UNIVERSIDADE D
COIMBRA

Ana Margarida da Silva Pinho

EFFECT OF NEUROPEPTIDE Y INTRANASAL DELIVERY IN AUTISTIC-LIKE BEHAVIORS IN A MOUSE MODEL OF AUTISM SPECTRUM DISORDER

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

Setembro de 2022

Faculdade de Medicina
da Universidade de Coimbra

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This work was supported by FLAD Life Sciences Ed 2 2016, FCT Strategic plan UIDP/04950/2021 (CIBIT), and ICNAS-P.

ACKNOLEDGEMENTS

Com a conclusão da presente Dissertação, queria deixar o meu agradecimento a todos aqueles que contribuíram de forma direta ou indireta para a concretização desta etapa.

Agradeço primeiramente aos meus orientadores, Professor Doutor Miguel Castelo-Branco e a Doutora Joana Gonçalves, por toda a ajuda, ensinamentos, assim como por todo o suporte e disponibilidade no decorrer deste projeto. Um agradecimento especial à Doutora Joana Gonçalves, pelo apoio contínuo e por me ter ajudado a crescer neste período não só no trabalho, como a nível pessoal. Também não poderia esquecer de agradecer ao Doutor João Martins, que esteve sempre pronto a ajudar e quando preciso dar na cabeça.

Aos meus colegas de laboratório, um obrigada do fundo do coração. Se houve algo que aprendi neste período foi em saber ajudar, pedir ajuda e trabalhar para o bem da nossa equipa. Apesar de cada um ter o seu trabalho individual, houve sempre momentos de amizade e interajuda, dentro e fora do trabalho.

A toda a minha família, agradeço pelo apoio incondicional ao longo de toda esta jornada, o que foi imprescindível.

E por fim, mas não menos importante aos meus amigos e em especial ao meu namorado, obrigada pela companhia, compreensão e apoio.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ADDM - Autism and Developmental Disabilities Monitoring

ADHD- Attention deficit hyperactivity disorder

AMPA- α-amino-3-hydroxy-5- methyl-4-isoxazolepropionic acid

ANOVA- One-Way analysis of varience

ARND- Alcohol-related neurodevelopmental disorder

AS- Asperger's syndrome

ASD- Autism spectrum disorder

B2M - Beta-2-Microglobulin

BD- Bipolar depression

CA- Closed arm

CD- Communication disorder

CNS- Central nervous system

DNA- Deoxyribonucleic acid

DSM- Diagnostic and Statistical Manual of Mental Disorders

EEG- Electroencephalogram

E/I – Excitation/ inhibition

EMB- Extreme Male Brain

EPM- Elevated plus maze

F- Female

FPE- Female Protective Effect

FAS- Fetal alcohol syndrome

FASD- Fetal alcohol spectrum disorders

GABA - Gamma-amino-butyric acid

GAD- Glutamate decarboxylase

GAP- GTPase-activating protein

GBA- Gut-brain axis

GI- Gastrointestinal

Glu- Glutamate

ICV- Intracerebroventricular

iGluRs- Glutamate receptor ion channels

IL- Interleukin

ID-Intellectual disability

LE- Lissencephaly

M- Male

MD- Motor disorders

ME- Microencephaly

mTOR- Mammalian target of rapamycin

mTORC1- Mammalian target of rapamycin complex 1

mTORC2- Mammalian target of rapamycin complex 2

NDD- Neurodevelopmental disorders

NMDA- N-methyl D- aspartate

NPY- Neuropeptide Y

OA- Open arm

PCR- Polymerase chain reaction

PDD-NOS- Pervasive developmental disorder-not otherwise specified

PDDs- Pervasive Developmental Disorders

PND- Postnatal day

PP- Pancreatic polypeptide

PYY- Peptide YY

qPCR- Quantitative polymerase chain reaction

Ref - Reference

RNA- Ribonucleic acid

rt-PCR- Real-time polymerase chain reaction

SZ- Schizophrenia

TD- Tic disorders

TEA- Transtorno do espectro do autismo

TNF- Tumor necrosis factor

TSC- Tuberous sclerosis complex

WT- Wild type

Y_1 R- Y_1 receptor

Y_2 R- Y_2 receptor

LIST OF TABLES

LIST OF FIGURES

RESUMO

O transtorno do espectro do autismo (TEA) é uma doença complexa do neuro desenvolvimento caracterizada por défices na interação e comunicação social, bem como comportamentos repetitivos. Cada vez mais, a investigação tem como objetivo definir as causas subjacentes à TEA. Apesar dos esforços, esta ainda continua por determinar. No entanto, existe consenso científico que as anomalias comportamentais que caracterizam o TEA se devem a perturbações na função cerebral bem como a outros fatores. Estes incluem fatores hereditários, ambientais e vulnerabilidade genética assim como outras causas desconhecidas. Estudos recentes têm demonstrado que a microbiota intestinal poderá ser fundamental no desenvolvimento do TEA, uma vez que problemas gastrointestinais estão associados à maioria dos casos de autismo. Por outro lado, o desequilíbrio excitatório/ inibitório (E/I) é um mecanismo comum no TEA. Alterações na aprendizagem e memória, défices cognitivos, sensoriais, motores, e convulsões estão associados a este desequilíbrio e presentes no TEA. No entanto, a ligação entre a disfunção gastrointestinal e os distúrbios cerebrais nesta doença, ainda permanece desconhecida. Esta tese de mestrado formulou a hipótese de que neuropeptídeos, como o neuropeptídeo Y(NPY), poderiam estar implicados na mediação desta ligação, tendo um papel crucial no eixo intestino-cérebro.

Esta tese de Mestrado tem como objetivo explorar o efeito da administração crónica intranasal de NPY no comportamento característico do TEA. Recorrendo a um modelo animal de autismo já estabelecido, modelo murganho de complexo de esclerose tuberosa 2 (*Tsc2*$^{+/-}$), investigámos o impacto do tratamento com NPY no microbioma bem como no sistema GABAérgico. Alterações no sistema NPYérgico, especificamente nos níveis de NPY, recetores Y_1 e Y_2 também foram avaliadas.

Realizámos um estudo longitudinal desde o período pós-natal até jovens adultos. O tratamento com NPY teve uma duração de 20 dias, com testes comportamentais realizados logo após o tratamento.

Neste estudo, não foi possível demonstrar o papel do NPY como modelador do comportamento autista nem eixo intestino-cérebro. No entanto, observámos um comportamento mais ansioso em animais WT (estirpe selvagem) administrados com NPY em comparação com WT tratados com salino, e um repetitivo mais acentuado em murganhos *Tsc2*$^{+/-}$ tratados com NPY. No entanto, no futuro, de forma a compreender

melhor o papel do NPY, teremos de aumentar o número de animais no estudo e segregar por sexo.

Palavras-chave: Transtorno do espectro do autismo, Complexo de esclerose tuberosa 2, Neuropeptídeo Y, Razão Excitação/Inibição

ABSTRACT

Autism spectrum disorder (ASD) is complex neurobiological disorder characterized by young age onset, impairments in social interaction and communication together with repetitive behavior. There is scientific consensus that autism involves a disorder in brain function and leads to behavioral abnormalities. However, the causes that underlie this disorder are still unclear. So far, it is known that there are several factors that might influence on the development, prognosis of the disease and can lead to the behavioral abnormalities. These include hereditary factors, environmental factors, genetic vulnerability and also unknown causes. Recently, gut microbiota has been postulated as pivotal player in ASD, since gastrointestinal (GI) problems seen are associated with most of the autism cases. On the other hand, and the excitatory/ inhibitory (E/I) imbalance is a common mechanism in ASD, being indicated as a cause to learning and memory, cognitive, sensory, motor deficits, and seizures occurring in these disorders. Nevertheless, is still unknown the link between GI dysfunction with brain disorders in ASD. This Master thesis hypothesized that neuropeptides, such as neuropeptide Y(NPY) could be implicated in mediating this link, being a key-player in gut-brain axis.

This Master Thesis explored the effect of chronic NPY intranasal administration in ASD-like behavior using a mouse model of tuberous sclerosis complex 2 (*Tsc2*$^{+/-}$), a well-established animal model of syndromic ASD. We also intended to investigate how NPY treatment impacts microbiome as well as the GABAergic system. Additionally, changes in NPYergic system, namely NPY, Y_1 and Y_2 receptors levels, were evaluated.

For that, a longitudinal study from an early age to young adulthood was conducted. NPY treatment was administered for 20 days, followed by behavioral tests.

Here, we were not able to show NPY has a prominent contributor in the relieve of autistic behavior and in gut-brain-axis. However, we observed a more anxious behavior in wild-type (WT) administered with NPY compared to WT treated with saline, and a higher repetitive behavior in *Tsc2*$^{+/-}$ mice treated with NPY. Nevertheless, in the future we will have to increase the number of animals studied and segregate by sexes to better understand the role of NPY.

Keywords: Autism spectrum disorder, Tuberous Sclerosis Complex 2, Neuropeptide Y, Excitation/Inhibition ratio

CHAPTER 1 – STATE-OF-THE-ART

1.1 Neurodevelopmental Disorders

Neurodevelopmental disorders (NDDs) comprise of a broad group of conditions characterized by alterations in the development of the central nervous system (CNS), resulting in an inability to reach cognitive, emotional, communication and motor developmental milestones [1]. The onset of NDDS occurs at birth or during infancy, before puberty [2].

Several conditions are grouped under the diagnosis of NDD in the proposed framework for the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5). Conditions such as intellectual developmental disorders, communication disorders, autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), specific learning disorder, and motor disorders are described as NDDs (Figure 1) [3].

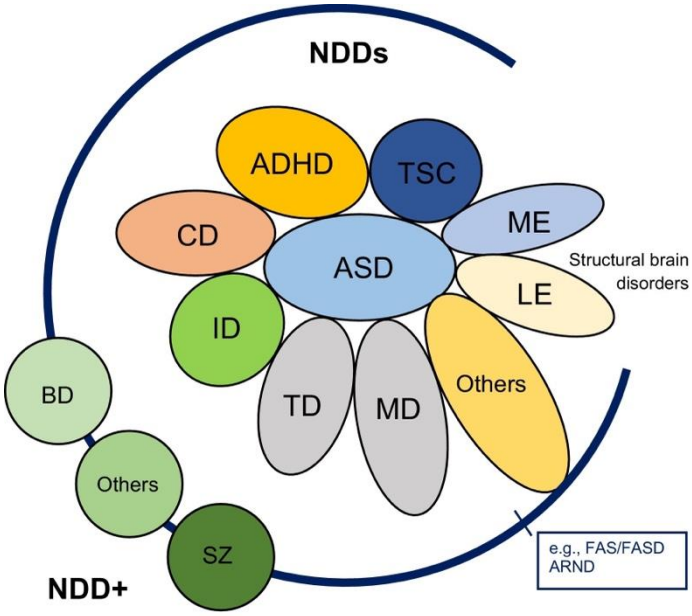


Figure 1. A scheme of major neurodevelopmental disorders (NDDs) and the larger 'NDD+' spectrum. NDDs form a heterogeneous group of illnesses with varying severity and clinical manifestations, these include ASD, ADHD, CD, ID, TD, MD, LE, ME, TSC.

The larger NDD+ spectrum of associated neuropsychiatric disorders include FAS/FASD and ARND. Additional disorders with neurodevelopmental trajectories comprise of BD and SZ.

Abbreviations: NDDs – neurodevelopmental disorders, ASD – autism spectrum disorder, ID – intellectual disability, CD – communication disorder, ADHD – attention deficit hyperactivity disorder, TSC – tuberous sclerosis complex, TD – tic disorders, MD – motor disorders, ME – microcephaly, LE –lissencephaly, FAS – fetal alcohol syndrome, FASD – fetal alcohol spectrum

disorders, ARND – alcohol-related neurodevelopmental disorder, BD – bipolar depression, SZ – schizophrenia. Adapted from Homberg et al.,2016

The identification of potential causes of NDDs is crucial for understanding the mechanisms responsible for the onset of these disorders. It is clear that genetics play an important causative role in mediating disorders of neurodevelopment, both by de novo and inherited variants. However, nongenetic causes also have an impact on the occurrence of such events. For instance, various studies showed that birth asphyxia, perinatal infections, central nervous system injuries, prematurity, and drug and tobacco exposure, represent risk factors that may lead to the onset of NDDs [4].

Concerning the clinical features of these disorders, not only co-occurrence and heterogeneity of symptoms and syndromes but also diagnostic overlap, are very common [3,5]. Studies suggest that the existence of these comorbidities (patient is diagnosed with two or more NDDs) might be associated with shared molecular pathways and genetic causes between the disorders [1].To corroborate this, several studies show that 22% to 83% of children with ASD have symptoms that satisfy the DSM-IV criteria for ADHD, and vice versa, 30% to 65% of children with ADHD have clinically significant symptoms of ASD [6].

Authors throughout studies, highlight the necessity of a multidisciplinary team of experts in evaluating and designing the best approach of therapy to the patient and its needs, taking part specialists in child psychiatry, psychology, and speech, motor therapy alongside with others [3,7].

At present, there are no specific treatments for NDD, the available therapies consist of a combination of behavioral approaches and drugs approved for ameliorating comorbidities such as irritability and anxiety. Nonetheless in many cases the core symptoms remain unsolved. However, from a genetic point of view, new strategies are being developed based on molecular pathways involved in NDDs that were targeted [8].

NDDs entail a major global burden in terms of individual and family suffering, educational and health care expenditure, and lost productivity, that lasts throughout their lives [9,10]. Consequently, these implications show the importance to study these disorders in the future in order to ameliorate the quality of life of the patient.

1.2 Autism Spectrum Disorder

In the past few decades, great efforts were made to understand ASD, that used to be a barely defined rare disorder of childhood onset to now a well-publicized, advocated, and researched lifelong condition. However, many questions still remain to be answered.

The word "Autism" is derived from the Greek word "autós", which means "self". In 1908, Eugen Bleuler, a Swiss psychiatrist created this term to describe withdrawal from reality in patients with schizophrenia. Fast forward to 1943, Leo Kanner redefined the term to describe symptoms of social isolation and linguistic disorders in children without schizophrenia or other known psychiatric disorders. The children that made part of the study presented difficulty in communicating and interacting with others and displayed repetitive behaviors and loss of interest in social activities unlike other children of the same age [11].

In 1994, the fourth edition of the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) described five Pervasive Developmental Disorders (PDDs), which included Autistic disorder, Asperger's syndrome (AS), pervasive developmental disorder-not otherwise specified (PDD-NOS), Rett's disorder and child disintegrative disorder [12]. Children diagnosed with these disorders typically showed deficits in three domains: social interaction, communication, and repetitive/restricted behaviors. However, wide variations in the severity of symptoms both within and across the group of disorders were observed, making it more difficult to accurately distinguish one disorder from the other [13]. This has led to the proposed formal changes of the taxonomy to autism spectrum disorder, first described in DSM-5 which reflects a scientific consensus that the four separate disorders are in fact a single condition with different levels of symptom severity in two core domains [14].

ASD is typically diagnosed within the first 3 years of life and is defined by persistent deficits in social communication and interaction, as well as restrictive and repetitive behaviors [3]. The prevalence of ASD has been steadily increasing in the past two decades. In the early 2000s, the Center for Disease Control's Autism and Developmental Disabilities Monitoring (ADDM) Network estimated the incidence of ASD to be 1 in 110 children. In 2012, the ADDM estimated to be 1 in 68 children [15]. In the latest prevalence rate report by ADDM, in 2018, a new record was met, citing ASD could be found in as

many as 1 in 44 children [16] . This ratio is thought to be the same across all racial, ethnic, or socioeconomic backgrounds, however gender variations exist [13].

It has been found in all populations studied throughout the years a strong and consistent male bias in ASD, with being recently established a ratio of 3 males to 1 female [17]. The neurobiological mechanisms responsible for the higher ratio of ASD in males are thought to be responsible for alterations observed in cognitive profiles, autistic symptoms, and coexisting behavior problems [18]. This pattern of unequal prevalence suggests the action of sex-differential risk factors for ASD that might act to either increase males' vulnerability and/or protects females [19]. Concerning the male's vulnerability theory, the so called "Extreme Male Brain (EMB) theory", studies conducted by Baron-Cohen propose that ASD individuals present a more masculinized brain and that there are two dimensions for understanding human sex differences: "empathizing" and "systemizing." Based on these studies, the male brain is defined as the one in which systematization fits better than empathy. On the other hand, females generally have a more empathizing brain, are better at discriminating emotions from expression in the eyes and social queues [20], [21]. Fetal testosterone exposure levels have been linked to EBM and the different autistic traits observed in ASD individuals [22]. An alternative hypothesis is the Female Protective Effect (FPE), this hypothesis is composed of two models, one hypothesizing that males have greater genetic variability due to their XY chromosomes, leading to a higher incidence but lower severity of the disease. Additionally, the other model states the additional X chromosome in females contributes to a higher threshold for the development of a neurodevelopmental disorder, as the second X chromosome acts as a buffer or "backup" [23,24].

Despite the knowledge in the neurobiology and genetic field of ASD, the diagnosis of this disorder continues to be based on identifying and reporting behaviorally defined clinical symptoms [25]. Making an accurate ASD diagnosis is a multi-stage process that requires a multidisciplinary team of experts and the help from the parents and teachers. Yet, the diagnostic in girls is harder as there is growing evidence of a camouflaging effect among females with ASD. It has been hypothesized that females can mask socio-communicative impairments due to increased sensitivity to social pressure to fit in, gendered expectations for social behavior, and strengths in some social-communication skills. This led to females being underdiagnosed or diagnosed at significantly later ages and also experience inaccurate diagnoses prior to the actual diagnosis [26].

The assessment should consider the symptomatology, the history and progress of the disorder and must adequately rule out other NDDs [27]. Observation of specific behavior forms the basis of diagnosis, with criteria focused on three main core traits: impairments in social interaction and communication – both verbal and nonverbal communication is affected, abnormalities in understanding the intent of eye contact, difficulty in evaluating facial expressions and gestures, atypical development of social communication and pretend play as well as lack of interest in other children and restricted/repetitive behaviors – this includes motor specific behaviors like hand flapping and finger flicking, the repetitive use of objects and speech (Table 1) [3,25,28].

Table 1. Diagnostic criteria for autism spectrum disorder. Adapted from DSM-5

	Social interaction and communication	Restricted repetitive behavior
Criteria of evaluation	Persistent deficits in social communication and social interaction across multiple contexts, currently or by history (1) Social-emotional reciprocity deficits- presents abnormal social approach, failure in initiating or respond to social interactions and having normal back-and-forth conversations, reduced interests, emotions. (2) Nonverbal communicative deficits- poor verbal and nonverbal communication, failure to do eye contact and body language, total lack of facial expressions. (3) Deficits in developing, maintaining, and understanding relationships- difficulties adjusting behavior in various social contexts, struggles in sharing imaginative play and in making friends, absence of interest in others	Restricted, repetitive patterns of behavior, interests, or activities, as manifested by at least two of the following: (1) Stereotyped or repetitive motor movements, use of objects, or speech. (2) Insistence on sameness, inflexible adherence to routines, or ritualized patterns of verbal or nonverbal behavior (3) Highly restricted, fixated interests that are abnormal in intensity or focus (4) Hyper- or hyporeactivity to sensory input or unusual interest in sensory aspects of the environment

The DSM-5 also introduced an approach to severity rating, which is summarized in Figure 2. Severity rating reflects the impairment of the ASD symptoms and the required levels of support to assist with the different needs of the individual [29].

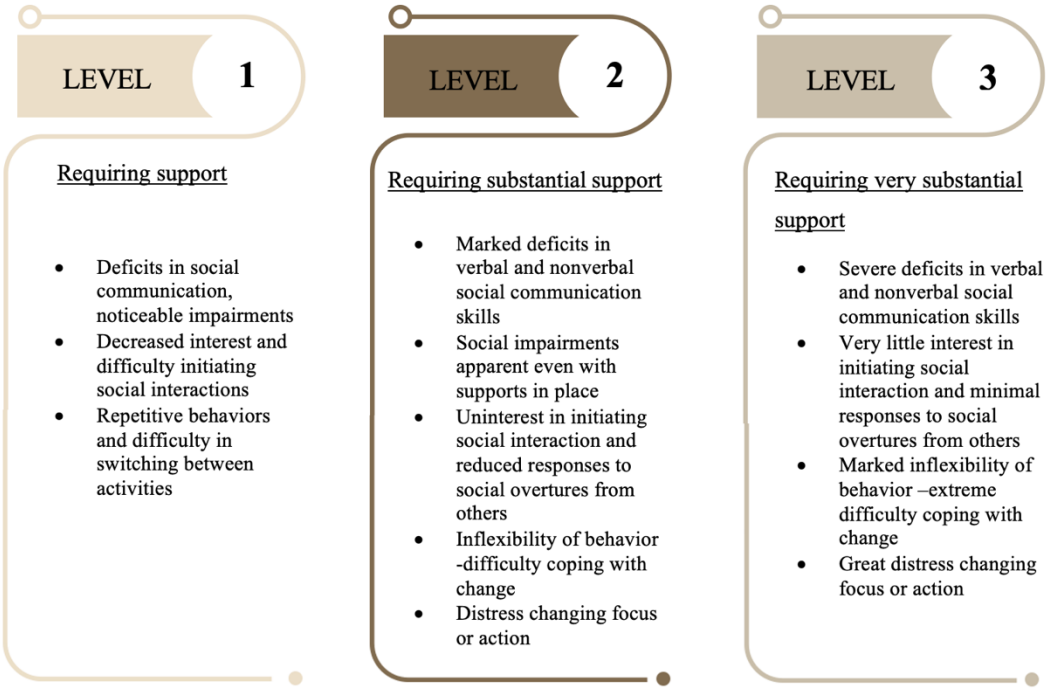


Figure 2. Graphical scheme of ASD levels of severity and their characteristics. Severity rating consists of three levels: level 1– requires support, level 2 – the patient needs substantial support, and level 3 – requires very substantial support. Adapted from DSM-5.

Previous studies have shown that ASD is a pathology with an unclear and a diverse etiology. However, there are several factors that might have an influence on development and prognosis of ASD, such as genetic anomalies, dysregulation of the immune system, inflammatory processes, and environmental factors, and more recently studies highlight the dysregulation of the gut microbiota (Figure 3) [30, 31].

In addition, clinicians have long been aware that ASD is often accompanied by other difficulties. Intellectual disability, ADHD, epilepsy, anxiety, mood disorders, seizures, gastrointestinal problems (GI), sleep and eating problems are a few of the comorbidities present in ASD [32].

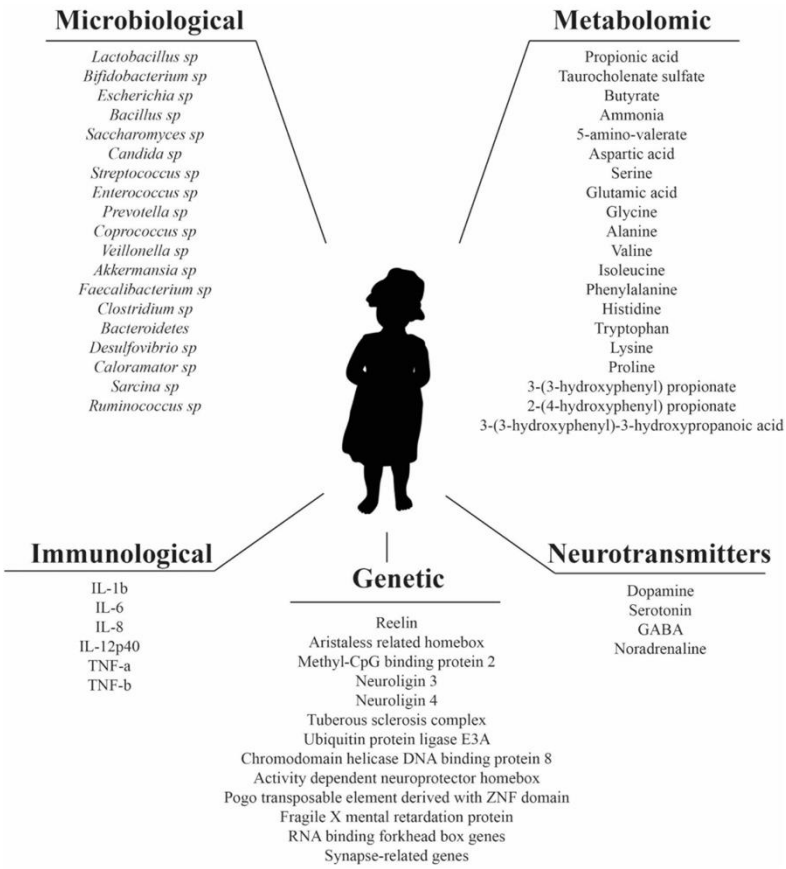


Figure 3. Scheme of the factors that might have an involvement on development and prognosis of ASD. These factors include microbiological, metabolomic, immunological, genetic factors and neurotransmitters that have reported in the literature as altered in ASD. Abbreviations: IL – interleukin, TNF – tumor necrosis factor, RNA – ribonucleic acid, DNA –deoxyribonucleic acid. Adapted from Garcia-Gutierrez et al., 2020.

1.3 Tuberous Sclerosis Complex Disorder

Being ASD a very complex disorder, its symptoms heterogeneity could be related to the heterogeneity of the genetic factors that underlie it. Studies have identified specific mutations in different genes that lead to autistic–like syndromes in subsets of children with an array of genetic diseases including Fragile X syndrome, Tuberous Sclerosis complex and Rett syndrome [33].

