI and colleagues (SCATTONI et al., 2008) that suggested a ten-category categorization, based on the shape of each call (Figure 6). Later with these ten categories have been joined into three possible main categories to evaluate their level of complexity:

- Single call, where only one call is formed and with no sudden frequency changes;
- Multi-syllabic call, that corresponds to two calls one right after the other with a sudden change in frequency but no overlap between them;
- Stacked call, in which two independent calls are produced at the same time (not necessarily the same duration, but with temporal overlap) (YOUNG et al., 2010).

With all this in mind, analysis of USVs allows the study of the evolution and maturing of the vocal repertoire of animals, of utmost importance in situations of developmental deficits.

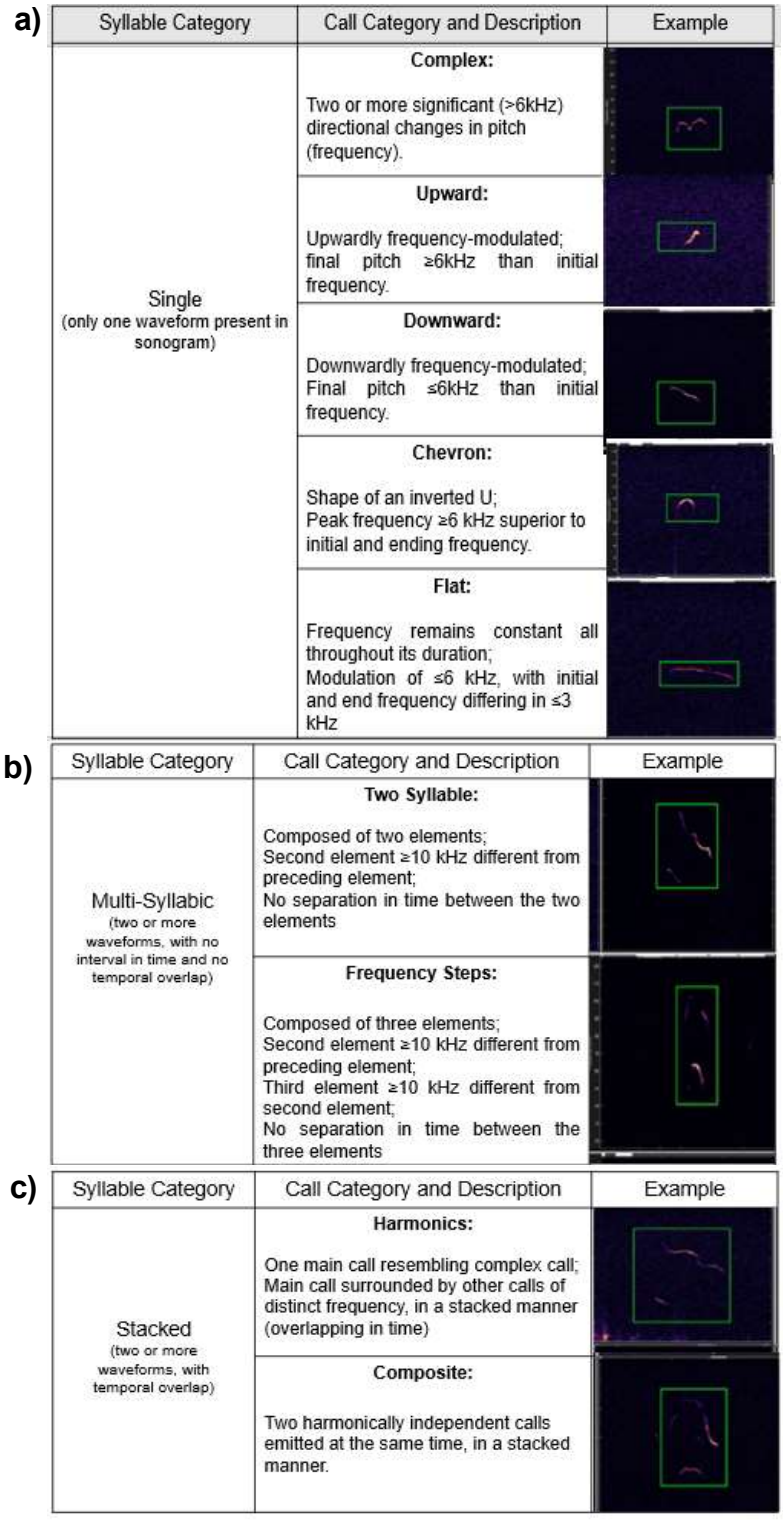

Figure 6. Characterization of Ultrasonic Vocalizations. Representation of typical USVs waveforms produced by mice classified into 9 categories based on Scattoni et al, 2008; Grimsley, Monaghan & Wenstrup, 2011. USV classification according to their complexity (Young et al, 2010) as well as waveform (Scattoni et al, 2008; Grimsley, Monaghan & Wenstrup, 2011). a) single category including complex, upward, downward, chevron and flat calls; b) multi-syllabic category including two syllable and frequency staps calls; c) stacked group including harmonics and composite calls. All sonograms were obtained using DeepSqueak version 2.6.2. Abbreviations: USVs, ultrasonic vocalizations.

1.6 Periaqueductal Gray (PAG)

The periaqueductal gray (PAG) is a very small brain region - about 14mm long and 4-5mm wide -, located in the midbrain and represents a functional interface between the forebrain and the brainstem involved in several behaviours such as social, maternal and aggressiveness (LINNMAN et al., 2012; O'CONNEL and HOFMANN, 2011; GREGG and SIEGEL 2001; BENARROCH, 2012). The PAG is not a clean homogenous region, being divided into dorsomedial (dmPAG), dorsolateral (dlPAG), lateral (lPAG) and ventrolateral (vlPAG), but without cytoarchitectural boundaries receiving specific inputs from others brain regions such as amygdala and hypothalamus (CARRIVE et al., 2012; LINNMAN et al., 2012; BENARROCH, 2012; KEAY et al., 2015; BANDLER et al., 1994; BEHBEHANI et al., 1995). The PAG is also responsible for cardiovascular, motor, respiratory and pain responses and contributes to thermoregulation (BENARROCH, 2012). This brain structure has been associated to response to stressors, namely threats or pain (BENARROCH, 2012; KIM et al., 2013).

Imminent threats caused midbrain regions, namely dlPAG, to be dominant and respond by forming active defensive reactions (BANDLER et al., 2000). Concerning pain responses, PAG is involved in the modulatory network that exerts inhibitory or excitatory control on nociceptive transmission (BENARROCH, 2012).

Nevertheless, PAG is a fundamental brain region to produce USV (GRAEFF et al., 1993; ARRIAGA et al., 2013), since vocal circuits in mammalian brains causally incorporate the PAG (Figure 7) (ARRIAGA et al., 2013). Importantly, it has also been described that a blockage of the PAG with a muscimol injection, a strong agonist for GABA(A) receptors, resulted in an elimination of the production of innate vocalizations in squirrel monkeys by vocalization sites in the forebrain (SIEBERT & JÜRGENS, 2003). Other studies have found that electrolytic lesions of the PAG would cause different mammals, such as cats, dogs and even in humans to go mute (SKULTETY et al., 1962; ADAMETZ et al., 1959; ESPOSITO ET AL., 1999). These results suggest that the PAG is a key region for USV production gating (SIEBERT & JÜRGENS, 2003). It has been suggested that vocalization production by the PAG relies mostly on vocalization-controlling neurons controlled by both a strong inhibitory command from GABAergic neurons, and a glutamatergic input (JÜRGENS, 1994). Indeed, a more recent study has shown, in a more molecular analysis, that in the PAG, neurons activated by vocalizations where approximately 75% glutamatergic and 25% GABAergic (TSCHIDA et al., 2019).

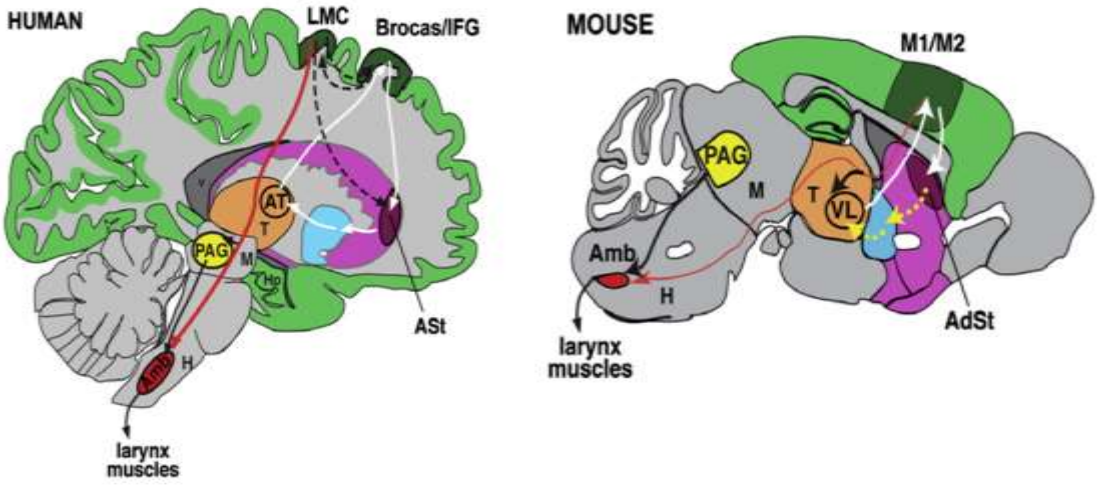

Figure 7. Human and mouse vocal communication. Representative image of the location of brain regions connected to the vocal repertoire, especially the location and comparable size of the midbrain periaqueductal gray in the human and mouse brain. Adapted by Arriaga et al., 2013. Abreviations: LMC, laryngeal motor cortex, IFG, inferior frontal gyrus, AdSt, anterodorsal striatum, AdSt, subjacent anterodorsal striatum, M1, primary motor cortex, M2, premotor cortex, H, hindbrain, M, midbrain, T, thalamus, VL, ventral lateral, PAG, periaqueductal gray.

1.7 Excitation/Inhibition neural ratio

In the human brain, the balance between excitatory and inhibitory neurotransmission is very strictly controlled depending on levels of GABA and glutamate for inhibitory and excitatory neurotransmission, respectively. GABA is the major inhibitory neurotransmitter in mammalian adult brain playing key roles in neural migration, maturation, and synapse formation. This neurotransmitter through its receptors $GABA_A$ (ionotropic) and $GABA_B$ (metabotropic) inhibits neuronal firing (Figure 8) (BOWERY et al., 1989; SESARINI, 2015).

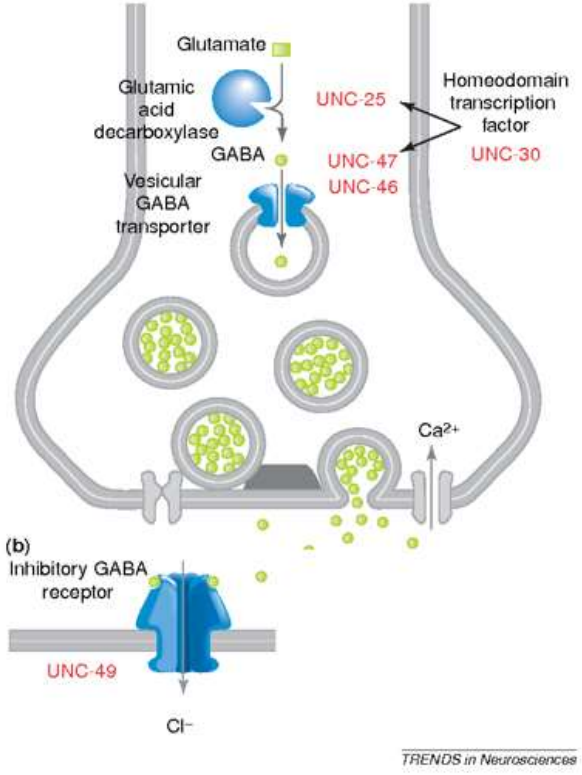

Figure 8. Graphic schemes of GABA neurotransmission. Scheme of GABAergic neurotransmission and GABA synaptic characteristics including auxiliary proteins and molecules. GABA is synthetised by glutamic acid decarboxylase, packed into vesicles by the vesicular GABA transporter and then neurotransmission in mediated by different types of receptors (LAGRANGE et al., 2012). Adapted by Jorgensen et al., 2005. Abbreviations: GABA, gamma-aminobutyric acid, UNC, GABA receptor subunits.

On the other hand, glutamate, the major excitatory neurotransmitter in mammalian adult brain, exerting its functions through several receptors divided into α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, kainate receptors and N-methyl D-aspartate (NMDA) receptors (Figure 9) (ORREGO et al., 1993; FONNUM, 1984).

A balanced interaction between GABA and glutamate is essential to brain homeostasis. A prolonged imbalance of this ratio is the source of several diseases, making excitation/inhibition (im)balance a growing subject of interest in neuroscience field (HAMPE et al., 2018). Indeed, changes in excitatory/inhibitory (E/I) ratio, have been described to underlie cognitive deficits and brain dysfunction in neurodevelopmental disorders, namely ASD (RAMAMOORTHI et al., 2011; CANITANO et a., 2017).

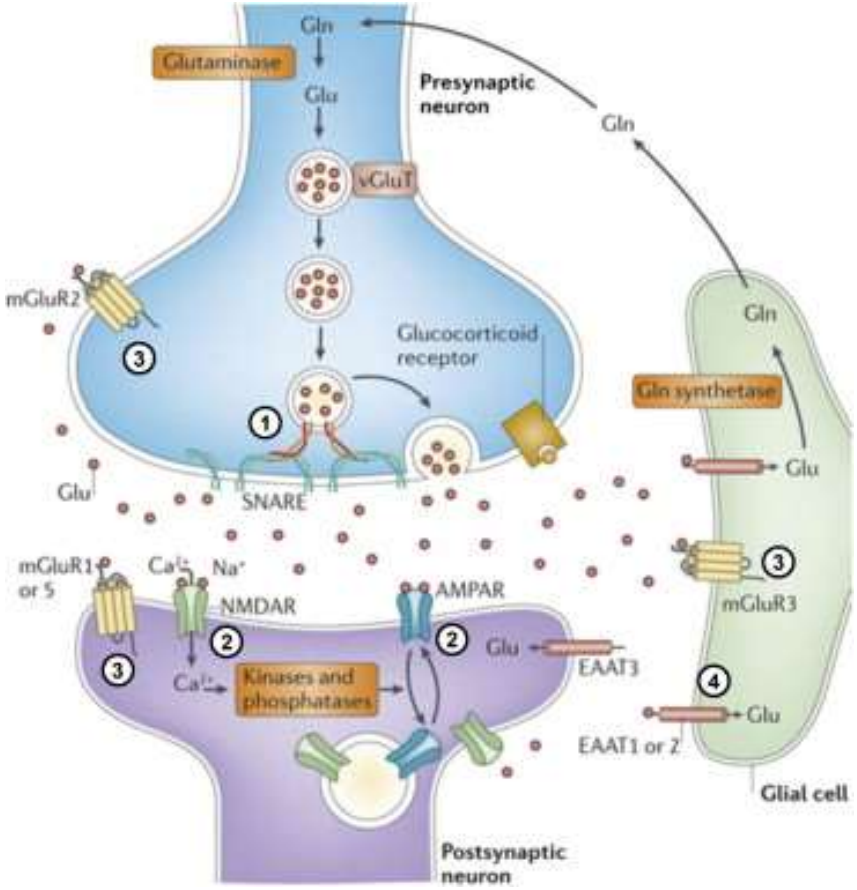

Figure 9. Graphical schemes of glutamate neurotransmission. Scheme of glutamatergic neurotransmission and glutamate synaptic characteristics including auxiliary proteins and molecules. Glutamate is an amino acid neurotransmitter, after synthesis it is transported into vesicles by glutamate vesicular transporters (BAUER et al., 2012). Glutamate receptors are divided in ionotropic, ion channels, and metabotropic, interactive with G-proteins (NICIU et al., 2012). Adapted from Musazzi et al., 2013. Abbreviations: Glu, glutamate, VGLUT, vesicular glutamate transporter, mGLUr, metabotropic glutamate receptor, AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, NMDA, N-methyl D-aspartate, EAAT, excitatory amino acid transporter, Gln, glutamine.

1.7.1 Excitation/inhibition imbalance in ASD and NF1

Human and animal studies indicated that ASD showed a clearly E/I imbalance in several brain regions. A clinical study showed that ASD patients (children aged 2-11 years) have reduced GABA levels and, consequently, GABA/glutamate ratio in the frontal lobe (HARADA et al., 2011). Other work performed in post-mortem brain of ASD patients has shown low density levels of both GABA receptors binding sites in brain regions related to socio-emotional behaviour (OBLAK et al., 2009, 2010). Rojas and colleagues (2013), have found, through analysis of proton magnetic resonance spectroscopy (^{1}H-MRS), that ASD subjects (approximately 14 years old) showed decrease of GABA levels (ROJAS et al., 2013). Other reported similar results, namely Kubas and colleagues (2012) and Puts and colleagues (2017) that described reduced GABA levels in sensorimotor regions and frontal lobe of children with ASD (PUTS et al., 2017; KUBAS et al., 2012).
In another study performed in adults with idiopathic ASD show a significant reduction of glutamate in the striatum (HORDER et al., 2018). In agreement, it was observed, in brain samples of autistic individuals, increased levels of cerebellar messenger ribonucleic acid (mRNA) of genes of glutamate system-related protein, such as the excitatory amino acid transporter 1 and the glutamate receptor α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA 1) (PURCELL et al., 2001).

