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Mother-Offspring Relationship in Autism Spectrum Disorder

Influence on Development and Behavior

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

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À minha irmã, a quem dedico esta tese.

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LIST OF ABBREVIATIONS

ADDM	Autism and developmental disabilities monitoring
ANOVA	Analysis of variance
ASD	Autism spectrum disorder
CNS	Central nervous system
CNV	Copy number variation
CUMS	Chronic unpredictable mild stress
dB	decibel
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunoassay
F	Female
FOXP2	Forkhead box P2
GABA	Gamma-aminobutyric acid
GAP	Guanosine triphosphatase activating protein
Glx	Glutamate+glutamine
GTP	Guanosine triphosphate-binding protein
M	Male

mTOR	mammalian target of rapamycin
mTORC	mammalian target of rapamycin complex
PCR	Polymerase chain reaction
PND	Postnatal day
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TE	Tris-ethylenediamine tetraacetic acid
TSC	Tuberous sclerosis complex
TPH2	Tryptophan hydroxylase 2
USV	Ultrasonic vocalization
WT	Wild-type

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RESUMO

O transtorno do espetro do autismo (TAE) é uma condição do neurodesenvolvimento que afeta 1 em cada 59 crianças. Esta patologia caracteriza-se por défices na comunicação e interação social, e por comportamentos repetitivos e restritivos. Adolescentes e adultos com TAE enfrentam geralmente desafios em vários aspetos das suas vidas, desde educação e emprego, até à criação de relações sociais. A etiologia do TAE envolve fatores genéticos, epigenéticos e ambientais, que se relacionam de uma forma complexa e heterogénea. No entanto, alguns aspetos permanecem por compreender, nomeadamente, a influência da mãe no desenvolvimento da descendência, e como as suas relações interpessoais poderão modular tanto o comportamento da descendência como o instinto maternal.

Neste trabalho, realizámos um estudo longitudinal, utilizando o murganho com complexo de esclerose tuberosa tipo 2 (*Tsc2*$^{+/-}$), um modelo animal estabelecido para o estudo do TAE. Formámos quatro grupos experimentais distintos: mães *wild type* (WT, estirpe selvagem) com ninhadas WT, mães WT com ninhadas *Tsc2*$^{+/-}$, mães *Tsc2*$^{+/-}$ com ninhadas WT, e mães *Tsc2*$^{+/-}$ com ninhadas *Tsc2*$^{+/-}$. Foi realizada uma bateria de testes ao longo das idades neonatal e juvenil da descendência para avaliar o seu desenvolvimento e comportamento. Ainda, o instinto maternal das mães foi avaliado, com o objetivo de compreender a sua influência no desenvolvimento da descendência. Finalmente, os níveis de serotonina e oxitocina no plasma foram medidos, para compreender como é que os seus sistemas moleculares poderão estar relacionados com os comportamentos observados.

Nos grupos experimentais onde mãe e ninhada partilhavam o mesmo genótipo, a descendência apresentou um desenvolvimento motor e vestibular mais rápido. Curiosamente, as mães pertencentes a estes grupos experimentais apresentaram níveis mais reduzidos de cuidados maternais, o que sugere que o instinto maternal foi menos eficiente em resposta ao estado mais desenvolvido da ninhada. Assim, poderemos sugerir que o cuidado maternal é ditado pelas necessidades da própria

ninhada.

De notar que, ainda que os grupos experimentais com genótipos partilhados tenham mostrado níveis semelhantes de desenvolvimento motor e vestibular, as suas capacidades sociais eram opostas, tendo assim permanecido até à adolescência. De facto, apenas o grupo mãe *Tsc2*$^{+/-}$ - ninhada *Tsc2*$^{+/-}$ mostrou uma tendência para ser mais social, apresentando melhor capacidade comunicativa. Estes resultados sugerem assim que apesar de os níveis de cuidados maternais serem semelhantes entre os grupos WT x WT e mutante x mutante, as suas relações mãe-ninhada poderão ser distintas, afetando de forma contrastante o desenvolvimento das suas capacidades sociais.

Além disso, o genótipo maternal *Tsc2*$^{+/-}$ levou a um aumento em comportamentos repetitivos, com maior impacte na descendência feminina, o que está de acordo com resultados obtidos previamente no nosso grupo. No que concerne à capacidade cognitiva, não conseguimos concluir em relação à influência do genótipo maternal na memória a curto prazo da descendência.

Finalmente, descendência mutante com mães mutantes mostraram um decréscimo nos níveis plasmáticos de serotonina. No entanto, são necessários estudos mais pormenorizados sobre os níveis no sangue e no cérebro de serotonina e oxitocina, ao longo da vida das mães e da descendência, para ser possível concluir sobre a influência destas vias moleculares no desenvolvimento e comportamento.

Em suma, este trabalho dá-nos uma visão importante das dinâmicas complexas que envolvem a relação mãe-descendência, e do seu impacto no desenvolvimento e comportamento da descendência.

Palavras-chave | *Transtorno do espetro do autismo; relação mãe-descendência; desenvolvimento; comportamento social; instinto maternal; serotonina e oxitocina.*

ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental condition affecting 1 in every 59 children, characterized by impairments in social communication and interaction and by restricted, repetitive behaviors. Adolescents and adults with ASD usually face challenges in multiple aspects of their lives, from education and employment to social relationships. Genetic, epigenetic and environmental factors have been implicated in ASD, creating a complex, heterogenous picture on the etiology of the disorder. However, some aspects remain to be understood, namely, the maternal influence on the development of the offspring, and how their interpersonal relationships may modulate both offspring's behavioral outcomes and maternal instinct.

In this work, we performed a longitudinal study using tuberous sclerosis complex 2 (*Tsc2*$^{+/-}$) mice, an established animal model of ASD, and formed four distinct experimental groups: WT (wild-type) mothers with WT litters, WT mothers with *Tsc2*$^{+/-}$ litters, *Tsc2*$^{+/-}$ mothers with WT litters and *Tsc2*$^{+/-}$ with *Tsc2*$^{+/-}$ litters. A battery of developmental milestones and behavioral tests were performed in each litter across the offspring's neonatal and juvenile age. Additionally, maternal instincts of the dams were assessed as well, to understand their link with the offspring's development. Finally, plasma and brain serotonin and oxytocin levels were measured, to understand how their molecular systems may be related to the observed behavioral outcomes.

We found that in experimental groups where genotypes were shared between dam and pups, there was an earlier motor and vestibular development in the offspring. Curiously, the mothers belonging to these groups also displayed lower scores in maternal care measures, suggesting that maternal instinct was less effective as a response to the more developed state of the litter. This finding suggests that maternal care is dictated by the litter's own needs.

Importantly, we found that although same-genotype experimental groups showed similar profiles of motor and vestibular development, their social skills were strikingly opposite and long-lasting until adolescence. Indeed, only the *Tsc2*$^{+/-}$ x *Tsc2*$^{+/-}$ group

showed a tendency for a more social and pro-social behavior, and displayed higher communicative scores. This suggests that even though maternal care levels were similar between WT x WT and *Tsc2*$^{+/-}$ x *Tsc2*$^{+/-}$ groups, their mother-pup relationship might have been contrasting, thus affecting the development of their social skills in a different way.

Moreover, we found that a *Tsc2*$^{+/-}$ maternal genotype leads to an increase in repetitive behaviors, with a specific impact on female offspring, which is in accordance to previous results obtained in our lab. Regarding cognitive ability, we could not conclude on the influence of maternal genotype on short-term memory.

Finally, we found that *Tsc2*$^{+/-}$ pups with *Tsc2*$^{+/-}$ mothers had a decrease in plasma serotonin levels. However, further, more detailed studies on blood and brain serotonin and oxytocin levels over the course of the mother's and offspring's lives should be performed to conclude on the influence of these molecular pathways on development and behavior.

Overall, this work provides important insight on the complex dynamics surrounding early mother-offspring relationship, and how this has a striking impact on early development and later behavioral outcomes of the offspring.

Keywords | *Autism spectrum disorder; maternal-offspring relationship; development; social behavior; maternal instinct; serotonin and oxytocin.*

CHAPTER 1

STATE OF THE ART

1.1.

AUTISM SPECTRUM DISORDER

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental condition characterized by impairments in social communication and social interaction, as well as by restricted, stereotyped behaviors and interests (American Psychiatric Association, 2013) (Figure 1).

The Autism and Developmental Disabilities Monitoring (ADDM) Network estimated a prevalence of ASD of 1 in every 59 children aged 8 years old in 2014, with a ratio of 4 boys:1 girl (Baio et al., 2018) (Figure 1). These conclusions were corroborated by the Early ADDM, which has found a prevalence of 17 per 1,000 children aged 4 years old, also with a male bias in diagnosis (Christensen et al., 2019). However, a systematic review found a global prevalence of 1 in every 132 persons (Baxter et al., 2015). It is important to note that these reports deal with relatively small geographic surveillance groups, scarcity of data regarding adult patients, or lack of information from developing countries. Therefore, such estimations must be taken with caution.

ASD symptoms start appearing at around 1.5 – 2 years of age, and present with great variability in severity and extent of disability (Mukherjee, 2017; Hyman et al., 2020). At this time, children may present hindered social communication and/or interaction, specifically, underdeveloped speech, difficulties in understanding the intent of others, reduced eye contact, and inadequate patterns of behavior and play. This problematic behavior can include aggressive and destructive behavior, self-injury, tantrums, difficulty in recognizing danger, in adjusting to a new daily routine and in transitioning activities, or fear of harmless items. ASD children may also carry cognitive limitations, inappropriate emotional responses, impaired daily living skills, or repetitive behaviors. Such stereotypies can be the result of compulsion, function as a coping mechanism in the face of impaired processing of sensory information, and/or be used to obtain control in one's environment (Jang et al., 2011; Mukherjee, 2017; Hyman et al., 2020; Kodak & Bergmann, 2020).

Diagnosis of ASD usually begins with identification of "red flags" suggestive of the condition, such as reduced eye contact and response to name, regression of social

and language skills, rigidity, extremely focused interests, or difficulty in understanding emotions. A comprehensive assessment includes interviews with parents/caregivers, direct observation of the child's language and cognitive functions by a specialized medic, a complete physical exam and use of screening tests (Huerta & Lord, 2012; Randall et al., 2018; Hodges et al., 2020). Algorithms and toolkits are also available for pediatricians to help obtain an earlier diagnosis and design a management strategy (Johnson & Myers, 2007).

In spite of the wide, thorough research on ASD, treatment of the condition is still limited and focuses on symptom management. Behavioral/psychological interventions such as cognitive behavioral therapy, social behavioral therapy, neurofeedback training or musical therapy, are the benchmark treatment options to ameliorate ASD-related symptomatology, by reducing aggression, increasing cognitive skills, and improving communication and social interaction. Alternatively, or as a complement, pharmacological treatment strategies are also used, and include psychostimulants, atypical antipsychotics, antidepressants and alpha-2 adrenergic receptor agonists (Bhat et al., 2014; Masi et al., 2017; Sharma et al., 2018).