Tuberous Sclerosis complex (TSC) is a multisystemic neurocutaneous genetic condition with autosomal-dominant inheritance. Uncontrolled abnormal tissue growth-hamartomas, that affect multiple organs represent a key feature of the disease [34]. The disease occurs due to a loss-of-function mutation of tumor suppressor gene TSC1 or TSC2, which encode for hamartin and tuberin proteins, respectively [35]. These two proteins form the TSC1-TSC2 complex and together with TBC1D7 form a heterotrimeric complex that serves as the main negative regulator of the mechanistic mammalian target of rapamycin (mTOR) signaling cascade [36]. The mTOR pathway is responsible for cell proliferation, autophagy, and apoptosis. The heterotrimeric complex regulates the activation of the mTOR pathway functioning as a tumor suppressor. The TSC protein complex inhibits mTOR activation through the action of the GTPase-activating protein (GAP) domain in TSC2. The mTOR forms two structurally and functionally distinct complexes called the mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 2 (mTORC2). mTORC1 is comprised of mTOR, raptor, GβL and deptor, and is selectively sensitive to rapamycin. On the other hand, mTORC2 is composed of mTOR, Rictor, GβL, PRR5, deptor, and SIN1 and contrary to mTORC1, is largely unaffected by rapamycin. mTORC1 integrates signals from multiple growth factors, nutrients, and energy supply to promote cell growth when energy is sufficient and catabolism when the body is hungry. mTORC1 mainly regulates cell growth and metabolism, while mTORC2 mainly controls cell proliferation and survival (Figure 4) [37, 38].

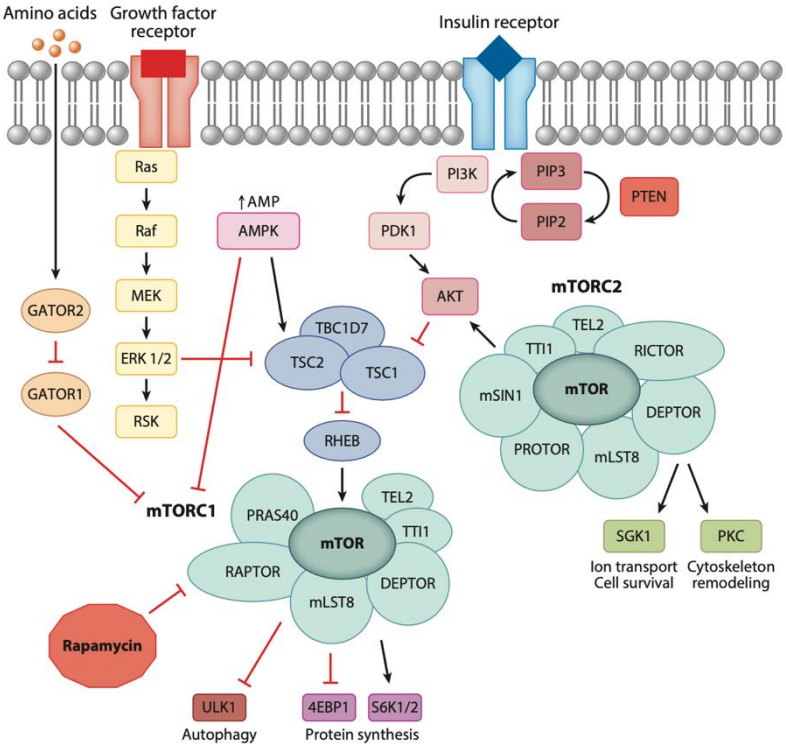


Figure 4. The mTOR signaling pathway. Three major upstream modulators—amino acids, energy (AMP), and growth factors and nutrients—regulate the pathway and its two complexes, mTORC1 and mTORC2. The presence of amino acids stimulates mTORC1 activity to promote cell growth and proliferation, whereas low-energy states activate AMPK, resulting in the inhibition of mTORC1 via the TSC protein complex and inhibition of RAPTOR. Growth factors and nutrients activate the PI3K/AKT and Ras/MEK/ERK pathways, resulting in TSC1/2-mediated disinhibition of mTORC1. The two mTOR complexes share four common components: mLST8, DEPTOR, TTI1, and TEL2. mTORC1 is defined by RAPTOR (a scaffolding protein essential to mTORC1 and sensitive to rapamycin) and PRAS40 (an inhibitor of mTORC1). mTORC2 is formed by mSIN1 (a molecule that is important for mTORC2-mediated activation of AKT), RICTOR (a scaffolding protein that is insensitive to rapamycin), and PROTOR (a scaffolding molecule that mediates activation of SGK1). Adapted from Salussoia et al., 2019.

Most individuals affected by TSC seek medical attention due to seizures or skin lesions. Skin lesions represent the most common finding observed in TSC patients. Neurological and renal complications are the main cause of morbidity and mortality associated to the condition (Figure 5 and 6) [39].

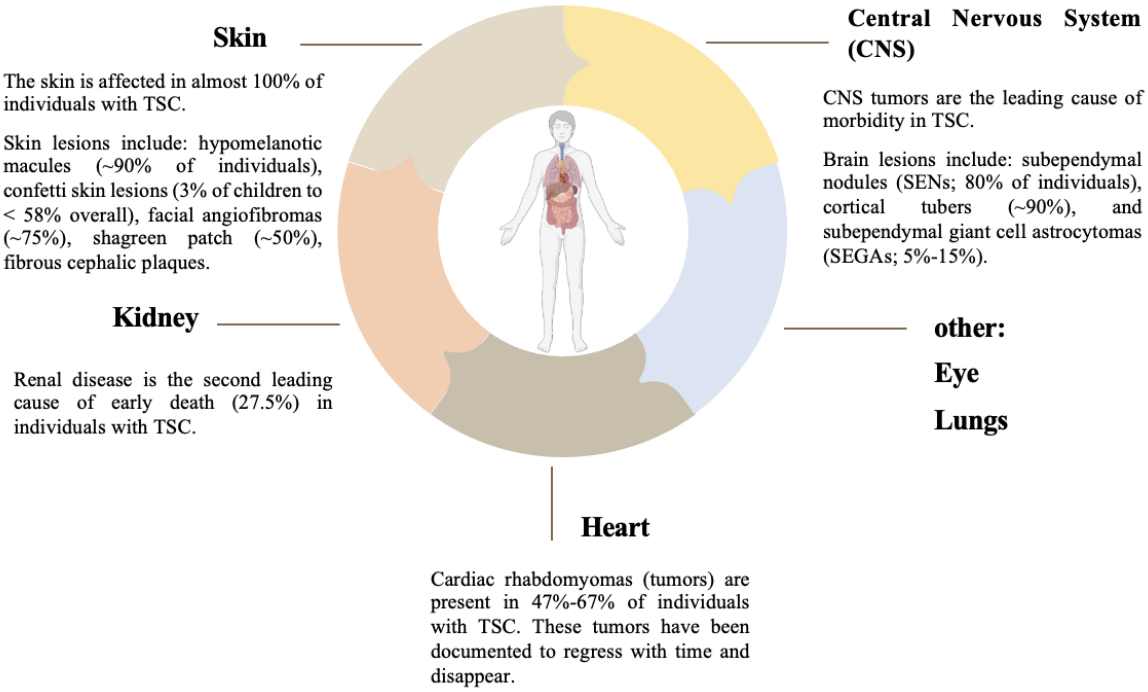


Figure 5. Graphical scheme of the clinical characteristics of tuberous sclerosis complex. The skin and the central nervous system are the most affected by the disease. Other organs like the kidneys, heart, eyes and lungs have also been widely reported to be affected.

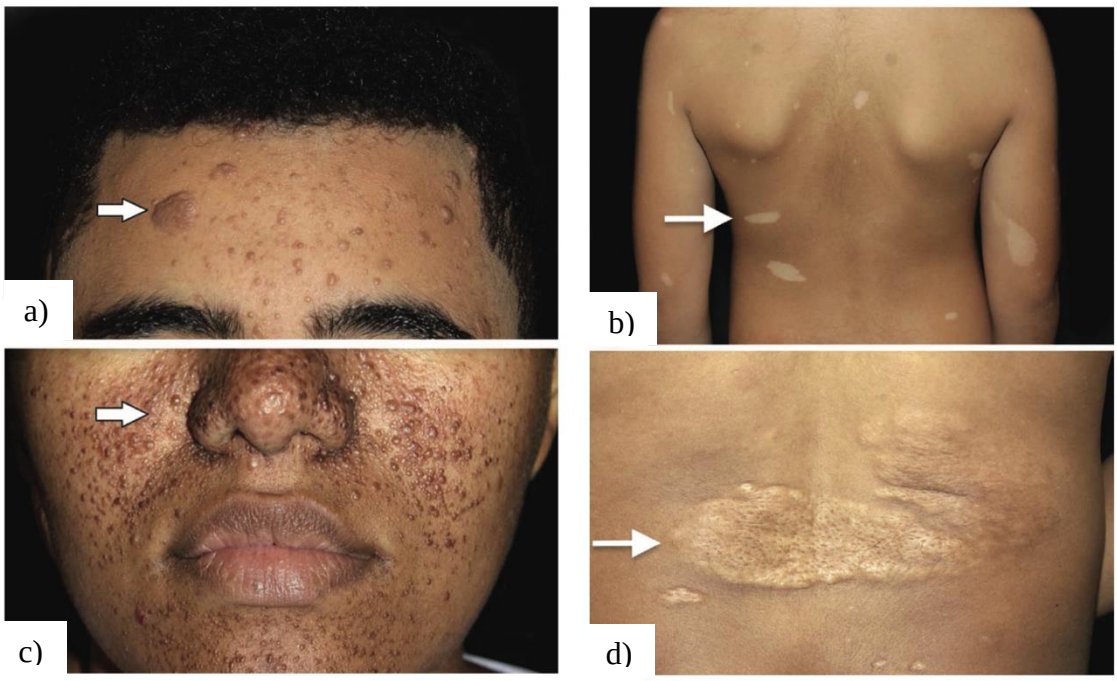


Figure 6. Skin lesions characteristic of Tuberous sclerosis complex (TSC). Skin lesions present in TSC patients include fibrous cephalic plaques (a), hypomelanotic macules (b), confetti skin lesions (c) and shagreen patches (d). Abbreviations: TSC – tuberous sclerosis complex. Adapted from Portacarrero et al., 2018.

A crucial aspect of the pathophysiology of the disorder appears to be an early deviation from typical neurodevelopment, through structural abnormalities. Epileptic seizures are one of the primary early manifestations of the disease in the CNS, occurring in 70 to 90% of the individuals with the condition [35]. These are considered to be a result of the genetic mutation leading to an imbalance between excitation and inhibition neurotransmission [40]. This condition is the sign that most frequently leads to the diagnosis of the syndrome, followed by intellectual deficits and ASD [41]. The disturbances in the autism spectrum, among other behavioral problems, can be found in 40-50% of the patients [41]. In agreement, our group recently published that female *Tsc2*$^{+/-}$ mouse showed a decreased in cortical GABA levels together with an imbalance in GABA/glutamate ratio [42].Dysregulation of the neurotransmission of gamma-amino-butyric acid (GABA) has been proposed as a neurobiological link between epilepsy and ASD in TSC patients. Evidence suggests that both epilepsy and ASD are linked with mutations on the *TSC1* and *TSC2* genes. Mutations in the *TSC2* gene are thought to be more related to epilepsy and ASD [40].

Rodents that harbor a defect in the Tsc1 or Tsc2 gene have been extensively investigated. Homozygous mutants are embryonically lethal, and heterozygotes develop tumors in various organs (Table 2) [43].

Table 2. Characteristic of model for ASD- *Tsc2*$^{+/-}$

Model /Specificity	Morphology and Behavior	Synaptic Transmission
Tsc2$^{+/-}$	No major morphological defects – Do not display brain lesions, Tsc2+/−rats feature cortical tubers, but not mice [44]	Altered synaptic transmission and plasticity
	No spontaneous seizures were detected in *Tsc2*$^{+/-}$ rats [44]	
	Mouse model of *Tsc2*$^{+/-}$ show deficits in learning and memory [45]	
	Impaired social behavior was observed in *Tsc2*$^{+/-}$ mice [43]	

1.4 Excitation/Inhibition Imbalance

In the human brain, regulated and balanced excitatory and inhibitory inputs are the key to an optimal functional brain. Glutamate and GABA, the two main neurotransmitters involved in excitatory and inhibitory signaling in the brain [46].

GABA is the main inhibitory neurotransmitter in the brain, a non-protein amino acid produced through α-decarboxylation of glutamate in a reaction catalyzed by glutamate decarboxylase (GAD) [47]. It inhibits neuronal firing by activating two different classes of receptors, GABA(A) and GABA(B). GABA(A) receptors are classified as ionotropic while GABA(B) receptors are metabotropic (guanidine nucleotide protein-coupled receptors). Seizure, threshold, anxiety, and panic are associated with $GABA_A$ receptors and GABA(B) receptors are associated with memory, mood, and pain [47]. The decrease in GABA(A) signaling caused by mutations or environmental factors leads to an increase in the brain's excitatory tone, contributing to hyperexcitable states (i.e., epilepsy) and cognitive dysfunction [48].

Glutamate is the most prevalent excitatory neurotransmitter in mammalian adult brain. The glutamate receptor ion channels (iGluRs) are abundantly expressed in the brain and spinal cord and mediate responses at most excitatory synapses, these include four major families, α-amino-3-hydroxy-5- methyl-4-isoxazolepropionic acid (AMPA) receptors, kainate receptors, N-methyl D- aspartate (NMDA) receptors and delta receptors [49]. Mutations (or potentially, environmental factors) that increase glutamate signaling increase excitatory tone. Mutations that result in the increase of the activity or number of glutamate receptors, or the amount of glutamate in the synapse lead to increase in the excitatory state of the brain [48].

A balanced interaction between these neurotransmitters is required to maintain the physiological homeostasis, as prolonged imbalance can result in disease [50]. In 2003, Rubenstein and Merzenich hypothesized that some forms of ASD could be caused by a reduction in signal-to-noise in key neural circuits, resulted by changes in the circuit's E/I balance [51].

Direct evidence that supports the E/I imbalance hypothesis in ASDs derives from: genetic observations, *in vitro* analysis of post-mortem brain tissues and *in vivo* studies on patients affected mainly by idiopathic forms of ASDs [52].

GABA receptor genes have been associated with autism in linkage and copy number variation studies. Post-mortem tissue of autistic patients show a reduction in GABA receptor subunits, and GABAergic signaling is disrupted across heterogeneous mouse models of autism [53]. Similarly, alpha1-$GABA_A$ receptor expression was reduced in tuberal tissue resected from TSC patients [44].

However, studies from our group using the NF1 mouse model showed regional phenotypes of E/I imbalance, with region-specific GABAergic changes. Enhanced GABA inhibition was observed in the cortex and levels of GABA receptors expression increased in the hippocampus [54]. Contrary to the NF1 mouse model, a study also conducted in our lab, showed a decrease GABA/Glu ratio TSC2 females, in the prefrontal cortex. The same analysis was performed in the hippocampus, but no significant differences were observed in this mouse model [42].

1.5 Microbiota

The GI tract is the largest surface in the body composed of trillions of microorganisms separated by the gut barrier. The gut barrier is composed of the commensal gut microbiota, a mucus layer and epithelial cells connected through tight junctions [55]. The term microbiota describes the complex and diverse population of microbes living in a mutualistic relationship with the host, and it is mainly composed of bacteria, viruses, fungi, protozoa, and archaea. As for the microbiome, it comprises of all the genes expressed in all these microorganisms [56].

The healthy adult gut microbiota is composed of 4 major phyla being: Bacteroidetes (Gram negative such as the Bacteroides and Prevotella genera), Firmicutes (Gram positive aerobic and anaerobic bacteria such as Lactobacillus, Clostridium and Ruminococcus), Proteobacteria (e.g., Enterobacter species) and Actinobacteria (e.g., Bifidobacterium), followed by the minor phyla Fusobacteria and Verrucomicrobia (Figure 7) [57].

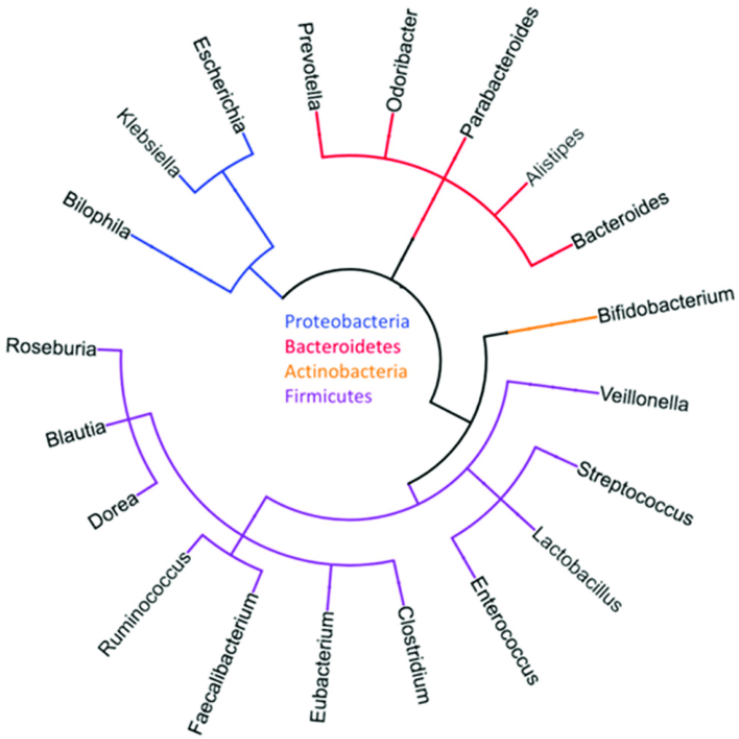


Figure 7. Major phyla of a healthy adult gut microbiome. Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes represent the 4 major phyla. Adapted from Cimadamore et al., 2019

The microbiota plays a critical function in the development of the intestinal architecture as well an important role in digestion, nutrient assimilation, vitamin production, and metabolism. Studies also found that the microbiota influences the bi-directional signaling that takes place between the gut and the nervous system- the microbiota-gut-brain axis [58].

1.5.1 The gut-brain axis

The term "gut-brain axis" (GBA) refers to the bidirectional communication between the central and the enteric nervous system, linking emotional and cognitive centers of the brain with peripheral intestinal functions. [59]. This bidirectional communication happens both in health and disease.

The gut-brain axis uses four major information carriers for the communication between the gut and the brain:

- neural messages carried by vagal and spinal afferent neurons,
- immune messages carried by cytokines,
- endocrine messages carried by gut hormones and
- microbial factors that may directly reach the brain via the blood stream but can also interact with the other three transmission pathways [60].

Microbiota may interact with GBA through different mechanisms resumed in Figure 8 [61].

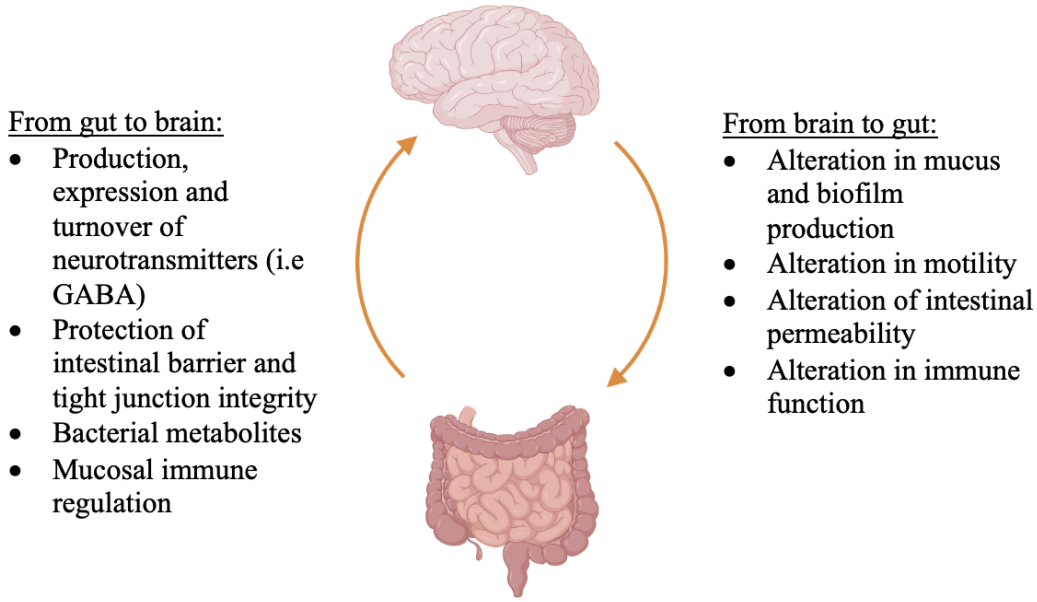


Figure 8. Main principal mechanisms of the bidirectional gut-brain axis. Mechanisms of how the gut interacts with the brain and how the brain interacts with the gut.

The communication pathways between the GI and CNS may involve neuropeptides and structurally related signaling molecules [60]. Neuropeptides such as substance P, calcitonin gene-related peptide and neuropeptide Y (NPY) are expressed at all levels of the GBA and thought to be produced by certain microbes. Furthermore, gut microbiota can respond to neuropeptides and gut hormones [56, 57].

1.5.2 The microbiota and ASD

Increasing evidence points to a considerable number of patients with ASD who display gastrointestinal dysfunctions, predominantly altered bowel habits and chronic abdominal pain, that complement their neurological alterations. The GI symptoms in individuals with ASD are four times more prevalent in children with ASD compared to normal population [62]. This changes in the gut microbiota can modulate the gastrointestinal physiology, immune function, and even behavior through the GBA and appear to be linked to the severity of the disorder phenotype [63, 64]

Accordingly, these co-occurring GI symptoms led researchers to analyze the gut microbiota composition of ASD patients and determine not only the potential consequences of these alterations in the symptomatology of ASD but also understand the relationship between the brain and the microbiota in disease (Figure 9).

A regularly observed phenomenon in the metagenomic analysis of ASD-gut microbiome show a significantly decreased ratio between the phyla *Bacteroidetes* to *Firmicutes*, which pointed to elevated numbers of *Firmicutes* in contrast to decreased levels of *Bacteroidetes* [52, 62].

The absence of microbial colonization in animal models results in abnormalities in a variety of complex behaviors, pointing to the possibility of a role of the microbiota in modulating behavioral outcomes in animal models of neurodevelopmental and neurological disorders. Two independent studies demonstrate that germ-free mice exhibit decreased sociability- preference to interact with a novel mouse rather than a non-social object and reduced social preference to interact with an unfamiliar versus familiar mouse. Also, a male bias was observed, the social behavioral abnormalities were more prevalent in male mice [66].