Regarding animal studies, it was demonstrated in VPA-induced model of ASD that male rats display disruptions in the NMDAR, AMPAR and mGluR5 pathways in the prefrontal cortex (Kim et al., 2016). Deficits in the NMDAR pathways have been linked to multiple mouse models of ASD such as the Pcdh10, Shank-2, Shank-3 and TSC (WON et al., 2012; BURKET et al., 2015; DUFFNEY et al., 2015; SCHOCH et al., 2017). The study performed by Horder and colleagues (2018) observed a decrease in striatal glutamate in six different rodent models of ASD – mouse models, namely prenatally exposed to VPA mice, BTBR T+tf/J mice, 15q11-13 patDP mice, *Shank3* knockout (KO) mice, *Nlgn3*^{R451C} KI mice; and rat models, namely *Nlgn3* KO rats - which was in agreement with human data (HORDER et al., 2018).

In NF1, E/I imbalance is also evident. Our group demonstrated that children and adolescents showed reduced GABA levels in the medial frontal cortex, occipital cortex and frontal eye fields (VIOLANTE et al., 2013; VIOLANTE et al., 2016).

It has been previously reported Ras-dependent increases in GABA release in the prefrontal cortex in a *Nf1*$^{+/-}$ mouse model (SHILYANSKY et al., 2010). Further, Cui and

colleagues (2008) provided support that imbalance of the E/I ratio is associated with learning deficits (CUI et al., 2008). More recently, our group have reported that NF1-related E/I was region-specific with specific pre- and post-synaptic changes (GONÇALVES et al., 2017). This study has shown increased GABA receptor levels in the hippocampus and overall increased GABA/glutamate ratio in the cortex and striatum as well as an increased inhibitory tone (Figure 10) (GONÇALVES et al., 2017).

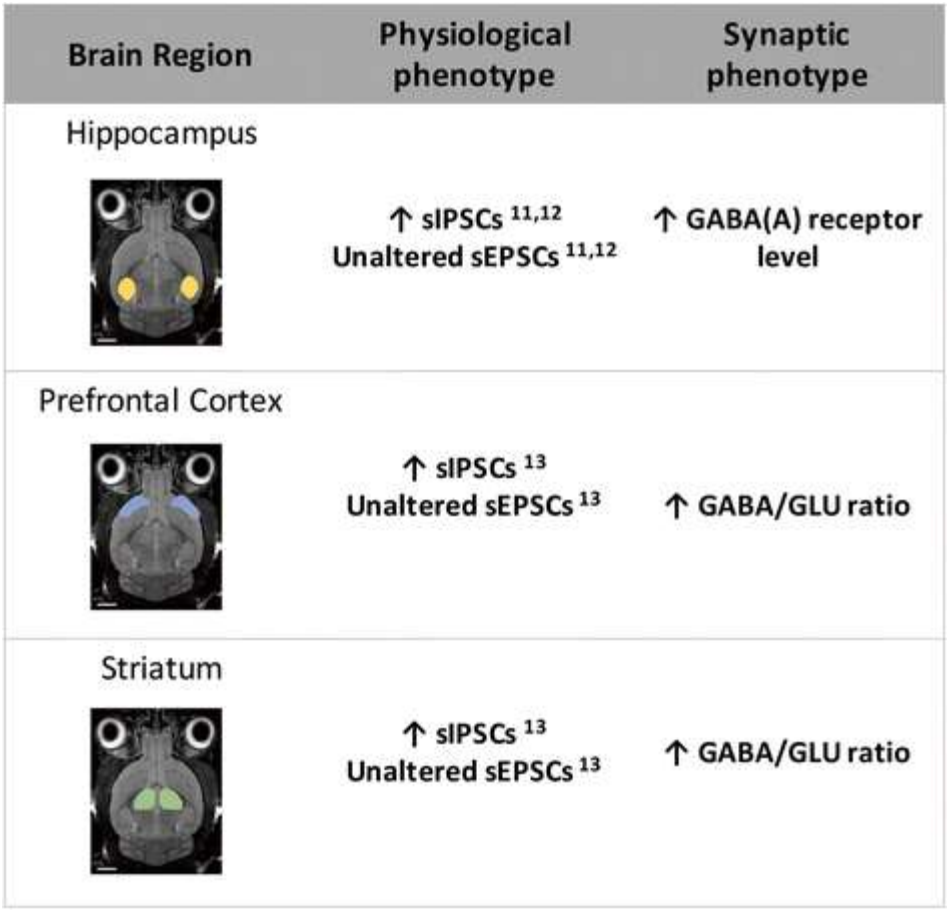

Figure 10. Region Specific Excitation/Inhibition Imbalance. Schematic representation of regional phenotypes of E/I imbalance showing region-specific GABAergic changes. In the hippocampus there are increased levels of GABA receptor levels, in the prefrontal cortex and striatum there is an increase in the GABA/glutamate ratio. Besides this, consistent increase in sIPSCs when compared to sEPSCs result in an inhibitory drive. Adapted by Gonçalves et al., 2017. Abbreviations: GABA, γ-aminobutyric acid; GLU, glutamate; sEPSCs, spontaneous excitatory post-synaptic currents; sIPSCs, spontaneous inhibitory post-synaptic currents.

Chapter 2 **| AIMS OF THE STUDY**

The sex dimorphism presents in ASD, specifically male bias, remains unclear. Understanding how sex bias influences the progression and/or severity of ASD symptoms is an important issue for the discovery of new therapies.

Recent studies demonstrated that rodent communication is impaired in ASD. Moreover, it is known that E/I ratio is changed in ASD mouse model, such as *Nf1*$^{+/-}$ mice. So, we proposed to understand how USV differ between ASD male and female and to clarify the role of PAG in this process with a focus on E/I (im)balance.

NF1 mice will be used to investigate how biological sex influences USVs during development, and during juvenile social and repetitive behaviour. By performing a longitudinal study, we intent to evaluated developmental milestones and maternal-separation induced USV production in the perinatal period, as well as USVs produced during social context and repetitive/persistent behaviour in the juvenile phase. We also aimed to determine the role of biological sex in changes in GABA and glutamatergic networks in PAG brain regions.

We expect found link between ASD symptoms and sex to understand distinct manifestations for disease and to help discovery new targets for futures sex-tailored therapies.

Chapter 3 | MATERIALS AND METHODS

3.1 Animals

One hundred and one mice were used in the experiments. These were obtained from animals backcrossed to C57BL/6 mice at least 10 times and bred once with 129T2/SvEmsJ before experiments. This breeding procedure was put in place to keep *Nf1*$^{+/-}$ mice genetic background constant across experiments and studies (SILVA et al., 1997). In the end of *in vivo* studies, at postnatal day (PND) 60, animals were sacrificed, and the PAG was isolated for analysis. Throughout the experiments, littermates are used as a control group. Furthermore, all experiments were performed and analysed by blind experimenters. Animals were group housed (2–5) on a 12-h light/dark cycle in animal facilities at ICNAS, University of Coimbra. The experiments were carried out in accordance with the European Union Council Directive (2010/63/EU), the National Regulations, and ORBEA board of the ICNAS. All included animals were healthy (discomfort score 0), and all efforts were made to minimize the number of animals used and their suffering.

3.2 Behavioural tests

3.2.1 Ultrasonic vocalization recordings and analysis

A 55 cm x 50 cm x 70 cm (H x D x W) anechoic chamber was assembled with 1.5 thick acrylic sheets, and fully covered with absorbing foam on the inside, to block external sound (Figure 11a,b). USVs were recorded using a recording system with Avisoft CM16/CMPA condenser microphone placed 28cm above the bottom of the test container, UltrasoundGate 416H amplifier and Avisoft Recorder software (Avisoft Bioacoustics, Glienicke/Nordbahn, Germany) (Figure 11c). Sonograms were formed with FFT – length 512 points, 16-bit format, sampling frequency 250kHz, time resolution 1ms, frequency resolution 488 Hz and an overlap of 50%. USV's recordings were analysed using the MATLAB toolbox DeepSqueak version 2.6.2, applying the Mouse Call_Network_V2 neural network with a chunk length of 6 seconds, overlap of 0.1 seconds, high frequency cut off of 125 kHz and no score threshold. Each USV was manually classified and separated into three different classes, single, multi-syllabic and stacked according to their complexity (YOUNG et al., 2010).

Figure 11. Ultrasonic Vocalization Recording system. Representation of the anechoic chamber used to record ultrasonic vocalizations (USVs) during behavioural tests. (a) System of regulation of the microphones' volume; (b) Anechoic chamber with acrylic sheets and covered with absorbing foam on the inside used to record vocalizations; (c) Example of a recording of a mouse pup inside the box covered in paper to control the animal's body temperature.

3.2.1.1 Maternal separation-induced USV - USVs were recorded during the neonatal period from postnatal day (PND) 6 to 16 individually. The recordings were performed during the light period (8:AM – 11:00AM). Each mouse pup was removed from the home cage and placed into a 15 x 10 x 8 plastic container, padded with tissue paper to help maintaining body temperature and the microphone placed 10cm above. The pup was acclimated to the box for 2 minutes, and USVs was recorded for 5 minutes. At the end of the test, the pup was returned to its home box.

3.2.1.2 Juvenile USV recording - During the juvenile period at PND 25, 30 and 35, the arena correspondent to each test was placed inside the anechoic chamber to allow the recording of USV's during the full course of each test with the microphone placed approximately 10cm above the top of the container. The recordings were performed during the light period (8:AM – 11:00AM). Operators were blind to genotype and sex during the test and further analyses of USVs.

3.2.2 Developmental Milestones

The battery of development milestones tests - seven developmental and five reflex tests - was performed at PND 6, 8, 10, 12, 14 and 16. Importantly, order of tests remained constant throughout the study. The developmental milestones tests were performed during light period (8:AM – 11:00AM) and, always, after USV recorded.

3.2.2.1 Surface righting - The animal was placed on its back on a flat surface and was held in that position by the operator for 5 seconds. The time taken by the animal to return to a four-limb position after release was registered (Figure 12). Cut-off time of 30 seconds (VANRYZIN et al., 2016).

Figure 12 Surface righting. Representation of the surface righting task with the animal held in the surface on its back by the operator.

3.2.2.2 Negative geotaxis reflex - The animal was placed facing down on a ramp with an inclination of about 35º and covered with fabric to allow traction. The time taken to rotate and orient its forepaws to the top was measured (Figure 13). The cut-off time was 30 seconds. In case the animal fell or rolled down the platform, it was given 2 extra trials maximum, if it still was not able to perform the task the time attributed would be the cut off (VANRYZIN et al., 2016).

Figure 13 Negative Geotaxis Reflex. Representation of the negative geotaxis reflex test, where animal in placed face down in a ramp covered in fabric.

3.2.2.3 Locomotion - The animal was placed in the centre of a 13 cm circle. The times the mouse took to cross the limit of the circle with both their front paws was measured (Figure 14). The cut-off time was 30 seconds (VANRYZIN et al., 2016).

Figure 14. Locomotion. Representation of the locomotion test with the animal placed in the centre of a 13cm circle.

3.2.2.4 Cliff aversion - The animal was placed with snout and digits of forepaws hanging over the edge of a flat surface elevated 10 cm from the bench. The time to turn snout and front paws away from the edge was measured (Figure 15). The cut-off time was 10 seconds. In case the animal fell from platform, it was given 2 extra tries, if it still was not able to perform the task the time attributed would be the cut-off (VANRYZIN et al., 2016).

Figure 15. Cliff Aversion. Representation of the cliff aversion task with the animal placed with nose and front paws on the edge of a flat surface elevated 10cm from the ground.

3.2.2.5 Forelimb grasp suspension - The animal was placed hanging by its forepaws on a translucent wire 10 cm above the padded surface. The time mediating release of the animal in that position and its fall was measured (Figure 16). The cut-off time was 10 sec. In case the animal fell immediately or not grasp the wire, 2 extra tries were given, after which cut-off score was attributed (VANRYZIN et al., 2016).

Figure 16. Forelimb Grasp Suspension. Representation of the forelimb grasp suspension test, where animal being carefully lifted by the operator and placed with its two front paws hanging from a wire.

3.2.2.6 Hindlimb grasp suspension - The animal as placed hanging by hind paws on a falcon tube and the time it took to either fall inside the tube or bring itself up and outside the tube was measured (Figure 17). The cut-off time was 30 seconds (FEATHER-SCHUSSLER et al., 2016).

Figure 17 Hindlimb Grasp Suspension. Representation of the hindlimb grasp suspension methodology, with the animal hanging from its rear paws from a falcon tube. Adapted from Feather-Schussler et al., 2016.

3.2.2.7 Nest seeking - A rectangular plastic arena (25cm x 10 cm) was divided into 3 compartments. Home bedding was placed on the left compartment, fresh bedding in a similar amount was placed on the right compartment. A goal line was traced in each compartment 6.5 cm from the centre. The animal was placed in the middle compartment, at a 90° angle from the bedding compartments, and allowed to explore the arena (Figure 18). Two trials were performed, with a 30 second intertrial between them, during which the operator held the animal. In each trial, latency to cross home bedding goal line with both forepaws was measured. Cut-off time of 120 seconds. If the animal did not perform or crossed fresh bedding goal line with both forepaws, a cut off score was attributed. Final score was considered the amount of time each animal took to reach home bedding. In each trial the animal was positioned facing opposite sides of the arena to even out possible head turning preferences. Along the experiment with all the animals, first trial and second trial position of the animal in the arena was alternated (VANRYZIN et al., 2016).

Figure 18. Nest Seeking. Representation of the nest seeking task, where animals were placed in the middle of the plastic arena, with fresh bedding on the left compartment and house-cage bedding on the right compartment.

3.2.3 Juvenile social play test

To study social behavioural, juvenile social play test was performed at PND25 during light period (8:AM – 11:00AM). At PND24, animals were isolated, housed individually into a new mouse cage, for 24h. In the next day, mice were acclimatised to the testing room 1h before the start of the test (PELEH et al., 2019). Then, pairs of animals of the same sex and genotype were formed from the same litter (COX & RISSMAN et al., 2011) and inserted on a normal, clean and empty rat cage, inside the anechoic chamber, and their behaviour and USV production recorded for 30 min (Figure19a,b). After the test, all animals returned to their respective home cage. The number and duration of each social and non-social behaviour were registered and an average for each calculated.

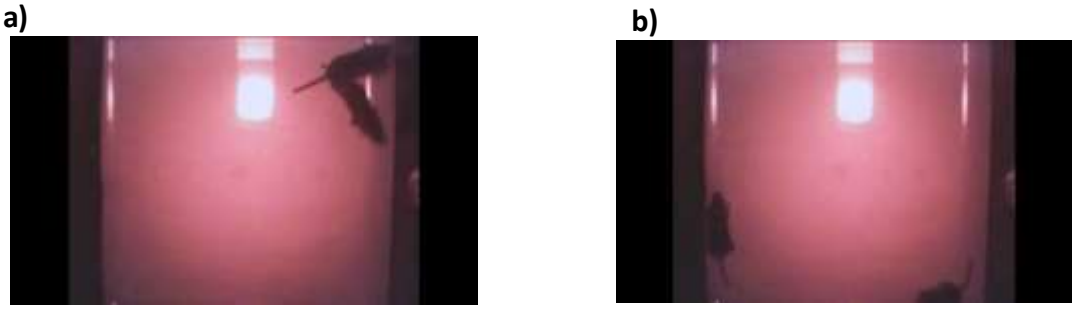

Figure 19. Juvenile Social Play Test. Representation of the juvenile social test inside the anechoic chamber for USV and video recording, simultaneously. (a) Photo of a social play test with two mice engaged on social interaction; (b) Photo of the juvenile social play test with two mice in a non-social context.

3.2.4 Empathy Test

This test was based on a resident-intruder paradigm to induce production of USV's from the tested animals ('resident' animals) (IVANENKO et al., 2020). The 'intruders' used were wild-type mice (129/SV or C57BL/6N) of the same sex and approximate age of tested animals. Further, "intruders" were anesthetised to ensure that USV production would come exclusively from tested animals. At PND30 during the light period (8:AM – 11:00AM), animals were acclimatized to the room for 1h before the start of the test. Each mouse was put inside a clean mouse cage with bedding filling approximately half the height of the cage, inside the anechoic chamber, for 2 min. After this the wild-type mouse anesthetised with an intraperitoneal (i.p) injection of 80 mg/5 mg/kg ketamine/xylazine (Nimatek 100 mg/ml, Dechra; Sedaxylan 20 mg/ml, Dechra), was placed in one of the corners of the arena. Behaviour of the resident mouse and USV production were recorded for 5 min (Figure 20a,b). The placement of the tested animals was always

consistent whereas the one of the anesthetised animals was alternated between corners to prevent possible position preference bias. The number and duration of each interaction were registered and an average for each calculated.

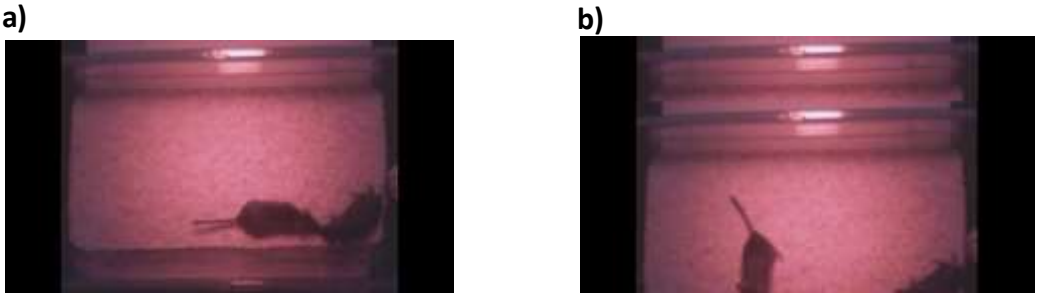

Figure 20. Empathy Test. Representation of the juvenile empathy test inside the anechoic chamber for USV and video recording. (a) Representation of the empathy test with the tested mouse showing interactive behaviour with the anesthetised animal; (b) Representation of the empathy test in a non-interactive context.