Long-term outcomes for adolescents and adults with ASD are poor, regarding education, employment, social relationships, sexuality, dependency of others for care and adaptive functioning, and mental health (Howlin & Magiati, 2017; Wisner-Carlson et al., 2020). Indeed, in spite of moderate amelioration of symptoms for some individuals, they continue to face challenges as functioning does not achieve normal range (Seltzer et al., 2004). Therapy in adult ASD persons targets social skills, with the goal to increase social and emotional awareness, build empathy and improve conversational skills (Ratto & Mesibov, 2015). Nevertheless, the authors alert for the need of more, stronger studies focusing on ASD individuals throughout their lives, to investigate how the condition affects the different aspects of their daily living and their overall quality of life.

Neuroimaging and postmortem studies have revealed that the individuals who live with the condition carry abnormalities in the brain, resulting from altered regulation of developmental processes (DiCicco-Bloom et al., 2006). In particular, growing evidence reports frontal-posterior and frontal-temporal long-distance, inter-regional, under connectivity, paired with short distance, local, over connectivity in the visual areas, for instance (Parellada et al., 2014). Additionally, altered neurotransmitter levels have also been implicated in the disorder, with consequences in the excitation/inhibition (im)balance in the brain of ASD individuals (Canitano & Palumbi, 2021) Indeed, our group has previously reported an unbalance in the Glx/total N-acetylaspartate ratio in ASD patients (Pereira et al., 2018). Moreover, reduced GABA concentrations, decreased $GABA_A$ receptor binding and normal levels of Glx/total creatine have also been found in patients with neurofibromatosis type 1, a neurodevelopmental disorder

with high comorbidity with ASD (Ribeiro et al., 2015; Violante et al., 2016), indicating an abnormal excitation/inhibition balance in this condition. Interestingly, this imbalance was also reported in an animal model of NF1 (Gonçalves et al., 2017). Overall, the several reported macroscopic and microscopic deficiencies lead to abnormalities in the anatomy and function of neural networks.

Thus far, there is no single unifying cause for ASD. Rather, an extensive list of possible altered processes and pathways have been indicated to be underlying the development of the condition (Figure 1).

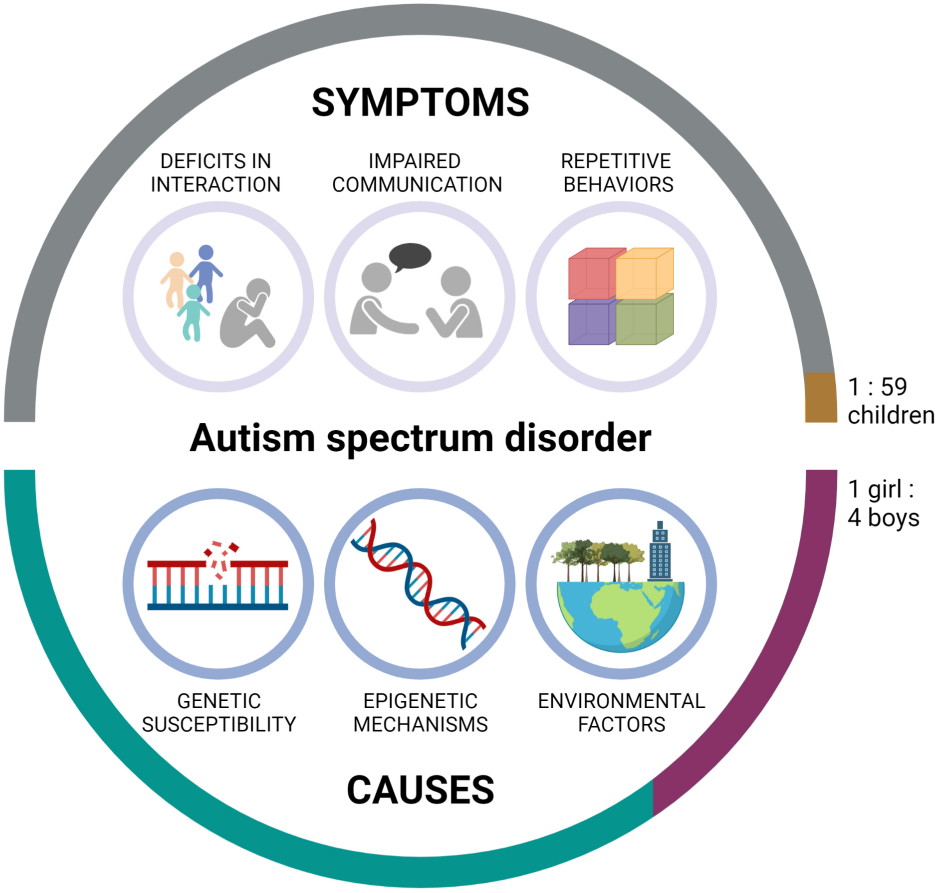


Figure 1 | Autism spectrum disorder's epidemiology, main symptoms and causes
Autism spectrum disorder is a neurodevelopmental disorder affecting 1 in every 59 children, with a 4 boys:1 girl diagnosis ratio. It is characterized by deficits in social interaction and in social communication, and by repetitive, stereotyped behaviors and interests. There is no one single cause for ASD, but rather a plethora of altered processes that increase susceptibility to the condition, which can stem from abnormal genetic and epigenetic mechanisms, and/or from environmental risk factors. Created with BioRender.com

Several inherited and *de novo* mutations, and copy number variations (CNVs) have been associated with ASD (Lyall et al., 2017; Manoli & State, 2021). Genetic factors that increase susceptibility to ASD include genes involved in brain development, such as abnormal signaling of the protein coded by the Reelin gene, important for the formation of brain structure and neural connections, or mutation in the FOXP2 gene, which codes for a transcription factor involved in the development of neural tissue; neurotransmission and neural excitability, such as mutations in the genes coding for the glutamate receptor ionotropic kainite 2, or the GABA receptor GABRB3, which affect the excitatory state of the brain; and neuroinflammation, such as overactivation of transcription factor NF-kB, resulting in cytokine production, or decreased levels of transforming growth factor β, hindering the ability to control inflammation (Rubenstein & Merzenich, 2003; Noriega & Savelkoul, 2014; Yoon et al., 2020).

Moreover, altered epigenetic mechanisms also mediate ASD etiology, such as abnormal DNA methylation (i.e., abnormal methylation of the promotor of MeCP2, which regulates several genes with synaptic roles, hypermethylation of the oxytocin receptors, and altered methylation patterns of SHANK3, which codes for a scaffolding protein involved in spine morphology and synaptic transmission), abnormal histone modification (i.e., mutations in genes coding for chromatin remodelers, such as chromodomain helicase DNA-binding proteins) and deregulated micro-RNAs, affecting synaptic function (Hodges et al., 2020; Yoon et al., 2020).

Increasing the already complex, heterogenous etiology of ASD, the environment is also a key contributor for the development of the condition. In fact, the interplay between both environmental and genetic risk factors increases susceptibility to the disorder. Exposure to heavy metals, air pollution or endocrine disruptors during neurodevelopment can lead to the condition, as well as maternal infection and immune activation (Lyall et al., 2017; Cheroni et al., 2020).

An important line of research is looking into the role of biological sex in the etiology, symptom manifestation and treatment response in ASD. In fact, girls seem to present less stereotyped, restricted behaviors and interests (Wijngaarden-Cremers et al., 2014), increased impairment in social communication and social functioning, and are more likely to deal with sleep problems, depression and anxiety (Carter et al., 2007; Hartley & Sikora, 2009). On the other hand, boys show increased language and motor skills (Carter et al., 2007), as well as hyperactivity and impulsivity (May et al., 2016). Interestingly, the male bias of ASD was found to be 3:1 in a meta-analysis (Loomes et al., 2017), rather than the more often reported 4:1 ratio (Baio et al., 2018). This difference may be due to a sex bias in the criteria for the diagnosis of ASD, as well as to the use of the male phenotype as reference for ASD-type behavior, leading to the underdiagnosis of girls and/or to a later age of formal diagnosis compared to boys (Giarelli et al., 2010; Wijngaarden-Cremers et al., 2014). Nevertheless, sex-

specific and sex-dependent mechanisms have been identified regarding the extreme male brain hypothesis, female protective effect, female camouflage effect, maternal immune activation and sex chromosomal abnormalities, which lead to an increased susceptibility to the disorder in males (Santos et al., 2022).

Research on ASD relies a great deal on animal experimentation with rodents. Indeed, these are highly active, social species, which facilitates the study of stereotyped, repetitive behaviors, social interactions and communication skills and their underlying pathways. As a result, multiple rodent ASD models have shown ASD-like behaviors, which allows the translation into the clinical domain and the investigation of therapeutic targets (DiCicco-Bloom et al., 2006; Ey et al., 2011; Pasciuto et al., 2015).

1.2.

TUBEROUS SCLEROSIS COMPLEX

Tuberous sclerosis complex (TSC) is a rare, autosomal, dominant condition with an incidence of 1 in 5,800 births (Osborne et al., 1991), and affecting an estimated total of 2 million people worldwide (Henske et al., 2016).

TSC is caused by mutations in either the *Tsc1* or the *Tsc2* genes, which encode the hamartin and tuberin proteins, respectively. The two proteins form the Tsc1-Tsc2 complex, in which tuberin functions as a GTPase-activating protein (GAP) targeting the Ras homolog enriched in brain (Rheb). In turn, Rheb mediates the phosphorylation of the mammalian target of rapamycin (mTOR), a serine/threonine kinase which forms the mTOR complex 1 (mTORC1). As such, the Tsc1-Tsc2 complex is an inhibitor of the Rheb-mTORC1 pathway, and a loss-of-function mutation in either hamartin or tuberin will lead to its hyperactivation (Inoki et al., 2003; Switon et al., 2016) (Figure 2).

The mTORC1 signaling pathway is involved in multiple cellular processes, controlling the cellular environment in various aspects, such as translation and protein synthesis, transcription and autophagy (McMahon et al., 2012; Malik et al., 2013; Lipton & Sahin, 2014). Further, mTOR also forms the mTORC2, targeted by the Tsc1-Tsc2 complex as well, and responsible for protein synthesis and maturation, autophagy, dynamics of the actin cytoskeleton and cell migration (Oh & Jacinto, 2011) (Figure 2).

Finally, mTORC-independent Tsc1-Tsc2-Rheb mechanisms have also been described, responsible for formation of cellular structures, regulation of transport, secretion and signaling across the plasma membrane, and regulation of the actin cytoskeleton (Neuman & Henske, 2011) (Figure 2). Overall, the Tsc1-Tsc2 complex is involved in a myriad of mechanisms and pathways which become affected by its abnormal functioning, resulting in disease.