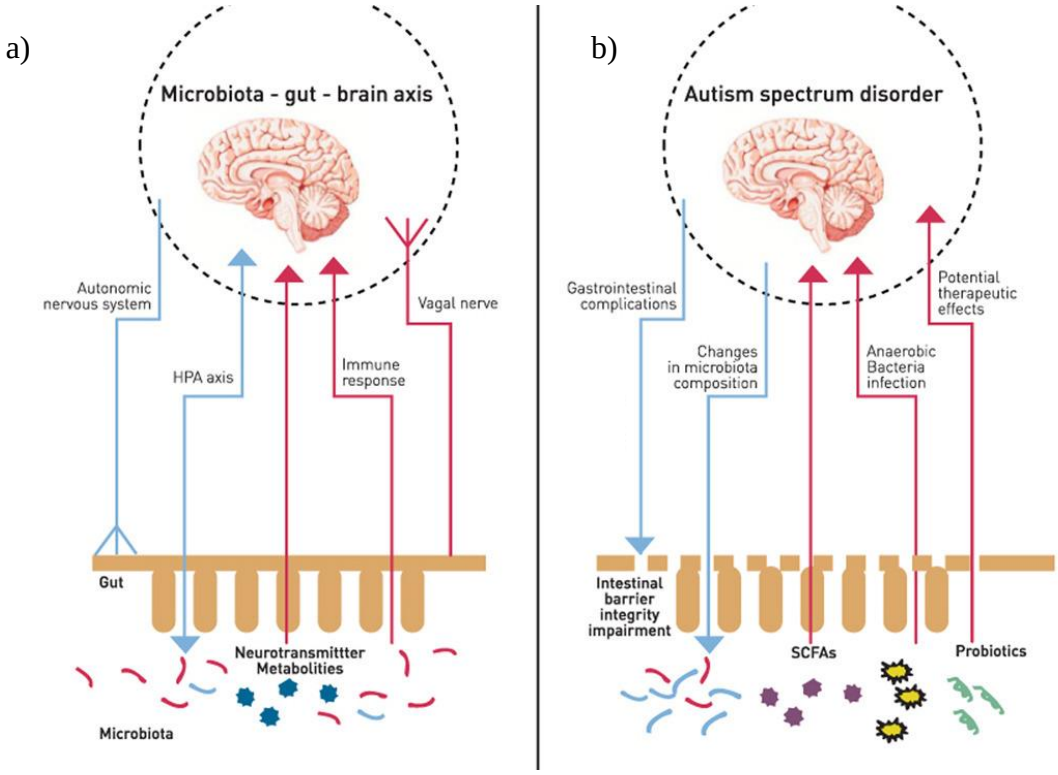


Figure 9. The bidirectional communication within in microbiota-gut-brain axis in health and in autism spectrum disorder. (a) The bidirectional communication occurs mainly through the autonomic nervous system, hypothalamic-pituitary-adrenal (HPA) axis, neuroendocrine and neuroimmune pathways. (b) The disturbances in the microbiota-gut-brain axis in ASD. Autistic deficits are associated with gastrointestinal complications and changes in microbiota composition. Abbreviations: HPA - hypothalamic-pituitary-adrenal, SCFA- short-chain fatty acid. Adapted from Principi et al., 2016

1.6 Neuropeptide Y

Although, several neurotransmitters are widely distributed throughout the nervous system, specific neuropeptides and their receptors are expressed in subsets of neurons due to their important role in mediating specific behaviors [48]. NPY is a tyrosine-rich 36 amino-acid peptide belonging to the pancreatic polypeptide family, which also involves peptide YY (PYY) and pancreatic polypeptide (PP). This peptide has been highly conserved throughout evolution, reflecting its important role in the body. Moreover, NPY is one of the most abundant and widely distributed neuropeptides in the CNS playing a role in numerous physiologic processes [67, 68]. Similar to other neuropeptides, NPY is synthesized in neuronal cell bodies, especially GABAergic neurons and predominantly expressed in a multitude of brain areas, the neocortex, hippocampus, striatum, and amygdala [69, 70]. The numerous and diverse roles of NPY are related to its expression in this brain areas. It plays a pivotal part in many physiological functions such as food intake, energy homeostasis, circadian rhythm, intestinal secretion, cognition and also is a key component in stress response, having anxiolytic properties [71]. NPY acts via G protein- coupled receptors, Y_1, Y_2, Y_4, Y_5 that mediate a wide range of physiological effects of this peptide and each of them has a distinct expression pattern in the brain (Table 3) [69, 72]

Table 3. The characteristics of NPY receptors [72]

NPY receptors	Expression	Amino acids	Agonists	Function
Y_1	Periphery, hypothalamus, hippocampus, neocortex, thalamus	384	NPY, PYY	Vasoconstriction, anxiolysis, food intake, heart rate, anxiety
Y_2	Brain, hippocampus, thalamus, hypothalamus	381	NPY, PYY	Memory, circadian rhythm, angiogenesis, epilepsy, secretion, bone formation
Y_4	Brain, gastrointestinal tract, pancreas, prostate	375	PP, NPY, PYY	Feeding, circadian ingestion, energy homeostasis, colonic transit
Y_5	Hypothalamus, hippocampus	445-455	NPY, PYY	Food intake, epilepsy, circadian rhythm

Abbreviations: NPY- Neuropeptide Y, PYY- peptide YY, PP- pancreatic polypeptide

1.6.1 Role of NPY and Gut

Scientific reports have implicated the NPY receptors in numerous roles in the GI tract including adaptation to diet, GI motility, electrolyte balance, nutrient/water uptake, intestinal growth and gastric emptying and in the pathophysiology of numerous gastrointestinal diseases. However, direct evidence that this neuropeptide contributes to the communication between the gut microbial community and the central nervous system is sparse [73]. NPY is one of the most potent orexigenic peptides found in the brain, their orexigenic effect is primarily mediated by Y_1 receptors, although Y_5 receptors also play a role [74].

1.6.2 NPY and Excitatory/Inhibitory Imbalance

NPY is mainly produced and released by GABAergic interneurons and inhibits glutamatergic neurotransmission. Under pathological conditions characterized by hyperexcitability, such as epilepsy, there is an increase in the expression of NPY and NPY receptors mainly in the granular and pyramidal cells. This contributes to the tonic

inhibition of glutamate release and consequently to control the spread of excitability into other parts of the brain [75]. Early in vitro studies demonstrated that NPY applied to rat hippocampus slices reduced synaptic excitation. This reduction was only mediated by glutamate in a selective way, not affecting synaptic inhibition. Subsequently, in vivo studies showed that intracerebroventricular (ICV) administration of NPY inhibited hippocampal seizures and wet dog shakes, through behavioral assessment and electroencephalogram (EEG)[67].

1.6.3 NPY regulates anxiety, stress, learning and memory

Recent clinical studies of NPY have broadened the understanding of the physiological effects of the peptide in anxiety and stress and pinpointed to possible relation between this peptide and these conditions, being its expression and its receptors in brain regions that regulate these phenomena. Studies where NPY was administered exogenously, anti-anxiety actions were observed. NPY is believed to tone down CNS activity by inhibiting the activity of pro-stress transmitters, thereby controlling stress and anxiety responses [76, 77] .

Several reports observed that the IVC administration of NPY and NPY injection directly to the amygdala or hippocampus, resulted in less anxious mice. Also, studies that tested NPY knockout mice showed that behaved in an anxious way. Further, hippocampal or amygdalar NPY overexpression renders animals less anxious, confirming the anxiolytic properties of endogenous NPY in these brain regions [71]. Given that NPY promotes stress resilience and aids the recovery from stress, an emerging hypothesis states that the NPY system may also influence the impact of stress on the gut–brain axis.

A high expression of NPY and NPY receptors in the cortex and hippocampus supports the role in cognitive processing, learning and memory [76] Behavioral studies with Y_2 knock out mice demonstrated that this animal model display poor performance in the Morris water maze and object recognition test. Results suggest that Y_2 receptors might play an integral role in spatial memory and nonspatial working memory processing [78].

CHAPTER 2- AIMS OF THE STUDY

The emerging microbial knowledge has pointed to a potential link between gut microbiota dysbiosis and ASD. In contrast, the excitatory/inhibitory imbalance has been described to be a common mechanism of ASD and associated with behavioral abnormalities. However, it remains unclear the link between GI dysfunction and brain disorders in ASD. Neuropeptides, such as NPY have been implicated in mediating specific mechanisms both in the brain and in the gut. Understanding the role of NPY in ASD, could lead to identifying a new therapeutic molecular target, refining the diagnosis and treatment of the disorder.

We will conduct a study to explore the following objectives:

1. Study the effect of chronic intranasal administration of NPY in ASD-like behavior, with a focus on anxiety, repetitive behaviors and social impairments.
2. Investigate the impact of the administration in the microbiome as well as the hippocampal and cortical GABAergic system (GABA receptors).
3. Explore changes in the hippocampal and cortical NPYergic system, specifically NPY, Y_1 and Y_2 receptors, caused by chronic administration NPY treatment.

CHAPTER 3- MATERIALS AND METHODS

3.1 Animals

Thirty-eight mice total were used in the experiments. *Tsc2*$^{+/-}$ mice were generated and backcrossed to a C57BL/6N. Mice for experiments were generated by crossing *Tsc2*$^{+/-}$ animals with mice with C57BL/6J background. All pups were housed together with the mom until postnatal day 21 (PND21) and segregated by sex and treatment from PND21 onwards. All animals were maintained in a housing room with a 12 h light/12h dark cycle, at 21 ± 2 °C. At PND5 dentification of pups was performed with permanent tattoos on the toes and tail tips were collected for posterior genotyping. All experiments were carried out in accordance with the European Union Council Directive (2010/63/EU) and the National Regulations, approved by the Internal Review Board of ICNAS and conducted under the authority of the Project License (1/2017).

Four experimental groups were established for this study: wild type (WT) with saline (vehicle) treatment; WT with NPY treatment; *Tsc2*$^{+/-}$ with saline (vehicle) treatment; and *Tsc2*$^{+/-}$ with NPY treatment.

Animals were weighted every two days of treatment and food consumption measured each week:

$$Food\ consumption\ per\ animal = \frac{initial\ amount\ of\ food - final\ amount\ of\ food}{nº\ of\ animals\ per\ cage}$$

The amount of food given to each cage was done according to the formula:

$$Food\ per\ cage = nº\ of\ days\ x\ nº\ of\ animals\ \ x\ 6g\ (average\ daily\ food\ consumption)$$

3.2 Intranasal administration of Neuropeptide Y

The study was conducted following the subsequent timetable (Figure 10).

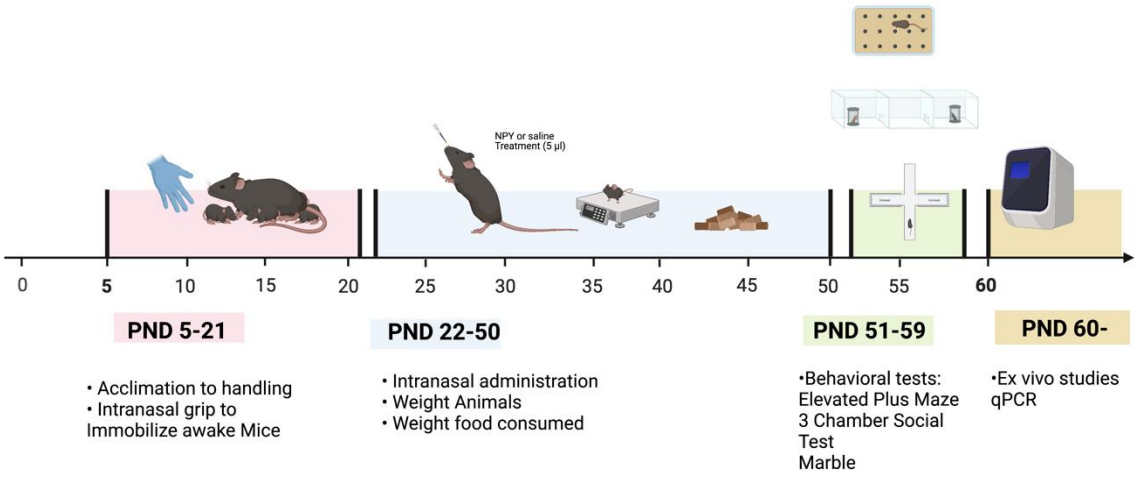


Figure 10. Schematic representation of the timetable. Starting at postnatal day 5 (PND), animals are handled by caretaker until PND21. After separation from their mother, at PND22 starts the intranasal administration of treatment, that last for 5 days a week/4 weeks. Behavioral tests are performed after treatment. Ex vivo studies start at ~PND60.

3.2.1 Handling

Before administration, it was important to acclimate the mice to the handling. This procedure helped to ensure correct body position for maximum effectiveness of awake intranasal drug delivery and to induce minimum anxiety level. We started handling the animals at PND5 up to PND21, accordingly, to Table 4. The pups at PND21 were separated into their designated cage, in accordance with their sex- male or female, and assigned treatment- NPY or saline (control group).

Table 4. Description of handling procedure

Handling Day	Procedure
Day 1	Put both hands in the cage for 5 min
Day 2	Put both hands in the cage for 5 min
Day 3	Hold for 2-3 min
Day 4	Hold for 2-3 min
Day 5	Hold and pet for 2-3 min
Day 6	Hold and pet for 2-3 min
Day 7	Lightly pinch or scruff
Day 8	Lightly pinch or scruff
Day 9	Intranasal grip
Day 10	Intranasal grip
Day 11	Intranasal grip and invert
Day 12	Intranasal grip and invert
Day 13	Intranasal grip, invert, place pipette tip near nose
Day 14	Intranasal grip, invert, place pipette tip near nose
Day 15	Intranasal grip, invert, place pipette tip near nose

3.2.2 Administration

Neuropeptide Y (human, rat) trifluoroacetate salt (1mg) (Bachem, Bubendorf, Switzerland) was resuspended in 0.9% NaCl, accordingly, to manufacture instructions. Using the intranasal grip as previously performed in the animal, 5 µl of NPY solution (0.20 mg/kg of body weight/day), or vehicle in the control group (0.9% NaCl) was administered. The treatment was infused into the nares with a pipetman and disposable plastic tip, 5 days a week, for 4 weeks (total of 20 administrations). Care was taken to avoid direct tip contact with intranasal mucosa. After administration the head of the mouse was held in the same position for approximately 10 s to prevent loss of the solution from the nares.

3.3 Behavioral tests

3.3.1 Elevated plus maze

The elevated plus maze (EPM) was performed the day after the last treatment (~PND 51). EPM consists of four elevated arms which radiate from a central platform, forming a plus shape (Figure 11). Two of the opposed arms are walled (apart from the ceiling, entrance and exit points)- closed arms, and the remaining two opposed are open apart from the platform itself- open arms. Before the test, the animals were placed in the test room for 1 hour for acclimation. For the behavioral session, mice were positioned in the center part of the elevated plus maze and were allowed to explore the maze for 5 min [79]. The exposure to light was measured before each test in different spots of the elevated plus maze, to ensure it was between 80- 100 lux. Video recordings were performed using the Microsoft life cam hd3000.

a) b)

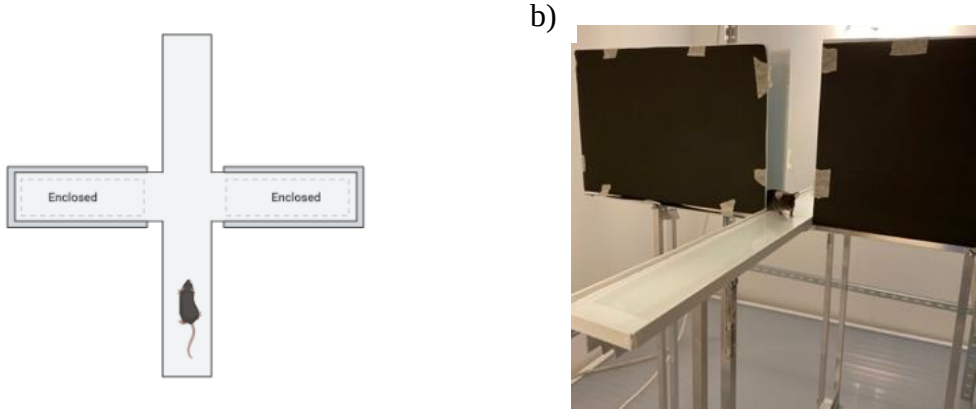


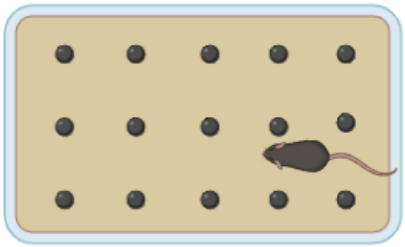
Figure 11. Elevated plus maze test. (a) Representation of the configuration of the test, two open arms and two closed arms opposed to each other. (b) Example of an animal during the elevated plus maze test.

3.3.2 Marble burying test

After one day of rest, the marble burying test was conducted. A standard mouse cage was walled with acrylic sheet and filled with unscented mouse bedding material to a depth of approximately 5cm and levelled out. Glass toy marbles were gently placed on the surface of the bedding evenly in 3 columns x 4 rows- 12 marbles (Figure 12). Before each test, previous bedding was replaced with new one and the marbles disinfected and dried. The

animal was placed in a corner with no marbles and left for the duration of the test, 30 minutes. Every 5 minutes, the number of marbles buried were counted, blinded from the sex, genotype and treatment of the animal. Marbles were considered buried if more than 75% of its volume was submersed in the bedding [80]. The exposure to light was measured before each test to ensure it was between 80- 100 lux. Video recordings were performed using the Microsoft life cam hd3000.

a)



b)

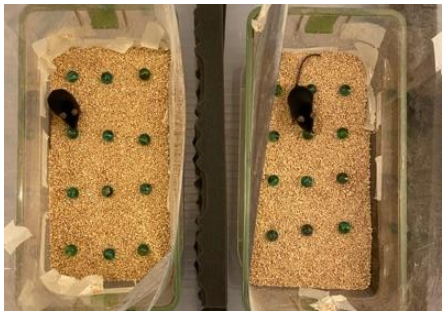


Figure 12. Marble burying test. (a) Representation of the configuration of the test, twelve marbles placed on the surface. (b) Example of two animals during the test.

3.3.2 Three-chamber social test

The three-chamber social test was performed a day after the last test and lasted for two days. The apparatus for the three-chamber social test is comprised of a rectangular three chamber box (with open ceiling). Each chamber is 19 x 45 cm and the dividing walls are made from clear Plexiglas, with an open middle section, which allows free access to each chamber.

The testing procedure consists of three chronological phases: Habituation, sociability, and social novelty (Figure 13). The first two take place in the first day of the test, and the social novelty test after 24h of the sociability test. A week prior to the test, the mice that are going to be placed in the cylindric containers during the social preference and social novelty test, are habituated to the apparatus for 10 minutes every day.

In the habituation phase the testing mouse is placed into the middle chamber and allowed to explore all three chambers freely for 10 min. In the sociability phase an age-, genotype,

sex-matched novel mouse is placed into one of the two identical, wire cup-like containers with removable lids that large enough to hold a single mouse.

In the second day, social novelty phase, a second age- and gender-matched novel mouse is added in the container that was empty- novel mouse- during the sociability phase. The mouse from the sociability test, now the familiar mouse is placed in the other container. The tester mouse's preference in social novelty is quantified by measuring the time spent in the "interaction zone" near the second novel mouse versus the time spent in the "interaction zone" near the now-familiar first novel mouse. The tests were all done under red light [81]. Video recordings were performed using the Microsoft life cam hd3000, for further analysis. All videos were manually analyzed by a blinded operator.

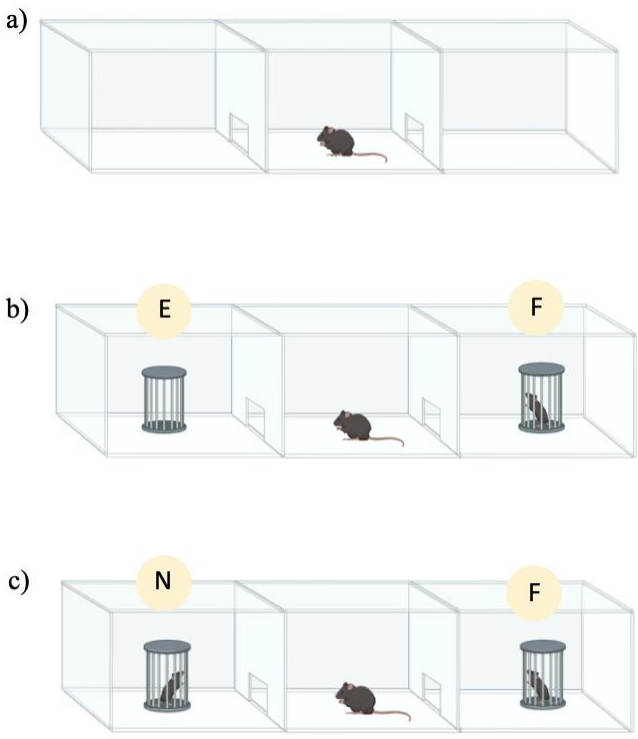


Figure 13. Three-chamber Social Test. (a) Representation of the configuration for the first part of the test- 10-minute habituation to the field. (b) Illustration of the sociability test, one wire container empty and another one with an animal. (c) Social novelty test representation, with one familiar animal in one of the wire containers and a novel animal in a wire container in the opposite chamber. E- Empty, F-Familiar, N-Novel

3.4 Molecular analysis

3.4.1 Sample collection

Following *in vivo* studies, at PND 60, animals were sacrificed, and hippocampus, prefrontal cortex and stool samples were isolated. The samples were preserved in an (ribonucleic acid) RNA stabilization Solution- RNAlater (Invitrogen by Thermo Fisher Scientific, Vilnius, Lithuania) and kept at -80ºC until used.

3.4.2 RNA extraction

3.4.2.1 RNA extraction of brain samples

The extraction of total RNA from brain samples, hippocampus and prefrontal cortex, was performed using the RNeasy Lipid Tissue Mini Kit (ref: 74804, Qiagen, Hilden, Germany). Tissue samples are homogenized in QIAzol Lysis Reagent (ref: 79306, Qiagen, Hilden, Germany), that facilitates lysis of fatty issues and inhibit RNases together. After addition of chloroform, the homogenate is separated into aqueous and organic phases by centrifugation. Once finished, three distinct phases are observed, RNA separates into the upper, aqueous phase while (deoxyribonucleic acid) DNA partitions to the interphase and proteins to the lower, organic phase. The upper, aqueous phase is collected, ethanol (70%) is then added to provide appropriate binding conditions. The sample is transferred to a RNeasy Mini spin column, where the total RNA binds to the membrane, with the help of different buffers and any contaminants present are washed away. Finally, high-quality RNA is eluted in RNase-free water (Figure 14).

The concentration of RNA is determined by measuring the absorbance at 260 nm (A_{260}) in the Nanodrop One (ND-ONE-W, Thermo Fisher Scientific, Vilnius, Lithuania). To ensure significance, A_{260} readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 μg of RNA per ml ($A260 = 1 \rightarrow 44$ μg/ml). This relation is valid only for measurements at a neutral pH. Thus, there is the need to dilute RNA sample with a neutral pH buffer, TrisHCl 10 mM (pH=7,5).

RNA quantification calculation:

$$Dilution\ of\ sample = 10\ \mu l\ of\ RNA\ sample + 490 \mu l\ of\ TrisHCl\ (1/50\ \ dilution)$$

$$Concentration\ of\ RNA\ sample = 44\ \ \mu g\ /ml\ x\ A_{260}\ x\ dilution\ factor$$

$$Total\ amount = concentration\ of\ RNA\ sample\ \ x\ volume\ (ml)$$

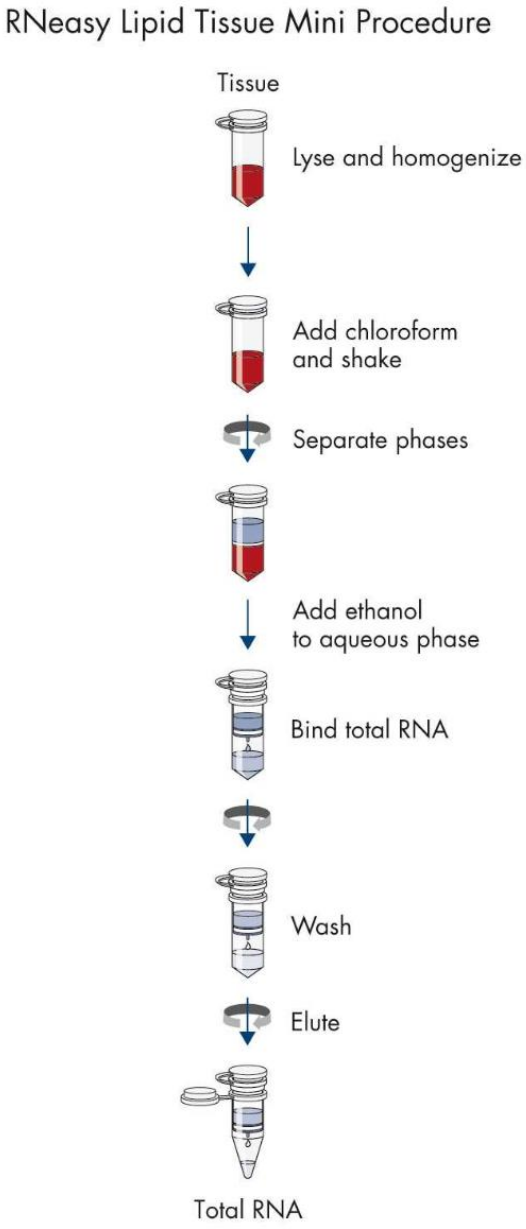


Figure 14. RNA extraction protocol with RNeasy Lipid Tissue Mini Kit. Tissue samples are first homogenized in QIAzol Lysis Reagent and lysis of tissue is performed. Chloroform is then added and after several centrifugations and washes, total RNA is obtained. Adapted from kit manufacturer.