3.2.5 Novel object exploration test

At PND35, during the light period (8:AM – 11:00AM), novel object exploration test was performed to analyse restricted and repetitive behaviours. Each mouse was placed in a new, filled up to half of its height and empty mouse cage, inside the anechoic, for a 10 min acclimatization period, and video recorded. After, four different objects in shape and colour - green Lego, red dice, yellow cheese magnet and blue 'H' letter magnet - but similar in size, were placed at approximately 3 cm from each corner. The animal's behaviour as well as USV production were recorded for 10 minutes (STEINBACH, et al. 2016). The position of the objects changed, and all objects were cleaned between trials with an antiseptic without a strong smell (Figure 21).

Each position is provided with a number from 1 to 4 and data were analysed by adapting formula previous described (MIEDEL et al. 2017).

The preference of each animal for each individual object and each individual position:

$$Object\ or\ Position\ preference = \frac{Object\ or\ Position\ Interaction}{Total\ number\ of\ interactions}\ x\ 100 \quad (1)$$

The number of times each mouse interacts with an object for every three consecutive interactions:

$$Spontaneous\ Alternation = \frac{Sequences\ Total}{Total\ Object\ Interactions - 2}\ x\ 100 \quad (2)$$

The level of activity of the animals, using three unrepeated digit sequences:

$$Sequence\ repeat\ index = \frac{Most\ Frequent\ Pattern}{Total\ number\ of\ patterns}\ x\ 100 \quad (3)$$

Figure 21. Novel Object Exploration Test. Representation of the juvenile novel exploration test's arena. Photo of the box used, as well as the four objects placed randomly (blue letter H, red dice, yellow cheese and green Lego), Position numbers were steady throughout the test, for later analysis.

3.2.6 Video Recordings

Videos from the juvenile social play test, empathy test and novel object exploration test, were recorded using a Logitech C170 video camera placed on top of the chamber while a red light was on. All videos were manually analysed by a blinded operator.

3.3 Molecular Analysis

3.3.1 GABA quantification by ELISA

Mice were sacrificed at PND60 by decapitation, brain was removed and PAG dissected on ice, washed with ice cold PBS, remove excess blood and weighted. Brains were then homogenised in PBS with a protease inhibitor tablet (in a volume/weight ratio of 1:30) and centrifuged at 10000g 5 minutes at 4ºC. Supernatants were quantified using the BCA method, to obtain concentration of total protein, and stored at -80ºC until use. The ELISA protocol for GABA was performed according to the manufacturer's instructions (ABIN1118057, Antibodies online, Aachen, Germany). Briefly, samples and standards in the precoated plate were firstly incubated for 1h at 37ºC with Detection Reagent A. Then, they were washed following incubation with Detection Reagent B for 30 minutes at 37ºC.

Samples were again washed and incubated for 15 minutes with Substrate Solution at 37ºC. Finally, the Stop Solution was added, and the optical density of the wells was measured using a plate reader (Gen5, Biotek) at 450nm and 570nm. A standard curve was drawn and concentrations of GABA in each sample were determined.

3.3.2 Western blot

At PND60, mice were sacrificed, brain was removed and PAG was dissected on ice. PGA was then homogenised in a lysis buffer (2M NaCl, 1M Tris-Base, 0.05M EGTA, pH 7.5 in 10% Triton, 10% sodium deoxycholate (DOC) and 10% sodium dodecyl sulphate (SDS) and a protease inhibitor tablet) and centrifuged t 13000 rotations per minute (rpm) for 15 minutes at 4˚C. Supernatants were quantified using BCA method and stored at -20ºC until use.

Total PAG protein (5µg/µL) were separated by electrophoresis on SDS-polyacrylamine gel electrophoresis, transferred onto polyvinylidine difluoride membrane (PVDF; Millipore, Madrid, Spain), and then blocked with 5% non-fat milk in Tris-Buffered Saline (TBS) (200Mm Tris-Base, 1.37M NaCl pH7.6) for 1h at room temperature (RT). Afterwards, membranes were incubated with the primary antibodies 1.30h at RT, as described in Table 3. Membranes were then washed with 0.5%Tween 20-TBS, incubated for 1h with alkaline phosphatase-conjugated secondary antibodies (anti-mouse, 31326, Thermo Fisher Scientific; anti-rabbit, 31341, Thermo Fisher Scientific, Massachusetts, U.S.A) and visualized using ECF reagent (RPN5785, GE Healthcare, Chicago, Illinois, U.S.A) on the Typhoon (FLA 9000, GE Healthcare, Chicago, Illinois, U.S.A). Immunoblots were reprobed with anti-GAPDH antibody (1:10000; EPR1689, Abcam, Cambridge, UK) to ensure equal sample loading, and densitometric analyses were performed using the Image Studio Lite 5.2 analysis software.

Table 3: List of primary antibodies used in western blot analysis.

Primary Antibody	Molecular Weight (kDa)	Dilution	Reference	Company
Rabbit anti-GABA(A) α1 Receptor	50	1:500	AGA-001	Alomone labs
Mouse anti-VGLUT 1	62	1:1000	135 011	Synaptic Systems
Mouse GAD1/GAD67	67	1:500	198 211	Synaptic Systems
Rabbit anti-Glutamine Synthetase	42	1:3000	Ab73593	Abcam
Mouse anti-VGAT	57	1:1000	131 011	Synaptic Systems

Abbreviations: MW, molecular weight; VGAT, vesicular GABA transporter; GAD, Glutamate decarboxylase; VGLUT, vesicular glutamate transporter.

3.4 Statistical Analysis

Data are expressed as mean values ± SEM. We used Mann-Whitney tests or a one-way analysis of variance (ANOVA) in the GraphPad Prism 7.04 (GraphPad Software, Inc., La Jolla California USA), as indicated in the figure legends Data were considered as statistically significant at $p<0.05$.

Chapter 4 **| RESULTS**

4.1 Nf1$^{+/-}$ males display an early-on developmental delay

To study the natural development of motor and cognitive functions, several developmental milestones were evaluated in mice of both sex and genotype. The used developmental milestones were based on motor abilities, namely surface righting, forelimb grasp and hindlimb grasp, and on cognitive ability, namely locomotion, cliff aversion and nest seeking. Overall, we observed a delay in development of Nf1$^{+/-}$ males on a, mostly, motor level (Figure 22). Surface righting test revealed that transgenic males at postnatal day 6 (PND6), comparing to their wild-type (WT) littermates - *Nf1*$^{+/+}$ - spent more time to reach an upright position (3.079 ± 0.3115 male WT vs 9.673 ± 2.362 male *Nf1*$^{+/-}$; p = 0.0006; Figure 22a). Also, *Nf1*$^{+/-}$ males showed a delay in upper strength development at PND12 during forelimb grasp task, when compared to their WT littermates (4.698 ± 0.6448 male WT vs 3.242 ± 06067 male *Nf1*$^{+/-}$; p = 0.0327; Figure 22b). On the other hand, negative geotaxis test showed that both transgenic male and female mice perform significantly better the task than their corresponding WT littermates at PND8 (20.83 ± 3.13 male WT vs 10.77 ± 2.497 male *Nf1*$^{+/-}$; p = 0.0481; 10.11± 1.247 female WT 5.413 ± 0.5166 female *Nf1*$^{+/-}$; p = 0.0038; Figure 22c). This finding demonstrated that *Nf1*$^{+/-}$ mice have improved motor coordination. Nevertheless, we observed that female WT mice have a better performance in negative geotaxis task than male WT animals (20.83 ± 3.13 male WT vs 10.11 ± 1.247 female WT; p = 0.0149; Figure 22c), which was not observed in transgenic animals. During execution of hindlimb grasp, we detected that male Nf1$^{+/-}$, compared to female *Nf1*$^{+/-}$, completed better the task early-on at PND6 (28.1 ± 1.705 male *Nf1*$^{+/-}$ vs 19.7 ± 2.877 female *Nf1*$^{+/-}$; p = 0.0204; Figure 22d) and PND8 (21.83 ± 2.403 male *Nf1*$^{+/-}$ vs 13.74 ± 1.886 female *Nf1*$^{+/-}$; p = 0.0460; Figure 22d).

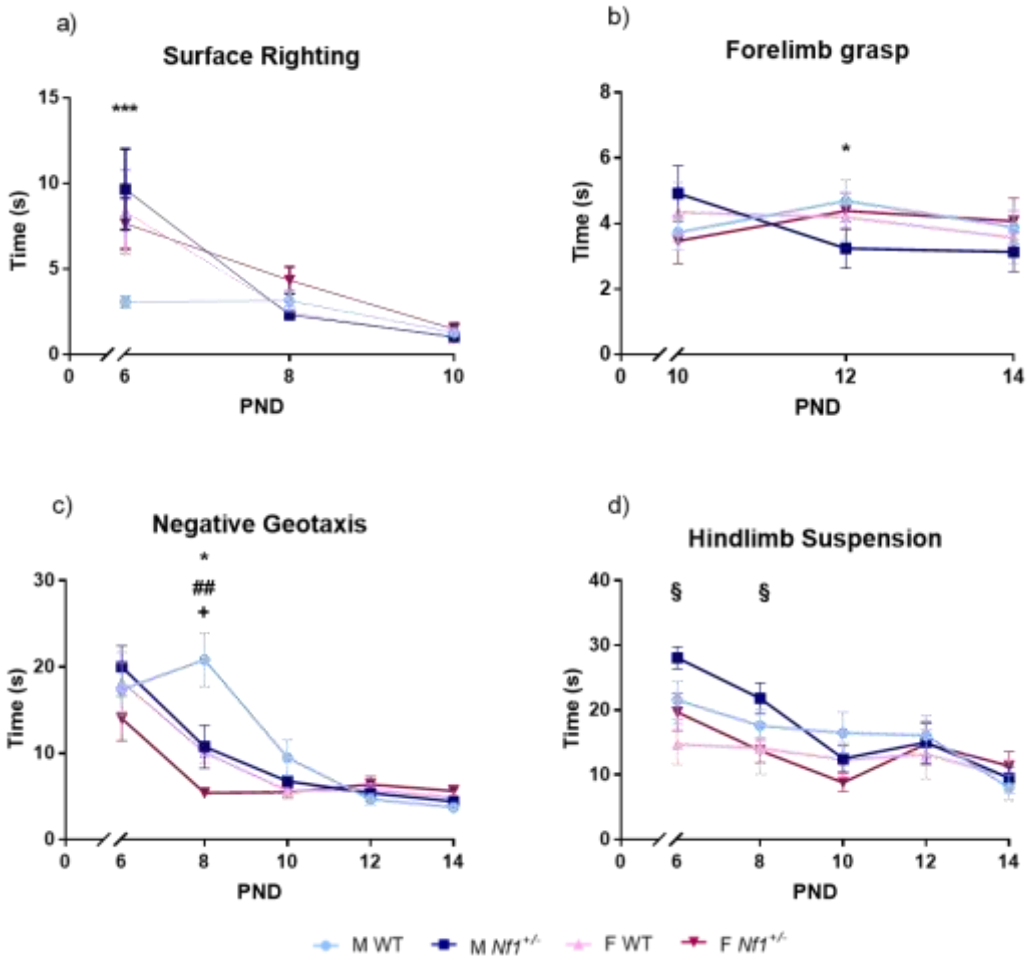

Figure 22. Developmental Milestones: Motor skills. The battery of development milestones tests was performed during light period (8:AM – 11:00AM) at PND 6, 8, 10, 12, 14 and 16. (a) Surface righting, (b) Forelimb grasp, (c) Negative geotaxis and (d) Hindlimb suspension. Latencies of each test is represented by mean ± SEM. The number of animals used were: WT males (n=19); WT females (n=20); *Nf1*$^{+/-}$ males (n=19); *Nf1*$^{+/-}$ females (n=22). $^{*}p < 0.05$, $^{***}p < 0.001$ - Mann-Whitney post-test, significantly different between male WT and male *Nf1*$^{+/-}$. $^{\#\#}p < 0.01$ - Mann-Whitney post-test, significantly different between female WT and female *Nf1*$^{+/-}$. $^{+}p < 0.05$ - Mann-Whitney post-test, significantly different between male WT and female WT. $^{\S}p < 0.05$ - Mann-Whitney post-test, significantly different between male and female *Nf1*$^{+/-}$. Abbreviations: M, male, F, female, WT, wild-type.

The analysis of developmental milestones based on cognitive skills, only locomotion showed significant results, without differences between groups in cliff aversion (Figure 23a) and nest seeking (Figure 23b) tests. Indeed, locomotion tests showed that male *Nf1*$^{+/-}$ spent more time lost to rotative behaviour, namely at PND10, comparing with male WT, which is also evident for female WT when compared to male WT (20.77 ± 2.711 male WT vs 29.56 ± 04423 male *Nf1*$^{+/-}$; $p = 0.0012$; Figure 23c; 20.77 ± 2.7111 male WT vs 29.18 ± 0.8158 female WT; $p = 0.0031$; Figure 23c). Interestingly, seems that male *Nf1*$^{+/-}$ animals at PND12 demonstrated a better performance when compared to female *Nf1*$^{+/-}$ (8.002 ± 2.263 male *Nf1*$^{+/-}$ vs 14.77 ± 2.408 female *Nf1*$^{+/-}$; $p = 0.0377$; Figure 23c).

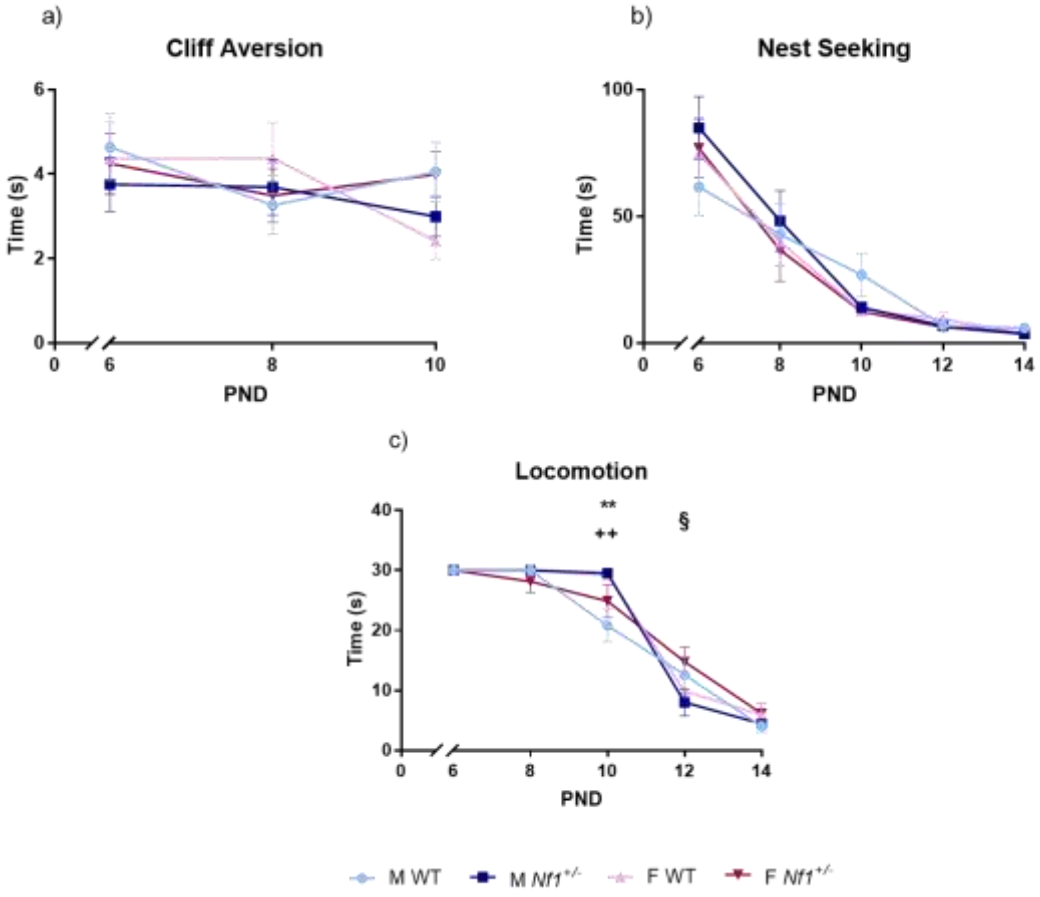

Figure 23. Developmental Milestones: Cognitive skills. The battery of development milestones tests was performed during light period (8:AM – 11:00AM) at PND 6, 8, 10, 12, 14 and 16. (a) Cliff aversion, (b) Nest Seeking and (c) Locomotion. Latencies of each test is represented by mean ± SEM. The number of animals used were: WT males (n=19); WT females (n=20); *Nf1*$^{+/-}$ males (n=19); *Nf1*$^{+/-}$ females (n=22). $^{**}p < 0.01$ - Mann-Whitney post-test, significantly different between male WT and male *Nf1*$^{+/-}$. $^{++}p < 0.01$ - Mann-Whitney post-test, significantly different between male WT and female WT. $^{§}p < 0.05$ - Mann-Whitney post-test, significantly different between male and female *Nf1*$^{+/-}$. Abbreviations: M, male, F, female, WT, wild-type.