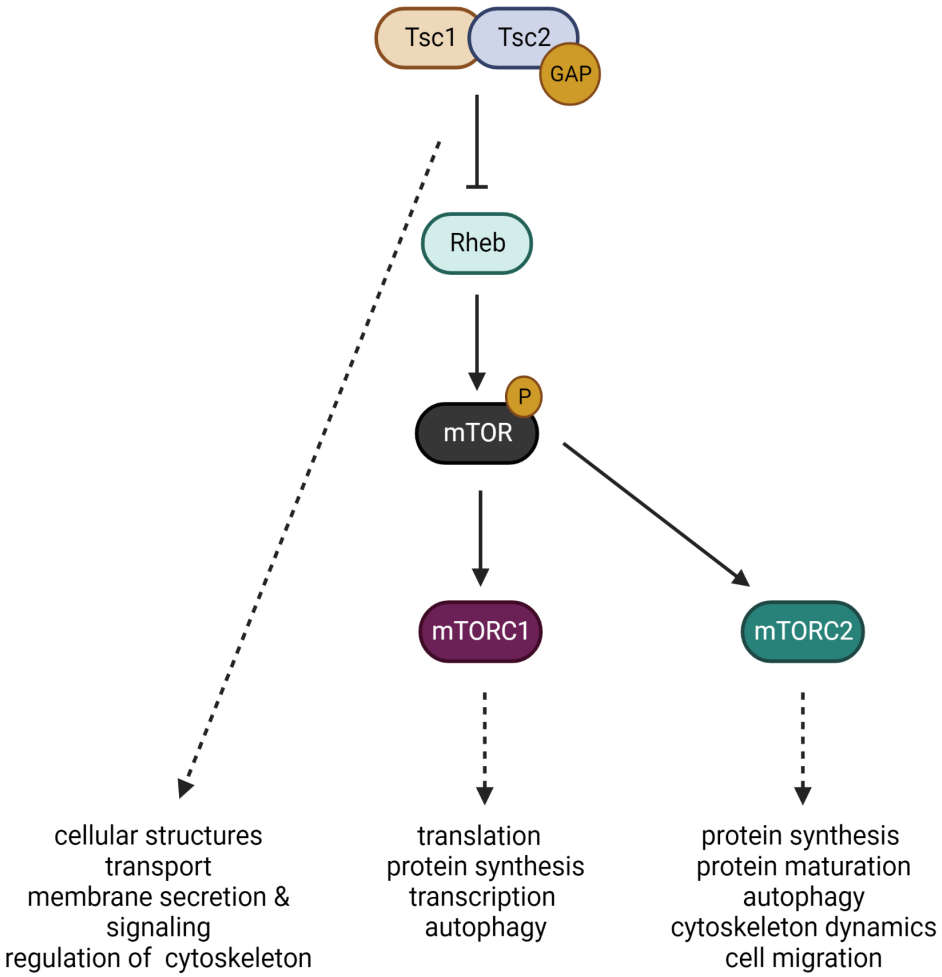


Figure 2 | Tsc1-Tsc2 complex cellular pathways

The Tsc1-Tsc2 complex, formed by the proteins hamartin (encoded by the Tsc1 gene) and by tuberin (encoded by the Tsc2 gene), targets the Ras homolog enriched in brain (Rheb), which in turn phosphorylates mTOR. mTOR forms the mTOR complex 1 (mTORC1) and 2 (mTORC2), both involved in many essential cellular functions. mTORC-independent Tsc1-Tsc2-Rheb mechanisms have also been described. As such, loss of function of either hamartin or tuberin will result in hyperactivation of these pathways, leading to abnormal cellular function. Created with BioRender.com

Hallmarks of TSC include the growth of benign tumors (hamartomas) in different organs, and dermatologic manifestations, namely, facial angiofibroma, hypomelanotic macules, ungual fibromas and shagreen patches. Patients also suffer from renal, cardiac, pulmonary and retinal problems, making this a multisystemic, life-long condition (Crino et al., 2006; McEneaney & Tee, 2019) (Figure 3).

Importantly, TSC is also characterized by neurological abnormalities, such as cortical tubers, subependymal nodules and subependymal giant cell astrocytomas. Patients may suffer from epilepsy, intellectual disability and/or ASD (Winden et al., 2018) (Figure 3).

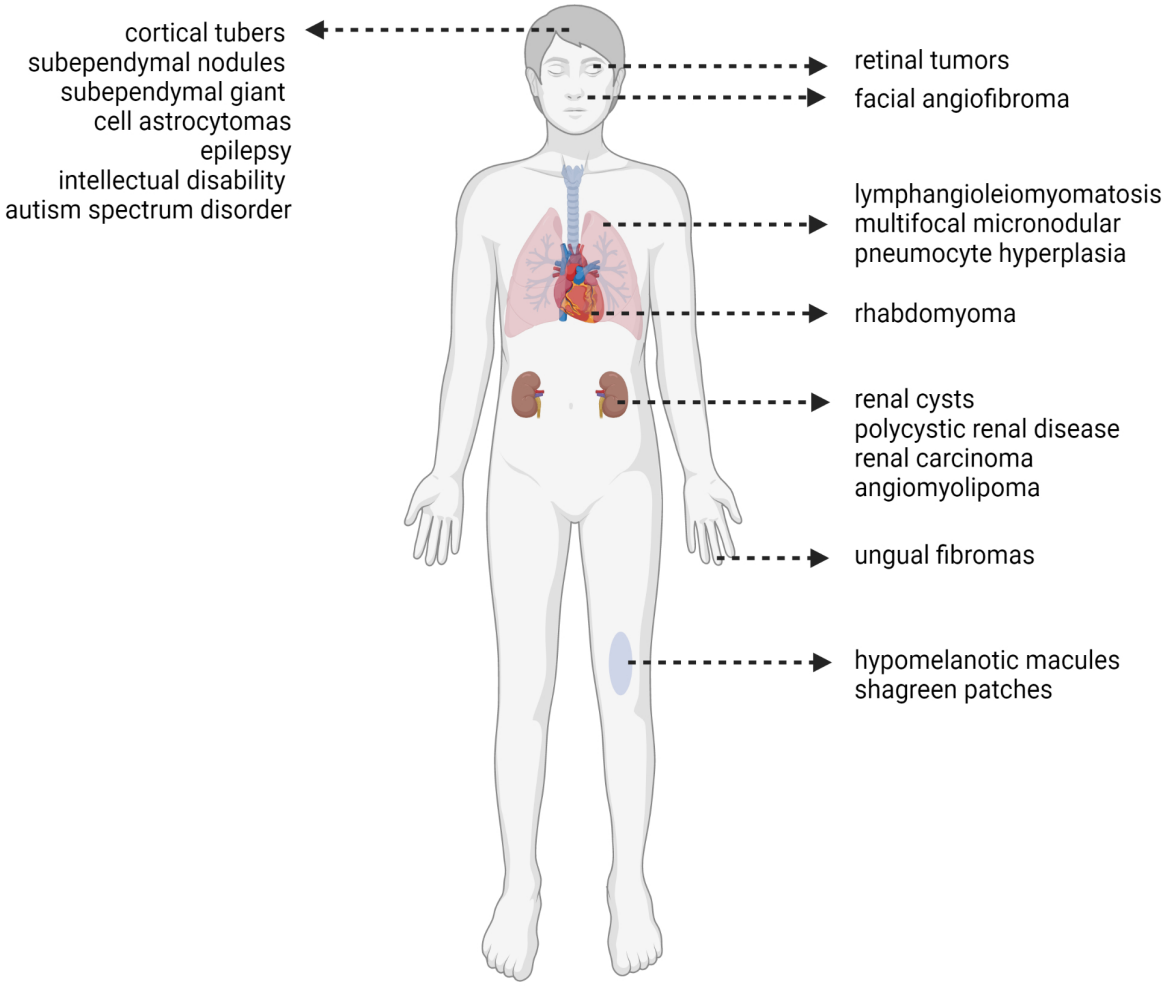


Figure 3 | Hallmarks of tuberous sclerosis complex

TSC is a multisystemic, chronic condition. Patients will suffer from growth of benign tumors in multiple organ systems, dermatological symptoms, renal, cardiac, pulmonary and retinal dysfunctions, as well as neurological abnormalities, including ASD. Created with BioRender.com

Indeed, it is estimated a prevalence of ASD in the TSC population of 21 - 36% (Richards et al., 2015, Vries et al., 2018). In particular, mutations on *Tsc2* gene were reported as a risk factor for the development of ASD, with 85.6% of patients with TSC and ASD carrying this mutation (Vignoli et al., 2015; Specchio et al., 2020).

ASD symptom profiling studies reported that children who presented TSC and comorbid ASD dealt with deficiencies in social communication in a similar way as children with non-syndromic ASD, highlighting the importance of early observation for the diagnosis of ASD in children with TSC (Jeste et al., 2016). Further, authors have claimed that children with TSC and ASD are at a higher risk to present cognitive impairments (Jeste et al., 2008).

Animal models with disruption in the mTOR pathway have been widely used to study the behavior and underlying mechanisms of TSC and ASD. Indeed, *Tsc2*$^{+/-}$ mice have shown spine pruning defects, deficient autophagy and phagocytosis, inhibition of oligodendrocyte differentiation mediated by microglia, hyperexcitability in the prefrontal cortex, and ASD-like social behavior and stereotypies (Tang et al., 2014; Pagani et al., 2021; Bassetti et al., 2020; Takanezawa et al., 2021; Ferreira et al., 2022). Additionally, loss of Tsc2 in the cerebellar Purkinje cells is associated with both repetitive behavior and social deficits (Reith et al., 2013). These results are in line with the deficient sociability, impaired communication and stereotyped behavior found with *Tsc1*$^{+/-}$ and *Tsc1*$^{-/-}$ in the Purkinje cells (Tsai et al., 2012).

Overall, the amount of evidence collected with animal models with mTOR-related pathologies, particularly with *Tsc2*$^{+/-}$ rodents, renders considerable translational potential, from the preclinical to the clinical scenario, thus motivating the study of ASD's manifestations and underlying mechanisms.

1.3.

MATERNAL INFLUENCE

The influence brought by maternal risk factors in the development of ASD is a widely discussed subject among the scientific community. Research focuses mainly on prenatal events, such as maternal diabetes, depression, hypertension or infection, as well as in utero chemical exposure and maternal hormone imbalance (Lu et al., 2022).

Nevertheless, beyond the solely molecular and/or genetic aspects of maternal influence in the development of their child, the social and interpersonal relations between them also ought to be considered. In fact, increased maternal warmth was associated with better outcomes in adulthood (Bishop-Fitzpatrick et al., 2016). This is in line with previous studies which found that ASD-associated behaviors were improved over time under a positive maternal environment of praise (Woodman et al., 2015; Woodman et al., 2016), thus highlighting the relevance of early child-parental relations in ASD outcomes.

An interesting line of research stemming from the study of mother-child relationships in ASD concentrates on the effect of maternal influence on communication skills. Indeed, behavior therapy in mothers was associated with improved communication abilities in their ASD children (Ijaz et al., 2020). Additionally, investigation on pragmatics[1] of maternal communication have shown that decreased rates of maternal pragmatic violations result in an increase in expressive language in their children (Stern et al., 2017). Further, the quality of mother-child interactions, with appropriate emotional and behavioral responses, as well as lower levels of parenting stress were predictive of treatment efficacy (Fu et al., 2020). This underlies the important contribution of early maternal influence on the development of the child's communicative skills.

Interestingly, the reverse has also been under study: the effect of raising an ASD child on the parents' well-being and mental state. Indeed, physical, emotional and cognitive stress are common among parents of ASD individuals (DePape & Lindsay, 2015). In this line, it has been reported that mothers whose children ameliorate their

1 Pragmatics is a branch of linguistics that studies the use of language within a certain context and in a given social interaction; pragmatic violations are then the result of misunderstandings of the context and/or of the social aspect of the communicative act, i.e., an apology being interpreted as an excuse (Luo & Gao, 2011).

social and communicative skills through therapy present decreased psychological distress and more parenting satisfaction (Ozturk et al., 2016; Laister et al., 2021).