3.4.2.2 RNA and DNA extraction of stool samples

RNA extraction from gut samples was executed using the RNeasy PowerMicrobiome Kit (Qiagen, Hilden, Germany) with the solutions and instructions provided by the manufacturer. Briefly, stool samples are initially processed through a lysis step that uses bead beating and a strong chemical lysis buffer- phenol-chloroform-isoamyl alcohol. After that, solutions from the kit are added and go through several centrifugations and washes. Lastly, RNA and DNA are obtained in a solution with RNAse-free water (Figure 15). DNA obtained, ng/µl, was quantified using the Nanodrop One (ND-ONE-W, Thermo Fisher Scientific, Vilnius, Lithuania), RNase-free water was used as the blank.
Sample preparation was done after quantification, according to the following:

$ci_{(sample)} = quantification\ obtained\ (ng/\mu l)$

$cf = 50\ ng/\mu l$

$vf = 50\ \mu l$

$$vi_{(sample)} = \frac{cf \times vf}{ci}$$

$$v_{H_2o} = 50 - vi_{(sample)}$$

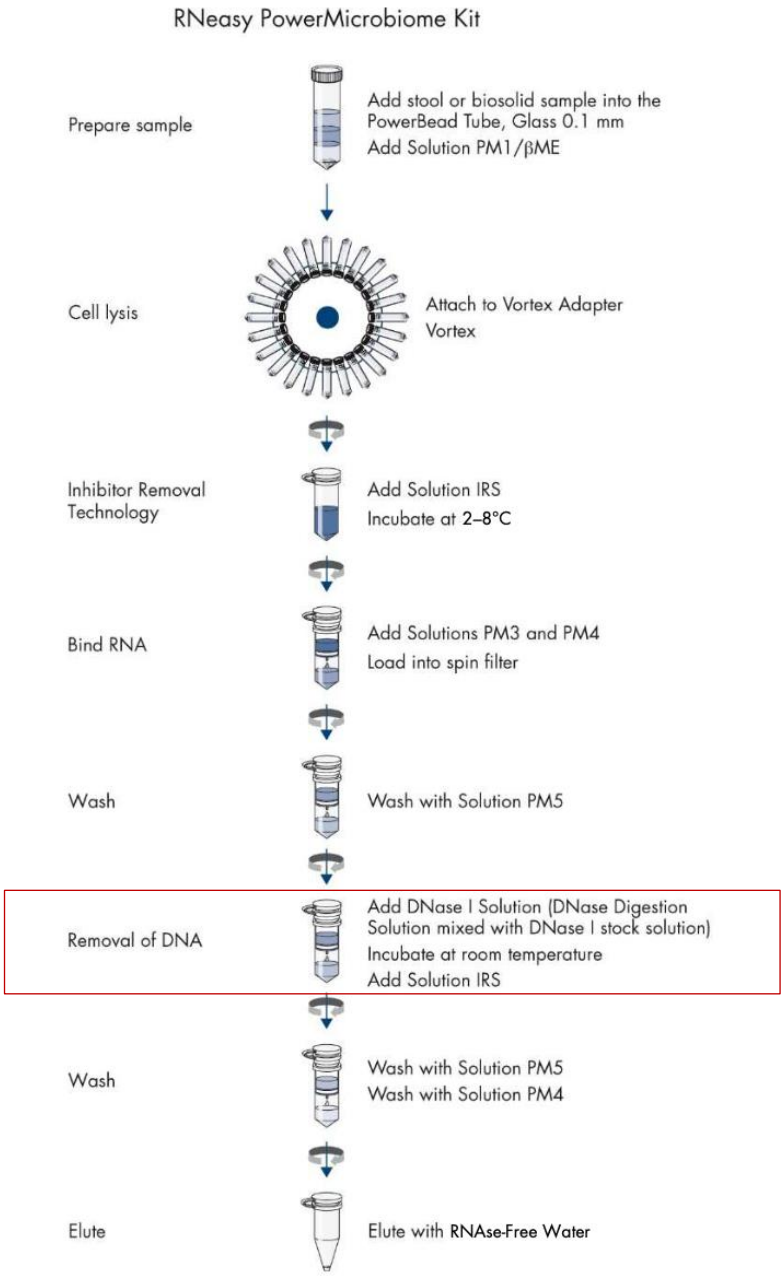


Figure 15. DNA and RNA extraction protocol with RNeasy Power Microbiome Kit. Stool samples are initially processed through a lysis step followed by vortex, samples are added to a Powerbead Tube and a chemical lysis buffer- phenol-chloroform-isoamyl alcohol. Several solutions and centrifugations are performed after. The removal of DNA is an optional step, and was not executed, as we wanted the final product to be both DNA and RNA. The final product is eluted in RNAse-Free Water. Adapted from kit manufacturer

3.4.3 cDNA conversion

RNA is easily prone to cleavage by RNAses leading to degradation. As a result, many gene expression protocols have been developed to use a more stable cDNA product that has been directly synthesized from the RNA. iScript cDNA Synthesis Kit (Bio-Rad Laboratories, California, USA) was used to perform the conversion of RNA obtained from the extraction of brain samples to DNA. Preparation of samples went as the following:

$$4\ \mu l\ 5x\ iScript\ Reaction\ Mix + v\ RNA\ template\ (1\mu g) + v\ Nuclease\ free\ water + 1\ \mu l\ iScript\ Reverse\ Transcriptase = 20\ \mu l$$

$$v\ RNA\ template\ (1\mu g) + v\ Nuclease\ free\ water = 15\ \mu l$$

The Polymerase Chain Reaction (PCR) technique was executed after preparation of samples, in the PCR T100 Thermal Cycler (Bio-Rad Laboratories, California, USA). The protocol had already been optimized to the subsequent conditions (Figure 16):

Volume = 20 μl

1. 25ºC, 5:00 (min)
2. 46ºC, 20:00 (min)
3. 95ºC, 1:00 (min)

Figure 16. PCR T100 Thermal Cycler (Bio-Rad Laboratories, California, USA) and protocol of cDNA conversion.

3.4.4 Real-Time Polymerase Chain Reaction

The cDNA of brain samples and the DNA from the stool samples were analyzed by quantitative real-time PCR (qPCR), with Sybr Green (Bio-Rad Laboratories, California, USA):

$$10\ \mu l\ Sybr\ Green + 6\ \mu l\ Nuclease\ free\ water + 2\ \mu l\ sample + 1\ \mu l\ Primer\ Forward + 1 \mu l\ Primer\ Reverse = 20\ \mu l$$

The primers used are listed in the Table 5 below. Importantly, each primer had already been designed and optimized in the laboratory earlier. For DNA of stool samples, 16S gene was used as housekeeping gene to normalize gene expression levels and Lactobacillus as a gene of interest. As for cDNA of brain samples, hippocampus and prefrontal cortex, the housekeeping gene selected was B2M (Beta-2-Microglobulin) and the genes of interest analyzed were NPY, Y_1 receptor (Y_1R), Y_2 receptor (Y_2R), GABA(A)R, GABA(B)R. qPCR was performed in a Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, California, USA).

Table 5. List of primers and respective sequence

Primer	Primers Sequence (5'-3')	Length (BP)
16S - FWD	ACG TCR TCC MCN CCT TCC TC	20
16S- REV	GTG STG CAY GGY YGT CGT CA	20
LACTO- FWD	GAG GCA GCA GTA GGG AAT CTT	21
LACTO- REV	GGC CAG TTA CTA CCT CTA TCC TTC TTC	27
B2M - FWD	CAT GGC TCG CTC GGT GAC	18
B2M - REV	CAG TTC AGT ATG TTC GGC TTC C	22
NPY - FWD	CAC CAG ACA GAG ATA TGG CAA GA	23
NPY - REV	TGT TCT GGG GGC GTT TTC TG	20
Y_1R - FWD	CCC ATC TGA CTC TCA CAG GC	20
Y_1R - REV	AGC GAA TGT ATA TCT TGA AGT AGC A	25
Y_2R - FWD	CGC AAG AGT CAA TAC AGC CAA	21
Y_2R - REV	CCC ATA GGG CTC CAC TTT CA	20
GABA(A)R- FWD	CAG ATT CAA AGC CAC TGG AGG	20
GAB(A)AR- REV	ATG TTA GCC AGC ACC AAC CT	21
GABA(B)R- FWD	CCC GTC ATG GTT GCT ATG GT	20
GABA(B)R- REV	TAT GCT GGC GAC ATC GAT CC	20

3.5 Quantification of molecular analysis

The quantitative analysis of RT-qPCR is obtained through analysis of the quantification of threshold cycle (C_T= C_q) values. The C_T is the cycle at which the amplification plot crosses the threshold (i.e., there is a significant detectable increase in fluorescence) (Figure 17).

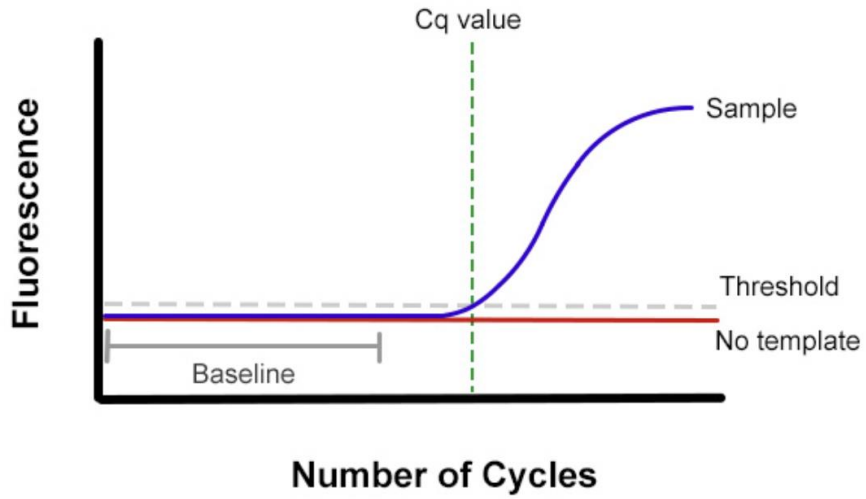


Figure 17. Determining of Cq value. Cq is defined in relation to the baseline level and the beginning of the exponential phase of the reaction curve

Relative quantification determines the ratio between the amount of target and the amount of a control. This ratio is then compared between different samples, the formulas are presented below.

First, we normalized the C_T of the target gene to the one of the endogenous reference (ref) gene, for both the test sample and the calibrator sample (WT treated with saline- control group):

$$\Delta C_{T(test)} = C_{T(target\ gene,test)} - C_{T(endogenous\ ref\ gene)}$$

$$\Delta C_{T(calibrator)} = C_{T(calibrator)} - C_{T(endogenous\ ref\ gene)}$$

Second, we calculate the $\Delta\Delta C_T$ value, the $\Delta\Delta C_T$ describes the difference between the average ΔC_Tvalue of the sample of interest and the average ΔCT value of a calibrator

sample. The calibrator sample and all other samples will be normalized to this when performing relative quantification:

$$\Delta\Delta C_T = average\ \Delta C_{T(sample\ of\ interest)} - \ average\ \Delta C_{T(calibrator\ sample)}$$

Finally, we calculated the expression ratio:

$$2^{-\Delta\Delta C_T} = Normalized\ expression\ ratio$$

The result obtained is the fold increase/decrease of the target gene in the sample relative to the calibrator sample and is normalized to the expression of the endogenous reference gene. Fold change of the calibrator is $\cong 1$ [82].

3.6 Data analysis

Data is expressed as mean values ± SEM. Kruskal-Wallis tests or two-way analysis of variance (ANOVA) were used for the analysis of the video results in the GraphPad Prism (version 8.0, GraphPad Software, San Diego, CA, USA) as indicated in the figure legends Data were considered as statistically significant at $p<0.05$. As for the qPCR analysis, the fold variation in gene expression levels was calculated following a mathematical model, using the formula $2^{-\Delta\Delta Ct}$. The statistical significance was determined using the non-parametric statical Kruskal-Wallis test, with $p<0,05$. Analysis and figures were executed in GraphPad Prism (version 8.0, GraphPad Software, San Diego, CA, USA).

CHAPTER 4- RESULTS

A longitudinal study was designed to investigate the impact of NPY intranasal treatment in core symptoms of ASD using a genetic mouse model of ASD. For that, we established four experimental groups: wild type (WT) with saline (vehicle) treatment; WT with NPY treatment; *Tsc2*$^{+/-}$ with saline (vehicle) treatment; and *Tsc2*$^{+/-}$ with NPY treatment. These animals were handled from an early age and started NPY administration at PN22 until young adulthood. Several behavioral and cognitive tests were performed after the treatment to understand the possible changes induced by NPY.

4.1 Intranasal administration of NPY does not affect body weight and food consumption levels

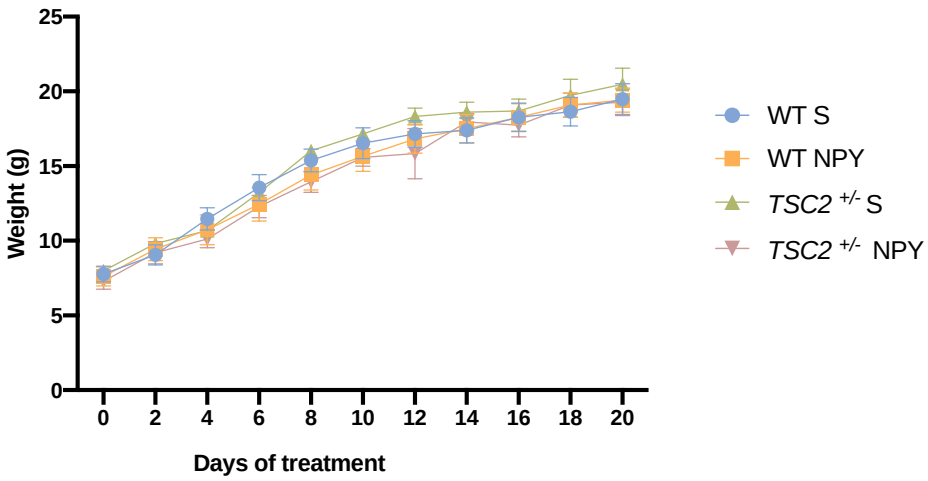
NPY is one the most potent orexigenic peptides found in the brain playing an important role in controlling food intake and body weight.

To investigate the impact of chronic NPY intranasal treatment on body weight, mice were measured every 2 days over 20 days of treatment. NPY treated WT and *Tsc2*$^{+/-}$ mice did not present significant body weight differences compared to control groups (Figure 18; $p>0.05$; n= 8-11).

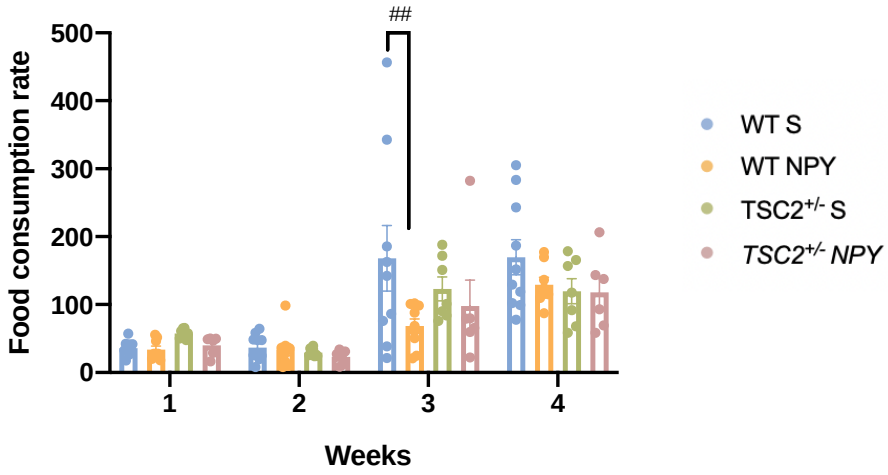
Moreover, no significant differences in food consumption between groups was observed (Figure 18b; $p>0.05$; n= 8-11). Also, no significant differences in food consumption rate between treated and non-treated, transgenic or wild-type animal groups were observed on week 1,2 and 4 ($p>0.05$; n= 8-11). However, saline WT animals display a significantly increased food consumption in comparison to WT administered with NPY (168.01 ± 144.89 WT S vs 68.42 ± 31.63 WT NPY, $p= 0.0012$; n=11) on week 3 (Figure 18c).

The absence of significance between treated and non-treated, transgenic or WT animal groups could be the result of the lack of NPY capability to stimulate food intake.

a)



b)



c)

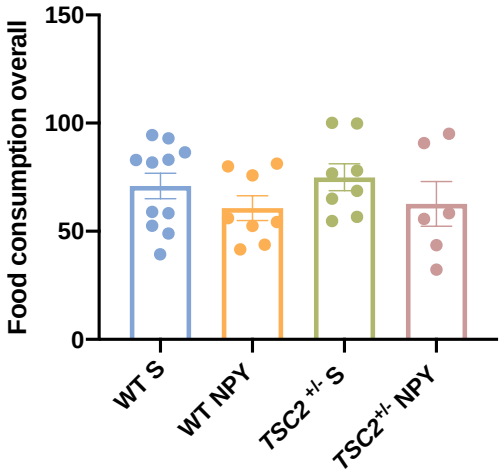


Figure 18. NPY administration does not affect body weight and weekly food consumption. (a) Body weight measured every 2 days of treatment for a total of 20 days. (b) Food consumption rate calculated per week based on the initial amount of food and the final amount of food left and the number of animals per cage. (c) Food consumption overall based on the food intake and the weight

gained. Results are presented by mean ± SEM. The number of animals used was: 11 WT treated with saline, 11 WT treated with NPY, 8 *Tsc2*$^{+/-}$ treated with saline, 8 *Tsc2*$^{+/-}$ treated with NPY. $^{\#\#}p>$ 0.01- 2way ANOVA, significantly different between WT treated with saline and WT treated with NPY. Abbreviations: WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, *Tsc*2$^{+/-}$ S - *Tsc*2$^{+/-}$ treated with saline; *Tsc*2$^{+/-}$ NPY - *Tsc*2$^{+/-}$ treated with neuropeptide Y.

4.2 Administration of NPY in wild-type animals results in a behavior less exploratory and more anxious

Following NPY treatment, a battery of behavioral tests was performed. First, the elevated plus maze test, was performed to assess anxiety-like behavior.
Comparing the entries, time and distance in both open arm and closed arm, all groups showed a significant increased number of entrances on the closed arm (Table 6).

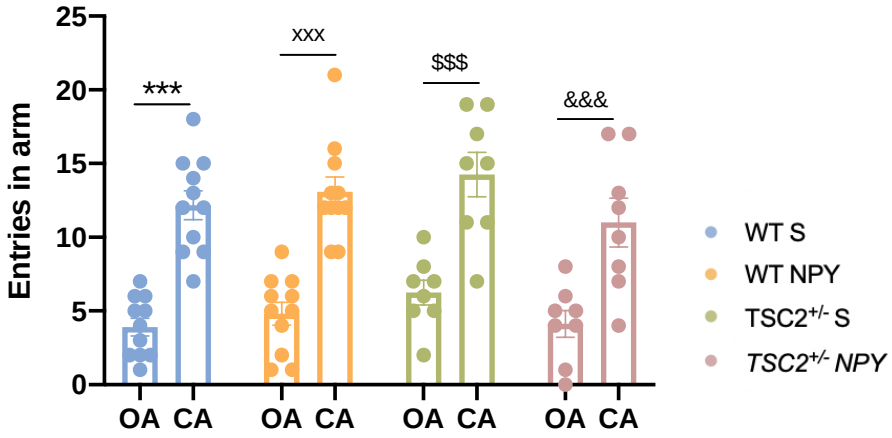
Table 6. Elevated plus maze results- number of entries, time in zone and distance in zone

EPM	Open Arms (mean ± SEM)	Closed Arms (mean ± SEM)	p
Entries	WT S = 3.91 ± 2.02	WT S = 12.18 ± 3.25	<0.0001
	WT NPY = 4.82 ± 2.60	WT NPY = 13.09 ± 3.36	<0.0001
	Tsc2$^{+/-}$ S = 6.25 ± 2.38	*Tsc2*$^{+/-}$ S = 14.25 ± 4.27	0.0001
	Tsc2$^{+/-}$ NPY = 4.13 ± 2.59	*Tsc2*$^{+/-}$ NPY = 11.00 ± 4.66	0.0013
Time in zone	WT S = 32.89 ± 17.80	WT S = 225.42 ± 33.29	<0.0001
	WT NPY = 29.94 ± 24.81	WT NPY = 212.36 ± 36.59	<0.0001
	Tsc2$^{+/-}$ S = 38.37 ± 12.76	*Tsc2*$^{+/-}$ S = 202.12 ± 27.82	<0.0001
	Tsc2$^{+/-}$ NPY = 30.98 ± 18.04	*Tsc2*$^{+/-}$ NPY= 18.04 ± 47.03	<0.0001
Distance in zone	WT S = 173.95 ± 48.84	WT NPY = 66.52 ± 47,18	0,0302
	WT S = 173.95 ± 48.84	WT S = 519.31 ± 98.53	<0.0001
	WT NPY = 93.25 ± 42.42	WT NPY = 490.50 ± 124.68	<0.0001
	Tsc2$^{+/-}$ S = 178.58 ± 73.26	*Tsc2*$^{+/-}$ S = 522.15 ± 211.45	<0.0001
	Tsc2$^{+/-}$ NPY = 131.10 ± 64.01	*Tsc2*$^{+/-}$ NPY= 456.01 ± 109.10	<0.0001

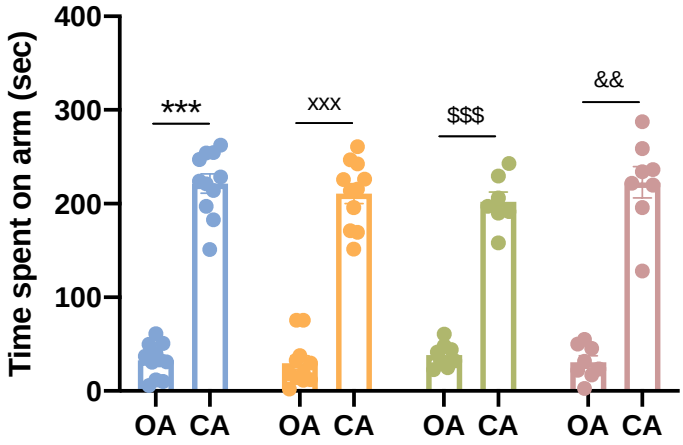
Abbreviations: EPM- Elevated plus maze

Moreover, we observed there are no differences between treatment and genetic groups concerning entries and time spent in both open and closed arms (Figure 19a and b; $p>0.05$; n=8-11). Concerning, distance traveled in open and closed arms, we found that WT mouse administered with NPY explored less the open arm than saline WT mouse NPY (Figure 19c; 173.95 ± 48.84 WT S OA vs. 66.52 ± 47,18 WT NPY OA, $p= 0,0302$; n=8).

a)



b)



c)

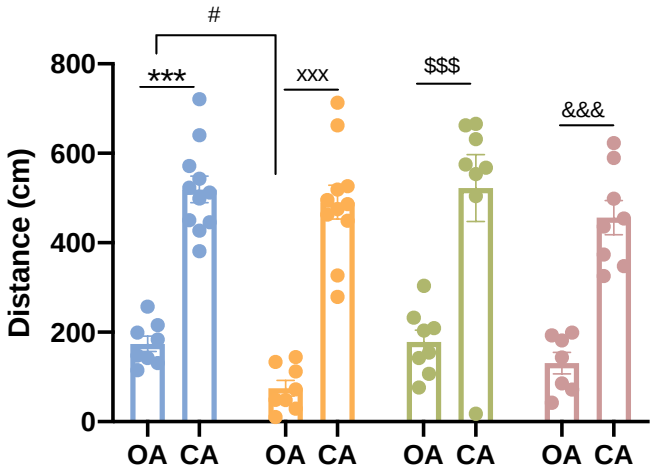


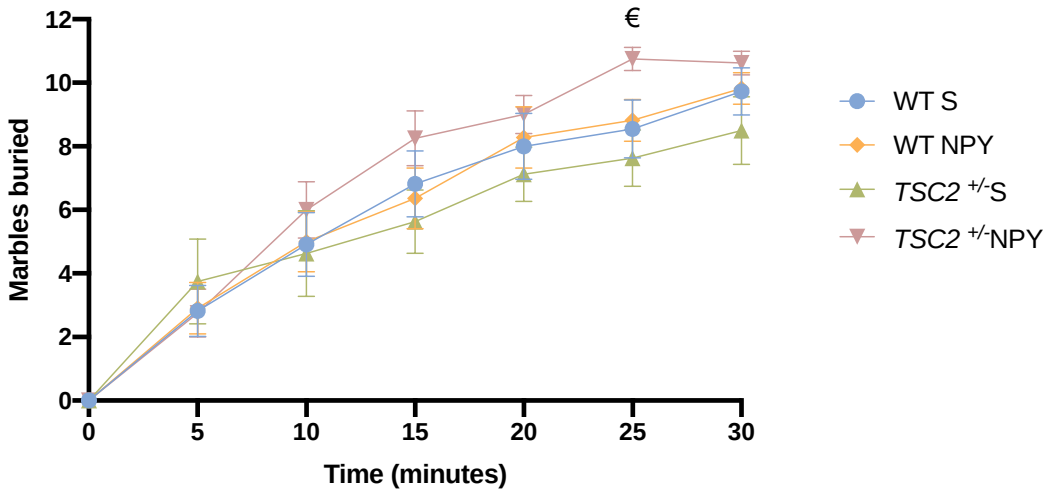
Figure 19. Elevated plus maze test show that WT mice treated with NPY travelled less in the open arms, showing a more anxious behavior. (a) Number of entries in open arm and closed arm (b) Time spent in open arm and closed arm. (c) Distance travelled in open arm and closed arm. Results are presented by mean ± SEM. The number of animals used was: 11 WT treated with saline, 11 WT treated with NPY, 8 *Tsc2*$^{+/-}$ treated with saline, 8 *Tsc2*$^{+/-}$ treated with NPY. $^{***}p$> 0.001- 2way ANOVA, significantly different in number of entries, time spent and distance travelled between WT treated with saline in OA and WT treated with saline in CA. ^{xxx}p> 0.001- 2way ANOVA, significantly different in number of entries, time spent and distance travelled between WT treated with NPY in OA and WT treated with NPY in CA. $^{\$\$\$}p$> 0.001- 2way ANOVA, significantly different in number of entries, time spent and distance travelled between *Tsc2*$^{+/-}$ treated with saline in OA and *Tsc2*$^{+/-}$ treated with saline in CA. $^{\&\&\&}p$> 0.001- 2way ANOVA, significantly different in number of entries and distance traveled between Tsc2$^{+/-}$ treated with NPY in OA and *Tsc2*$^{+/-}$ treated with NPY in CA. $^{\&\&}p$> 0.01- 2way ANOVA, significantly different in the time spent between *Tsc2*$^{+/-}$ treated with NPY in OA and *Tsc2*$^{+/-}$ treated with NPY in CA. $^{\#}p$> 0.05- 2way ANOVA, significantly different in distance travelled between WT treated with saline in OA and WT treated with NPY in OA. Abbreviations: OA- open arm, CA-closed arm, WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, *Tsc2*$^{+/-}$ S - *Tsc2*$^{+/-}$ treated with saline; *Tsc2*$^{+/-}$ NPY - *Tsc2*$^{+/-}$ treated with neuropeptide Y.