Longitudinally, and on the same days that the developmental milestones are performed, maternal-separation-induced USVs were analysed. Our data showed no immediate relation between delays in perinatal development and USV patterns (Figure 24). At PND6 there are no significant differences shown between type of vocalization, sex nor genotype (Figure 24a). At PND8 WT females produce significantly more multi-syllabic vocalizations when compared to their *Nf1*$^{+/-}$ littermates (32.812 ± 3.349 female WT vs 18.582 ± 1.694 female *Nf1*$^{+/-}$; $p = 0.0053$; Figure 24b). We only found again significant differences shown between group at PND14, without changes at PND10 (Figure 24c), PND12 (Figure 24d) and PND16 (Figure 24f). At PND14, *Nf1*$^{+/-}$ males produce significatively more single vocalizations when compared to their WT littermates (53.036 ± 6.474 male WT vs 63.662 ± 3.152 male *Nf1*$^{+/-}$; $p = 0.0360$; Figure 24e).

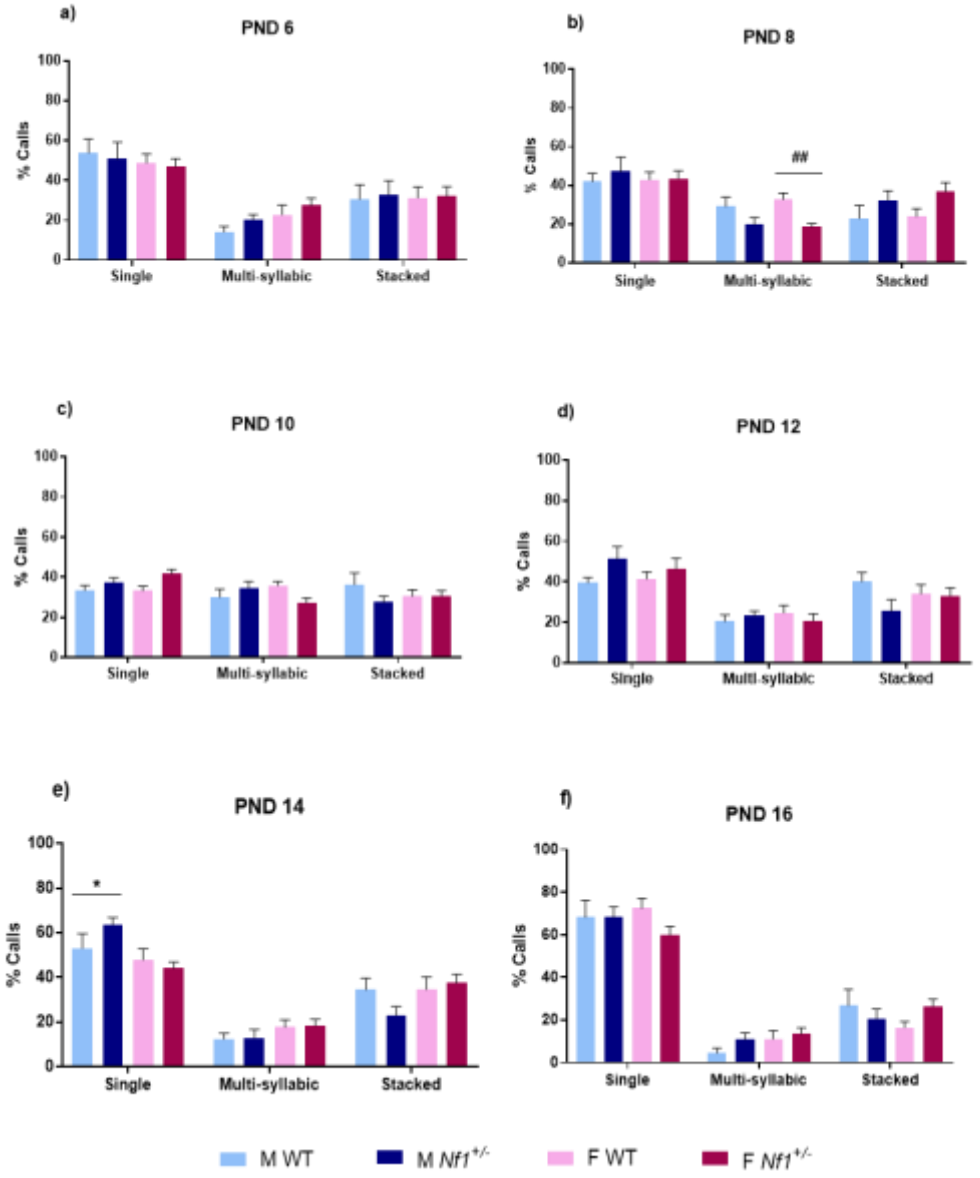

Figure 24. Pups Ultrasonic Vocalizations. Mice were put in an anechoic chamber and USVs were recorded with an acclimatization period of 2 minutes and test recording of 5 minutes. (a) PND6, (b) PND8, (c) PND10, (d) PND12, (e) PND14 and (f) PND16. Recordings were analysed using the MATLAB toolbox Deep Squeak v3. Results are presented by mean ± SEM of percentage of calls of each category - single, multi-syllabic and stacked. The number of animals used was: WT males (n=11) and WT females (n=10) and *Nf1*$^{+/-}$ males (n=10) and *Nf1*$^{+/-}$ females (n=12). $^{*}p < 0.05$ - Mann-Whitney post-test, significantly different between male WT and male *Nf1*$^{+/-}$. $^{\#\#}p < 0.01$ - Mann-Whitney post-test, significantly different between female WT and female *Nf1*$^{+/-}$. Abbreviations: M, male, F, female, WT, wild-type.

4.2 Female Nf1$^{+/-}$ showed preference for social interaction

To evaluate social interaction, a juvenile social play test was performed. We observed that mutant female spent more time socializing with pairs compared to mutant males (1357 ± 102.7 female *Nf1*$^{+/-}$ vs 1017 ± 79.14 male *Nf1*$^{+/-}$; p = 0.0280; Figure 25a). Although, both male and female of different genotypes showed a preference to spend time socializing (59.665 ± 2.169 social male WT vs 40.333 ± 2.169 non-social male WT p = 0.0022; 60.027 ± 3.347 social male *Nf1*$^{+/-}$ vs 39.805 ± 3.4 non-social male *Nf1*$^{+/-}$; p = 0.0022; Figure 25b), female Nf1$^{+/-}$ stand out (73.67 ± 6.003 social male *Nf1*$^{+/-}$ vs 27.886 ± 6.224 non-social male *Nf1*$^{+/-}$; p = 0.0023; Figure 25b).

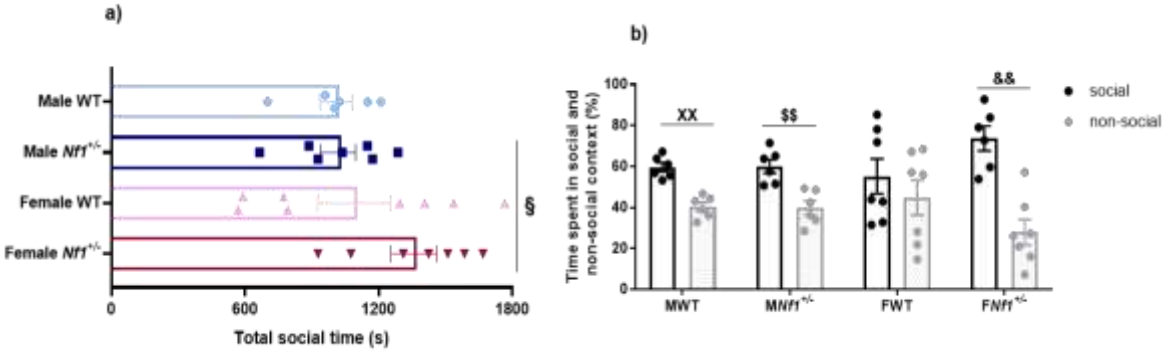

Figure 25. Juvenile Social Play Test: social interaction. At PND25 – juvenile period -, pairs of same sex and same genotype animals were inserted on a normal, clean and empty rat cage and their behaviour recorded for 30min. (a) Total social time as well as (b) comparison between social and non-social time between the groups are presented with mean ± SEM. We observed a preference for the social context from WT males, *Nf1*$^{+/-}$ males and especially *Nf1*$^{+/-}$ females. The number of animals used was: WT males (n=14) and WT females (n=10) and *Nf1*$^{+/-}$ males (n=10) and *Nf1*$^{+/-}$ females (n=13). $^{XX}p < 0.01$- Mann-Whitney post-test, significantly different between male WT social context and male WT non-social context. $^{\$\$}p < 0.01$ - Mann-Whitney post-test, significantly different between male *Nf1*$^{+/-}$ social context and male *Nf1*$^{+/-}$ non-social context. $^{\&\&}p < 0.01$ - Mann-Whitney post-test, significantly different between female *Nf1*$^{+/-}$ social context and female *Nf1*$^{+/-}$ non-social context. $^{\S}p < 0.05$ - Mann-Whitney post-test, significantly different between male and female *Nf1*$^{+/-}$. Abbreviations: M, male, F, female, WT, wild-type.

Further, we recorded USV resulting from juvenile social play test. Our data demonstrated that, even though female WT do not show particular preference for neither social nor non-social behaviour, it produces a significant higher USV percentage in a social context (75.006 ± 8.333 social female WT vs 24.99 ± 8.333 non-social female WT; $p = 0.0286$, Figure 26). This result was not observed when the mutation is present, with female *Nf1*$^{+/-}$ vocalizing in a similar way in social and non-social situations (Figure 26). On the other hand, male *Nf1*$^{+/-}$ showed a higher percentage of calls in a non-social context (9.090 ± 5.749 social male Nf1$^{+/-}$ vs 90.907 ± 5.751 non-social male *Nf1*$^{+/-}$; $p = 0.0022$, Figure 26). Overall, juvenile mice prefer social contexts, even though mutant females tend to spend a lot more time with their pairs than any other group, especially mutant males. In addition, mutant male mice tend to vocalize a lot more in the non-social environment, which leads us to infer that, mutant males have difficulty in social interaction and communication.

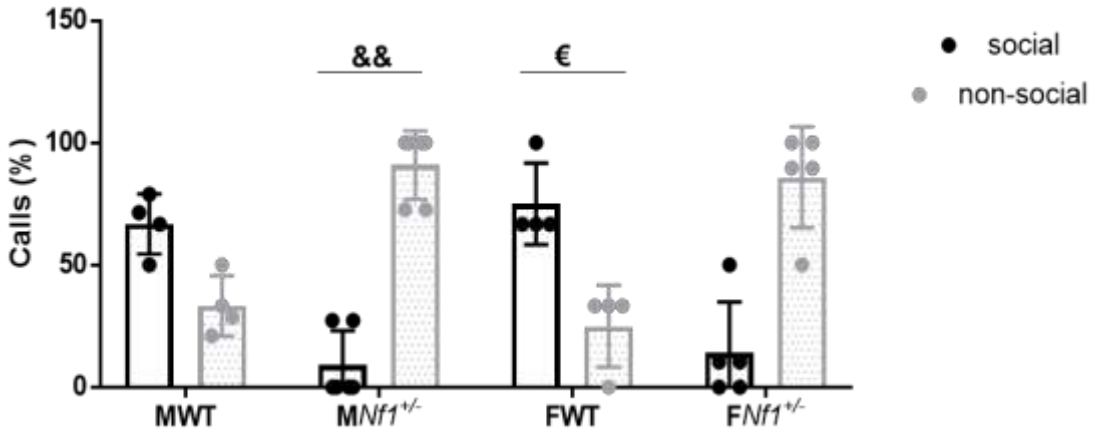

Figure 26. Social Play-induced Ultrasonic Vocalizations. During the 30 minutes of the social play test, animals' cage was inside the anechoic chamber and USVs were recorded. Percentage of calls recorded in a social and non-social context throughput the test of the groups is presented with mean ± SEM. We observed a preference of WT females to vocalize in a social context and the opposite for *Nf1*$^{+/-}$ males who vocalized a lot more in a non-social context. Recordings were analysed using the MATLAB toolbox Deep Squeak v3. Results are presented by mean ± SEM of percentage of calls of each category - single, multi-syllabic and stacked. The number of animals used was: WT male (n=5) and WT females (n=4) and *Nf1*$^{+/-}$ males (n=6) and *Nf1*$^{+/-}$ females (n=8). $^{\&\&}p < 0.01$ - Mann-Whitney post-test, significantly different between female *Nf1*$^{+/-}$ social vocalizations and female *Nf1*$^{+/-}$ non-social vocalizations. $^{€}p < 0.05$ - Mann-Whitney post-test, significantly different between WT females' social vocalizations and WT females' non-social vocalizations. Abbreviations: M, male, F, female, WT, wild-type.

4.3 Nf1$^{+/-}$ males display a more empathetic behaviour

To analyse empathic behaviour, we performed a test based on resident-intruder paradigm. Here, all groups demonstrated that they spend similar time interacting with the anesthetized animal (Figure 27a). However, *Nf1*$^{+/-}$ males took less time to perform their first interaction with the stranger animal when compared to their WT littermates (12.52 ± 2.164 male WT vs 5.568 ± 1.561 male *Nf1*$^{+/-}$; p = 0.0129; Figure 27b). Accordingly, to less period of latency to interact with strange animal, transgenic males also display more interactions within the first minute than transgenic females (30.664 ± 3.47 male *Nf1*$^{+/-}$ vs 20.133 ± 3.0463 female *Nf1*$^{+/-}$; p = 0.0312; Figure 27c).
In females there are no changes in time spent in interaction, but neither in latency of first interaction nor in the percentage of interactions within the first minute (Figures 27a-c). In fact, their interest in the strange animal is only demonstrated by the fact that for both WT and *Nf1*$^{+/-}$ females, their first interaction is a lot longer than the second one (24.842± 1.6789 first interaction female WT vs 4.9525 ± 1.239 second interaction female WT; p = 0.0286; 17.8016 ± 2.03 first interaction female *Nf1*$^{+/-}$ vs 7.82 ± 1.44 second interaction female *Nf1*$^{+/-}$; p = 0.0087; Figure 27d). In addition, *Nf1*$^{+/-}$ males also show a must longer first interaction when compared to the second one (18.819 ± 1.779 first interaction male *Nf1*$^{+/-}$ vs 8.053 ± 0.67267 second interaction male *Nf1*$^{+/-}$; p = 0.0002; Figure 27d) which only solidifies their apparent interest in their surrounding environment (Figure 27d). At this age, PND30, the animals studied did not produce any USVs. Overall, this test shows that females do not really show interest in the strange, anesthetized animal, contrary to males that show immediate attentiveness towards the animal.

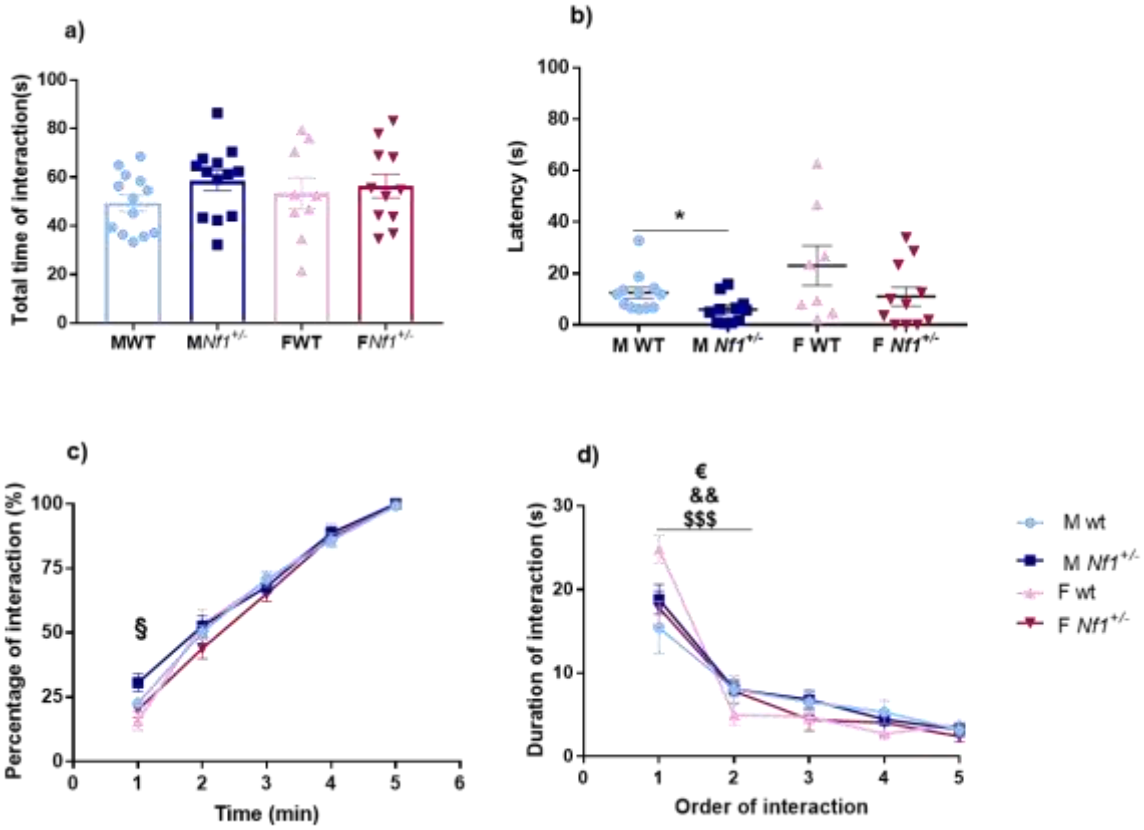

Figure 27. Empathy Test: interactive behaviour. At PND30, animals were put inside a clean mouse cage with an acclimatization period of 2 minutes and a recording period of 5 minutes. (a) Total time of interaction, (b) latency of first interaction, (c) percentage of interaction per minute, and (d) and duration of the first 5 interactions of the animals of the groups are presented with mean ± SEM. We observed that *Nf1*$^{+/-}$ males take less time to perform the first interaction and have more interactions within the first minute with the strange animal, demonstrating an empathetic behaviour. The number of animals used was: WT male (n=15) and WT females (n=9) and *Nf1*$^{+/-}$ male (n=14) and *Nf1*$^{+/-}$ females (n=12). $^{*}p < 0.05$ - Mann-Whitney post-test, significantly different between male WT and male *Nf1*$^{+/-}$. $^{€}p < 0.05$ - Mann-Whitney post-test, significantly different between WT females' first interaction and WT females' second interaction. $^{\&\&}p < 0.01$ - Mann-Whitney post-test, significantly different between female *Nf1*$^{+/-}$ first interaction and female *Nf1*$^{+/-}$ second interaction. $^{\$\$\$}p < 0.001$ - Mann-Whitney post-test, significantly different between male *Nf1*$^{+/-}$ first interaction and male *Nf1*$^{+/-}$ second interaction. Abbreviations: M, male, F, female, WT, wild-type.