In parallel, maternal care and instinct are also object of research in mouse models, with similar conclusions being drawn. Indeed, a thought-provoking cross-fostering experiment showed that pups born from a mother exposed to trauma during pregnancy but raised by a healthy female were less anxious than pups born from a healthy mother and raised by a female with maternal trauma (Golub et al., 2016). This shows very clearly that maternal behavior is essential in the development of the litter, and may even be of greater importance than *in utero* environmental factors. Accordingly, pups raised by mothers with hearing deficits, causing the maternal response to pups' USVs to be limited, presented abnormal social behavior, which was rescued when the offspring was raised by healthy females (Wu et al., 2009).

Furthermore, the alteration of female-pup early relationship through either maternal separation or limited bedding caused shifts in the development of acoustic parameters and USV types in the offspring (Yin et al., 2016; Granata et al., 2021). Again, this underscores how the quality of mother-pup interactions impacts offspring development, and specifically, communication skills over time.

The amount of evidence here presented indicates that mother-child, or female-pup relationship may very well be a key contributor for the development of ASD symptoms, or ASD-like manifestations, and for later outcomes in adolescence and adulthood, thus deserving close attention from researchers and clinicians alike.

1.4.

SEROTONIN AND OXYTOCIN

Serotonin (5-hydroxytryptamine) is a signaling molecule produced from the essential amino acid tryptophan. It is found both in the brain, where it functions as a neurotransmitter, and in a greater proportion in the periphery. Peripheral serotonin is mainly produced by enterochromaffin cells in the lumen of the gastrointestinal tract, and is then taken up into the blood stream through the enteric circulatory system (Muller et al., 2016) (Figure 4). Importantly, this peripheral production of serotonin is increased during pregnancy, with a placental source of serotonin and maternal tryptophan precursor (Bonnin et al., 2011). This hints towards the role of serotonin in the early stages of brain development.

In fact, the serotonergic system has been implicated in ASD since increased blood serotonin was reported in 1961 (Schain & Freedman, 1961), making hyperserotonemia the first molecular biomarker identified for the disorder. Indeed, a meta-analysis found that 28.3% of ASD patients have increased levels of whole blood serotonin (Gabriele et al., 2014).

Regarding brain serotonin (which exists independently from peripheral serotonin, as the blood-brain barrier prevents its passage), it is produced by TPH2-expressing serotonergic neurons located in the midbrain and hindbrain, which are organized into clusters that form the raphe nuclei. From there, serotonergic neurons enervate a wide number of brain regions (Muller et al., 2016) (Figure 4).

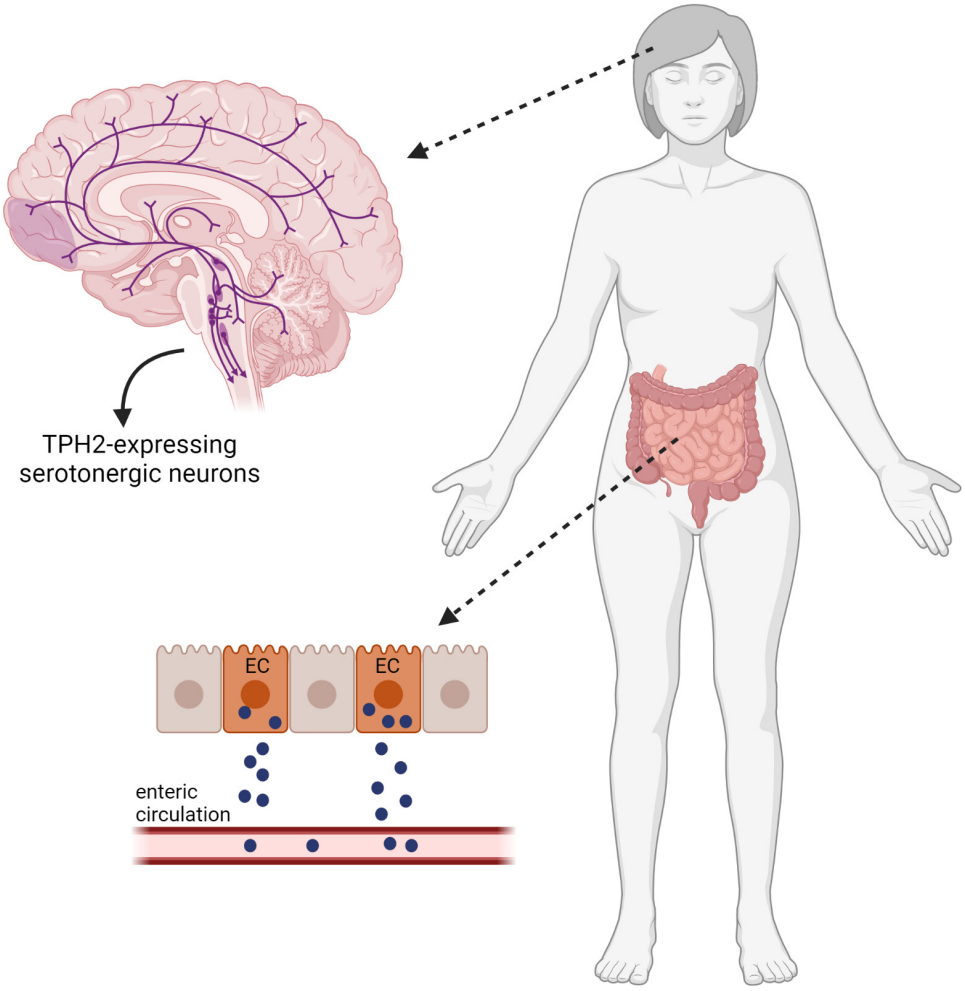


Figure 4 | Peripheral and brain serotonin

Serotonin exists in a greater proportion in the periphery, where it is mainly produced by enterochromaffin cells of the intestinal tract and is taken up in the blood stream through the enteric circulation. Brain serotonin functions as a neurotransmitter, and is produced by serotonergic neurons organized in the raphe nuclei, in the midbrain and hindbrain. Serotonergic neurons enervate through a wide range of brain regions. Created with BioRender.

Oxytocin is a peptide mainly produced in the magnocellular neurons of the hypothalamic paraventricular and supraoptic nuclei, and released from the posterior pituitary into peripheral circulation upon stimuli such as stress, suckling or during birth. Additionally, oxytocinergic neurons are broadly projected over the central nervous system (Gimpl & Fahrenholz, 2001) (Figure 5).

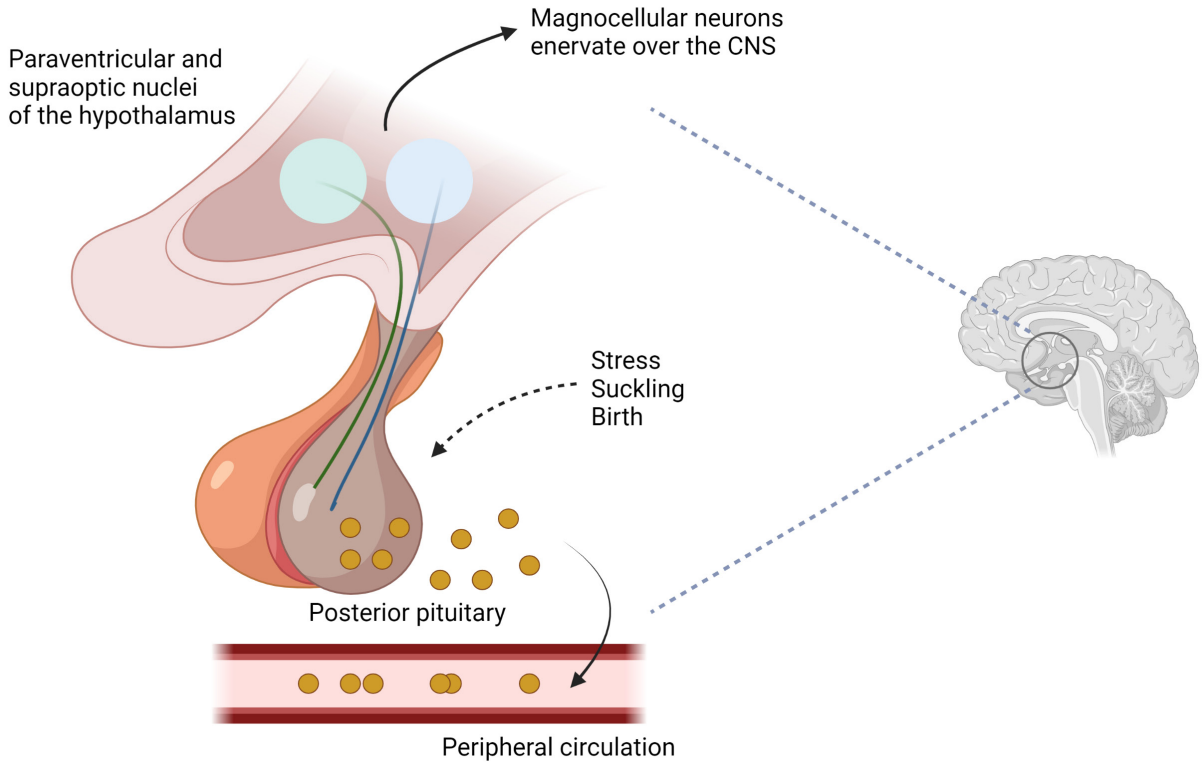


Figure 5 | Production and release of oxytocin

Oxytocin is produced in the paraventricular and supraoptic nuclei of the hypothalamus by magnocellular neurons, which broadly enervate through the central nervous system. Oxytocin is released into peripheral circulation from the posterior pituitary, under stimuli such as stress, suckling or birth. Created with BioRender.com

Both serotonin and oxytocin are implicated in the mechanisms underlying social behaviors. In fact, a serotonin-environment interaction was reported, where not only serotonin modulates social behavior, but the behavioral responses also create an effect in the serotonergic system (Kiser et al., 2012). Serotonin impacts on parental attachment, social play, aggressive, cooperative and sexual behavior (Kiser et al., 2012), and oxytocin has been proposed as a therapeutic option for social anxiety and dysfunction (Jones et al., 2017). Interestingly, authors found that depletion of

serotonergic neurons from the raphe nuclei stopped the oxytocin-elicited social reinforcement signal in the nucleus accumbens. Moreover, activation of serotonin receptors 5-HT1b was required for oxytocin-induced synaptic plasticity. In this sense, a mechanism of serotonin-oxytocin interaction was proposed, which can modulate social reward (Dölen et al., 2013; Dölen, 2015).

The role of serotonin and oxytocin in communication has also been under study. Genetic and pharmacological studies have concluded that modulation of the serotonergic system results in altered separation-induced USVs in pups, although the authors warn that conclusions are to be drawn with caution, as complex interactions between several factors may confound the results. Interestingly, the influence of serotonin in social USVs produced in adult mice is not so clear (Wöhr et al., 2015). However, a thought-provoking study found that USVs number, variety and sequence structure in infancy were predictive of anxiety behaviors in adulthood, in a 5-HT1a deficient mouse model (Budylin et al., 2019). Furthermore, intranasal oxytocin in female mice increased USV production when in contact with their pups, as well as caused greater maternal care (Guoynes & Marler, 2021).