4.3 Tsc2$^{+/-}$ mice administered with NPY display significant repetitive behaviors

Next, we explored how the treatment on each of the groups affected repetitive/ stereotyped behavior by performing the marble burying test.

Overall analysis of the number of marbles buried over time indicates significant differences between *Tsc2*$^{+/-}$ administered with NPY when compared to *Tsc2*$^{+/-}$ saline treatment (Figure 20a and b; 7.63 ± 2.50 *Tsc2*$^{+/-}$ S vs. 10.75 ± 1.04 *Tsc2*$^{+/-}$ NPY, *p*= 0,0286; n=8).

a)



b)

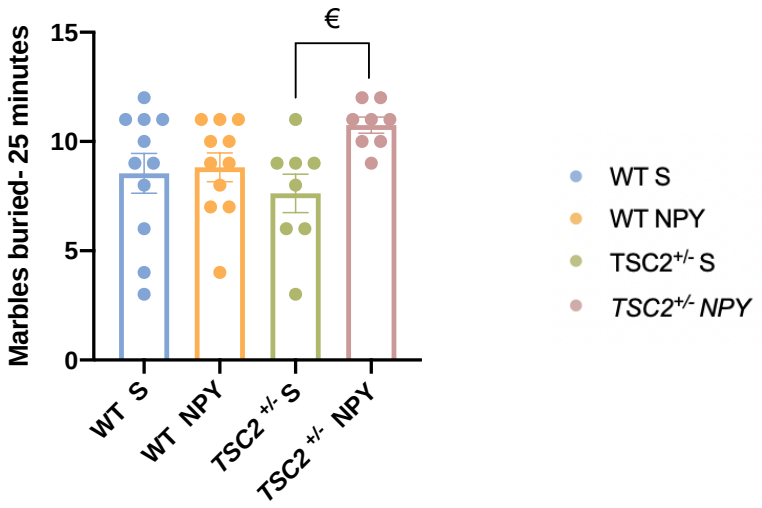
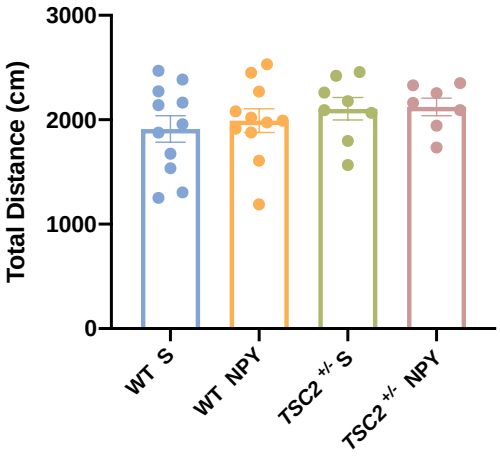


Figure 20. Repetitive behavior assessment show that at 25 minutes, *Tsc2*$^{+/-}$ mice treated with NPY significantly buried more marbles than *Tsc2*$^{+/-}$ treated with saline. (a) Number of marbles buried overtime (b) Marbles buried at 25 minutes. Results are presented by mean ± SEM. The number of animals used was: 11 WT treated with saline, 11 WT treated with NPY, 8 Tsc2$^{+/-}$ treated with saline, 8 Tsc2$^{+/-}$ treated with NPY. $^{€}p$> 0.05- Kruskal-Wallis, significantly different between *Tsc2*$^{+/-}$ treated with saline and *Tsc2*$^{+/-}$ treated with NPY. Abbreviations: WT S- wild type treated with saline, WT

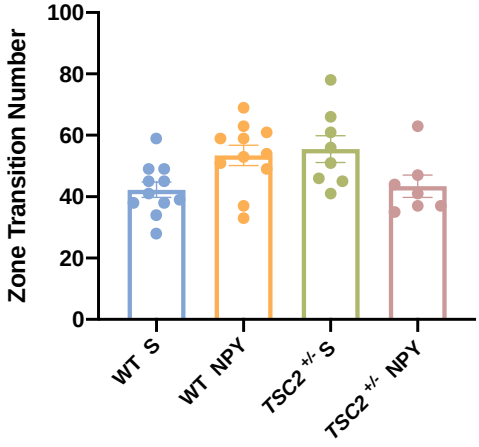
NPY- wild type treated with neuropeptide Y, *Tsc2*$^{+/-}$ S - *Tsc2*$^{+/-}$ treated with saline; *Tsc2*$^{+/-}$ NPY - *Tsc2*$^{+/-}$ treated with neuropeptide Y

Three-chamber apparatus was used as open filed during the habituation phase. Data recorded, was analyzed for total distance travelled, zone transition number, rearing and stretching number and duration. Concerning the total distance travelled no significant changes were noted between groups (Figure 21a; $p>0.05$; n= 7-11). Zone transitions between groups were not significant (Figure 21b; $p>0.05$; n= 7-11). Rearing duration was not significant between groups ($p>0.05$; n= 7-11), nonetheless *Tsc2*$^{+/-}$ NPY displayed a significantly higher number of rearing compared to WT NPY (Figure 21c and d; 265.5 ± 71.37 *Tsc2*$^{+/-}$ NPY vs. 137.2 ± 86.31 WT NPY, $p= 0.0495$; n=6-10). *Tsc2*$^{+/-}$ NPY exhibited a significantly higher duration of stretching compared to WT NPY (Figure 21c and d; 112.49 ± 46.79 *Tsc2*$^{+/-}$ NPY vs. 42.53 ± 32.09WT NPY, $p= 0.0406$; n=6-11). Contrary to stretching duration, the number of stretching was not significant between groups (Figure 21e and f; $p >0.05$; n= 7-11).

a)



b)



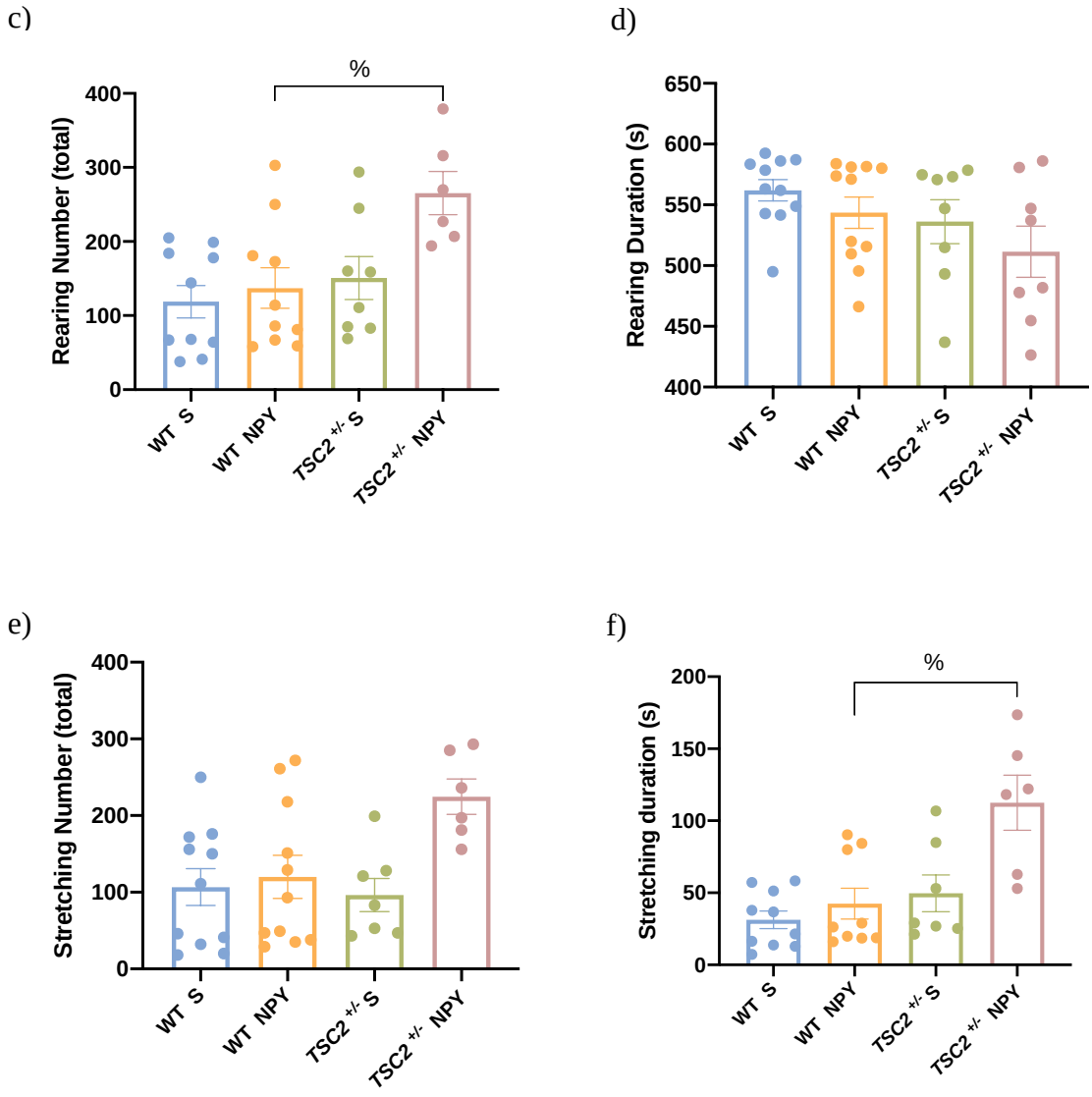
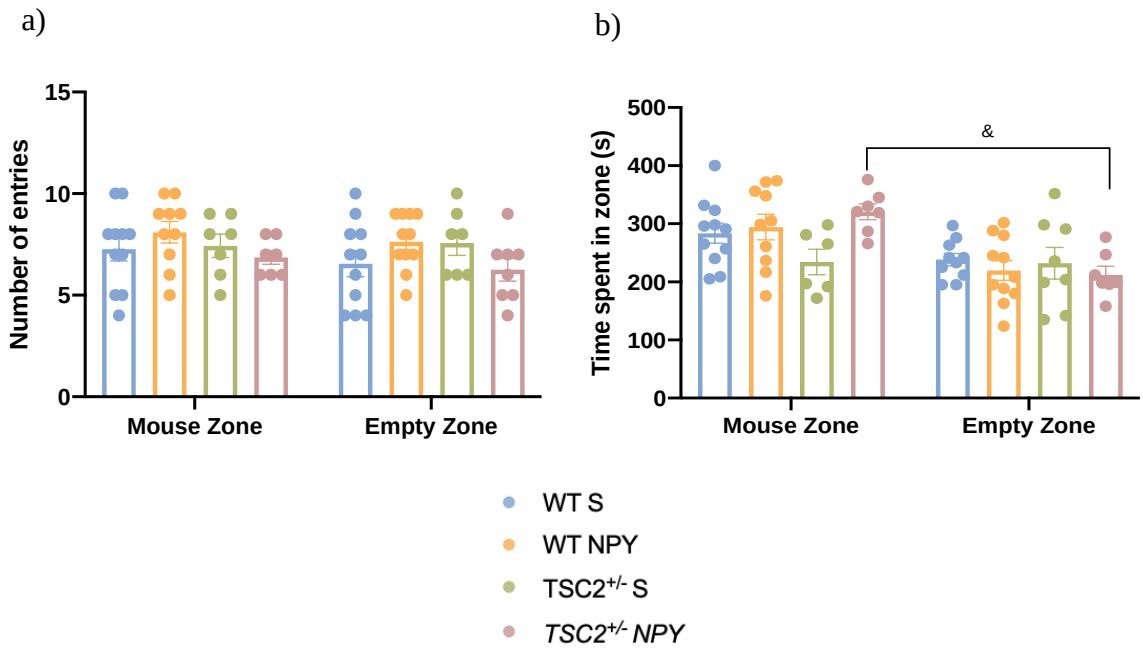


Figure 21. Open field test performed on habituation phase of three-chamber social test, shows a more anxious behavior in *Tsc2*$^{+/-}$ NPY. (a) Total distance traveled during the 10 minutes (b) Number of zone transitions (c) Rearing number (d) Duration of the rearing (e)Stretching number (f) Stretching duration. Results are presented by mean ± SEM. The number of animals used was: 11 WT treated with saline, 11 WT treated with NPY, 8 Tsc2$^{+/-}$ treated with saline, 8 *Tsc2*$^{+/-}$ treated with NPY. $^{\%}p>$ 0.05-Kruskal-Wallis, significantly different between WT treated with NPY and *Tsc2*$^{+/-}$ treated with NPY. Abbreviations: WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, *Tsc2*$^{+/-}$ S - *Tsc2*$^{+/-}$ treated with saline; *Tsc2*$^{+/-}$ NPY - *Tsc2*$^{+/-}$ treated with neuropeptide Y

4.4 Tsc2$^{+/-}$ mice administered with NPY showed an increase in social interaction

For assessing social impairment phenotypes in our experimental groups, we performed the three-chamber social test. The testing procedure consisted of three chronological phases: habituation (results showed above), sociability, and social novelty.

Concerning the sociability phase, there were no significant differences between groups in the number of entries on the mouse zone vs empty zone (non-social zone) (Figure 22a; p>0.05; n= 7-11). Concerning the time spent on one zone than the other, *Tsc2*$^{+/-}$ NPY spent significantly more time on the mouse zone than on the empty (320.57 ± 36.21 *Tsc2*$^{+/-}$ NPY social vs 212.43 ± 38.70 *Tsc2*$^{+/-}$ NPY empty, p= 0.0120; n=7). As for the rest of the groups, no significant changes were observed (Figure 22b; p>0.05; n= 7-11). Significant differences between the time spent interacting with mouse cage than the empty cage were observed in *Tsc2*$^{+/-}$ S (161.63 ± 53.74 *Tsc2*$^{+/-}$ S social vs 87.14 ± 27.28 *Tsc2*$^{+/-}$ S empty, p= 0.0297; n=8) and *Tsc2*$^{+/-}$ NPY (184.57 ± 59.66 *Tsc2*$^{+/-}$ NPY social vs 97.63 ± 42.66 *Tsc2*$^{+/-}$ NPY empty, p= 0.0057; n=7). Within the time spent interacting with the social stimulus, there were no significant differences between groups (Figure 22c; p>0.05; n= 7-11). Results regarding the social preference show no significant differences among experimental groups (Figure 22d; p>0.05; n= 7-11).



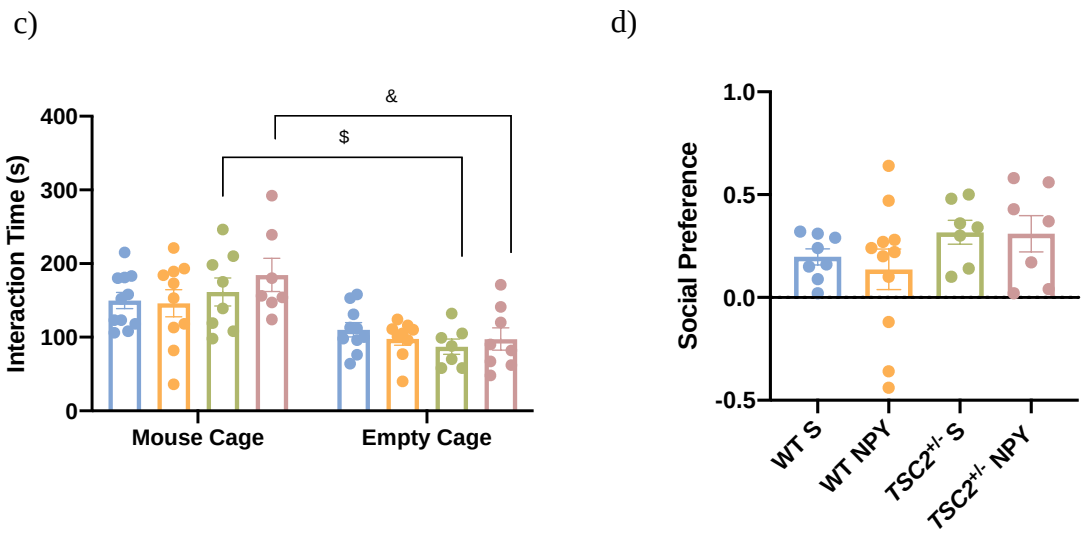


Figure 22. Time spent in mouse zone versus empty zone is significantly higher in *Tsc2*$^{+/-}$ mice treated with NPY in social preference test. (a) Time spent in the zone with the social stimulus vs in the zone with the non-social stimulus (b) Number of entries in each zone (c) Time spent interacting with the mouse or the empty cage (d) Social preference. Results are presented by mean ± SEM. The number of animals used was: 11 WT treated with saline, 11 WT treated with NPY, 8 *Tsc2*$^{+/-}$ treated with saline, 8 *Tsc2*$^{+/-}$ treated with NPY. $^{\&}p$> 0.05- Kruskal-Wallis, significantly different the time spent and interaction time between *Tsc2*$^{+/-}$ treated with NPY in the mouse and *Tsc2*$^{+/-}$ treated with NPY in the empty zone. $^{\$}p$> 0.05- Kruskal-Wallis, significantly different the interaction time between *Tsc2*$^{+/-}$ treated with saline in the mouse cage and *Tsc2*$^{+/-}$ treated with saline in the empty cage. Abbreviations: WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, *Tsc2*$^{+/-}$ S - *Tsc2*$^{+/-}$ treated with saline; *Tsc2*$^{+/-}$ NPY - *Tsc2*$^{+/-}$ treated with neuropeptide Y

As for the number of entries on the familiar zone and in the novel zone, no significant differences were observed between groups (Figure 23a; p>0.05; n= 7-11). Results from the time spent on each chamber, show no differences between groups spending more time on one chamber than the other (Figure 23b; p>0.05; n= 7-11).

Regarding the time that the experimental animal interacted with the novel or the familiar animal, no significant differences were observed among groups (Figure 23c; p>0.05; n= 7-11). The social novelty preference measured the experimental animal preference to interact with the novel stimulus over the familiar stimulus. The results obtained were quiet disperse and no significance was obtained between the groups (Figure 23d; p>0.05; n= 7-11).

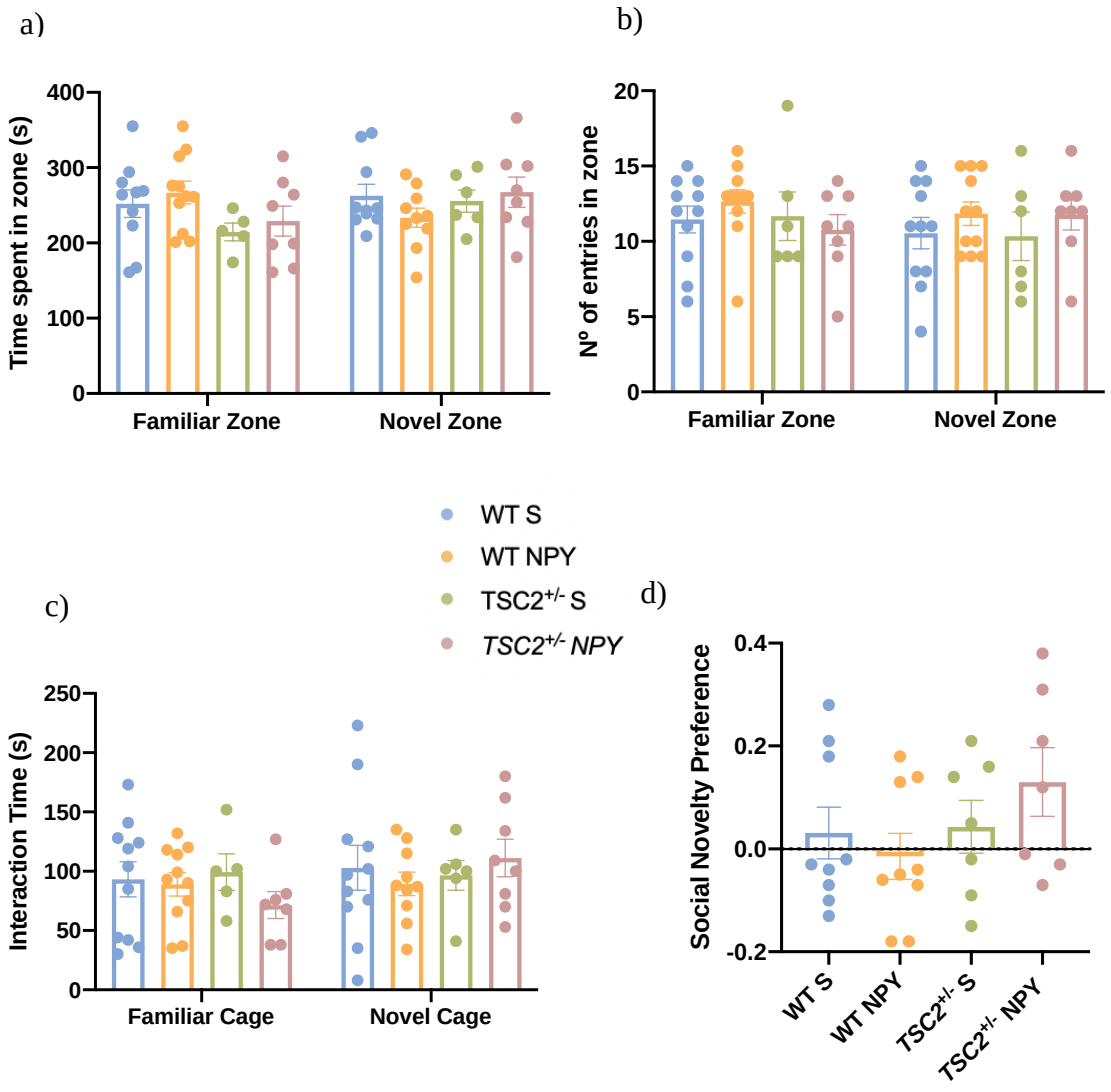


Figure 23. Social novelty preference test of 3 Chamber social test shows no significant differences. (a) Time spent in the zone with the familiar animal vs in the zone with the novel mouse (b) Number of entries in each zone (c) Time spent interacting with the familiar mouse or the novel mouse (d) Social novelty preference. Results are presented by mean ± SEM. The number of animals used was: 11 WT treated with saline, 11 WT treated with NPY, 8 *Tsc2*$^{+/-}$ treated with saline, 8 *Tsc2*$^{+/-}$ treated with NPY. $^{\%}p$> 0.05- Kruskal-Wallis, significantly different between WT treated with NPY and *Tsc2*$^{+/-}$ treated with NPY. Abbreviations: WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, *Tsc2*$^{+/-}$ S - *Tsc2*$^{+/-}$ treated with saline; *Tsc2*$^{+/-}$ NPY - *Tsc2*$^{+/-}$ treated with neuropeptide Y

4.5 Lactobacillus was not significantly changed between groups

Stool samples were analyzed for the genera *Lactobacillus*, since our group previously observed changes in these bacteria in another animal model for autism, mouse *Nf1*$^{+/-}$. Nevertheless, in this study, no significant differences were observed between groups (Figure 24; p>0.05; n= 6-10). Relative gene expression (fold change) of the genes of interest – WT with NPY treatment, *Tsc2* $^{+/-}$ with saline treatment, *Tsc2* $^{+/-}$ with NPY treatment (Group 2)- and the comparison to control group- WT with saline treatment (Group 1)- is present in table 7.

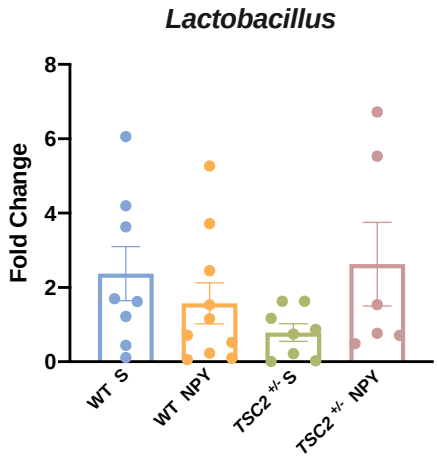


Figure 24. Real time PCR was performed on microbial DNA from the stool samples of transgenic and wild type mice for the dysregulation of Lactobacillus. Total 16S DNA was used as endogenous control. Results are presented by mean ± SEM. The number of animals used was: 8 WT treated with saline, 11 WT treated with NPY, 8 *Tsc2*$^{+/-}$ treated with saline, 8 *Tsc2*$^{+/-}$ treated with NPY. Kruskal-Wallis test for statistical analysis. Abbreviations: WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, *Tsc2*$^{+/-}$ S - *Tsc2*$^{+/-}$ treated with saline; *Tsc2*$^{+/-}$ NPY - *Tsc2*$^{+/-}$ treated with neuropeptide Y

Table 7. RT PCR on stool samples for the genera *Lactobacillus*

Gene of interest	Group 1 (mean ± SEM)	Group 2 (mean ± SEM)	Fold change	p
Lactobacillus	WT S = 1.46 ± 1.24	WT NPY= 1.58 ± 1.74	Increase	>0.05
		Tsc2$^{+/-}$ S= 0.79 ± 0.66	Decrease	>0.05
		Tsc2$^{+/-}$ NPY= 2.63 ± 2.76	Increase	>0.05

Abbreviations: WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, *Tsc2*$^{+/-}$ S - *Tsc2*$^{+/-}$ treated with saline; *Tsc2*$^{+/-}$ NPY - *Tsc2*$^{+/-}$ treated with neuropeptide Y, NPY – neuropeptide Y

4.6 Cortical Y_2 receptors are changed in wild-type animals after NPY administration

We performed rt-PCR on hippocampal and cortical samples for the following genes of interest: NPY, Y_1 and Y_2 receptors, GABA(A) and GABA(B) receptors. Hippocampal levels of NPY, Y_1 and Y_2 receptors did not show significant changes in these genes were observed between experimental groups (Figure 25a, b and c; p>0.05; n= 6-11). Also, GABA(A) R and GABA(B) R receptors presented no difference in this study. Nevertheless, *Tsc2*$^{+/-}$ mice administered with NPY tended to display a decreased in GABA(A) R expression compared to mutant mice with saline (Figure 25a; 1.44 ± 0.45 *Tsc2*$^{+/-}$ S vs 0.78 ± 0.39 *Tsc2*$^{+/-}$ NPY; p= 0.0538; n=7).