4.4 Repetitive and hyperactive behaviour was observed in Nf1$^{+/-}$ male mice

To assess repetitive behaviour, one of the three main core symptoms of ASD, an object exploration test, using different objects with different colours and shapes that were unknown to the animals, was performed. This test revealed that *Nf1*$^{+/-}$ males exhibit a repetitive and hyperactive behaviour, based on the results from the sequence alternation and sequence repeat index (Figure 28). The sequence of spontaneous alternation showed that *Nf1*$^{+/-}$ males have a more exploratory behaviour, since their alternation between objects is significantly higher that of *Nf1*$^{+/-}$ females (27.88 ± 0.3155 male *Nf1*$^{+/-}$ vs 25.3 ± 0.594 female *Nf1*$^{+/-}$; $p = 0.0022$; Figure 28a). In addition, *Nf1*$^{+/-}$ males display an increase in the sequence repeat index when compared to their WT littermates (26.52 ± 8.318 male WT vs 32.81 ± 1.924 male *Nf1*$^{+/-}$; $p = 0.0133$; Figure 28b), indicating a more repetitive behaviour.

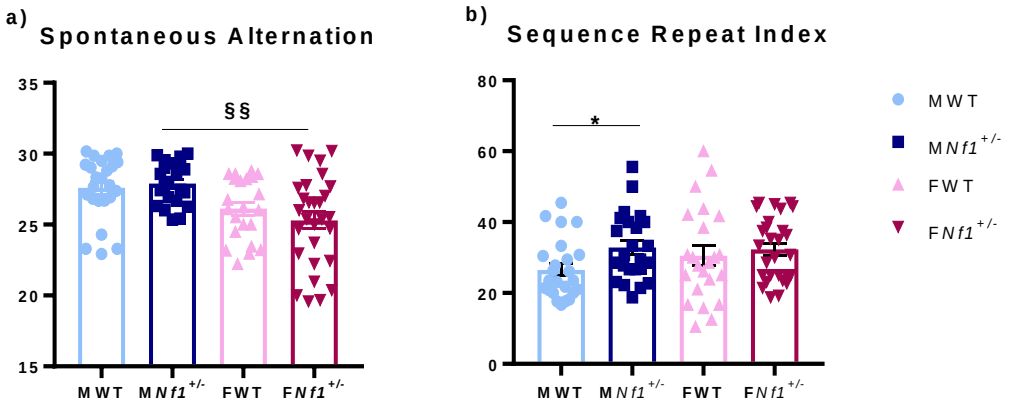

Figure 28. Novel Object Exploration Test: Spontaneous Alternation and Sequence Repeat Index. At PND35, each mouse was placed in a new mouse cage for a 10-minute acclimatization period, followed by a 10-minute recording with 4 different objects. (a) Spontaneous alternation and (b) sequence repeat index of the groups are presented with mean ± SEM. We observed that *Nf1*$^{+/-}$ males have a higher number of alternations between objects as well as a higher rate of sequence repetitions. The number of animals used was: WT male (n=25) and WT females (n=23) and *Nf1*$^{+/-}$ male (n=24) and *Nf1*$^{+/-}$ females (n=29). $^{§§}p < 0.01$ - Mann-Whitney post-test, significantly different between male and female *Nf1*$^{+/-}$. $^{*}p < 0.05$ - Mann-Whitney post-test, significantly different between male WT and male *Nf1*$^{+/-}$. Abbreviations: M, male, F, female, WT, wild-type.

When comparing either object or position preference there is no significant difference between sex or genotype, but only within the groups with some common preferences in all groups (Figure 29). All groups presented preference for the LEGO® piece, rather than the other objects. Male WT animals showed significant differences between the LEGO and the cheese (28.05 ± 1.94 LEGO male WT vs 21.16 ± 1.35 cheese male WT; p = 0.0010 Figure 29a) and a preference for the dice when compared to the cheese as well (27 ± 0.9139 dice male WT vs 21.16 ± 1.35 cheese male WT; p = 0.0065; Figure 29a). Male Nf1$^{+/-}$ animals showed significant preference for the LEGO when compared to the letter H (29.84 ± 1.235 LEGO male *Nf1*$^{+/-}$ vs letter H 23.18 ± 1.199 male *Nf1*$^{+/-}$; p = 0.0032; Figure 29a) as well as the cheese (29.84 ± 1.235 LEGO male Nf1$^{+/-}$ vs 21.91 ± 1.214 cheese male *Nf1*$^{+/-}$; p = 0.0004; Figure 29a). Female WT animals preferred the LEGO in detriment of the dice (29.78 ± 1.028 LEGO female WT vs 21.39 ± 1.299 dice female WT; p = 0.0006; Figure 29a) and the cheese (29.78 ± 1.028 LEGO female WT vs 22.11 ± 1.293 cheese female WT; p = 0.0001; Figure 29a). Finally, female Nf1$^{+/-}$ animals preferred the LEGO when compared to the cheese (28.18 ± 1.237LEGO female *Nf1*$^{+/-}$ vs 24.04 ± 1.227 cheese female *Nf1*$^{+/-}$; p-value= 0.0439; Figure 29a). It is important to note that all groups have shown significant preference for the LEGO when compared to the cheese piece.

Regarding position of object, we observed that all groups have preference for the position 3 regardless of the object present in it. As exception, WT males that did not show any preference between positions. *Nf1*$^{+/-}$ males preferred the position 3 (28.62 ± 1.19 position 3 male *Nf1*$^{+/-}$ vs position 2 20.63 ± 1.259 male *Nf1*$^{+/-}$; p = 0.0095; Figure 29b) and position 1 (26.68 ± 1.2 position 1 male *Nf1*$^{+/-}$ vs position 2 20.63 ± 1.259 male *Nf1*$^{+/-}$; p = 0.0001; Figure 29b) rather than the position 2. WT females preferred the position 3 when compared to the position 2 (28.31 ± 1.828 position 3 female WT vs position 2 21.82 ± 1.356 female WT; p = 0.0112; Figure 29b). Finally, Nf1$^{+/-}$ females preferred the position 3 when compared to the position 1 (28.11 ± 1.192 position 3 female *Nf1*$^{+/-}$ vs position 1 22.19 ± 1.228 female *Nf1*$^{+/-}$; p = 0.0076; Figure 29b).

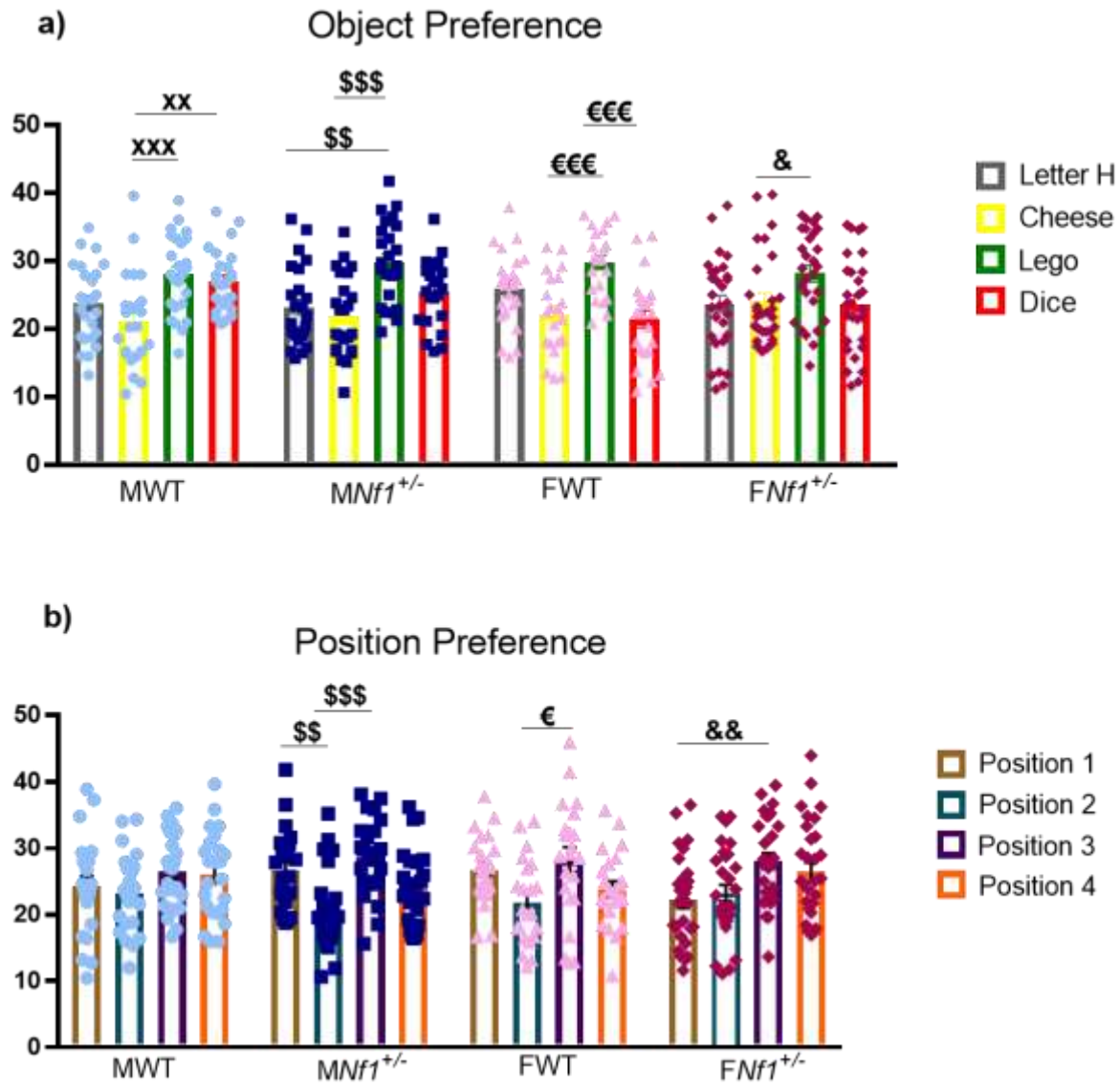

Figure 29. Novel Object Exploration Test: Object and Position Preference. At PND35, during the object novel exploration test, objects were placed randomly in the corners of the cage (a green Lego, a red dice, a yellow cheese and a blue H letter). (a) Object presence as well as (b) position preference of the groups are represented by mean ± SEM. We observed that all animals preferred the Lego piece and gave less attention to the yellow cheese. Almost all animals also preferred position 3 regardless of the object present at the time of testing. The number of animals used was: WT male (n=25) and WT females (n=23) and *Nf1*$^{+/-}$ male (n=24) and *Nf1*$^{+/-}$ females (n=29). $^{XX}p < 0.01$, $^{XXX}p < 0.01$- Mann-Whitney post-test, significantly different within the male WT group. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ - Mann-Whitney post-test, significantly different within the female *Nf1*$^{+/-}$ group. $^{€}p < 0.05$, $^{€€€}p < 0.0001$- Mann-Whitney post-test, significantly different within the female WT group. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ - Mann-Whitney post-test, significantly different within the female *Nf1*$^{+/-}$ group. Abbreviations: M, male, F, female, WT, wild-type.

Vocalization recorded during novel object exploration test, did not demonstrate significant differences between groups. So, we could postulate that there is no direct association between communication skills and repetitive and/or hyperactive behaviours (Figure 30).

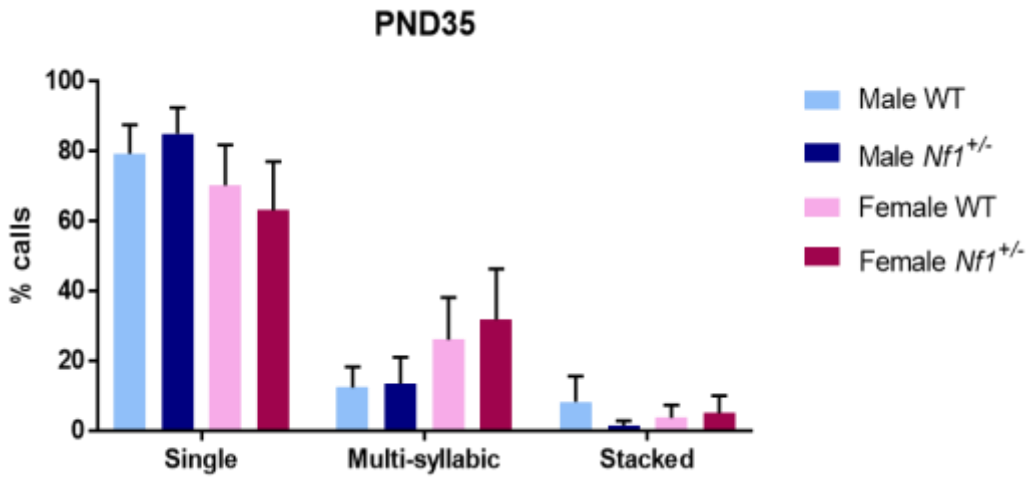

Figure 30. Novel Object Exploration Ultrasonic Vocalizations. During the 10 minutes of the repetitive behaviour assessment test, with the toys already in the arena, animals' cage was inside the anechoic chamber and USVs were recorded. We observed no difference between call type, sex nor genotype. Recordings were analysed using the MATLAB toolbox Deep Squeak v3. Percentage of calls vocalized during the test of the groups are represented with mean ± SEM of percentage of calls of each category - single, multi-syllabic and stacked. The number of animals used was: WT males (n=9) and WT females (n=9) and *Nf1*$^{+/-}$ males (n=10) and *Nf1*$^{+/-}$ females (n=4). Abbreviations: M, male, F, female, WT, wild-type.

Here, we concluded that mutant males, by presenting higher levels of sequence repetitions and alternating more between objects than the other groups, seem to have a marked presence of repetitive behaviours and hyperactivity, demonstrating the presence of ASD's hallmark in this group.

4.5 PAG showed changes in protein levels of GABAergic and glutamatergic systems

Molecular analysis by immunoblotting of GABA- and glutamate-related proteins have shown that in the PAG there were indeed changes in their levels between groups. Western blot was performed to analyse levels of proteins related to GABA namely GABA(A) receptor, VGAT and GAD, as well as proteins related to glutamate, namely glutamine synthetase and VGLUT. Analysis of the GABAergic system has shown an increase in male *Nf1*$^{+/-}$ GABA(A) receptor levels when compared to their WT littermates (100 ± 11.79 male WT vs 216.6 ± 15.97 male *Nf1*$^{+/-}$; $p = 0.0055$; Figure 31a). Levels of GABA(A) receptors have also shown an increase when comparing to *Nf1*$^{+/-}$ females (100 ± 11.79 male *Nf1*$^{+/-}$ vs 101.2 ± 11.76 female *Nf1*$^{+/-}$; $p = 0.0049$; Figure 31a). *Nf1*$^{+/-}$ males show exacerbated levels of GAD when compared to both WT males (100 ± 13.95 male WT vs 318 ± 81.1 male *Nf1*$^{+/-}$; $p = 0.0057$; Figure 31b) and *Nf1*$^{+/-}$ females (318 ± 81.1 male *Nf1*$^{+/-}$ vs 106.2 ± 19.04 female *Nf1*$^{+/-}$; $p = 0.0121$; Figure 31b). Besides this they also show higher levels of VGAT when compared to *Nf1*$^{+/-}$ females (142.3 ± 29.10 male *Nf1*$^{+/-}$ vs 63.55 ± 9.216 female *Nf1*$^{+/-}$; $p = 0.0120$; Figure 31c).