Interestingly, the maternal serotonergic system had already previously shown great influence in maternal behavior and offspring development. Indeed, in a mouse model of 5-HT1a deficiency, pups born to null or heterozygous females displayed reduced USV emission, and pups born to null females had a deficient physical development, which could be linked to poor maternal care (Van Velzen & Toth, 2010).

Naturally, the role of serotonin and oxytocin in the social and communicative symptoms of ASD has been under scrutiny recently. Deficient oxytocinergic signaling in the hypothalamus was found in mouse pups prenatally exposed to valproic acid, as well as abnormal qualitative and quantitative profiles of USVs over time, which were reversed upon acute administration of oxytocin (Tsuji et al., 2020; Tsuji et al., 2021). Additionally, administration of a 5-HT1b agonist reversed social abnormalities in six different mouse models of ASD (Walsh et al., 2021). Importantly, a positive association was found between maternal whole blood serotonin levels and better performance in non-verbal communication and adaptive function in ASD children (Montgomery et al., 2018).

These studies shed light on the relevant role of the serotonergic and oxytocinergic systems in the manifestations of ASD, and hint towards a complex interaction between mother, offspring, and molecular environment, which contributes towards the individual's social and communication skills.

CHAPTER 2

AIMS OF THE STUDY

AIMS OF THE STUDY

The influence of maternal genotype in ASD on the offspring's early development and later behavioral outcomes remains unclear. Understanding in greater detail the mother-offspring relationship could prove invaluable for the development of therapies in affected individuals.

We will address the following objectives:

1 – Investigate the influence of maternal genotype on neonatal offspring development with focus on physical and pro-social skills, and early communication;

2 – Understand the influence of maternal genotype on juvenile offspring behavior with focus on social skills, repetitive behavior, and memory/learning;

3 – Study how maternal care is linked to offspring's early development, and whether the exposure to mutant pups will impact maternal behaviors;

4 – Explore the effect of maternal genotype on levels of serotonergic and oxytocinergic systems of offspring.

CHAPTER 3

MATERIALS AND METHODS

3.1.

ANIMALS

Seventy-six animals (6 wild type dams, 6 *Tsc2*$^{+/-}$ dams, 6 litters of 31 wild type pups and 6 litters of 33 *Tsc2*$^{+/-}$ pups) were used in this study. *Tsc2*$^{+/-}$ mice were generated and backcrossed to a C57BL/6J background for at least 10 generations. Mice for experiments were generated by crossing animals from the reproduction pool previously described with a C57BL/6N animal (Ehninger et al., 2008).

Four experimental groups were used in this study: WT (wild-type) dam with WT pups; WT dam with *Tsc2*$^{+/-}$ pups; *Tsc2*$^{+/-}$ dam with WT pups; *Tsc2*$^{+/-}$ dam with *Tsc2*$^{+/-}$ pups. To do experimental groups, virgin WT females were mated with *Tsc2*$^{+/-}$ males, and virgin *Tsc2*$^{+/-}$ females were mated with WT males. At postnatal day (PND)3, pups were identified with permanent tattoos on toes, and tails were collected for genotyping. The experimental group attributed to each litter was decided based on the greater number of pups carrying each genotype. Pups of the excluded genotype were sacrificed.

All pups were housed together with the dam until PND21, at which point each litter was segregated by sex. All animals were maintained in a housing room with a 12h/12h light-dark cycle, at 21 ± 2 °C, in animal facilities at ICNAS, University of Coimbra. All experiments are in accordance with the European Union Council Directive (2010/63/EU), National Regulations and ORBEA board of ICNAS (1/2017).

3.2.

GENOTYPING

Tissue was digested on a lysis solution [10mM Tris, 5mM EDTA, 200mM NaCl, 0.3% SDS, 2.5% proteinase K (Invitrogen, Waltham, MA, USA)], for 4 hours at 55ºC. The resulting solution was centrifuged (1730R, Gyrozen, Gimpo, South Korea) at 12000 rpm for 15 min at 4ºC. Supernatant was collected, and DNA was precipitated with two sequential centrifugations at 4000 rpm for 15 min at 4ºC with 100% ethanol and 75% ethanol (Sigma-Aldrich, St. Louis, MO, USA). Pellet was dried overnight and resuspended in TE buffer (Tris 10Mm, EDTA1mM, pH 8.0). DNA strands were untangled with a 10-min 70ºC heating. DNA was quantified (NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA) and PCR was prepared with the following components: Taq Master Mix 50% (Bioron Diagnostics GmbH, Römerberg, Germany), primers 5% (Metabion, Planegg, Germany) (Table 1), PCR Water (Bioron Diagnostics GmbH, Römerberg, Germany) until volume was complete and DNA (400ng/uL). PCR was run (T100 Thermal Cycler, Bio-Rad Laboratories, Hercules, CA, USA) with the following amplification temperatures: 3 min 94ºC; 45 seconds 94ºC, 1 min 64ºC, 1 min 72ºC x 35 cycles; 10 min 72ºC; infinite hold 4ºC. Loading buffer 6x (Thermo Fisher Scientific, Waltham, MA, USA) was added to each PCR product and the resulting solutions were loaded into a 3% agarose (NZYTech, Lisboa, Portugal) gel in TAE (Tris 40mM, EDTA 5mM, acetic acid 5.71%, pH 8.0) with GreenSafe Premium (NZYTech, Lisboa, Portugal) for band visualization. Electrophoresis was run with TAE at 120V for 35 min (PowerPac, Bio-Rad Laboratories, Hercules, CA, USA). Results were visualized under UV light.

Table 1 | Primers used in genotyping

Primer	Sequence
TSC2-P605	5’ CAA ACC CAC CTC CTC AAG CTT C 3’
TSC2-P606	5’ AAT GCG GCC TCA ACA ATC G 3’
TSC2-P607	5’ AGA CTG CCT TGG GAA AAG CG 3’

3.3. BEHAVIORAL TESTS

3.3.1. Pup developmental milestones

Developmental milestones test was performed on PND6, 8, 10, 12 and 14, always in the same order, during the light period (8:00AM – 11:00AM), in a quiet room.

3.3.1.1. Surface righting reflex

The pup was placed on its back on a flat surface, held by the operator for 2 seconds and released. The time taken to return to a four-limb position after release was registered, with a cut-off time of 30 seconds (VanRyzin et al., 2016) (Figure 6A).

3.3.1.2. Nest seeking

A rectangular plastic arena (25cm x 10cm) was divided into 3 compartments, by tracing a goal line on each side, 6.5 cm from the center. Home bedding material was placed on the left compartment, and fresh bedding material in a similar amount was placed on the right compartment. The pup was placed in the center of the arena compartment, at a 90° angle from the bedding compartments, and allowed to explore the arena. Two 120-second trials were performed, with a 30-second intertrial interval between them, during which the operator held the pup. In each trial the pup was positioned facing opposite sides of the arena to even out possible head turning preferences, and along the experiment with all pups, first trial and second trial position of pup in the arena was alternated. The time taken to cross the home bedding material goal line with both forepaws was registered, with a cut-off time of 120 seconds. If the pup crossed the fresh bedding material goal line, cut-off time was scored. The final score was calculated by averaging the scored attributed in each trial (VanRyzin et al., 2016) (Figure 6B).

3.3.1.3. Locomotion

The pup was placed in the center of a 13cm diameter circle. The time taken to cross the limit of the circle with both forepaws was registered, with a cut-off time of 30 seconds (VanRyzin et al., 2016) (Figure 6C).

3.3.1.4. Negative geotaxis reflex

The pup was placed facing down on a ramp with a 35° inclination, covered with fabric to allow traction. The time taken to rotate and direct the forepaws to the top was registered, with a cut-off time of 30 seconds. If the pup fell, rolled or walked down the ramp, it was given a maximum of 2 extra tries, after which cut-off time was scored. (VanRyzin et al., 2016) (Figure 6D).

3.3.1.5. Cliff aversion

The pup was placed with snout and digits of forepaws hanging over the edge of a flat surface elevated 10-cm above the bench. The time taken to turn snout and forepaws away from the edge was registered, with a cut-off time of 10 seconds. If the pup fell from the platform, it was given a maximum of 2 extra tries, after which cut-off time was scored. (VanRyzin et a.l, 2016) (Figure 6E).

3.3.1.6. Forelimb grasp

The pup was held hanging by its forepaws on a horizontal string 10-cm above a padded surface. The time mediating release of the pup by the operator and its fall on the padded area was registered with a cut-off time of 10 seconds. If the pup did not grasp the wire or fell immediately after release, it was given a maximum of 2 extra tries, after which cut-off time was scored (VanRyzin et al., 2016) (Figure 6F).

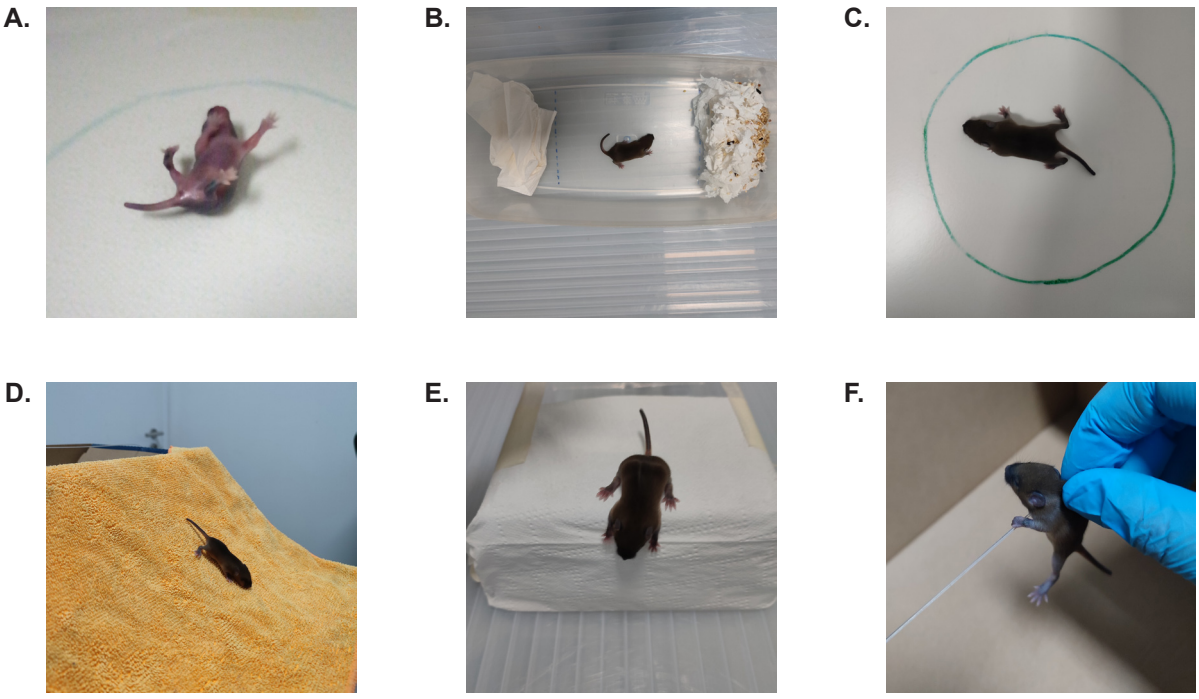


Figure 6 | Developmental milestones tests

Representation of **A**. surface righting reflex, **B**. nest seeking, **C**. locomotion, **D**. negative geotaxis reflex, **E**. cliff aversion, and **F**. forelimb grasp tests.