Table 8, compilates the different folds (average) in the samples of interest - WT with NPY treatment, *Tsc2*$^{+/-}$ with saline treatment, *Tsc2*$^{+/-}$ with NPY treatment (Group 2) in comparison to the control group- WT with saline treatment (Group 1) for samples of hippocampus.

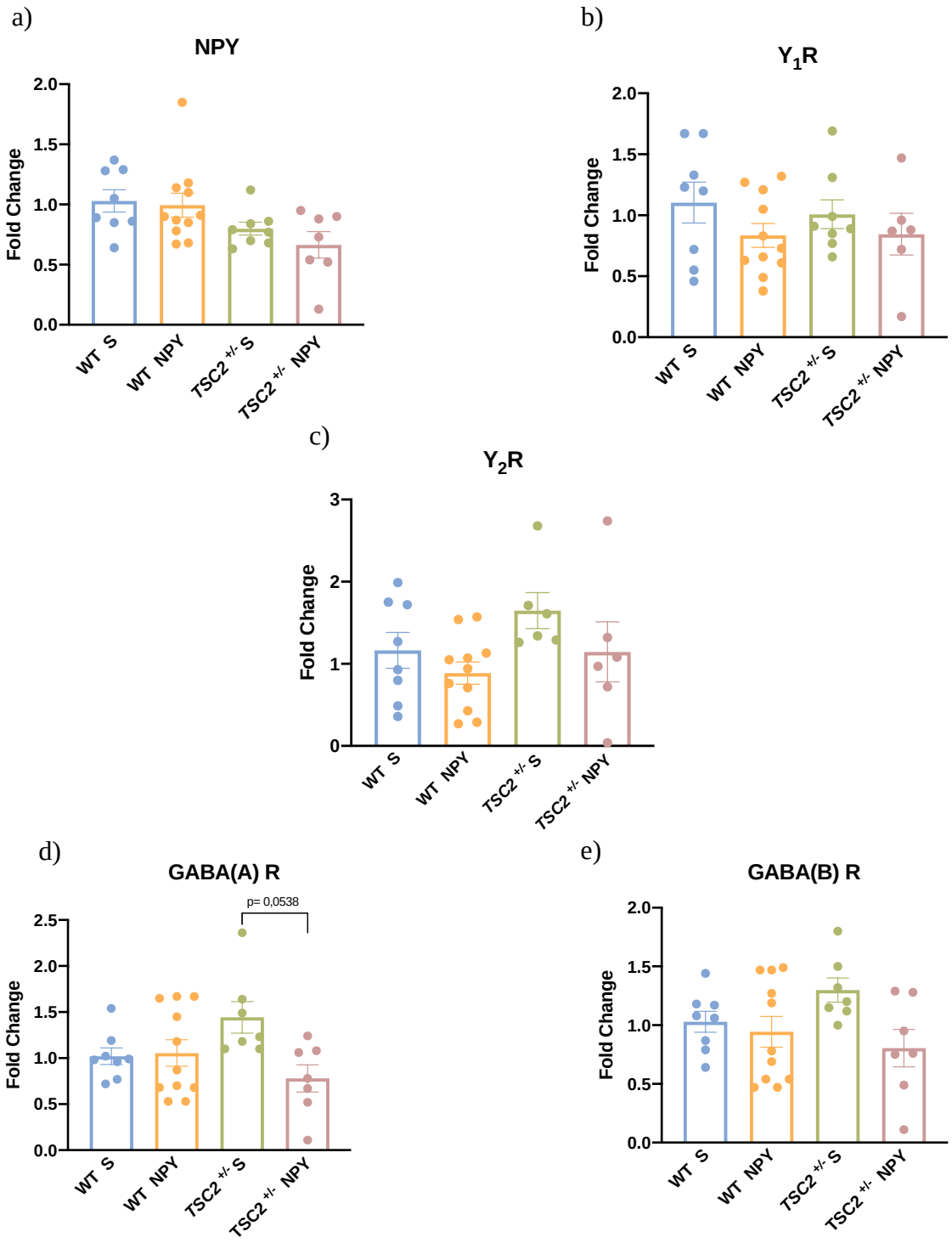


Figure 25. Real time PCR was performed on cDNA from the hippocampal samples of transgenic and wild type mice for changes in NPY, Y1R, Y2R, GABA(A) R and GABA(B) R. B2M was used as endogenous control. Results are presented by mean ± SEM. The number of animals used was: 8 WT treated with saline, 11 WT treated with NPY, 8 *Tsc2*$^{+/-}$ treated with saline, 8 *Tsc2*$^{+/-}$ treated with NPY. Kruskal-Wallis test for statistical analysis. p=0.0538- *Tsc2*$^{+/-}$ mice administered with NPY tend

to display a decreased in GABA(A) R expression compared to mutant mice with saline. Abbreviations: WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, *Tsc2*$^{+/-}$ S - *Tsc2*$^{+/-}$ treated with saline; *Tsc2*$^{+/-}$ NPY - *Tsc2*$^{+/-}$ treated with neuropeptide Y, NPY – neuropeptide Y, Y1R- Y1 receptor, Y2R- Y2 receptor, GABA(A) R- GABA(A) receptor, GABA(B) R- GABA(B) receptor

Table 8. Real Time PCR on hippocampal samples for genes of interest: NPY, Y_1R, Y_2R, GABA(A) R and GABA(B) R.

Gene of interest	Group 1 (mean ± SEM)	Group 2 (mean ± SEM)	Fold	p
NPY	WT S = 1.03 ± 0.26	WT NPY= 0.99 ± 0.33	Decrease	>0.9999
		Tsc2$^{+/-}$ S= 0.80 ± 0.15	Decrease	0.3189
		Tsc2$^{+/-}$ NPY= 0.66 ± 0.29	Decrease	0.2273
Y_1R	WT S = 1.10 ± 0.48	WT NPY= 0.86 ± 0.34	Decrease	>0.9999
		Tsc2$^{+/-}$ S= 1.01 ± 0.33	Decrease	>0.9999
		Tsc2$^{+/-}$ NPY= 0.85 ± 0.42	Decrease	>0.9999
Y_2R	WT S = 1.16 ± 0.61	WT NPY= 0.89 ± 0.45	Decrease	>0.9999
		Tsc2$^{+/-}$ S= 1.65 ± 0.54	Increase	>0.9999
		Tsc2$^{+/-}$ NPY= 1.15 ± 0.90	Decrease	>0.9999
GABA(A) R	WT S = 1.02 ± 0.26	WT NPY= 1.06 ± 0.48	Increase	>0.9999
		Tsc2$^{+/-}$ S= 1.44 ± 0.45	Increase	0.4742
		Tsc2$^{+/-}$ NPY= 0.78 ± 0.39	Decrease	>0.9999
GABA(B) R	WT S = 1.03 ± 0.25	WT NPY= 0.94 ± 0.43	Decrease	>0.9999
		Tsc2$^{+/-}$ S= 1.30 ± 0.27	Increase	0.9327
		Tsc2$^{+/-}$ NPY= 0.80 ± 0.42	Decrease	>0.9999

Abbreviations: WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, *Tsc2*$^{+/-}$ S - *Tsc2*$^{+/-}$ treated with saline; *Tsc2*$^{+/-}$ NPY - *Tsc2*$^{+/-}$ treated with neuropeptide Y, NPY – neuropeptide Y, Y1R- Y1 receptor, Y2R- Y2 receptor, GABA(A) R- GABA(A) receptor, GABA(B) R- GABA(B) receptor

Prefrontal cortex results were not significant between groups for NPY, Y_1 R, GABA(A) and GABA(B) receptors (Figure 26a, b, d and e; $p > 0.05$; n= 6-11). Yet, Y_2R results show

a significant decrease in fold change between WT NPY and WT S (Figure 26 c; 1.45 ± 0.30 WT S vs 0.80 ± 0.38 WT NPY, p= 0.0319). Table 9, compilates the different folds (average) in the samples of interest - WT with NPY treatment, *Tsc2*$^{+/-}$ with saline treatment, *Tsc2*$^{+/-}$ with NPY treatment (Group 2) in comparison to the control group- WT with saline treatment (Group 1) for samples of cortex.

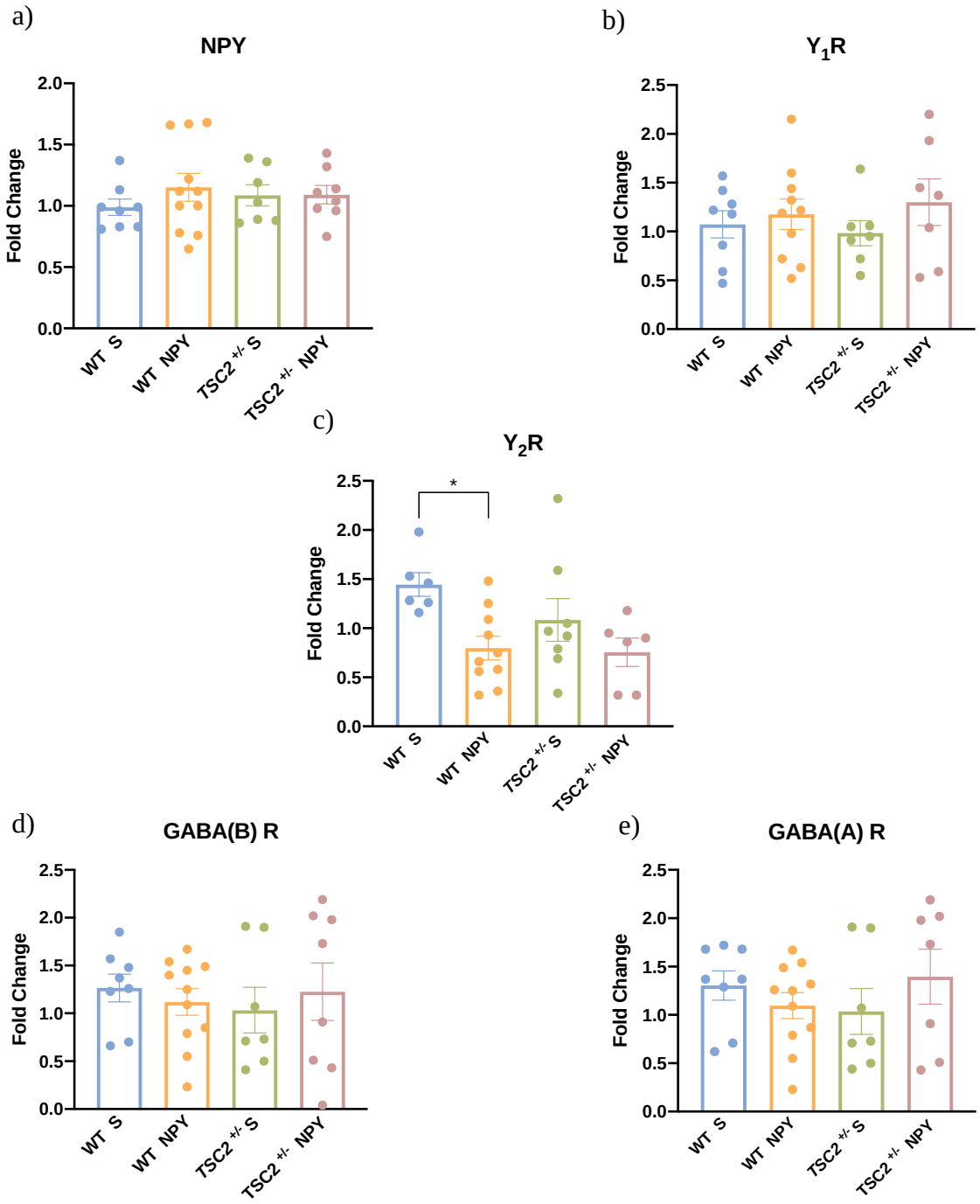


Figure 26. Real time PCR was performed on cDNA from cortex samples of transgenic and wild type mice for changes in NPY, Y1R, Y2R, GABA(A) R and GABA(B) R. B2M was used as

endogenous control. Results are presented by mean ± SEM. The number of animals used was: 8 WT treated with saline, 11 WT treated with NPY, 8 *Tsc2*$^{+/-}$ treated with saline, 8 *Tsc2*$^{+/-}$ treated with NPY. Kruskal-Wallis test for statistical analysis. $^{*}p<0.05$- Kruskal-Wallis, significantly different between WT treated with saline and WT treated with NPY in the Y_2R. Abbreviations: WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, *Tsc2*$^{+/-}$ S - *Tsc2*$^{+/-}$ treated with saline; *Tsc2*$^{+/-}$ NPY - *Tsc2*$^{+/-}$ treated with neuropeptide Y, NPY – neuropeptide Y, Y1R- Y1 receptor, Y2R- Y2 receptor, GABA(A) R- GABA(A) receptor, GABA(B) R- GABA(B) receptor

Table 9. RT PCR on cortical samples for genes of interest: NPY, Y_1R, Y_2R, GABA(A) R and GABA(B) R.

Gene of interest	Group 1 (mean ± SEM)	Group 2 (mean ± SEM)	Fold	p
NPY	WT S = 0.99 ± 0.19	WT NPY= 1.15 ± 0.37	Increase	>0.9999
		Tsc2$^{+/-}$ S= 1.09 ± 0.23	Increase	>0.9999
		Tsc2$^{+/-}$ NPY= 1.09 ± 0.21	Increase	>0.9999
Y_1R	WT S = 1.07 ± 0.39	WT NPY= 1.18 ± 0.49	Increase	>0.9999
		Tsc2$^{+/-}$ S= 0.98 ± 0.34	Decrease	>0.9999
		Tsc2$^{+/-}$ NPY= 1.30 ± 0.63	Increase	>0.9999
Y_2R	WT S = 1.45 ± 0.30	WT NPY= 0.80 ± 0.38	Decrease	0,0319
		Tsc2$^{+/-}$ S= 1.08 ± 0.61	Decrease	>0.9999
		Tsc2$^{+/-}$ NPY= 0.76 ± 0.35	Decrease	0.0429
GABA(A) R	WT S = 1.31 ± 0.43	WT NPY= 1.10 ± 0.44	Decrease	>0.9999
		Tsc2$^{+/-}$ S= 1.04 ± 0.63	Decrease	>0.9999
		Tsc2$^{+/-}$ NPY= 1.40 ± 0.76	Increase	>0.9999
GABA(B) R	WT S = 1.27 ± 0.41	WT NPY= 1.12 ± 0.46	Decrease	>0.9999
		Tsc2$^{+/-}$ S= 1.03 ± 0.63	Decrease	>0.9999
		Tsc2$^{+/-}$ NPY= 1.23 ± 0.85	Decrease	>0.9999

Abbreviations: WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, *Tsc2*$^{+/-}$ S - *Tsc2*$^{+/-}$ treated with saline; *Tsc2*$^{+/-}$ NPY - *Tsc2*$^{+/-}$ treated with neuropeptide Y, NPY – neuropeptide Y, Y1R- Y1 receptor, Y2R- Y2 receptor, GABA(A) R- GABA(A) receptor, GABA(B) R- GABA(B) receptor

4.7 Sex segregation did not show any significant differences between groups in behavior and molecular analysis

Sex segregation analysis was also performed to understand how the results could vary when separated by sexes. Because of the reduced number of animals in each group (table 10), the results were quiet disperse and no significant differences were observed on the behavioral tests and on the molecular analysis (Annexes p >0.05, n=2-7).

Table 10. Group of treatment, sex and number of animals

Group	Sex	Nº animal
WT S	M	4
	F	7
WT NPY	M	6
	F	5
Tsc2$^{+/-}$ S	M	6
	F	2
Tsc2$^{+/-}$ NPY	M	4
	F	4

Abbreviations: F- female, M-male, WT S- wild-type mice treated with saline, WT NPY- wild-type mice treated with neuropeptide Y, *Tsc2*$^{+/-}$ S - *Tsc2*$^{+/-}$ mice treated with saline, *Tsc2*$^{+/-}$ NPY - *Tsc2*$^{+/-}$ mice treated with neuropeptide Y.

CHAPTER 5- DISCUSSION

ASD research has focused on understanding the neural physiological basis of the disorder and eventually identify new therapeutic molecular targets to minimize the symptoms of the disease.

Emerging evidence indicates a therapeutic potential of intranasal administration of NPY to the brain, for managing stress-triggered disorders [83]. Intranasal administration is a method of delivering therapeutic agents rapidly to the CNS. It has several advantages such as being non-invasive, easy delivery, avoids hepatic first-pass elimination, and allows large molecules that do not cross the blood-brain barrier access to the CNS [84].

So, we explored how chronic intranasal delivery of NPY could improve behavioral traits of ASD, given that NPY has been reported to be a behavior modulator.

NPY is one of the most potent orexigenic peptides found in the brain, playing an important role in physiological control of food intake and body weight. It is responsible for a strong feeding response, by a decrease of latency to eat, increase motivation to eat and delayed satiety [85]. However, in our study, intranasal administration of NPY did not affect food intake and body weight between experimental groups [86, 87]. These results are in line with previous studies, in which NPY was also continuously administered intranasally. One of the studies was conducted by Duarte-Neves and colleagues, where intranasal administration of NPY was performed to mice for a period of 8 weeks, with the same concentration of NPY as used in this study. Body weight, food intake levels, white adipose tissue and dyslipidemia levels were measured and no significant differences between groups were reported [87].

In DSM-5, ASD is well described, being characterized by three main core traits- social and communication impairments and restricted or repetitive behaviors [29]. Here, we studied these core symptoms and their relief by administering NPY in a very well-established ASD mouse model, *Tsc2*$^{+/-}$. Ehninger and colleagues performed a study using *Tsc2*$^{+/-}$ mice, that showed that cognitive deficits- learning and memory impairments were present in this mouse model [45]. Also, studies conducted in our group have shown that this mouse model presents social deficiencies and an increase in repetitive behavior [42].

The EPM is centered on the natural aversion of mice for open and elevated areas, and their natural spontaneity to explore new environments. This test allowed us to measure the induced anxiety in the different groups [79]. Results showed that administration of NPY in WT mice alters anxiolytic state, when comparing to WT mice treated with saline. The distance traveled by WT treated with NPY in the open arm was significantly less, showing a more anxious and less exploratory behavior compared to other groups.

Accumulated evidence suggests that NPY is involved in anxiety by acting on Y_1, Y_2 and Y_5 receptors. To investigate the effect of Y_2R in anxiety, Tschenett and colleagues conducted a study with Y_2 knock out ($Y_2^{-/-}$) mice. EPM was performed, and the percentage of entries in open arms and time spent, was significantly higher in the knock out mice compared to controls $Y_2^{+/+}$, showing an anxiolytic effect after deletion of Y_2R [88]. It has also been shown that both ICV and intra-amygdala administration of Y_2-type receptor agonists, namely $NPY_{13–36}$ and C2-NPY, induced anxiogenic-like responses in the elevated plus maze and social interaction tests, respectively [89, 90]. NPY Y_1 and Y_5 receptors were reported to have an anxiolytic effect. It was showed that [D-His^{26}] NPY (Y1 agonist) ICV administration resulted in significant anxiolytic-like effect in EPM, at 0.8 and 3.0 nmol, on the percentage open arm entries parameter and at 0.8 nmol on the percent- age time in open arms parameter. Also, ICV administration of [cPP]hPP (Y5 agonist) caused significant anxiolytic-like effect at doses of 0.2, 0.8, and 3.0 nmol as revealed by significant increases in percentage open arm entries at the two higher doses and in percentage time in open arms at the two lower doses [91]. The question after seeing the results that we obtained is whether the administration of NPY could have resulted in a higher affinity of this peptide to Y_2 R resulting in a more anxiogenic effect rather than anxiolytic. Unlike humans and other mouse models with ASD, we did not see high levels of anxiety in *Tsc2*$^{+/-}$ mice treated with saline compared to the other experimental groups [92, 93]. This absence of anxiety behavior in *Tsc2*$^{+/-}$ mice treated with saline could be due to early life stress adaptative behavior after chronic administration and daily handling.

Repetitive and restrictive behaviors are among the core symptoms of ASD, marble burying is the behavior of burying marble scattered on the bedding into the bedding. It has been reported to be a little controversial to categorize marble burying as a repetitive behavior, since the behavior is also associated with anxiety to a novel context and exploration. Some ASD mouse models show an increased marble burying behavior, while others demonstrate a decrease [94]. In our lab, studies conducted using the marble burying test, with *Tsc2*$^{+/-}$ mice, showed increased number of total marbles buried by transgenic females, in comparison to WT, however the same was not observed in males [42]. Here, we observed significant differences in the number of marbles buried at 25 minutes. *Tsc2*$^{+/-}$ mice treated with NPY buried significantly more marbles than *Tsc2*$^{+/-}$ mice treated with saline. When comparing these results to the ones obtained using the same mouse model, a few possible factors can explain. Firstly, we did not have enough animals of each sex

to segregate and see whether the NPY administration causes any changes on females. But also, these animals were handled and chronically administered for a long period of time, and these can have repercussions on their behavior.

A study conducted by Özge Sungur and colleagues, set two experiments to assess repetitive behaviors using the SHANK1 mouse model. The first one was performed on juvenile mice (PND 42) Shank1$^{-/-}$, Shank1$^{+/-}$ and Shank1$^{+/+}$ (WT littermates control mice) and the results show no significant differences in self-grooming and digging behavior. However, results from the second experiment done in adult age (~PND 155) show significant differences in self-grooming and digging behavior between groups. Self-grooming was significantly increased in Shank1$^{+/-}$ compared to Shank1$^{+/+}$, however the number of marbles buried was significantly decreased in Shank1$^{-/-}$ and Shank1$^{+/-}$ [95]. This shows that the same test performed in different ages can have distinct outcomes in the same mouse model. Therefore, it is plausible to do future studies in older mice.

As previously mentioned, deficiencies in social interaction, including reduced interaction, abnormal social preference and lack of preference for social novelty, have been extensively described in ASD patients and animal models of the disorder. Social preference studies, that evaluated the preference for a social stimulus vs an object, found that Shank2$^{-/-}$ mice spent significantly less time than WT animals interacting with the social stimulus. Additionally, homozygous mice with the deletion of Shank3 exon 4–9 (Shank3^{e4-9}) were reported to also display significant deficits in social preference when tested [96, 97]. Social preference and social novelty preference was assessed in Shank3$\beta^{-/-}$, notably, Shank3$\beta^{-/-}$ mice exhibited a clear preference for interacting with the empty cage rather than with the social partner. When introduced a novel social partner to the previously empty cage, WT mice displayed preference for the novel animal, however, the Shank3$\beta^{-/-}$ mice markedly spent more time in the center chamber and a reduced amount of time closely interacting with either social partner [98]. However, a study conducted by Crawley and colleagues using two different lines of oxytocin knockout mice also evaluated social tendencies. On the social preference test, oxytocin null mutant (-/-), heterozygote (+/-), and wildtype littermate controls (+/+) displayed normal sociability. All three genotypes spent significantly more time in the chamber containing a stranger mouse than in the chamber that was empty, but no significant differences were observed.

Also in the social novelty preference test, oxytocin null mutants (-/-) and wild-type littermate controls (+/+) both displayed significant preference for social novelty, but no significant differences between the groups [99]. Our results from the social preference test show that *Tsc2*$^{+/-}$ mice treated with NPY spent significantly more time in the zone with social stimulus rather than the empty zone. Further, *Tsc2*$^{+/-}$ mice spend more time interacting with the social cage rather than the empty cage, however we did not observe the same with the WT mice. In contrast, no significant differences were found in social novelty preference test between groups. As described in bibliography, between different mouse models of autism, the social preference varies, in some, the mutation leads to a less social behavior, in others, to behavior similar to the control group. Given the discrepancy of values obtained in the different parameters, we could speculate that other factors might have played a role in the behavior of the experimental groups. Factors such as stress from the administration or from the previous tests performed could have led to these results.

Overall, our behavioral data showed no significance between the transgenic and WT groups as is would be expected. One possible explanation for this could be the constant stress that the animal has to go through during the treatment, inducing neuronal and cellular changes that promote stress resilience and enhance the flexibility of behavioral adaptations [100]. Therefore, we couldn't see the anxiolytic properties of administering NPY in the tests.

Functional differences in neural networks have been reported in ASD, however, the molecular pathways responsible remain unclear. In this thesis, we focused on the study of gut dysfunction, more precisely on the analysis of stool samples, and on the excitatory/ inhibitory (E/I) imbalance as a common mechanism in ASD. We also investigated the levels of NPY system in the gut-brain axis.