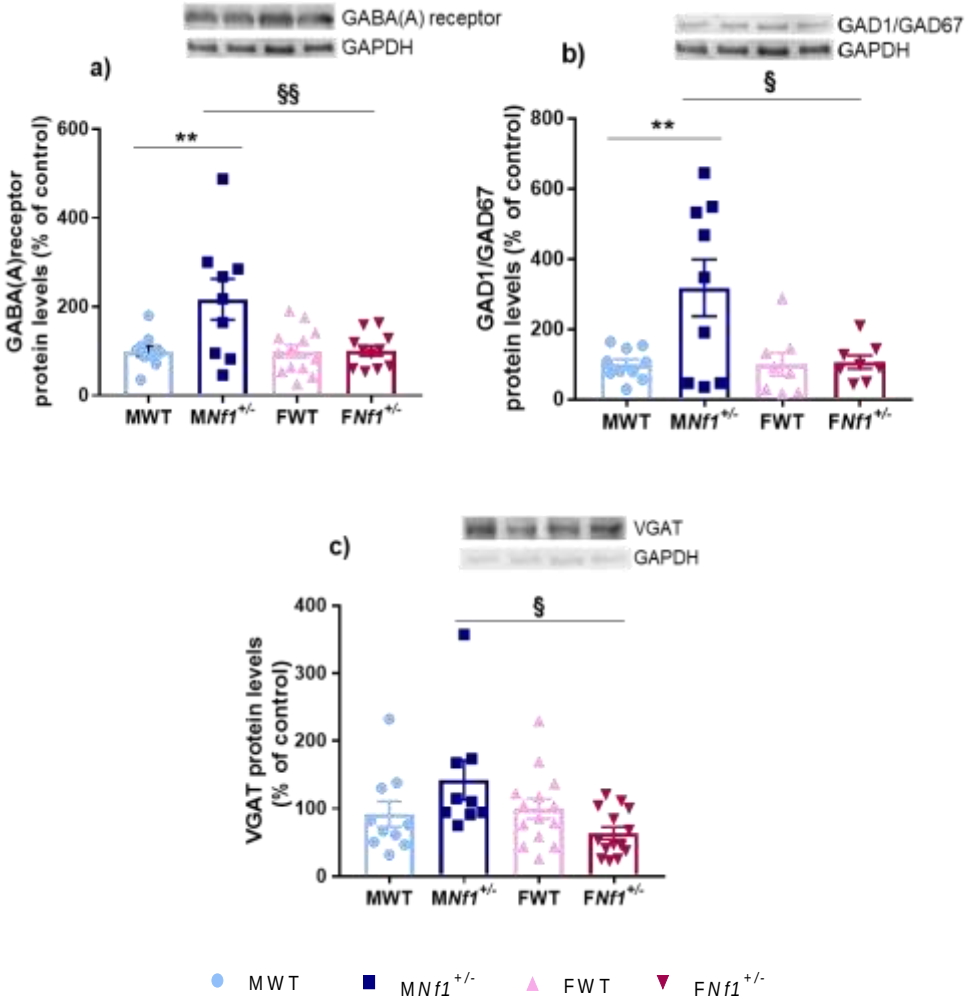

Figure 31. Western Blot: GABA. At PND60, brains from sacrificed mice were removed and the PAG isolated and lysed. PAG proteins (5µg/µL) were separated by electrophoresis on SDS and blocked with 5% non-fat milk in TBS (200Mm Tris-Base, 1.37M NaCl pH7.6) for 1h, probed with the primary antibodies 1,30h and then washed with 0.5%Tween 20-TBS, incubated for 1h with alkaline phosphatase-conjugated secondary antibodies and visualized using ECF reagent. Percentage of total protein levels are represented with mean ± SEM. (a) GABA(A)R – gamma-aminobutyric acid receptor, (b) GAD – glutamate decarboxylase and (c) VGAT- vesicular GABA transporter. The number of animals used was: WT males (n=15) and WT females (n=14) and *Nf1*$^{+/-}$ males (n=13) and *Nf1*$^{+/-}$ females (n=13). $^{\S}p < 0.05$, $^{\S\S}p < 0.01$ – ANOVA test, significantly different between male and female *Nf1*$^{+/-}$. $^{**}p < 0.01$ – ANOVA test, significantly different between male WT and male *Nf1*$^{+/-}$. Abbreviations: M, male, F, female, WT, wild-type.

Glutamatergic system analysis also shows *Nf1*$^{+/-}$ males' tendency to have increased levels of glutamine synthetase, when compared to both WT males (100 ± 12.72 male WT vs 180.3 ± 25.55 male *Nf1*$^{+/-}$; p = 0.0047; Figure 32a) and *Nf1*$^{+/-}$ females (180.3 ± 25.55 male *Nf1*$^{+/-}$ vs 86.28 ± 10.13 female *Nf1*$^{+/-}$; *p = 0.0008*; Figure 32b).

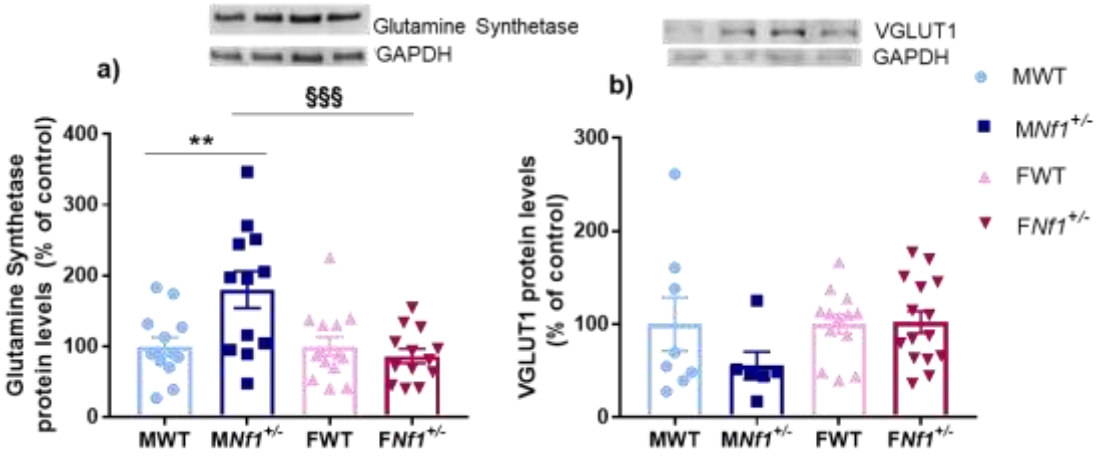

Figure 32. Western Blot: Glutamate. at the glutamatergic system was also assessed, by analysing levels of glutamate related proteins. a) glutamine synthetase protein levels. The process was the same as the one from GABA. PAG proteins (5µg/µL) were separated by electrophoresis on SDS and blocked with 5% non-fat milk in TBS (200Mm Tris-Base, 1.37M NaCl pH7.6) for 1h, probed with the primary antibodies 1,30h and then washed with 0.5%Tween 20-TBS, incubated for 1h with alkaline phosphatase-conjugated secondary antibodies and visualized using ECF reagent. Percentage of total protein levels are represented with mean ± SEM. (a) glutamine synthetase and (b) VGLUT – vesicular glutamate transporter. The number of animals used was: WT males (n=15) and WT females (n=14) and *Nf1*$^{+/-}$ males (n=13) and *Nf1*$^{+/-}$ females (n=13). $^{§§§}p < 0.001$ – ANOVA test, significantly different between male and female *Nf1*$^{+/-}$. $^{**}p < 0.01$ – ANOVA test, significantly different between male WT and male *Nf1*$^{+/-}$. Abbreviations: M, male, F, female, WT, wild-type.

We also performed enzyme linked immunoassay (ELISA) to investigate possible changes in GABA levels. Again, no significant changes were detected in the PAG (Figure 33).

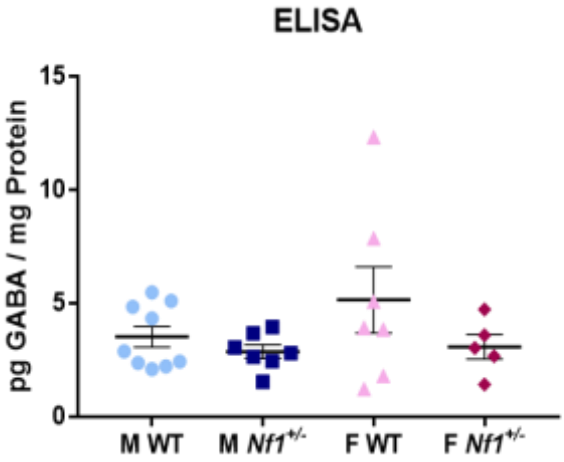

Figure 33. GABA analysis by ELISA. At PND60, brains from sacrificed mice were removed and the PAG isolated, weighted and homogenised. The ELISA protocol for GABA was performed according to manufactured instructions and the optical density of the wells was measured at 450nm and 570nm. We observed no differences in GABA concentration between the groups. The comparison between the GABA concentrations in the groups is represented with mean ± SEM. The number of animals used was: WT male (n=9) and WT females (n=7) and *Nf1*$^{+/-}$ male (n=7) and *Nf1*$^{+/-}$ females (n=5). Abbreviations: M, male, F, female, WT, wild-type.

Chapter 5 | **DISCUSSION**

Recently, ASD research has been increasingly focused on sexual dimorphism to explain behavioural differences between males and females and the respective implications for the current diagnosis of this disorder. Several studies have shown that males have delays in motor function, social contexts and repetitive behaviours, whereas these manifestations are masked in females (VAART et al., 2011; SHILYANSKY *et al.,* 2010a, b; GARG et al., 2016; MAY et al., 2014, 2016). So, a male oriented diagnostic bias could, in fact, be on the basis of ASD females underdiagnosis (GIARELLI et al., 2010; BARGIELA et al., 2016; LOOMES et al., 2017). However, it still remains to be disclosed behavioural biomarkers that can be used both to uncover early disease onset as well as the molecular pathways underlying sex differences.

ASD is characterized by three main core traits - social interaction deficits, impairment in communication and RRBs (DMS5, APA), which were studied in presented work. Also, ASD are commonly associated with monogenic comorbid disorders, such as NF1 (GARG et al., 2013; WALSH et al., 2013; PLASSCHAERT et al., 2015; GARG et al., 2015). Indeed, about 25% of NF1 children showed ASD symptomatology (ROSSER, et al., 2003). Furthermore, 80% of children with NF1 also exhibit high tendency to have cognitive symptoms, including learning disabilities, attention deficit disorder, social and communications deficits and motor delays (CHAMPION et al., 2014; WILLIAMS et al., 2009; ROSSER, et al., 2003). Academic impairments are indicated as the major consequence of cognitive delays, representing about 52%, affecting social abilities, with boys being more affected by academic impairments in the first four grades (COUDÉ et al., 2006; HYMAN et al., 2005).

In agreement with these findings, our results demonstrated significant male delays in developmental milestones in an early postnatal period, but mostly in a motor context. It is worth pointing out that motor coordination deficits are often present in ASD (FOURNIER et al., 2010). This is also in line with the findings by Vaart and colleagues (2011). These authors showed that males display motor impairments rather than cognitive (VAART et al., 2011). However, others have observed that *Nf1*$^{+/-}$ males have more learning disabilities (SHILYANSKY *et al.,* 2010a, b). Accordingly, Diggs-Andrews and collaborators (2014) reported that both human and mouse males were more likely to develop cognitive and learning deficits (DIGGS-ANDREWS et al., 2014).

In the presented work, we did not observe major changes in USV production during the early postnatal period. But USV production seems to be largely heterogeneous as also documented in existent bibliography. In fact, previous studies have shown that ASD mouse models have communication deficits mainly by observing different vocalization rates, fewer calls and shorter duration (LAI et al., 2014; SCATTONI et al., 2008;

MICHETTI et al., 2012; DOUGHERTY et al. 2013). However, Maloney and colleagues (2018) demonstrated that *Nf1*$^{+/-}$ mice exhibited more USV production compared with their WT littermates after maternal separation. Also, they observed that USV duration was not altered, but the mean frequency of calls was lower (MALONEY et al., 2018). Our data did not show these described alterations in percentage of calls or type of call produced in the groups studied. The inconsistency of findings leads us to speculate that USV production may not be a feasible early biomarker for ASD. Nevertheless, several factors might influence these results, such as ASD animal model used and the USV analysis method (detection method or classification method). Here, detection of calls was performed by DeepSqueak software followed by a manual call classification. Refining methods to analyse USVs seems to be essential for more concise and coherent findings.
Although three core symptoms of ASD are well defined, when we segregate their incidence by sex, some patterns of difference become clearer (GARG et al., 2015). Frazier and colleagues (2014) demonstrated that females with ASD exhibited impairments in social communication, lower RRB interests and lower cognitive ability (FRAZIER et al., 2014). However, this study included 2,114 males and only 304 females (FRAZIER et al., 2014). Additionally, others have stated that ASD females present a reduced RRBs and minor changes in social domains when compared with male ASD (MANDY et al., 2012; HOLTMANN et al., 2007; VAN WIJNGAARDEN-CREMERS et al., 2014). In 2016, a study with ASD patients (103 males and 91 females aged 4 to 18) showed that ASD males exhibited deficits in the social interaction and communication, namely NF1 children with ASD symptomatology (GARG et al., 2016). On the other hand, BA and colleagues (2004) demonstrated that children with NF1 had significantly less ability in social domains than their unaffected siblings, but without sex bias. Since sex bias in ASD is still controversial, it is important to clarify this with further studies in order to improve diagnostic approaches.
We demonstrated that male *Nf1*$^{+/-}$ mice have less interest in a social context. Female *Nf1*$^{+/-}$ mice spend more time in social interaction than males which is consistent with the results stated previously in studies with NF1 patients (GARG et al., 2016). Consistent with this finding, *Nf1*$^{+/-}$ males exhibited more USV calls in a non-social context, demonstrating their difficulty in social communication, and overall, in social domains.
Further, we observed that male *Nf1*$^{+/-}$ mice show a more repetitive and hyperactive-like behaviour. Accordingly, previous studies stated that ASD females present less RRBs, while males tend to develop externalising problems such as hyperactivity, impulsivity and aggression (MANDY et al., 2012; HOLTMANN et al., 2007; VAN WIJNGAARDEN-CREMERS et al., 2014; HARTLEY & SIKORA, 2009; MAY et al., 2014; MAY et al.,

2016). Indeed, data obtained from spontaneous alternation - a measure of exploratory behaviour when facing a new environment (WOZNIAK et al., 2013) – revealed that female *Nf1*$^{+/-}$ mice display less of this repeated behaviour. This leads us to hypothesize that transgenic females have an increase in anxiety levels, which is consistent with previous observation in human ASD females that develop more internalising problems, such as depression, anxiety, sleeping or other emotional symptoms (HARTLEY & SIKORA, 2009; May et al., 2014).

Regarding empathic behaviour, there is lack of information in ASD or even NF1 patients. However, Baron-Cohen (2005) proposed the idea of a hyper-masculinized brain in autistic people leading to a prioritization of systematization – Extreme Male Brain (EMB) theory. On the other hand, the female brain is believed to give more importance to empathy (Baron-Cohen et al, 2005). ASD individuals have showed lower levels of empathy and more systematic responses, which supports Baron-Cohen's theory of ASD individuals having a tending masculine brain, by prioritizing systematization (GREENBERG et al., 2018). However, our results do not corroborate the EMB theory. In fact, we observed that male *Nf1*$^{+/-}$ mice have a more empathetic behaviour than their WT littermates. Nonetheless, these results are not enough to sustain such a claim since *Nf1*$^{+/-}$ male mice interest was significant within first minute and mostly in the first interaction.

As mentioned previously, molecular pathways responsible for the sexual dimorphism in ASD are still unclear. However, promising data has been gathered concerning important pathways already established to be affected in ASD, namely GABA and glutamate pathways. Since our lab previously demonstrated that *Nf1*$^{+/-}$ mouse exhibited imbalanced E/I ratio (GONÇALVES et al., 2017), here we looked for proteins related with GABA and glutamate neurotransmission in the PAG region. Our molecular data demonstrated significant changes in GABAergic- and glutamatergic-related proteins in the PAG. However, since we did not perform protein level analysis at the same age that we performed social behavioural tests, we may be missing an important time-window.

Having our results into consideration, it is important to understand that GABAergic and glutamatergic systems were studied in the entire PAG, where only a portion is responsible for the production of USVs, this could also be masking our results regarding alterations in USV production. Nevertheless, the interesting differences seen in *Nf1*$^{+/-}$ mice lead us to suggest that this brain region is in fact associated with some ASD symptomatology. The alterations in GABA-related proteins consistently show that *Nf1*$^{+/-}$ males have increased GABA(A) receptor levels, production of GABA and vesicles to transport, whereas glutamate-related proteins showed an increase in glutamate

production. The fact that there were no alterations in GABA concentration in the PAG even though there was an indication for higher production, could mean there is a compensatory mechanism. Glutamate levels should also be measured in order to understand the basis of this imbalance and possible relation with behavioural responses. In fact, GABAergic and glutamatergic systems have already been connected to fear and anxiety behaviours (LOWERY-GIONTA et al, 2018; MORAES et al., 2008). Lowery-Gionta and colleagues' results in 2018 suggest that GABA neuronal activity in vPAG underlies emotional behavioural responses connected to anxiety and fear. Another study has also shown the involvement of NMDA receptors in the modulation of anxiety-related behaviour in the PAG (MORAES et al., 2008).

The present work investigated GABAergic and glutamatergic systems only in the PAG, and these systems could have alterations in other brain regions responsible for other functions. In agreement, GONÇALVES and colleagues (2017), using the same mouse model, found there was a region-specific disruption of the E/I balance, with a decrease in the cortex and striatum and the hippocampus showing increased GABA(A) receptor levels. In humans, patients with NF1 have shown reduced GABA concentration in the frontal eye fields and occipital cortex (VIOLANTE et al., 2016).

Chapter 6 | **CONCLUDING REMARKS**

The present Master Thesis demonstrated that *Nf1*$^{+/-}$ mouse model display an ASD-like behaviour characterized by impairments in social interaction, communication deficits and RRBs. Moreover, we concluded that in the animal model used USVs did not seem to be suitable as an early biomarker.

We have also shown that there is, in fact, a substantial difference in behaviour between males and females and that this should be taken into consideration in the future to improve diagnostic strategies and possible therapeutic approaches.