3.3.1.7. Auditory startle

A loud snap with a latex glove was produced next to the pup's head. The pup was observed to check whether it reacted to the noise by shaking or quickly turning its head.

3.3.1.8. Eye opening

Each pup was observed to check whether its eyes were open.

3.3.1.9. Pup USVs recording

A 55 cm x 50 cm x 70 cm (H x D x W) anechoic chamber was assembled with 1.5-cm thick acrylic sheets, and fully covered with absorbing foam on the inside, to block external sound (Figure 7A). Pup USVs were recorded on PND6, 8 and 10, following developmental milestones tests, during the light period (8:00AM – 11:00AM). The order in which each pup was tested for developmental milestones was the same as the order in which each pup's USVs were recorded. The pup was placed into a 15 cm x 10 cm x 8 cm plastic container padded with tissue paper to maintain body temperature, which was then placed inside the anechoic chamber. A 5-minute recording started immediately after the door of the chamber was closed. USVs were acquired using an ultrasound recording system with Avisoft CM16/CMPA condenser microphone placed 28 cm above the bottom of test container, UltrasoundGate 416H amplifier and Avisoft Recorder software (Avisoft Bioacoustics, Glienicke/Nordbahn, Germany) (Figure 7B). At the end of the test, the pup was returned to its home box.

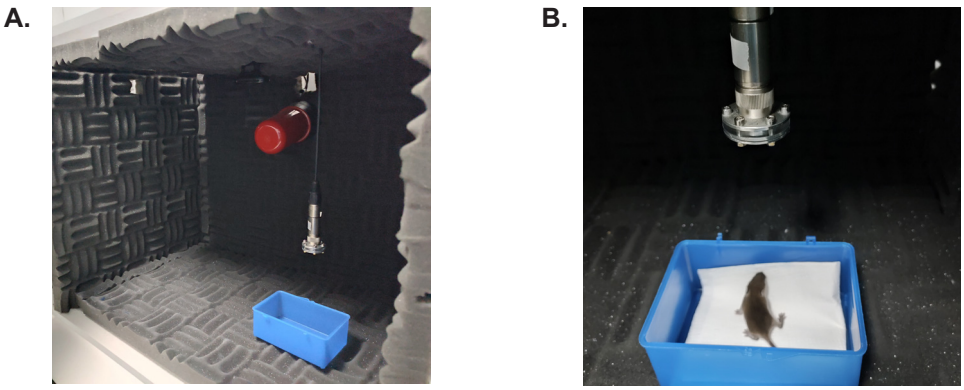


Figure 7 | Ultrasonic vocalizations recording equipment

A. Anechoic chamber fully lined with absorbing foam to block external sound. **B**. Condenser ultrasonic microphone placed above the test container.

3.3.2. Juvenile behavior tests

All juvenile behavior tests were performed during the light period (8:00AM – 13:00AM), in a quiet room. Tests were recorded for later analysis with a video camera (LifeCam HD-3000, Microsoft, Redmond, WA, USA) placed on the ceiling of the room. USVs were also recorded during all juvenile behavior tests. For that, an 80 cm x 50 cm x 40 cm (H x D x W) box was assembled, with bottom and walls fully covered with absorbing foam on the inside, to block external sound. Test apparatuses for the different juvenile behavior tests were placed inside the foamed box (Figure 8). An ultrasound recording system was used, with a Avisoft CM16/CMPA condenser microphone placed 20 cm above the bottom of the foamed box, UltrasoundGate 416H amplifier and Avisoft Recorder software (Avisoft Bioacoustics, Glienicke/Nordbahn, Germany).

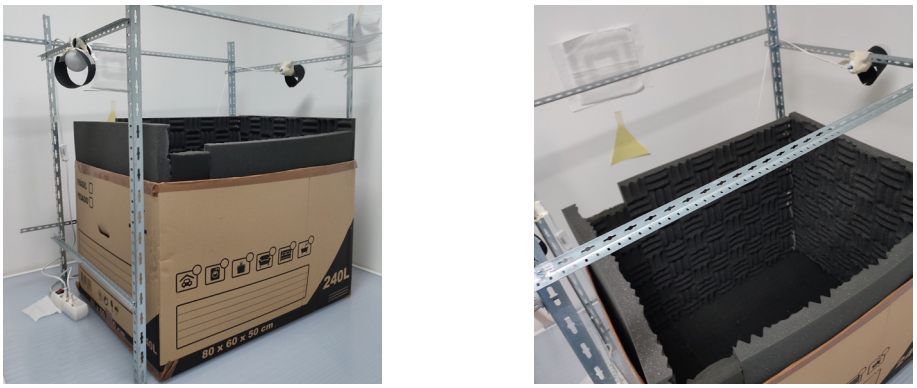


Figure 8 | Anechoic box for USVs recording
Box with walls and bottom lined with absorbing foam to block external sound.

3.3.2.1. Social odor discrimination test

On PND24, the animal was placed inside a 40 cm x 42 cm clean arena (Panlab, Barcelona, Spain) and let habituate for 10 minutes. On PND25, odor discrimination test was performed, during which the animal was exposed to both non-social and social odors.

A preliminary test was performed to determine which non-social and social aromas were more adequate; the animals engaged more with the almond non-social aroma, and with the C57BL/6J social aroma, so these were chosen to be used in this test (Figure 9).

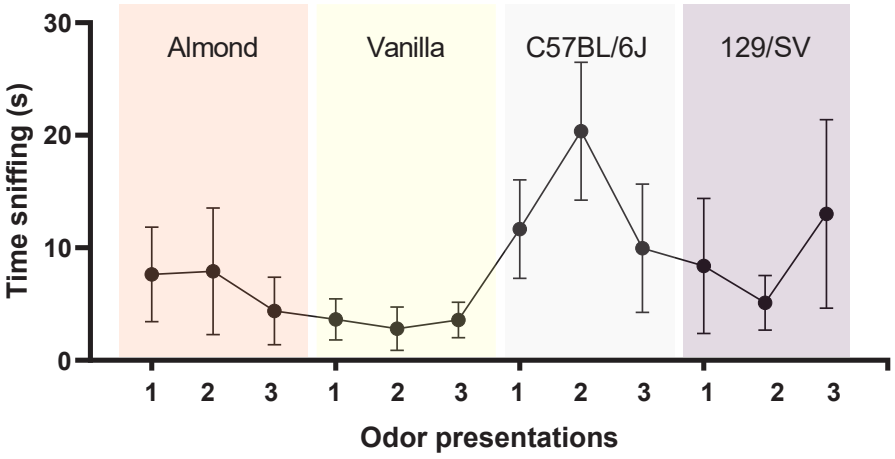


Figure 9 | Preliminary odor discrimination test

Time spent engaging with the non-social almond and vanilla aromas, and with the social odors of C57BL/6J and 129/SV mice. Each odor was presented 3 times for 2 minutes each time. n = 4 (2 males + 2 females).

Before each test, non-social odor was prepared by diluting almond liquid aroma (Condi Alimentar, S.A., Loures, Portugal) in water (1:100). Social odor was obtained by brushing clean cotton swabs on the bottom of a C57BL/6J mouse cage, which had not been cleaned for at least 3 days. Sex of the social odor was matched to the sex of the tested animal. The recipient with the non-social odor solution and the box with the social odor cotton swabs were kept tightly closed whenever possible to maintain the odor. The test room and test apparatus were clean and odorless. The animal was placed in the arena and explored freely for 10 minutes, to adjust to the odor-free environment. A cotton swab with 40uL of non-social odor solution was presented to the animal in a fixed place in the box, by one of the walls, for 2 minutes. Then, the swab was removed and after a 1-minute odor-free interval, a fresh non-social odor swab was presented for 2 minutes. Following another 1-minute interval, a third fresh non-social odor swab was presented for 2 minutes. After a 1-minute odor-free interval, 3 social odor cotton swabs were presented to the animal in the same fashion (Figure 10).

An operator observed the entire test and manually timed the amount of time the animal spent sniffing and/or directly interacting with the swab in each trial (Arbuckle et al., 2015).

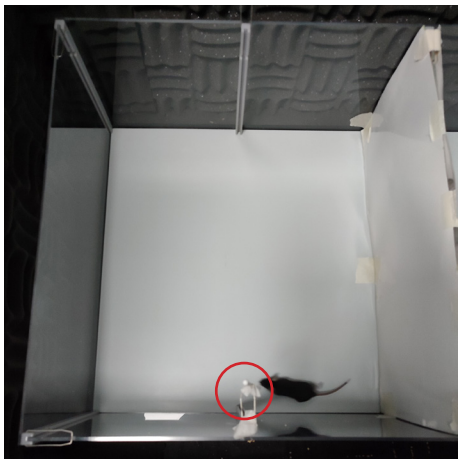


Figure 10 | Social odor discrimination test arena

Clean arena (40 cm x 42 cm) inside an anechoic box for simultaneous video and USV recording. The red circle represents the area where a cotton swab was presented to the animal with a social or non-social odor, 3 times each, for 2 minutes.

3.3.2.2. Marble burying test

A standard mouse cage was filled with an 8-cm layer of sawdust, and 12 marbles were placed equidistantly on top of the sawdust, in a 3x4 array. The walls of the cage were further extended with acrylic sheets, to avoid the animal exiting the apparatus (Figure 11). On PND30, the animal was placed on the cage and allowed to explore it freely for 20 minutes. The number of marbles buried at 5, 10, 15 and 20 minutes of the experiment were registered (Angoa-Pérez et al., 2013).

Figure 11 | Marble burying test arena

Standard mouse cage filled with sawdust, inside an anechoic box for simultaneous video and USV recording. Twelve marbles are placed equidistantly on top of the sawdust in a 3x4 array.

3.3.2.3. Y-maze test

On PND35, a clean Y-shaped arena (Panlab, Barcelona, Spain) was prepared with different visual cues placed above each of the three arms. A first trial was performed in which one of the arms of the apparatus was closed. The animal was placed in the center of the apparatus facing an open arm and let explore freely for 8 minutes (Figure 12A). At the end of the first trial, the animal was placed back in its cage which stayed inside the test room. The second trial was performed 90 minutes after the start of the first trial, with all arms of the apparatus open. The animal was placed in the apparatus in the same position as before and let explore freely for 8 minutes (Figure 12B) (Prieur & Jadavji, 2019). Animal behavior was tracked with the Smart Video Tracking Software version 3.0.06 (Panlab, Barcelona, Spain) to evaluate time, distance and mean speed on each zone for each trial.