Ongoing work from our lab, analyzed microbial DNA from stool samples of *Nf1*$^{+/-}$ mice by rt-PCR for several bacteria to study gut dysfunction in this mouse model of ASD. The relative of abundance of *Lactobacillus* was significantly decreased in this mouse model compared to WT (Martins et al, unpublished data). Based on this work, gene expression of *Lactobacillus* was explored in stool samples of our animal models. We followed the same protocol and performed rt-PCR on stool samples of our mouse model, to determine whether the changes observed in the other study were also present. Our results showed no significant differences between group. However, we found a decrease in fold change in the transgenic group treated with saline (*Tsc2*$^{+/-}$ S) in comparison to other groups. After

chronic administration of NPY, an increase in the levels of *Lactobacillus* in *Tsc2*$^{+/-}$mice is observed. These results although not significant, they align with the results obtained in the group and other studies [101]. In human studies, the fecal microbiota of autistic children showed a significant decrease of the *Bacteroidetes/ Firmicutes* ratio and elevation of the amount of *Lactobacillus* [23, 24]. Being these studies in humans, only a small number of subjects were studied, and variables such as diet were not thoroughly controlled. However, studies in animal models allow to have control in environment and diet, while some studies have identified an increase of *Bacteroidetes* in ASD, others have reported the contrary, the same was observed in *Firmicutes* genera [101]. Therefore, further studies on larger number of animals and a more thorough analysis of bacterial populations are necessary to gain a firm consensus regarding specific changes to the microbiome in ASD.

Regarding the NPYergic system analysis, we quantified gene expression of NPY and Y_1 and Y_2 receptors. Hippocampus analysis for the genes above mentioned showed no significant differences between experimental groups. As for the cortex, no significant differences were observed between experimental groups in the expression of NPY and Y_1R. However, we observed a significant decrease in the expression of Y_2R in the WT mice treated with NPY compared to the ones treated with saline. Y_2R are associated with an anxiogenic effect, and we observed an increase in anxious behavior in WT animals treated with NPY. However, no changes were detected in the expression of these receptors in the brain areas analyzed. Intranasal administration, contrary to ICV is not directed to a particular brain region, therefore the effects of NPY are general. The amygdala has a central role in anxiety and stress responses, as there were alterations in the behavioral tests that assayed these responses in WT mice treated with NPY, maybe the NPYsystem, more specifically the Y_2R are increased in this brain region. Furthermore, during this study, Y_5R levels were not studied, which could provide relevant information to understand behavioral changes.

Previous work from our lab showed that *Nf1*$^{+/-}$ mice exhibited imbalanced E/I ratio with an increase in GABAergic system levels on region-depended manner [54]. On the other hand, recently we discovered that *Tsc2*$^{+/-}$ mouse model display cortical E/I ratio dysfunction but with an increase and a reduction in glutamate and GABA levels, respectively. This effect is exclusive of female *Tsc2*$^{+/-}$ [42]. Here, we did not observe

significant differences in the expression of GABA receptors, GABA(A) and GABA(B), both in the hippocampus and cortex, between groups.

Even though mRNA expression levels are commonly used as a proxy for estimating functional differences that occur at the protein level, the relation between mRNA and protein expression is not well established. Therefore, protein levels should also be determined, as loss in protein function could be happening.

CHAPTER 6- CONCLUDING REMARKS

The present Master Thesis is a pioneer study in which chronic intranasal administration of NPY was performed in a mouse model of ASD, given its reported implications in mediating behavioral changes. For that, we used a well-established mouse model for ASD, that had already been described and tested in our group.

We have shown that the administration of NPY in WT mice led to a more anxious behavior, less exploratory, which was not observed in our transgenic mice. The conditions in which the study is conducted could cause alterations of behavior, since it was done a chronic administration. Factors such as handling performed from a very young age, the daily intranasal administration, that includes the grabbing, administration and weighting each mouse and finally the behavioral tests caused stress to the animals from an early age. To do a true comparison between the experimental groups, other controls could be added to our study, like WT and *Tsc2*$^{+/-}$ mice without any administration, that would only be weighted and perform behavioral tests. *Tsc2*$^{+/-}$ mice, could provide information on how the chronic administration, even saline, might change specific behaviors in those animals. Secondly, even though we separated the animals according to their designated treatment and sex, it would be ideal to also separate according to their genotype, since it can have an impact on their behavior and development.

Furthermore, the molecular evaluation of stool samples could also be performed in an array of bacteria, to better understand the alterations caused by NPY treatment. Regarding the molecular analysis of the brain regions, different brain regions could improve the understanding of the mechanisms of the disease, and also protein analysis.

CHAPTER 7- REFERENCES

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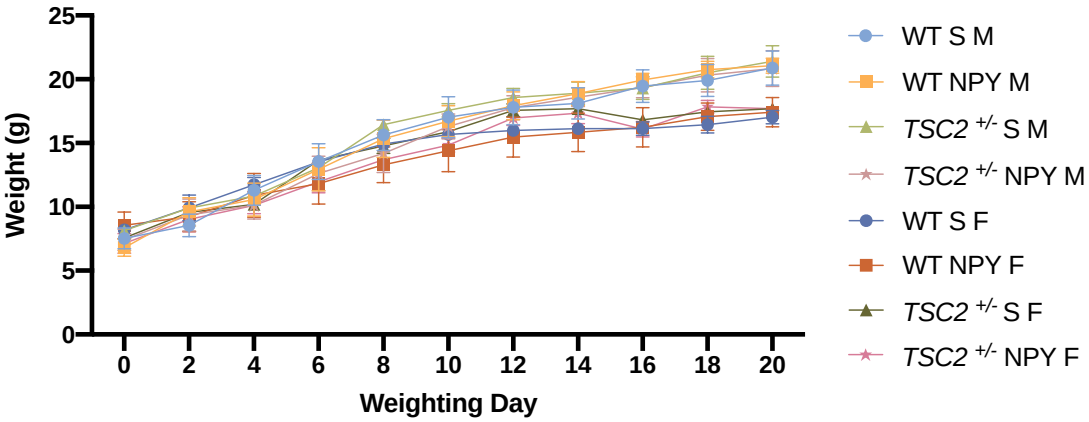
CHAPTER 8 – ANNEXES

a)


Weight (g)
25
20
15
10
5
0
0
2
4
6
8
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12
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16
18
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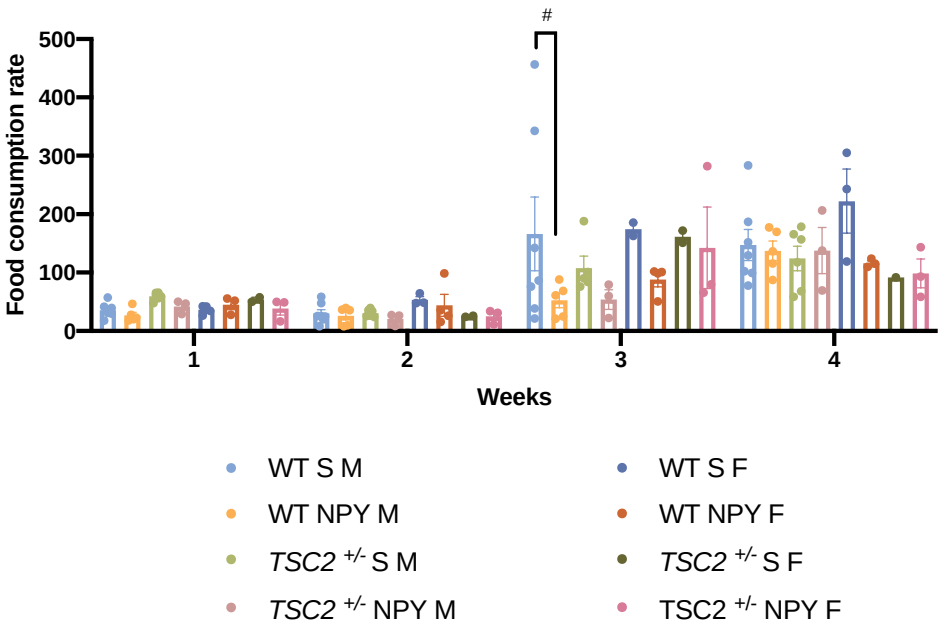
Weighting Day
WT S M
WT NPY M
TSC2 +/- S M
TSC2 +/- NPY M
WT S F
WT NPY F
TSC2 +/- S F
TSC2 +/- NPY F

b)


Food consumption rate
500
400
300
200
100
0
#
1
2
3
4
Weeks
WT S M
WT NPY M
TSC2 +/- S M
TSC2 +/- NPY M
WT S F
WT NPY F
TSC2 +/- S F
TSC2 +/- NPY F

c)

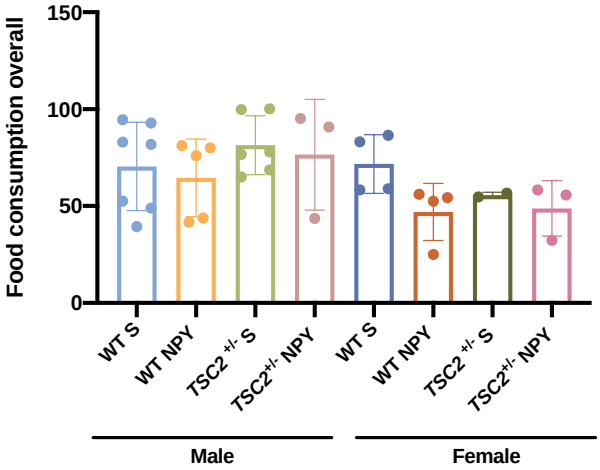
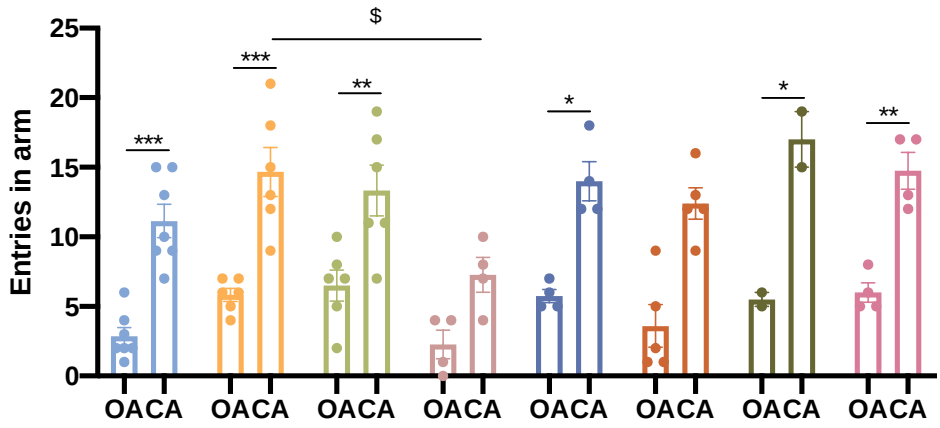

Food consumption overall
150
100
50
0
WT S
WT NPY
TSC2 +/- S
TSC2 +/- NPY
WT S
WT NPY
TSC2 +/- S
TSC2 +/- NPY
Male
Female

Figure 1A. NPY administration does not affect body weight and weekly food consumption. (a) Body weight measured every 2 days of treatment for a total of 20 days. (b) Food consumption rate calculated per week based on the initial amount of food and the final amount of food left and the number of animals per cage. (c) Food consumption overall based on the food intake and the weight gained. Results are presented by mean ± SEM. The number of animals used was: 7 WT male treated with saline, 6 WT male treated with NPY, 6 Tsc2+/- male treated with saline, 4 Tsc2+/- male treated with NPY, 4 WT female treated with saline, 5 WT female treated with NPY, 2 Tsc2+/- female treated with saline, 4 Tsc2+/- female treated with NPY. #p> 0.05- 2way ANOVA, significantly different between WT male treated with saline and WT male treated with NPY. Abbreviations: M- male, F- female, WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, Tsc2+/- S - Tsc2+/- treated with saline; Tsc2+/- NPY - Tsc2+/- treated with neuropeptide Y.

a)



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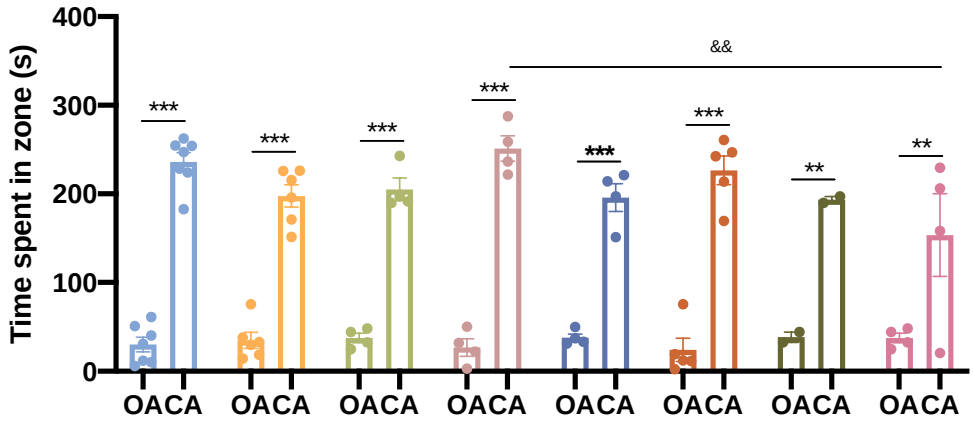

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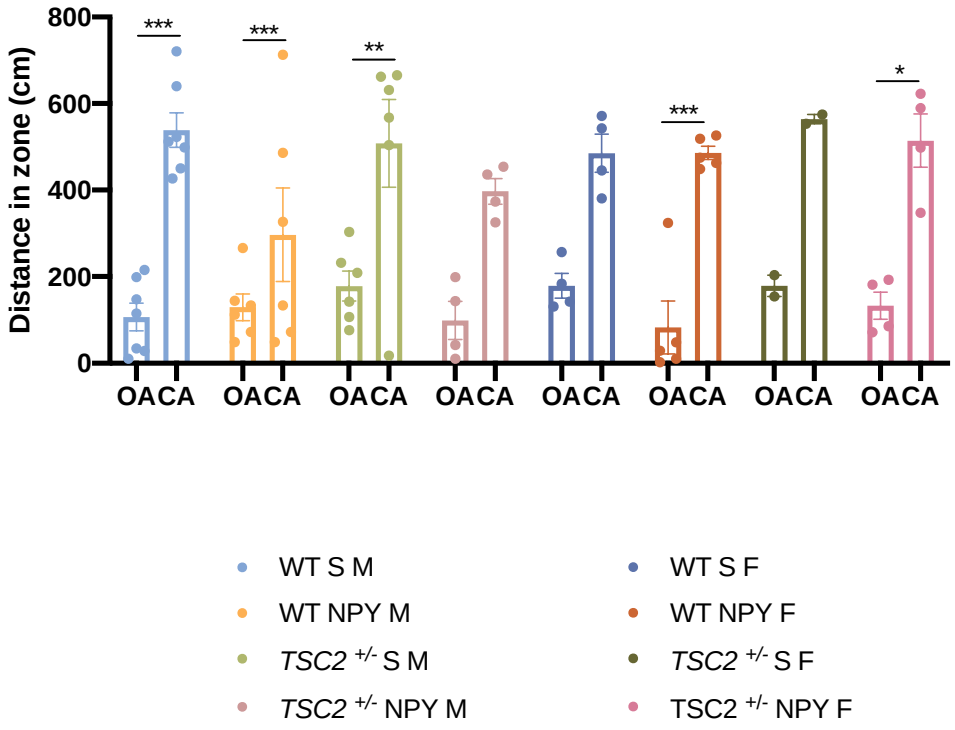


Figure 2A. Elevated plus maze test show that in general the groups spend more time in closed arms than in open arms. (a) Number of entries in open arm and closed arm (b) Time spent in open arm and closed arm. (c) Distance travelled in open arm and closed arm. Results are presented by mean ± SEM. The number of animals used was: 7 WT male treated with saline, 6 WT male treated with NPY, 6 Tsc2+/- male treated with saline, 4 Tsc2+/- male treated with NPY, 4 WT female treated with saline, 5 WT female treated with NPY, 2 Tsc2+/- female treated with saline, 4 Tsc2+/- female treated with NPY. *p> 0.05- 2way ANOVA, significantly different in number of entries, time spent and distance travelled between OA and CA. **p> 0.01- 2way ANOVA, significantly different in number of entries, time spent and distance travelled between OA and CA. ***p> 0.001- 2way ANOVA, significantly different in number of entries, time spent and distance travelled between OA and CA. $p> 0.05- 2way ANOVA, significantly different in number of entries in the CA between WT male treated with NPY and Tsc2+/- male treated with NPY. &&p> 0.01- 2way ANOVA, significantly different in the time spent in the CA between Tsc2+/- male treated with NPY and Tsc2+/-female treated with NPY. Abbreviations: OA- open arm, CA-closed arm, M- male, F- female, WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, Tsc2+/- S - Tsc2+/- treated with saline; Tsc2+/- NPY - Tsc2+/- treated with neuropeptide Y.

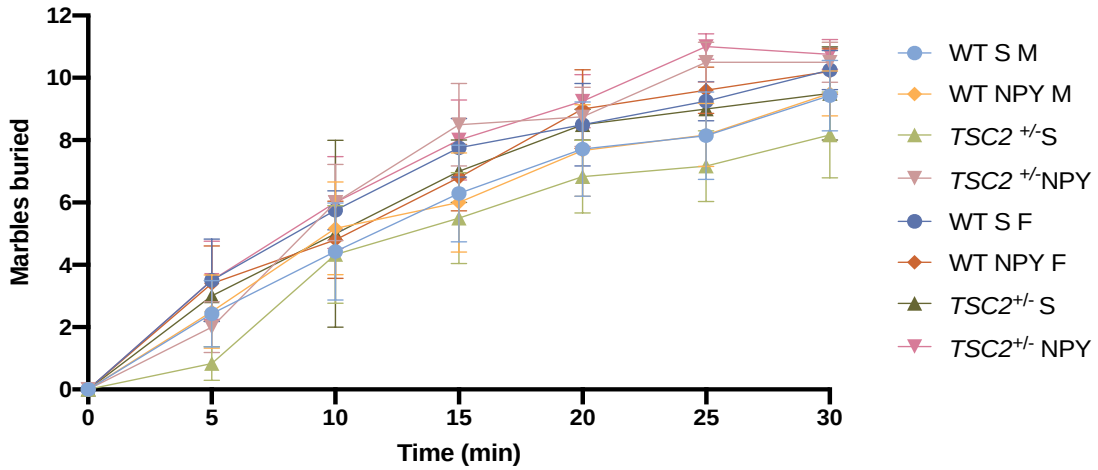
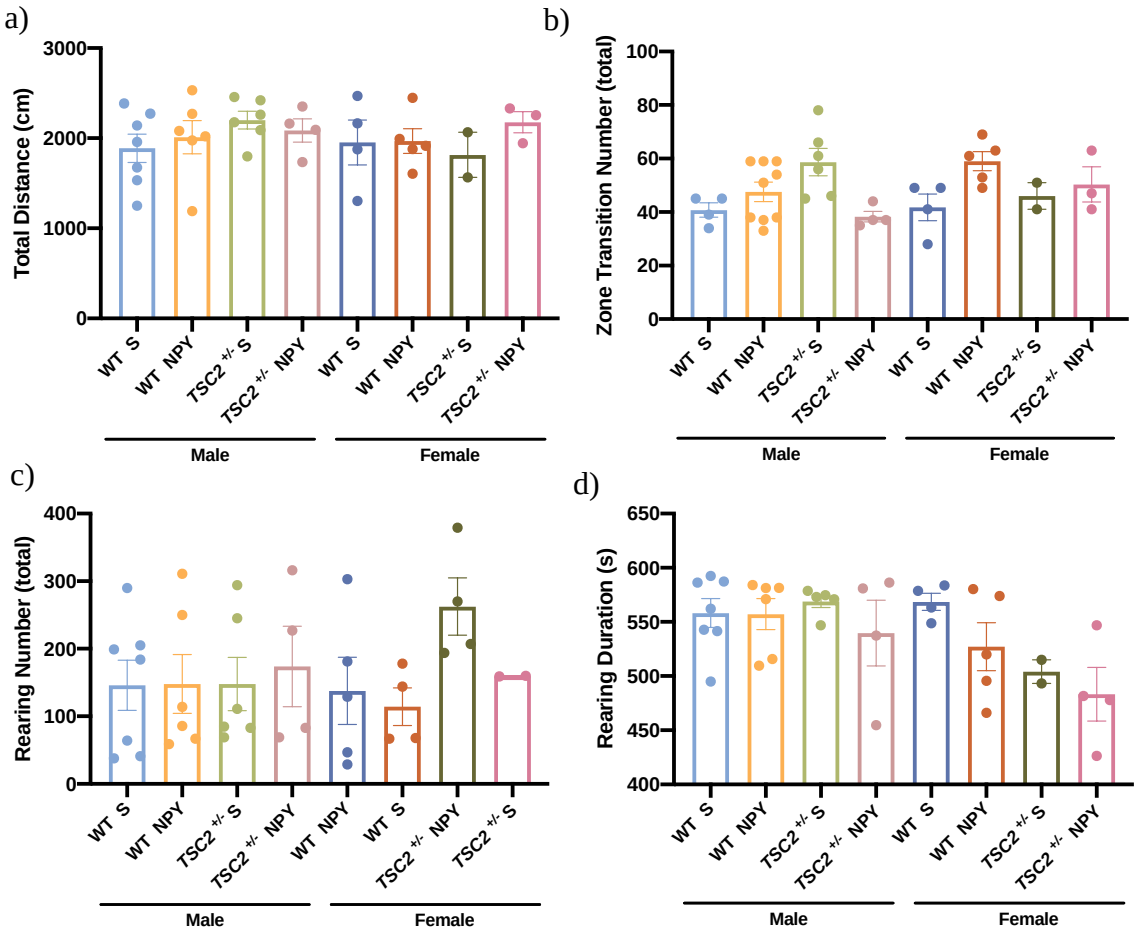


Figure 3A. Repetitive behavior assessment did not show significant differences among groups. Results are presented by mean ± SEM. The number of animals used was: 7 WT male treated with saline, 6 WT male treated with NPY, 6 Tsc2+/- male treated with saline, 4 Tsc2+/- male treated with NPY, 4 WT female treated with saline, 5 WT female treated with NPY, 2 Tsc2+/- female treated with saline, 4 Tsc2+/- female treated with NPY. Kruskal-Wallis test for statistical analysis. Abbreviations: M- male, F- female, WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, Tsc2+/- S - Tsc2+/- treated with saline; Tsc2+/- NPY - Tsc2+/- treated with neuropeptide Y.