Overall, the present work leads to the conclusion that males show behavioural impairments very early-on in life and those impairments are steady over time, extending to the juvenile period, whereas females do not share these impairments and also seem to adapt to social situations even when compared with unaffected individuals. This finding is of some significance, since it shows us the importance of finding new diagnostic methods than the ones used now, for they may naturally be a primary source of male bias. The discovery of biomarkers is of utmost necessity to prevent female diagnosis being overlooked.

Furthermore, the molecular evaluation of different brain regions, others than the one studied, and thought to be responsible for the functions affected in ASD patients, will help to clarify the underlying mechanisms of disease.

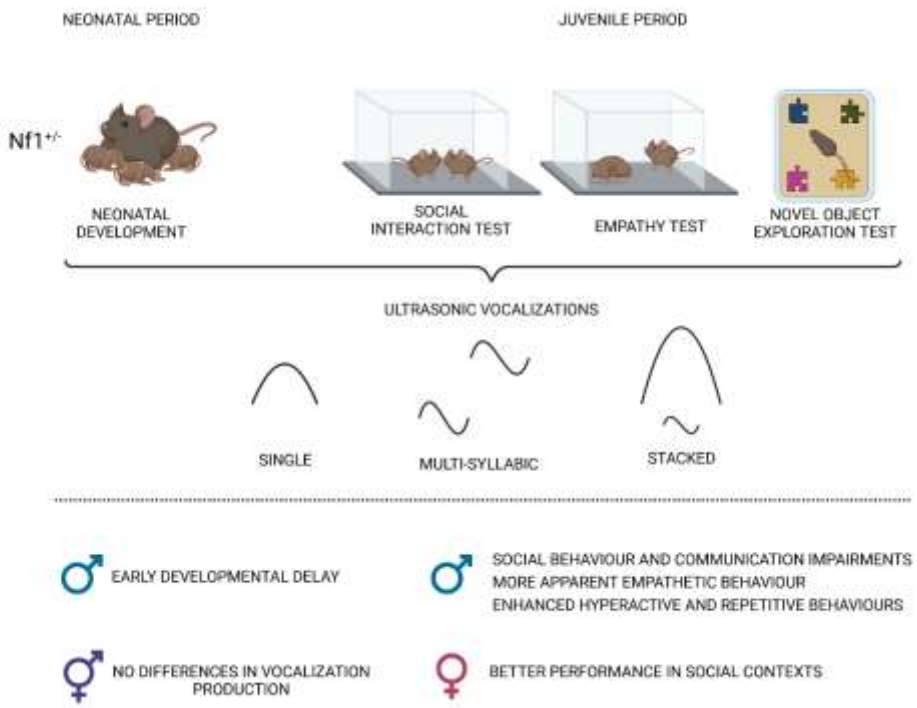

Figure 34 Graphical Abstract. Representation of the Master Thesis methods and general results. Pups during the neonatal period were subjected to developmental milestone tests, along with maternal separation-induced ultrasonic vocalization (USV) recordings. During the juvenile phase, mice were subjected to three different behavioural tests, namely, a social play test, an empathy test and a novel exploration test, in order to evaluate the two core symptoms of ASD, impairments in social interaction and communication and repetitive behaviours. Along with these tests, USVs were also recorded. USVs were analysed and separated into one of their three main categories (single, multi-syllabic and stacked). Overall, male *Nf1*$^{+/-}$ mice showed early developmental delay, more empathic behaviour and enhanced hyperactivity and repetitive behaviours. Besides this, male *Nf1*$^{+/-}$ mice have also shown impairments in social interaction and communication, whereas *Nf1*$^{+/-}$ female mice demonstrated to be relatively comfortable in social contexts, even more so that WT mice. Created with BioRender.com

Chapter 7 **| REFERENCES**

ADAMETZ, John H. Rate of recovery of functioning in cats with rostral reticular lesions: an experimental study. *Journal of Neurosurgery*, 1959, 16.1: 85-98.

AMERICAN PSYCHIATRIC ASSOCIATION, et al. *DSM-5: Manual diagnóstico e estatístico de transtornos mentais*. Artmed Editora, 2014.

ANDERSON, Jacqueline L.; GUTMANN, David H. Neurofibromatosis type 1. *Handbook of clinical neurology*, 2015, 132: 75-86.

ATIT, Radhika P., et al. The Nf1 tumor suppressor regulates mouse skin wound healing, fibroblast proliferation, and collagen deposited by fibroblasts. *Journal of Investigative Dermatology*, 1999, 112.6: 835-842.

ARRIAGA, Gustavo; JARVIS, Erich D. Mouse vocal communication system: are ultrasounds learned or innate?. *Brain and language*, 2013, 124.1: 96-116.

BA, Belinda Barton; NORTH, Kathryn. Social skills of children with neurofibromatosis type 1. *Developmental Medicine & Child Neurology*, 2004, 46.8: 553-563.

BALLESTER, Roymarie, et al. The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. *Cell*, 1990, 63.4: 851-859.

BANDLER, Richard, et al. Central circuits mediating patterned autonomic activity during active vs. passive emotional coping. *Brain research bulletin*, 2000, 53.1: 95-104.

BANDLER, Richard; SHIPLEY, Michael T. Columnar organization in the midbrain periaqueductal gray: modules for emotional expression?. *Trends in neurosciences*, 1994, 17.9: 379-389.

BARGIELA, Sarah; STEWARD, Robyn; MANDY, William. The experiences of late-diagnosed women with autism spectrum conditions: An investigation of the female autism phenotype. *Journal of autism and developmental disorders*, 2016, 46.10: 3281-3294.

BARON-COHEN, Simon, et al. Elevated fetal steroidogenic activity in autism. *Molecular psychiatry*, 2015, 20.3: 369-376.

BARON-COHEN, Simon; KNICKMEYER, Rebecca C.; BELMONTE, Matthew K. Sex differences in the brain: implications for explaining autism. *Science*, 2005, 310.5749: 819-823.

BARON-COHEN, Simon. The extreme male brain theory of autism. *Trends in cognitive sciences*, 2002, 6.6: 248-254.

BAUER, Deborah E., et al. The glutamate transporter, GLAST, participates in a macromolecular complex that supports glutamate metabolism. *Neurochemistry international*, 2012, 61.4: 566-574.

BEHBEHANI, Michael M. Functional characteristics of the midbrain periaqueductal gray. *Progress in neurobiology*, 1995, 46.6: 575-605.

BENARROCH, Eduardo E. Periaqueductal gray: an interface for behavioral control. *Neurology*, 2012, 78.3: 210-217.

BOLLAG, Gideon, et al. Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells. *Nature genetics*, 1996, 12.2: 144-148.

BÖLTE, Sven, et al. Sex differences in cognitive domains and their clinical correlates in higher-functioning autism spectrum disorders. *Autism*, 2011, 15.4: 497-511.

BOWERY, Norman. GABAB receptors and their significance in mammalian pharmacology. *Trends in Pharmacological Sciences*, 1989, 10.10: 401-407.

BOZZI, Yuri; CASAROSA, Simona; CALEO, Matteo. Epilepsy as a neurodevelopmental disorder. *Frontiers in psychiatry*, 2012, 3: 19.

BUCHBERG, Arthur M., et al. Sequence homology shared by neurofibromatosis type-1 gene and IRA-1 and IRA-2 negative regulators of the RAS cyclic AMP pathway. *Nature*, 1990, 347.6290: 291-294.

BURKET, Jessica A., et al. NMDA receptor activation regulates sociability by its effect on mTOR signaling activity. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 2015, 60: 60-65.

CAMPISI, Lisa, et al. Autism spectrum disorder. *British Medical Bulletin*, 2018, 127.1.

CANITANO, Roberto; PALLAGROSI, Mauro. Autism spectrum disorders and schizophrenia spectrum disorders: excitation/inhibition imbalance and developmental trajectories. *Frontiers in psychiatry*, 2017, 8: 69.

CARTER, Alice S., et al. Sex differences in toddlers with autism spectrum disorders. *Journal of autism and developmental disorders*, 2007, 37.1: 86-97.

CARRIVE, Pascal; MORGAN, Michael M. Periaqueductal gray. *The human nervous system*, 2012, 3: 368-401.

CARUSO, Angela; RICCERI, Laura; SCATTONI, Maria Luisa. Ultrasonic vocalizations as a fundamental tool for early and adult behavioral phenotyping of Autism Spectrum Disorder rodent models. *Neuroscience & Biobehavioral Reviews*, 2020, 116: 31-43.

CHABOUT, Jonathan, et al. Male mice song syntax depends on social contexts and influences female preferences. *Frontiers in behavioral neuroscience*, 2015, 9: 76.

CHAMPION, Joel A., et al. Relationship between cognitive dysfunction, gait, and motor impairment in children and adolescents with neurofibromatosis type 1. *Developmental Medicine & Child Neurology*, 2014, 56.5: 468-474.

CHEN, Christina; VAN HORN, John Darrell. Developmental neurogenetics and multimodal neuroimaging of sex differences in autism. *Brain imaging and behavior*, 2017, 11.1: 38-61.

CICHOWSKI, Karen, et al. Mouse models of tumor development in neurofibromatosis type 1. *Science*, 1999, 286.5447: 2172-2176.

COSTA, Rui M., et al. Learning deficits, but normal development and tumor predisposition, in mice lacking exon 23a of Nf1. *Nature genetics*, 2001, 27.4: 399-405.

COSTA, Rui M.; SILVA, Alcino J. Molecular and cellular mechanisms underlying the cognitive deficits associated with neurofibromatosis 1. *Journal of child neurology*, 2002, 17.8: 622-626.

COSTA, Rui M.; SILVA, Alcino J. Mouse models of neurofibromatosis type I: bridging the GAP. *Trends in molecular medicine*, 2003, 9.1: 19-23.

COUDÉ, François X., et al. Academic impairment is the most frequent complication of neurofibromatosis type-1 (NF1) in children. *Behavior genetics*, 2006, 36.5: 660-664.

COX, Kimberly H.; RISSMAN, Emilie F. Sex differences in juvenile mouse social behavior are influenced by sex chromosomes and social context. *Genes, Brain and Behavior*, 2011, 10.4: 465-472.

CUI, Yijun, et al. Neurofibromin regulation of ERK signaling modulates GABA release and learning. *Cell*, 2008, 135.3: 549-560.

DEAN, Michelle; HARWOOD, Robin; KASARI, Connie. The art of camouflage: Gender differences in the social behaviors of girls and boys with autism spectrum disorder. *Autism*, 2017, 21.6: 678-689.

DE RUBEIS, Silvia; BUXBAUM, Joseph D. Genetics and genomics of autism spectrum disorder: embracing complexity. *Human molecular genetics*, 2015, 24.R1: R24-R31.

DIGGS-ANDREWS, Kelly A., et al. Sex is a major determinant of neuronal dysfunction in neurofibromatosis type 1. *Annals of neurology*, 2014, 75.2: 309-316.

DOERNBERG, Ellen; HOLLANDER, Eric. Neurodevelopmental disorders (asd and adhd): dsm-5, icd-10, and icd-11. *CNS spectrums*, 2016, 21.4: 295-299.

DOUGHERTY, Joseph D., et al. The disruption of Celf6, a gene identified by translational profiling of serotonergic neurons, results in autism-related behaviors. *Journal of Neuroscience*, 2013, 33.7: 2732-2753.

DUFFNEY, Lara J., et al. Autism-like deficits in Shank3-deficient mice are rescued by targeting actin regulators. *Cell reports*, 2015, 11.9: 1400-1413.

DURAND, V. Mark. *Autism spectrum disorder: A clinical guide for general practitioners*. American Psychological Association, 2014.

ELSABBAGH, Mayada, et al. Global prevalence of autism and other pervasive developmental disorders. *Autism research*, 2012, 5.3: 160-179.

ESPOSITO, Antonio, et al. Complete mutism after midbrain periaqueductal gray lesion. *Neuroreport*, 1999, 10.4: 681-685.

FEATHER-SCHUSSLER, Danielle N.; FERGUSON, Tanya S. A battery of motor tests in a neonatal mouse model of cerebral palsy. *Journal of visualized experiments: JoVE*, 2016, 117.

FONNUM, Frode. Glutamate: a neurotransmitter in mammalian brain. *Journal of neurochemistry*, 1984, 42.1: 1-11.

FRAZIER, Thomas W., et al. Behavioral and cognitive characteristics of females and males with autism in the Simons Simplex Collection. *Journal of the American Academy of Child & Adolescent Psychiatry*, 2014, 53.3: 329-340. e3.

FOURNIER, Kimberly A., et al. Motor coordination in autism spectrum disorders: a synthesis and meta-analysis. *Journal of autism and developmental disorders*, 2010, 40.10: 1227-1240.

GARG, Shruti, et al. Autism spectrum disorder profile in neurofibromatosis type I. *Journal of autism and developmental disorders*, 2015, 45.6: 1649-1657.

GARG, Shruti, et al. Neurofibromatosis type 1 and autism spectrum disorder. *Pediatrics*, 2013, 132.6: e1642-e1648.

GARG, Shruti, et al. Sex bias in autism spectrum disorder in neurofibromatosis type 1. *Journal of neurodevelopmental disorders*, 2016, 8.1: 1-8.

GEIER, David A., et al. An evaluation of the role and treatment of elevated male hormones in autism spectrum disorders. *Acta Neurobiol Exp (Wars)*, 2012, 72.1: 1-17.

GESCHWIND, Daniel H. Genetics of autism spectrum disorders. *Trends in cognitive sciences*, 2011, 15.9: 409-416.

GIARELLI, Ellen, et al. Sex differences in the evaluation and diagnosis of autism spectrum disorders among children. *Disability and health journal*, 2010, 3.2: 107-116.

GONÇALVES, Joana, et al. Testing the excitation/inhibition imbalance hypothesis in a mouse model of the autism spectrum disorder: in vivo neurospectroscopy and molecular evidence for regional phenotypes. *Molecular Autism*, 2017, 8.1: 1-8.

GRAEFF, Frederico G., et al. Role of the amygdala and periaqueductal gray in anxiety and panic. *Behavioural brain research*, 1993, 58.1-2: 123-131.

GREENBERG, David M., et al. Testing the Empathizing–Systemizing theory of sex differences and the Extreme Male Brain theory of autism in half a million people. *Proceedings of the National Academy of Sciences*, 2018, 115.48: 12152-12157.

GREGG, Thomas R.; SIEGEL, Allan. Brain structures and neurotansmitters regulating aggression in cats: implications for human aggression. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 2001, 25.1: 91-140.

GRIMSLEY, Jasmine MS; MONAGHAN, Jessica JM; WENSTRUP, Jeffrey J. Development of social vocalizations in mice. *PloS one*, 2011, 6.3: e17460.

GUTMANN, David H., et al. Neurofibromatosis type 1. *Nature Reviews Disease Primers*, 2017, 3.1: 1-17.
GUTMANN, David H.; WOOD, Deborah L.; COLLINS, Francis S. Identification of the neurofibromatosis type 1 gene product. *Proceedings of the National Academy of Sciences*, 1991, 88.21: 9658-9662.

HALLMAYER, Joachim, et al. Genetic heritability and shared environmental factors among twin pairs with autism. *Archives of general psychiatry*, 2011, 68.11: 1095-1102.

HAMPE, Christiane S.; MITOMA, Hiroshi; MANTO, Mario. GABA and Glutamate: Their transmitter role in the CNS and pancreatic islets. *U: GABA And Glutamate-New Developments In Neurotransmission Research. Samardzic J, ur., London, IntechOpen*, 2018, 65-90.

HARADA, Masafumi, et al. Non-invasive evaluation of the GABAergic/glutamatergic system in autistic patients observed by MEGA-editing proton MR spectroscopy using a clinical 3 tesla instrument. *Journal of autism and developmental disorders*, 2011, 41.4: 447-454.

HARROP, Clare, et al. Sex differences in social attention in autism spectrum disorder. *Autism Research*, 2018, 11.9: 1264-1275.

HARROP, Clare, et al. Visual attention to faces in children with autism spectrum disorder: are there sex differences?. *Molecular Autism*, 2019, 10.1: 1-10.

HARTLEY, Sigan L.; SIKORA, Darryn M. Sex differences in autism spectrum disorder: an examination of developmental functioning, autistic symptoms, and coexisting behavior problems in toddlers. *Journal of autism and developmental disorders*, 2009, 39.12: 1715.

HECKMAN, Jesse, et al. Determinants of the mouse ultrasonic vocal structure and repertoire. *Neuroscience & Biobehavioral Reviews*, 2016, 65: 313-325.

HOLTMANN, Martin; BÖLTE, Sven; POUSTKA, Fritz. Autism spectrum disorders: Sex differences in autistic behaviour domains and coexisting psychopathology. *Developmental Medicine & Child Neurology*, 2007, 49.5: 361-366.

HOLY, Timothy E.; GUO, Zhongsheng. Ultrasonic songs of male mice. *PLoS biology*, 2005, 3.12: e386.

HORDER, Jamie, et al. Glutamate and GABA in autism spectrum disorder—a translational magnetic resonance spectroscopy study in man and rodent models. *Translational psychiatry*, 2018, 8.1: 1-11.

HULL, Laura, et al. “Putting on my best normal”: Social camouflaging in adults with autism spectrum conditions. *Journal of autism and developmental disorders*, 2017, 47.8: 2519-2534.

HYMAN, Shelley L.; SHORES, Arthur; NORTH, Kathryn N. The nature and frequency of cognitive deficits in children with neurofibromatosis type 1. *Neurology*, 2005, 65.7: 1037-1044.