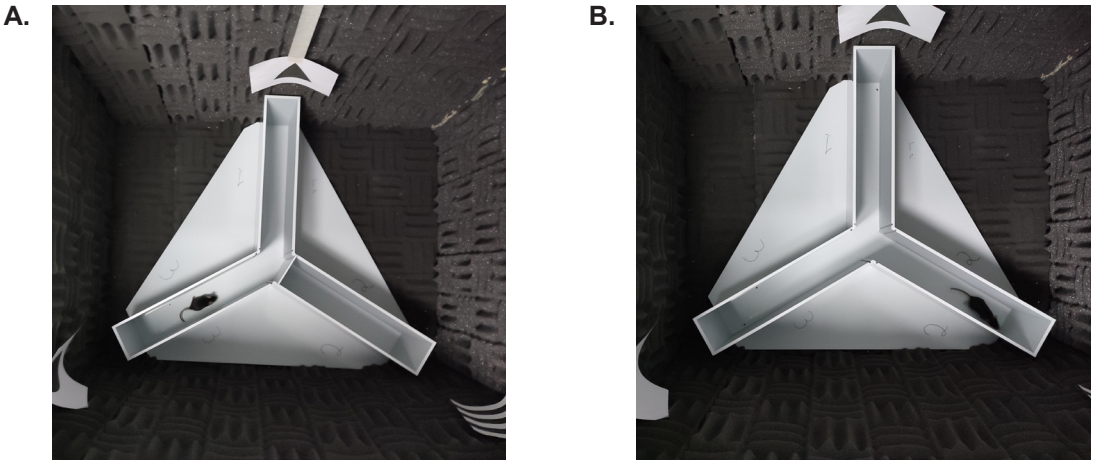


Figure 12 | Y-maze test arena

Y-shaped arena inside an anechoic box for simultaneous video and USV recording. Visual cues are placed above each of the arms of the arena. **A**. First trial performed with one arm of the arena closed. **B**. Second trial performed with all arms open.

3.3.3. Maternal behavior tests

All maternal behavior tests were performed during the light period (8:00AM – 13:00AM), in a quiet room.

3.3.3.1. Nest building

Each litter box was provided with a house-like shelter and nesting material. On the litter's PND6, 8, 10, 12, 14 and 16, photographs were taken of the nest and nesting ability was scored according to the following: 1 – paper scattered, with no visible formed nest-like structure; 2 – paper slightly scattered, with a nest-like structure; 3 – nesting

material forms a recognizable nest structure, with only minor quantities of scattered paper; 4 – nesting material is well organized to form a full nest. Representative images of each nesting score in Figure 13 (Yun et al., 2019).

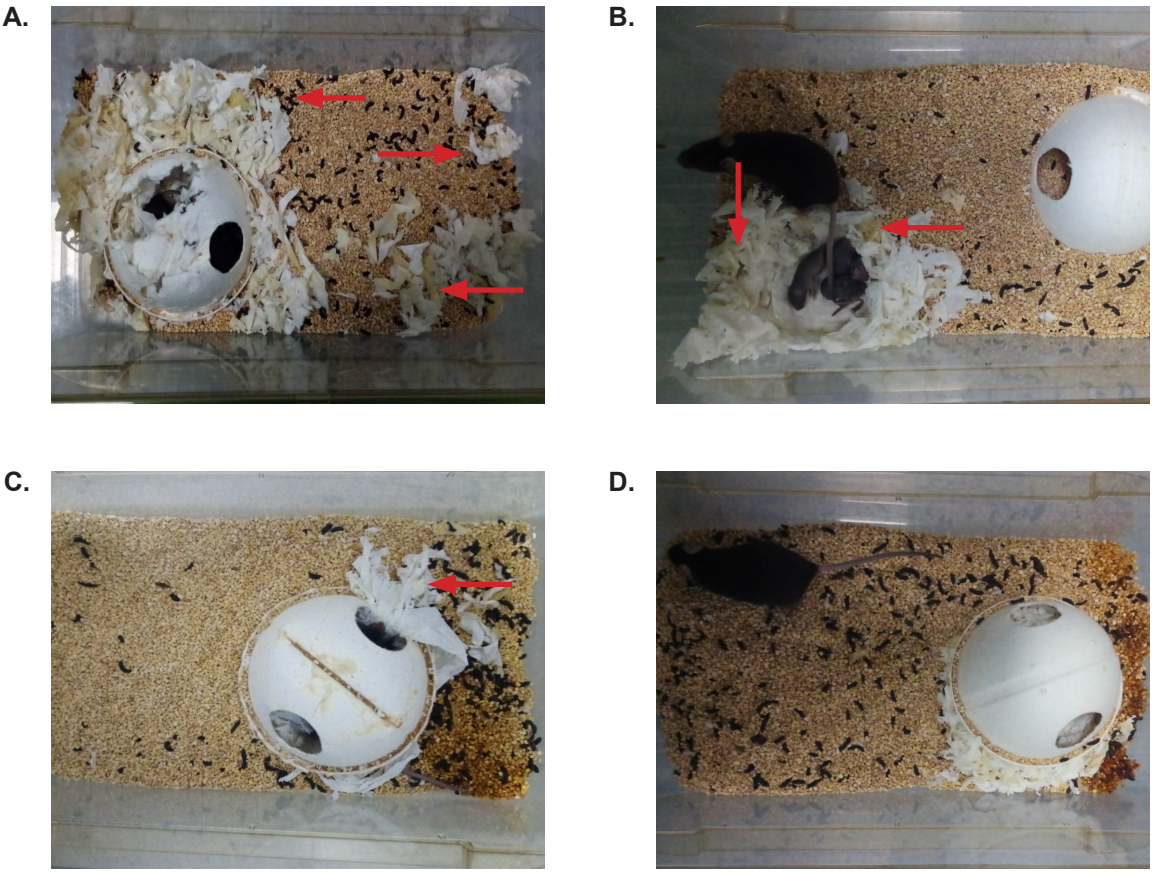


Figure 13 | Nest building scores

Representative images of the four possible nesting scores attributed to each litter over time. **A**. Score 1 (paper scattered, with no visible formed nest-like structure); **B**. Score 2 (paper slightly scattered, with a nest-like structure); **C**. Score 3 (nesting material forms a recognizable nest structure, with only minor quantities of scattered paper); **D**. Score 4 (nesting material is well organized to form a full nest). Red arrows indicate scattered paper.

3.3.3.2. Pup retrieval latency

On the litter's PND6, 8, 10, 12, 14 and 16, following developmental milestones assessment, each pup was placed back on the home cage, in the corner further away from the litter's nest. The time taken by the dam to collect the pup and retrieve it back to the nest was registered (Wu et al., 2009).

3.3.3.3. Reunion test

On the litter's PND9, a clean arena divided into two similar compartments of 20cm x 42 cm each with a removable, transparent partition between them (Panlab, Barcelona, Spain) was prepared (Figure 14A). The test was recorded for later analysis with a video camera (LifeCam HD-3000, Microsoft, Redmond, WA, USA) placed on the ceiling of the room. To record USVs, an 80 cm x 50 cm x 40 cm (H x D x W) box was assembled, with bottom and walls fully covered with absorbing foam on the inside, to block external sound. The test apparatus was placed inside the foamed box. An ultrasound recording system was used, with a Avisoft CM16/CMPA condenser microphone placed 20 cm above the bottom of the foamed box, UltrasoundGate 416H amplifier and Avisoft Recorder software (Avisoft Bioacoustics, Glienicke/Nordbahn, Germany).

The test was composed of 3 phases: the habituation phase, where dam and pups were placed on one side of the arena, with the partition closed, and let explore freely for 5 minutes (Figure 14B); then the separation phase, where the dam was placed for 3 minutes on the other compartment of the arena – the novel compartment - with the partition closed, thus being in visual, auditory and olfactory, but not physical, contact with its pups (Figure 14C); finally, the reunion phase, where the partition was removed and the dam was allowed to explore both compartments freely for 5 minutes (Figure 14D) (Guoynes & Marler, 2021).

Behavior was manually analyzed by an experienced operator blind to experimental group (Table 2) (Stanford School of Medicine, n.d.). Time spent exploring and grooming on each side of the arena, huddling the litter, or by the partition next to the litter during separation phase of the test was registered. USVs were considered to be all produced by the pups, and none by the dams, as adult mice produce USVs in a very reduced number. Total number of USVs and number of USVs of each category (Young et al., 2010) were normalized for number of pups in each litter.

Table 2 | Description of each behavior analyzed in reunion test

Behavior	Description
Exploring	The animal is investigating the local environment, adapting several possible postures such as upright (mouse rearing on hind legs), oblique (mouse is sitting up), or sideways (all paws on arena floor).
Huddling	Affiliative interaction to ensure warmth and safety when the mouse is pressing its body against another mouse.
Grooming	Maintenance behavior when the animal licks its fur, groom with forepaws and scratches a limb.

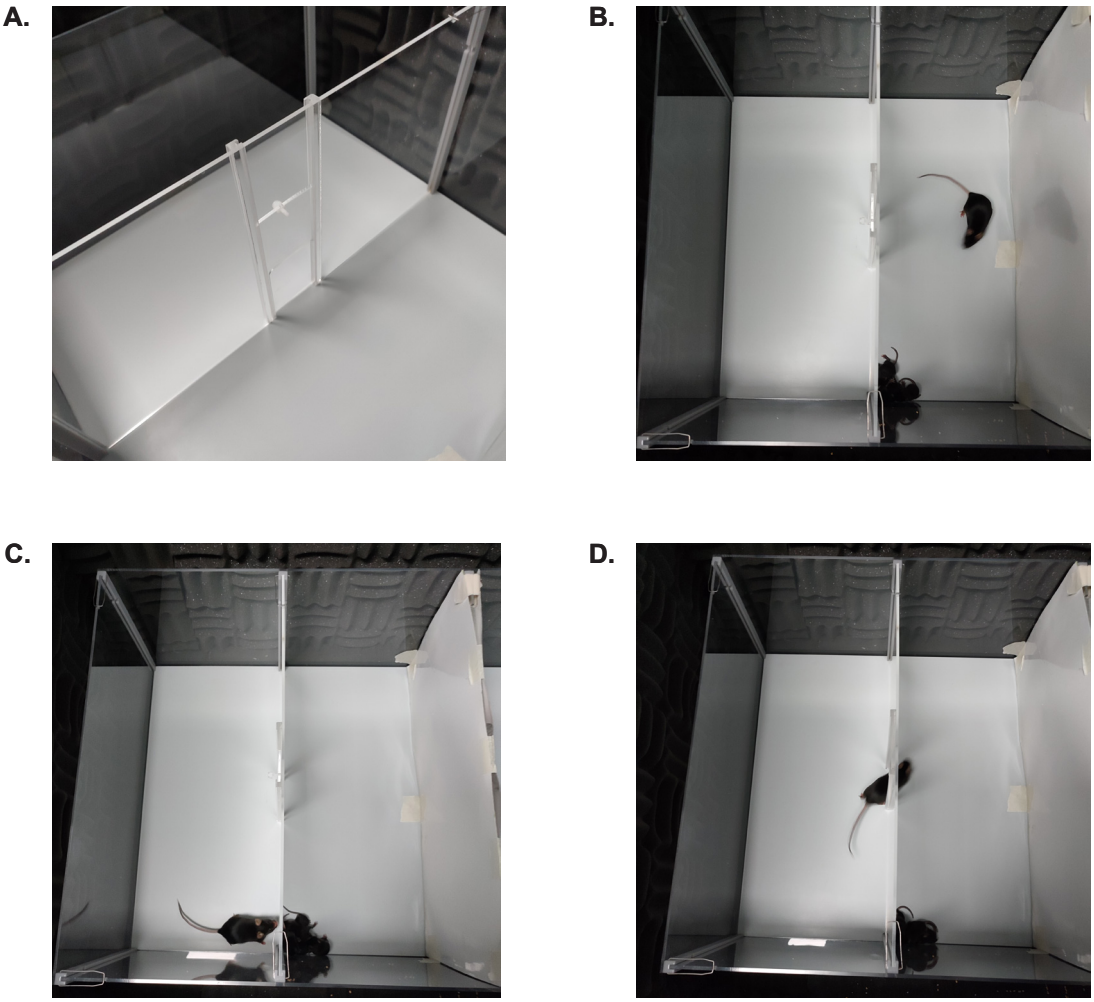


Figure 14 | Reunion test arena

A. Clean arena, with two 20 cm x 42 cm compartments divided with a transparent, removable partition. **B**. Representation of the first phase of the test – habituation phase – where both mother and pups are placed in one side of the arena. **C**. Representation of the second phase of the test – separation phase – where the mother is placed on the novel side of the arena, with the partition closed. **D**. Representation of the third phase of the test – reunion phase – where the partition is removed and the mother is allowed to explore both compartments freely.