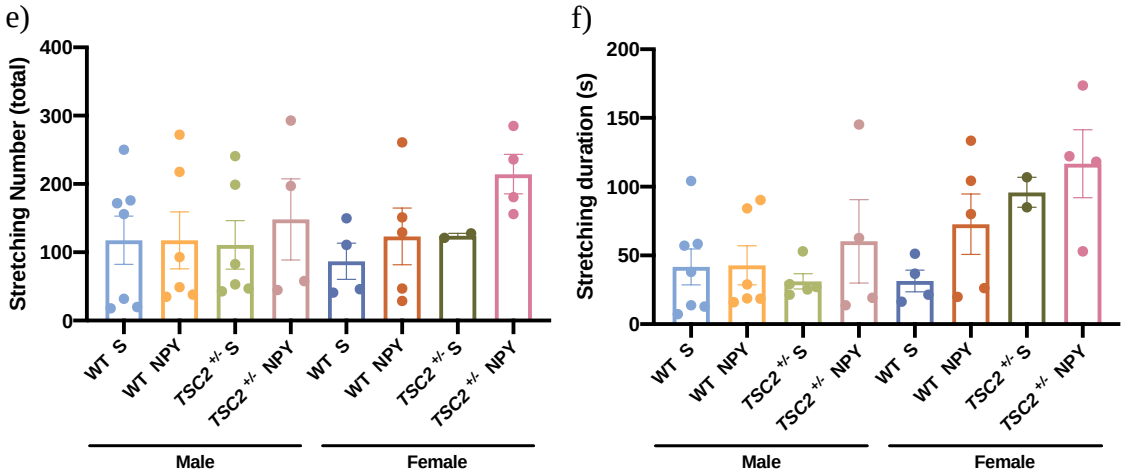
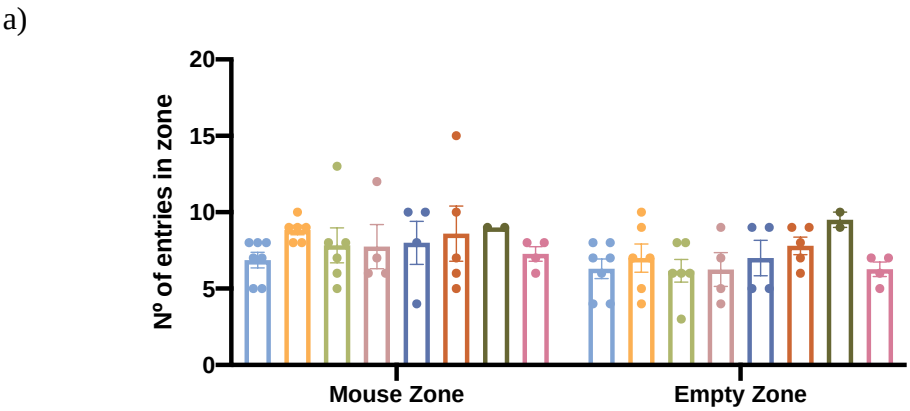
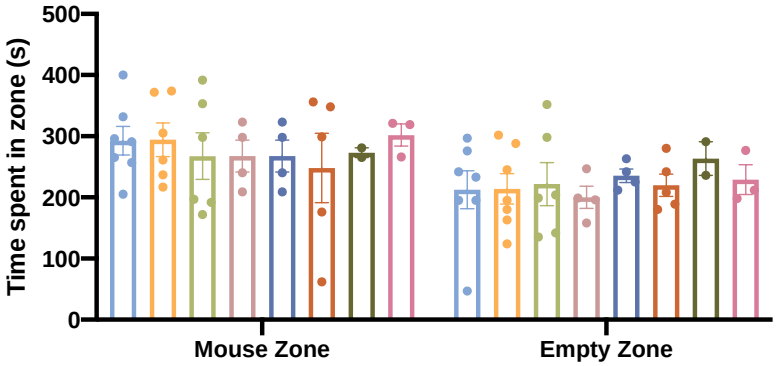


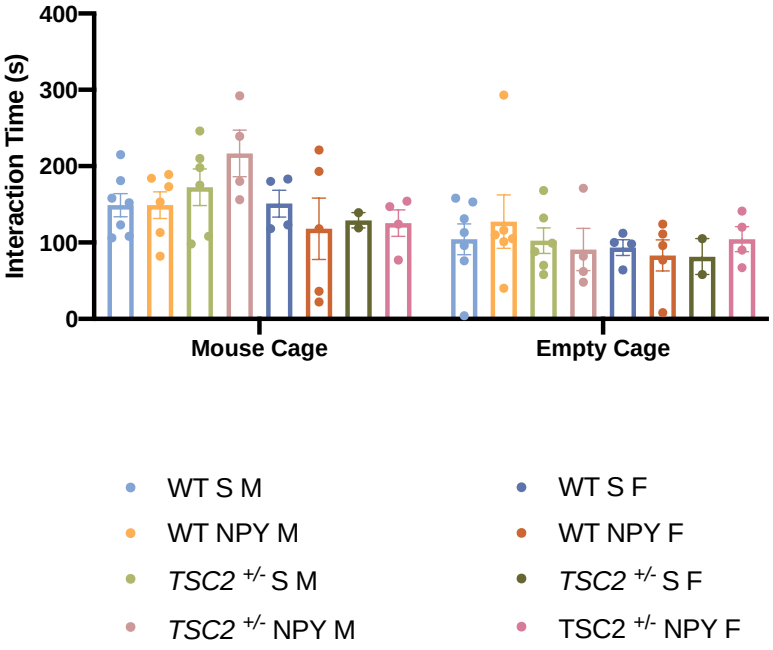
Figure 4A. Open field test performed on habituation phase of three-chamber social test, shows no significant differences between groups. (a) Total distance traveled during the 10 minutes (b) Number of zone transitions (c) Rearing number (d) Duration of the rearing (e) Stretching number (f) Stretching duration. Results are presented by mean ± SEM. The number of animals used was: 7 WT male treated with saline, 6 WT male treated with NPY, 6 Tsc2+/- male treated with saline, 4 Tsc2+/- male treated with NPY, 4 WT female treated with saline, 5 WT female treated with NPY, 2 Tsc2+/- female treated with saline, 4 Tsc2+/- female treated with NPY. Kruskal-Wallis test for statistical analysis. Abbreviations: M- male, F- female, WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, Tsc2+/- S - Tsc2+/- treated with saline; Tsc2+/- NPY - Tsc2+/- treated with neuropeptide Y.



b)


Time spent in zone (s)
500
400
300
200
100
0
Mouse Zone
Empty Zone

c)


Interaction Time (s)
400
300
200
100
0
Mouse Cage
Empty Cage
WT S M
WT NPY M
TSC2 +/- S M
TSC2 +/- NPY M
WT S F
WT NPY F
TSC2 +/- S F
TSC2 +/- NPY F

d)

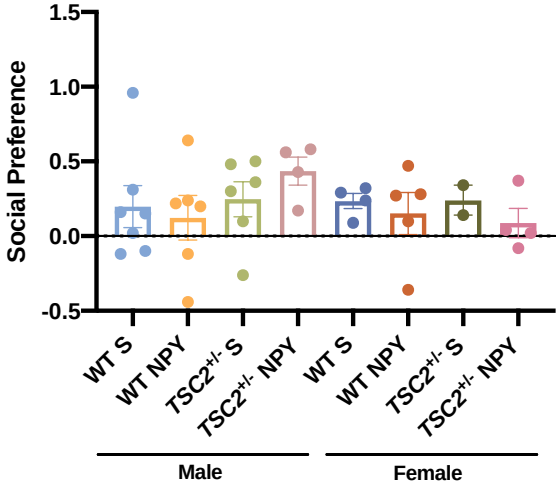
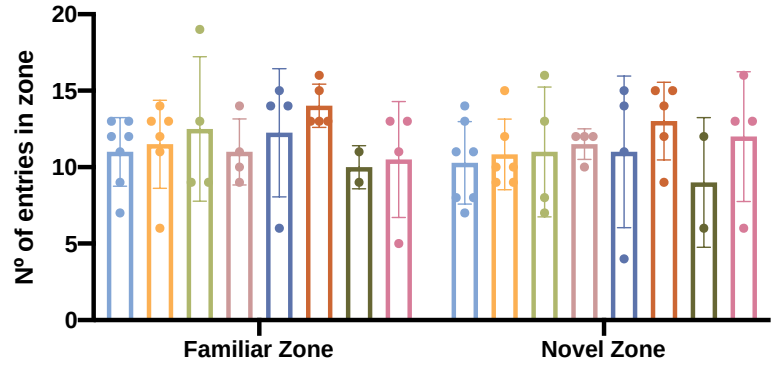
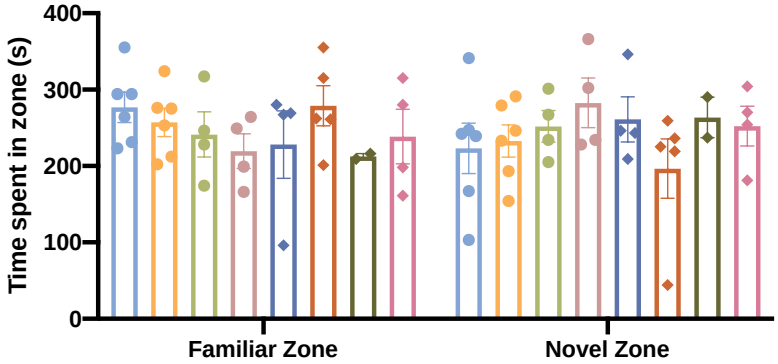

Social Preference
1.5
1.0
0.5
0.0
-0.5
WT S
WT NPY
TSC2+/- S
TSC2+/- NPY
WT S
WT NPY
TSC2+/- S
TSC2+/- NPY
Male
Female

Figure 5A. Time spent in social zone versus empty zone is not significantly different between experimental groups. (a) Time spent in the zone with the social stimulus vs in the zone with the non-social stimulus (b) Number of entries in each zone (c) Time spent interacting with the mouse or the empty cage (d) Social preference. Results are presented by mean ± SEM. The number of animals used was: 7 WT male treated with saline, 6 WT male treated with NPY, 6 Tsc2+/- male treated with saline, 4 Tsc2+/- male treated with NPY, 4 WT female treated with saline, 5 WT female treated with NPY, 2 Tsc2+/- female treated with saline, 4 Tsc2+/- female treated with NPY. Kruskal-Wallis test for statistical analysis. Abbreviations: M- male, F- female, WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, Tsc2+/- S - Tsc2+/- treated with saline; Tsc2+/- NPY - Tsc2+/- treated with neuropeptide Y.

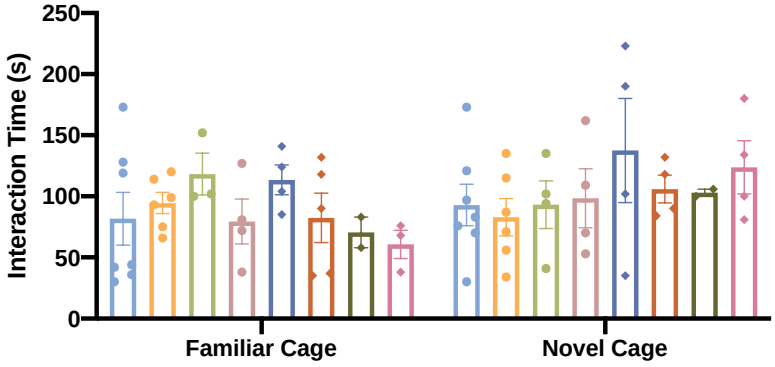
a)



b)



c)



d)

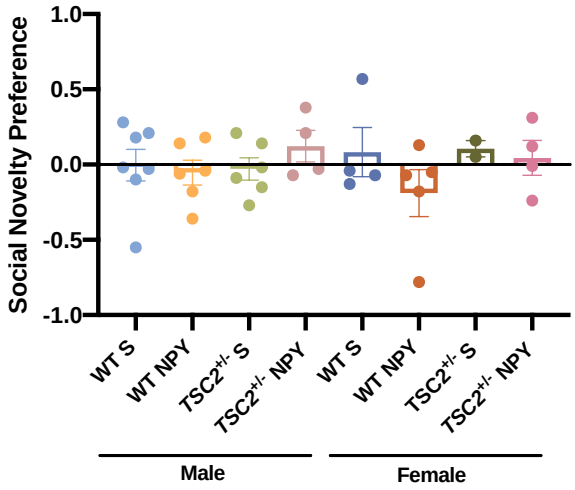


Figure 23. Social novelty preference test of 3 Chamber social test shows no significant differences. (a) Time spent in the zone with the familiar animal vs in the zone with the novel mouse (b) Number of entries in each zone (c) Time spent interacting with the familiar mouse or the novel mouse (d) Social novelty preference. Results are presented by mean ± SEM. The number of animals used was: 7 WT male treated with saline, 6 WT male treated with NPY, 6 Tsc2+/- male treated with saline, 4 Tsc2+/- male treated with NPY, 4 WT female treated with saline, 5 WT female treated with NPY, 2 Tsc2+/- female treated with saline, 4 Tsc2+/- female treated with NPY. Kruskal-Wallis test for statistical analysis. Abbreviations: M- male, F- female, WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, Tsc2+/- S - Tsc2+/- treated with saline; Tsc2+/- NPY - Tsc2+/- treated with neuropeptide Y.

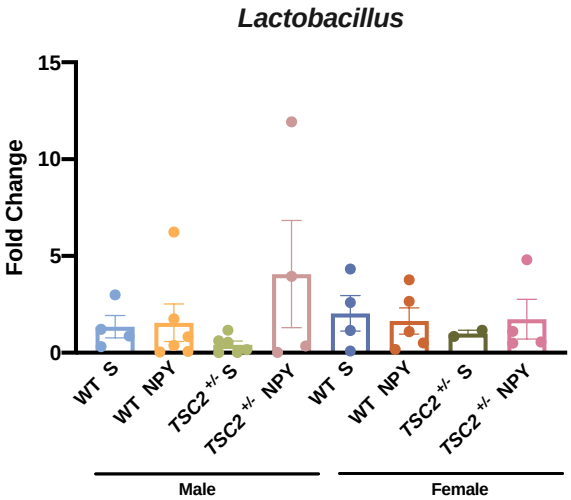
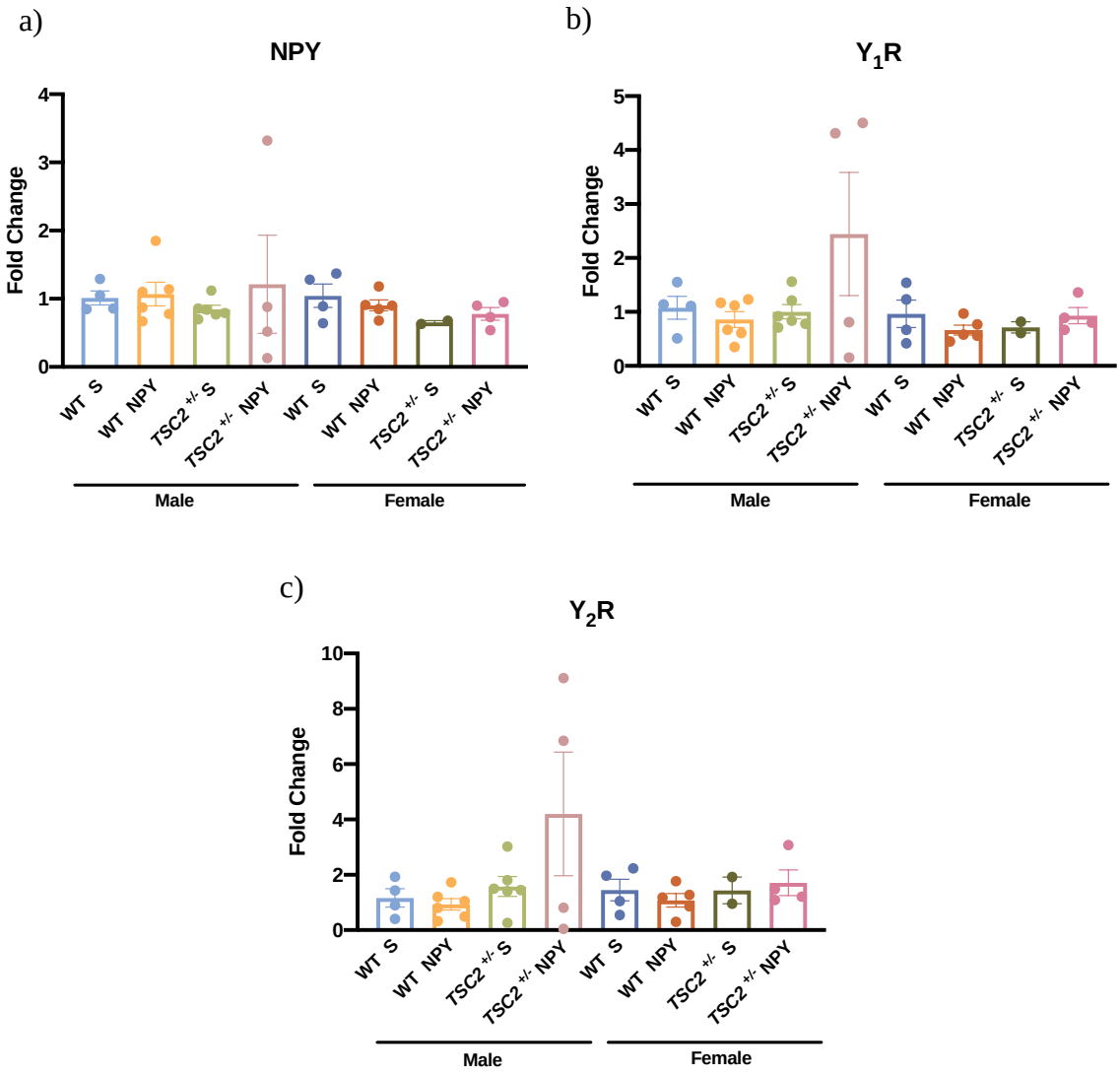


Figure 7A. Real time PCR was performed on microbial DNA from the stool samples of transgenic and wild type mice for the dysregulation of Lactobacillus. Total 16S DNA was used as endogenous control. Results are presented by mean ± SEM. The number of animals used was: 4 WT male treated with saline, 6 WT male treated with NPY, 6 Tsc2+/- male treated with saline, 4 Tsc2+/- male treated with NPY, 4 WT female treated with saline, 5 WT female treated with NPY, 2 Tsc2+/- female treated with saline, 4 Tsc2+/- female treated with NPY. Kruskal-Wallis test for statistical analysis. Abbreviations: M- male, F- female, WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, Tsc2+/- S - Tsc2+/- treated with saline; Tsc2+/- NPY - Tsc2+/- treated with neuropeptide Y.



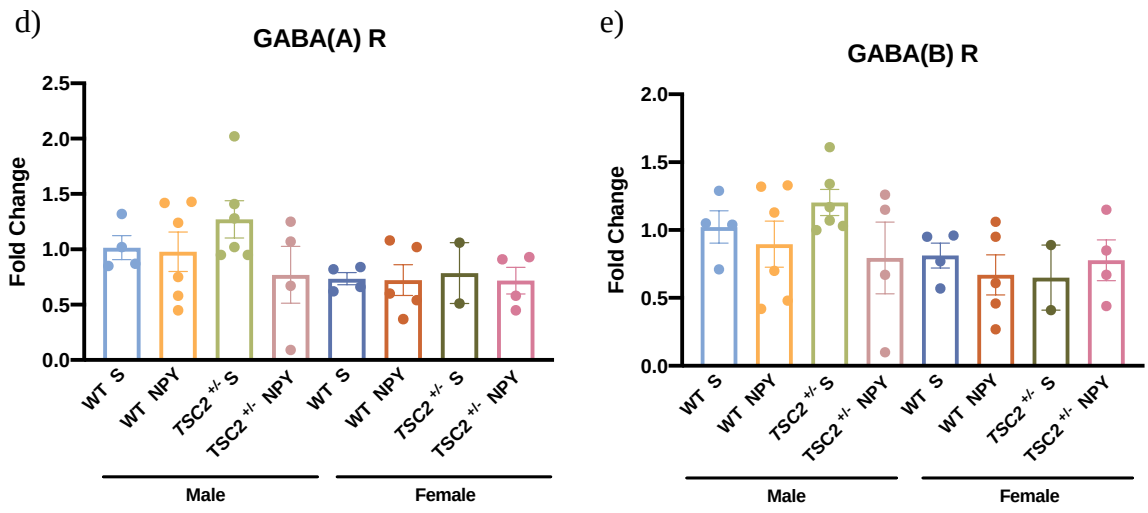
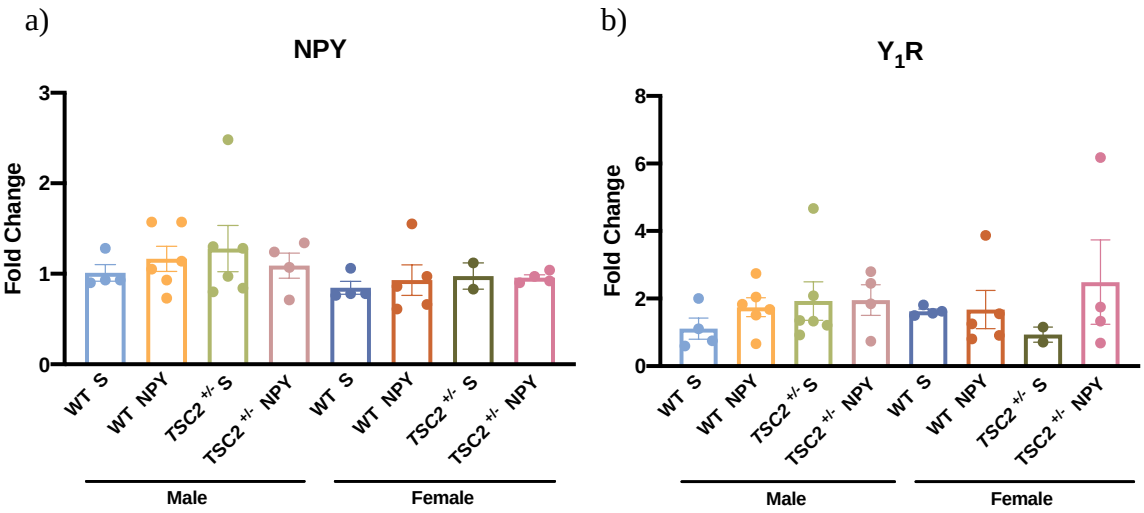


Figure 8A. Real time PCR was performed on cDNA from the hippocampal samples of transgenic and wild type mice for changes in NPY, Y1R, Y2R, GABA(A) R and GABA(B) R. B2M was used as endogenous control. Results are presented by mean ± SEM. Total 16S DNA was used as endogenous control. Results are presented by mean ± SEM. The number of animals used was: 4 WT male treated with saline, 6 WT male treated with NPY, 6 *Tsc2*$^{+/-}$ male treated with saline, 4 *Tsc2*$^{+/-}$ male treated with NPY, 4 WT female treated with saline, 5 WT female treated with NPY, 2 *Tsc2*$^{+/-}$ female treated with saline, 4 *Tsc2*$^{+/-}$ female treated with NPY. Kruskal-Wallis test for statistical analysis. Abbreviations: M- male, F- female, WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, *Tsc2*$^{+/-}$ S - *Tsc2*$^{+/-}$ treated with saline; *Tsc2*$^{+/-}$ NPY - *Tsc2*$^{+/-}$ treated with neuropeptide Y.



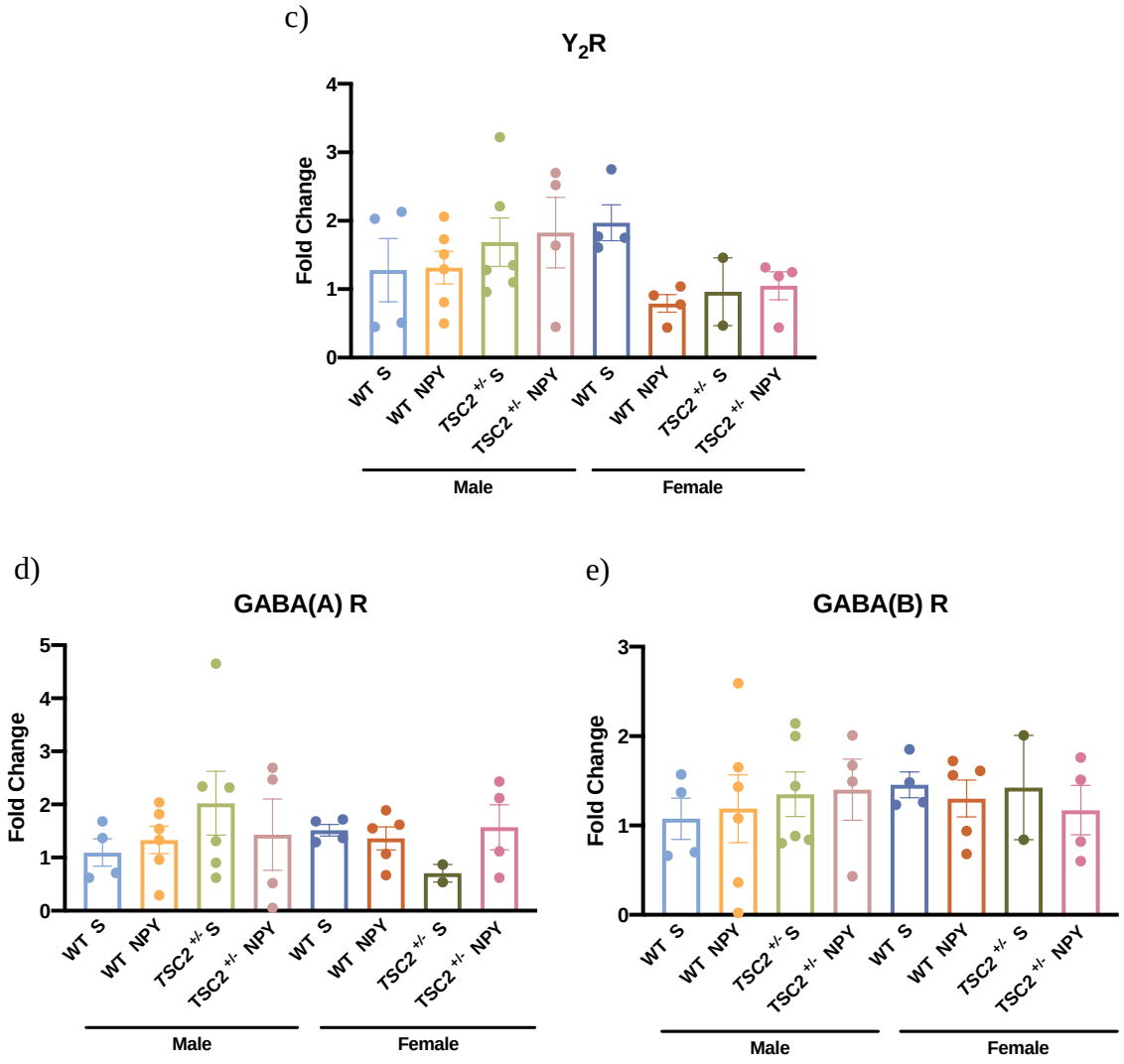


Figure 9A. Real time PCR was performed on cDNA from cortex samples of transgenic and wild type mice for changes in NPY, Y1R, Y2R, GABA(A) R and GABA(B) R. B2M was used as endogenous control. Results are presented by mean ± SEM. The number of animals used was: 4 WT male treated with saline, 6 WT male treated with NPY, 6 Tsc2+/- male treated with saline, 4 Tsc2+/- male treated with NPY, 4 WT female treated with saline, 5 WT female treated with NPY, 2 Tsc2+/- female treated with saline, 4 Tsc2+/- female treated with NPY. Kruskal-Wallis test for statistical analysis. Abbreviations: M- male, F- female, WT S- wild type treated with saline, WT NPY- wild type treated with neuropeptide Y, Tsc2+/- S - Tsc2+/- treated with saline; Tsc2+/- NPY - Tsc2+/- treated with neuropeptide Y.