IVANENKO, A., et al. Classifying sex and strain from mouse ultrasonic vocalizations using deep learning. *PLoS computational biology*, 2020, 16.6: e1007918.

JACKS, Tyler, et al. Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. *Nature genetics*, 1994, 7.3: 353-361.

JACQUEMONT, Sébastien, et al. A higher mutational burden in females supports a “female protective model” in neurodevelopmental disorders. *The American Journal of Human Genetics*, 2014, 94.3: 415-425.

JESSEN, Walter J., et al. MEK inhibition exhibits efficacy in human and mouse neurofibromatosis tumors. *The Journal of clinical investigation*, 2013, 123.1: 340-347.

JETT, Kimberly; FRIEDMAN, Jan M. Clinical and genetic aspects of neurofibromatosis 1. *Genetics in Medicine*, 2010, 12.1: 1-11.

JORGENSEN, Erik M. Gaba. *WormBook: The Online Review of C. elegans Biology [Internet]*, 2005.

JÜRGENS, Uwe. Neural pathways underlying vocal control. *Neuroscience & Biobehavioral Reviews*, 2002, 26.2: 235-258.

JÜRGENS, Uwe. The role of the periaqueductal grey in vocal behaviour. *Behavioural brain research*, 1994, 62.2: 107-117.

JÜRGENS, Uwe. The neural control of vocalization in mammals: a review. *Journal of Voice*, 2009, 23.1: 1-10.

KAZDOBA, Tatiana M., et al. Translational mouse models of autism: advancing toward pharmacological therapeutics. *Translational neuropsychopharmacology*, 2015, 1-52.

KEAY, Kevin A.; BANDLER, Richard. Periaqueductal gray. In: *The rat nervous system*. Academic Press, 2015. p. 207-221.

KIM, Eun Joo, et al. Dorsal periaqueductal gray-amygdala pathway conveys both innate and learned fear responses in rats. *Proceedings of the National Academy of Sciences*, 2013, 110.36: 14795-14800.

KLINGER, Laura Grofer, et al. Autism spectrum disorder. 2014.

KUBAS, B., et al. Metabolite alterations in autistic children: a 1H MR spectroscopy study. *Advances in medical sciences*, 2012, 57.1: 152-156.

LAGRANGE, Andre H.; GRIER, Mark D. GABAergic Neurotransmission. In: *Primer on the Autonomic Nervous System*. Academic Press, 2012. p. 109-111.

LAI, Jonathan KY, et al. Temporal and spectral differences in the ultrasonic vocalizations of fragile X knock out mice during postnatal development. *Behavioural brain research*, 2014, 259: 119-130.

LAI, Meng-Chuan, et al. Sex/gender differences and autism: setting the scene for future research. *Journal of the American Academy of Child & Adolescent Psychiatry*, 2015, 54.1: 11-24.

LAMSAL, Ramesh; ZWICKER, Jennifer D. Economic evaluation of interventions for children with neurodevelopmental disorders: opportunities and challenges. *Applied health economics and health policy*, 2017, 15.6: 763-772.

LEEKAM, Susan, et al. Repetitive behaviours in typically developing 2-year-olds. *Journal of Child Psychology and Psychiatry*, 2007, 48.11: 1131-1138.

LEHTONEN, Annukka, et al. Behaviour in children with neurofibromatosis type 1: cognition, executive function, attention, emotion, and social competence. *Developmental Medicine & Child Neurology*, 2013, 55.2: 111-125.

LI, Xiaohong; ZOU, Hua; BROWN, W. Ted. Genes associated with autism spectrum disorder. *Brain research bulletin*, 2012, 88.6: 543-552.

LINNMAN, Clas, et al. Neuroimaging of the periaqueductal gray: state of the field. *Neuroimage*, 2012, 60.1: 505-522

LOOMES, Rachel; HULL, Laura; MANDY, William Polmear Locke. What is the male-to-female ratio in autism spectrum disorder? A systematic review and meta-analysis. *Journal of the American Academy of Child & Adolescent Psychiatry*, 2017, 56.6: 466-474.

LORD, Catherine; SCHOPLER, Eric; REVICKI, Dennis. Sex differences in autism. *Journal of Autism and developmental disorders*, 1982, 12.4: 317-330.

LOWERY-GIONTA, Emily G., et al. GABA neurons of the ventral periaqueductal gray area modulate behaviors associated with anxiety and conditioned fear. *Brain Structure and Function*, 2018, 223.8: 3787-3799.

MAINBERGER, Florian, et al. Lovastatin improves impaired synaptic plasticity and phasic alertness in patients with neurofibromatosis type 1. *BMC neurology*, 2013, 13.1: 1-12.

MALONEY, Susan E., et al. Characterization of early communicative behavior in mouse models of neurofibromatosis type 1. *Autism Research*, 2018, 11.1: 44-58.

MANDY, William, et al. Sex differences in autism spectrum disorder: evidence from a large sample of children and adolescents. *Journal of autism and developmental disorders*, 2012, 42.7: 1304-1313.

MARTIN, George A., et al. The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. *Cell*, 1990, 63.4: 843-849.

MAUTNER, Victor-F., et al. Treatment of ADHD in neurofibromatosis type 1. *Developmental medicine and child neurology*, 2002, 44.3: 164-170.

MAY, Tamara; CORNISH, Kim; RINEHART, Nicole. Does gender matter? A one year follow-up of autistic, attention and anxiety symptoms in high-functioning children with autism spectrum disorder. *Journal of autism and developmental disorders*, 2014, 44.5: 1077-1086.

MAY, Tamara; CORNISH, Kim; RINEHART, Nicole J. Gender profiles of behavioral attention in children with autism spectrum disorder. *Journal of attention disorders*, 2016, 20.7: 627-635.

MAY, Tamara, et al. Sex differences in neurodevelopmental disorders. *Current opinion in neurology*, 2019, 32.4: 622-626.
MICHETTI, C.; RICCERI, L.; SCATTONI, M. L. Modeling social communication deficits in mouse models of autism. *Autism S1*, 2012, 7.10.4172: 2165-7890.

MIEDEL, Christian J., et al. Assessment of spontaneous alternation, novel object recognition and limb clasping in transgenic mouse models of amyloid-β and tau neuropathology. *Journal of visualized experiments: JoVE*, 2017, 123.

MORAES, Cladis LK; BERTOGLIO, Leandro J.; CAROBREZ, Antonio P. Interplay between glutamate and serotonin within the dorsal periaqueductal gray modulates anxiety-related behavior of rats exposed to the elevated plus-maze. *Behavioural Brain Research*, 2008, 194.2: 181-186.

MORRIS-ROSENDAHL, Deborah J.; CROCQ, Marc-Antoine. Neurodevelopmental disorders—the history and future of a diagnostic concept. *Dialogues in clinical neuroscience*, 2020, 22.1: 65.

MOY, Sheryl S., et al. Mouse models of autism spectrum disorders: the challenge for behavioral genetics. In: *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*. Hoboken: Wiley Subscription Services, Inc., A Wiley Company, 2006. p. 40-51.

MURDOCH, John D.; STATE, Matthew W. Recent developments in the genetics of autism spectrum disorders. *Current opinion in genetics & development*, 2013, 23.3: 310-315.

MUSAZZI, Laura, et al. The action of antidepressants on the glutamate system: regulation of glutamate release and glutamate receptors. *Biological psychiatry*, 2013, 73.12: 1180-1188.

NICIU, Mark J.; KELMENDI, Benjamin; SANACORA, Gerard. Overview of glutamatergic neurotransmission in the nervous system. *Pharmacology Biochemistry and Behavior*, 2012, 100.4: 656-664.

NORDLUND, Michael L., et al. Neurofibromin expression and astrogliosis in neurofibromatosis (type 1) brains. *Journal of Neuropathology & Experimental Neurology*, 1995, 54.4: 588-600.

NORTH, Kathryn. Neurofibromatosis type 1. *American journal of medical genetics*, 2000, 97.2: 119-127.

OBLAK, A.; GIBBS, T. T.; BLATT, GJ2762426. Decreased GABAA receptors and benzodiazepine binding sites in the anterior cingulate cortex in autism. *Autism Research*, 2009, 2.4: 205-219.

OBLAK, Adrian L.; GIBBS, Terrell T.; BLATT, Gene J. Decreased GABAB receptors in the cingulate cortex and fusiform gyrus in autism. *Journal of neurochemistry*, 2010, 114.5: 1414-1423.

O'CONNELL, Lauren A.; HOFMANN, Hans A. The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. *Journal of Comparative Neurology*, 2011, 519.18: 3599-3639.

ORREGO, F.; VILLANUEVA, S. The chemical nature of the main central excitatory transmitter: a critical appraisal based upon release studies and synaptic vesicle localization. *Neuroscience*, 1993, 56.3: 539-555.

OWEN, Michael J., et al. Neurodevelopmental hypothesis of schizophrenia. *The British journal of psychiatry*, 2011, 198.3: 173-175.

PASMANT, Eric, et al. Identification of genes potentially involved in the increased risk of malignancy in NF1-microdeleted patients. *Molecular Medicine*, 2011, 17.1: 79-87.

PELEH, Tatiana; ELTOKHI, Ahmed; PITZER, Claudia. Longitudinal analysis of ultrasonic vocalizations in mice from infancy to adolescence: Insights into the vocal repertoire of three wild-type strains in two different social contexts. *PLoS One*, 2019, 14.7: e0220238.

PERSICO, Antonio M.; BOURGERON, Thomas. Searching for ways out of the autism maze: genetic, epigenetic and environmental clues. *Trends in neurosciences*, 2006, 29.7: 349-358.

PLASSCHAERT, Ellen, et al. Prevalence of autism spectrum disorder symptoms in children with neurofibromatosis type 1. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, 2015, 168.1: 72-80.

PRIDE, Natalie A., et al. The genetic and neuroanatomical basis of social dysfunction: lessons from neurofibromatosis type 1. *Human brain mapping*, 2014, 35.5: 2372-2382.

PURCELL, A. E., et al. Postmortem brain abnormalities of the glutamate neurotransmitter system in autism. *Neurology*, 2001, 57.9: 1618-1628.

PUTS, Nicolaas AJ, et al. Reduced GABA and altered somatosensory function in children with autism spectrum disorder. *Autism Research*, 2017, 10.4: 608-619.

PYLAYEVA-GUPTA, Yuliya; GRABOCKA, Elda; BAR-SAGI, Dafna. RAS oncogenes: weaving a tumorigenic web. *Nature Reviews Cancer*, 2011, 11.11: 761-774.

RAMAMOORTHI, Kartik; LIN, Yingxi. The contribution of GABAergic dysfunction to neurodevelopmental disorders. *Trends in molecular medicine*, 2011, 17.8: 452-462.

ROJAS, Donald C., et al. Decreased left perisylvian GABA concentration in children with autism and unaffected siblings. *Neuroimage*, 2014, 86: 28-34.

ROSS, Judith L., et al. Behavioral and social phenotypes in boys with 47, XYY syndrome or 47, XXY Klinefelter syndrome. *Pediatrics*, 2012, 129.4: 769-778.

ROSSER, Tena L.; PACKER, Roger J. Neurocognitive dysfunction in children with neurofibromatosis type 1. *Current neurology and neuroscience reports*, 2003, 3.2: 129-136.

SCATTONI, Maria Luisa; CRAWLEY, Jacqueline; RICCERI, Laura. Ultrasonic vocalizations: a tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. *Neuroscience & Biobehavioral Reviews*, 2009, 33.4: 508-515.

SCHOCH, Hannah, et al. Sociability deficits and altered amygdala circuits in mice lacking Pcdh10, an autism associated gene. *Biological psychiatry*, 2017, 81.3: 193-202.

SESARINI, Carla V., et al. Association between GABA (A) receptor subunit polymorphisms and autism spectrum disorder (ASD). *Psychiatry research*, 2015, 229.1-2: 580-582.

SHILYANSKY, Carrie, et al. Neurofibromin regulates corticostriatal inhibitory networks during working memory performance. *Proceedings of the National Academy of Sciences*, 2010, 107.29: 13141-13146.

SHILYANSKY, C.; LEE, Yong-Seok; SILVA, AJ3063104. Molecular and cellular mechanisms of learning disabilities: a focus on NF1. *Annual review of neuroscience*, 2010, 33: 221-243.

SIEBERT, S.; JÜRGENS, U. Vocalization after periaqueductal grey inactivation with the GABA agonist muscimol in the squirrel monkey. *Neuroscience letters*, 2003, 340.2: 111-114.

SIMONYAN, Kristina, et al. New developments in understanding the complexity of human speech production. *Journal of Neuroscience*, 2016, 36.45: 11440-11448.

SILVA, Alcino J., et al. A mouse model for the learning and memory deficits associated with neurofibromatosis type I. *Nature genetics*, 1997, 15.3: 281-284.

SKULTETY, F. MILES. Experimental mutism in dogs. *Archives of neurology*, 1962, 6.3: 235-241.

SMITH, Rachel EW, et al. Sex differences in resting-state functional connectivity of the cerebellum in autism spectrum disorder. *Frontiers in human neuroscience*, 2019, 13: 104.

SPARKS, B. F., et al. Brain structural abnormalities in young children with autism spectrum disorder. *Neurology*, 2002, 59.2: 184-192.

STACY, Maria E., et al. Sex differences in co-occurring conditions of children with autism spectrum disorders. *Autism*, 2014, 18.8: 965-974.

STEINBACH, Jessica M.; GARZA, Elizabeth T.; RYAN, Bryce C. Novel object exploration as a potential assay for higher order repetitive behaviors in mice. *Journal of visualized experiments: JoVE*, 2016, 114.

SUPEKAR, Kaustubh; MENON, Vinod. Sex differences in structural organization of motor systems and their dissociable links with repetitive/restricted behaviors in children with autism. *Molecular Autism*, 2015, 6.1: 1-13.

SUZUKI, Katsuaki, et al. Microglial activation in young adults with autism spectrum disorder. *JAMA psychiatry*, 2013, 70.1: 49-58.

TAN, Diana Weiting, et al. Hypermasculinised facial morphology in boys and girls with autism spectrum disorder and its association with symptomatology. *Scientific reports*, 2017, 7.1: 1-11.

THAPAR, Anita; RUTTER, Michael. Neurodevelopmental disorders. *Rutter's child and adolescent psychiatry*, 2015, 31-40.

TSAI, Luke; STEWART, Mark A.; AUGUST, Gerald. Implication of sex differences in the familial transmission of infantile autism. *Journal of Autism and Developmental Disorders*, 1981, 11.2: 165-173.

TSCHIDA, Katherine, et al. A specialized neural circuit gates social vocalizations in the mouse. *Neuron*, 2019, 103.3: 459-472. e4.

VAN DER VAART, Thijs, et al. Motor deficits in neurofibromatosis type 1 mice: the role of the cerebellum. *Genes, Brain and Behavior*, 2011, 10.4: 404-409.

VANRYZIN, Jonathan W., et al. Temporary depletion of microglia during the early postnatal period induces lasting sex-dependent and sex-independent effects on behavior in rats. *Eneuro*, 2016, 3.6.

VAN WIJNGAARDEN-CREMERS, P. J., et al. Gender and age differences in the core triad of impairments in autism spectrum disorders: a systematic review and meta-analysis. *Journal of autism and developmental disorders*, 2014, 44.3: 627-635.

VASA, Roma A., et al. Assessment and treatment of anxiety in youth with autism spectrum disorders. *Pediatrics*, 2016, 137.Supplement 2: S115-S123.

VERLINSKY, Yury, et al. Preimplantation diagnosis for neurofibromatosis. *Reproductive BioMedicine Online*, 2002, 4.3: 218-222.

VIOLANTE, Inês R., et al. GABA deficiency in NF1: a multimodal [11C]-flumazenil and spectroscopy study. *Neurology*, 2016, 87.9: 897-904.

VIOLANTE, Ineˆs R., et al. GABA deficit in the visual cortex of patients with neurofibromatosis type 1: genotype–phenotype correlations and functional impact. *Brain*, 2013, 136.3: 918-925.
WILLIAMS, Virginia C., et al. Neurofibromatosis type 1 revisited. *Pediatrics*, 2009, 123.1: 124-133.

VOGEL, Kristine S., et al. Mouse tumor model for neurofibromatosis type 1. *Science*, 1999, 286.5447: 2176-2179.

WALSH, Karin S., et al. Symptomatology of autism spectrum disorder in a population with neurofibromatosis type 1. *Developmental Medicine & Child Neurology*, 2013, 55.2: 131-138.

WESSEL, Lauren E., et al. Longitudinal analysis of developmental delays in children with neurofibromatosis type 1. *Journal of Child Neurology*, 2013, 28.12: 1689-1693.

WILSON, C. Ellie, et al. Does sex influence the diagnostic evaluation of autism spectrum disorder in adults?. *Autism*, 2016, 20.7: 808-819.

WING, Lorna. Sex ratios in early childhood autism and related conditions. *Psychiatry research*, 1981, 5.2: 129-137.

WON, Hyejung, et al. Autistic-like social behaviour in Shank2-mutant mice improved by restoring NMDA receptor function. *Nature*, 2012, 486.7402: 261-265.

WOZNIAK, David F., et al. Motivational disturbances and effects of L-dopa administration in neurofibromatosis-1 model mice. *PloS one*, 2013, 8.6: e66024.

XU, Gangfeng, et al. The neurofibromatosis type 1 gene encodes a protein related to GAP. *Cell*, 1990, 62.3: 599-608.

YOUNG, David M., et al. Altered ultrasonic vocalizations in a tuberous sclerosis mouse model of autism. *Proceedings of the National Academy of Sciences*, 2010, 107.24: 11074-11079.