3.3.3.4. Dams' marble burying test

A standard mouse cage was filled with an 8-cm layer of sawdust, and 12 marbles were placed equidistantly on top of the sawdust, in a 3x4 array. The walls of the cage were further extended with acrylic sheets, to avoid the animal exiting the apparatus (Figure 15). To record USVs during the test, the test box was placed inside a 55 cm x 50 cm x 70 cm (H x D x W) anechoic chamber, with 1.5-cm thick acrylic sheets, and fully covered with absorbing foam on the inside, to block external sound. An ultrasound recording system was used, with a Avisoft CM16/CMPA condenser microphone placed 20 cm above the bottom of the chamber, UltrasoundGate 416H amplifier and Avisoft

Recorder software (Avisoft Bioacoustics, Glienicke/Nordbahn, Germany). The test was also video recorded, using a video camera (C170, Logitech, Lausanne, Switzerland) placed on the top of the anechoic chamber and a red light. On the litter's PND21, the dam was placed on the cage and allowed to explore it freely for 30 minutes. The number of marbles buried at 5, 10, 15, 20, 25 and 30 minutes of the experiment were registered (Angoa-Pérez et al., 2013).

Figure 15 | Dams' marble burying test arena

Standard mouse cage filled with sawdust, inside an anechoic chamber for simultaneous video and USV recording. Twelve marbles are placed equidistantly on top of the sawdust in a 3x4 array.

3.3.4. USVs analyses

Following USVs recording, sonograms were generated, with FFT-length 512 points, 16-bit format, sampling frequency 250 kHz, time resolution 1 ms, frequency resolution 488 Hz, overlap 50%. USVs were further analyzed using MATLAB toolbox DeepSqueak version 2.6.1., which allowed the extraction of individual mouse USV calls by applying the Mouse Call_Network_V2 neural network, with a chunk length analysis of 6 seconds, overlap of 0.1 seconds, high frequency cut-off of 125kHz and no score threshold. Principal frequency, lowest frequency, highest frequency, duration and power of each USV was extracted. Moreover, each USV was manually classified by an experienced operator into three categories, as described by Young et al. (2010). Characteristics of each USV category are summarized on Table 3 and representative USVs of each category are showed on Figure 16.

Table 3 | Description of each ultrasonic vocalization category

USV category	Description
Single	One waveform only present in the sonogram, with no sudden frequency jumps and with no waveforms overlapping in time
Multi-syllabic	Two or more waveforms that immediately follow one another with a frequency jump, with no temporal interval between them and with no waveforms overlapping in time
Stacked	Two or more waveforms that overlap in time

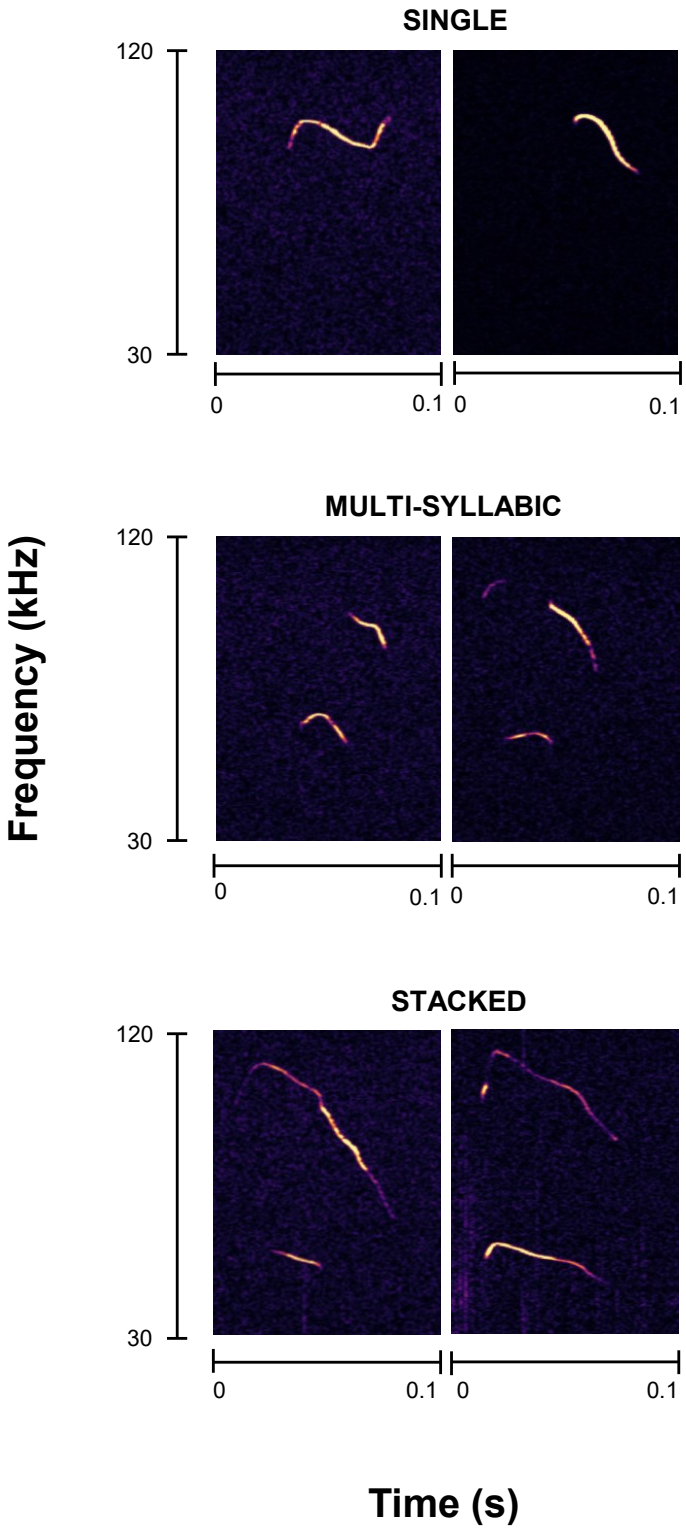


Figure 16 | Ultrasonic vocalizations categories

Representation of typical USVs waveforms produced by *Tsc2*$^{+/-}$ and WT pups, classified into 3 categories (Young et al, 2010). Sonograms obtained using MATLAB toolbox DeepSqueak version 2.6.1

3.4. ELISA

Litters were sacrificed at PND37 and dams were sacrificed after weaning of their litter. Blood was collected into EDTA-coated tubes (BD Vacutainer, Franklin Lakes, NJ, USA) and centrifuged at 1600 x g, for 15 min, at 4ºC. Plasma was collected and frozen at -80ºC until further use. Hypothalamus and amygdala of dams were dissected and frozen at -80ºC until further use.

3.4.1. Serotonin ELISA

For serotonin ELISA procedure, manufacturer's instructions were followed (Serotonin ELISA kit, Enzo Biochem, Farmingdale, NY, USA). Briefly, samples and serotonin standards were incubated with serotonin conjugate and serotonin antibody for 2 hours at room temperature with agitation. Then, wells were washed three times with wash buffer and incubated with substrate solution for 1 hour at room temperature under agitation. Stop solution was then added and plate was read at 405nm. Results were normalized with blank wells' mean absorbance and serotonin concentration was extrapolated from standard curve.

3.4.2. Oxytocin ELISA

For oxytocin ELISA procedure, manufacturer's instructions were followed (Oxytocin ELISA kit, Enzo Biochem, Farmingdale, NY, USA). Briefly, samples and oxytocin standards were incubated with oxytocin conjugate and oxytocin antibody for 24 hours at 4ºC. Then, wells were washed three times with wash buffer and incubated with substrate solution for 1 hour at room temperature. Stop solution was then added and plate was read at 405nm. Results were normalized with blank wells' mean absorbance and oxytocin concentration was extrapolated from standard curve.

3.5. STATISTICAL ANALYSIS

All data were analyzed using GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, CA, USA). Outliers were identified as all values outside the (mean ± standard deviation) interval, and excluded. Milestone development over time was analyzed with two-way analysis of variance (ANOVA), followed by Sidak's post-hoc comparisons. Composition of ultrasonic vocalizations was analyzed with unpaired t-test for genotype differences and with ordinary one-way ANOVA followed by Tukey's multiple comparisons test for sex differences. Behavioral tests were analyzed with mixed effects analysis followed by Sidak's multiple comparisons test, Kruskal-Wallis test followed by Dunn's multiple comparisons test, or Mann-Whitney test, according to data normality and study design. USVs in behavioral tests were analyzed with Kruskal-Wallis test followed by Dunn's multiple comparisons test. ELISA results were analyzed with Kruskal-Wallis test followed by Dunn's multiple comparisons test. All effects are reported as significant at $p<0.05$. Error bars are given as SEM.

CHAPTER 4

RESULTS

Here, we designed a longitudinal study to investigate the effect of maternal genotype in offspring's development, behavior and cognition. For that, we formed 4 experimental groups: WT female with WT pups; WT female with *Tsc2*$^{+/-}$ pups; *Tsc2*$^{+/-}$ female with WT pups; and *Tsc2*$^{+/-}$ female with *Tsc2*$^{+/-}$ pups. These animals performed several developmental, behavioral and cognitive tests throughout their lives to understand the influence of maternal genotype and of maternal care in the litter's ASD-like manifestations.

4.1. MATERNAL INFLUENCE ON OFFSPRING

To investigate the influence of maternal genotype in its offspring's development and behavior, we performed milestones tests in infancy to assess developmental profile. Further, at juvenile period, a social behavior test, a repetitive behavior test and a cognitive test were done to evaluate long-term impact of maternal genotype on behavior and cognition.

4.1.1. Maternal genotype influences development of motor, vestibular and communicative skills and of pro-social behavior

During this study, no significant differences were found regarding length and weight between any experimental groups (Figure 17A, B). Also, physical landmarks were registered for open eyes and startle response. We observed no significant differences on eye-opening day in the tested timepoints (Figure 18A). However, on PND14 and among the WT pups, those with a WT mother reacted to an auditory stimulus in a higher proportion than those with a heterozygote mother (WT mother & WT pups = 86.67 ± 13.33 vs *Tsc2*$^{+/-}$ mother & WT pups = 53.81 ± 9.271, p = 0.0269) (Figure 18B).

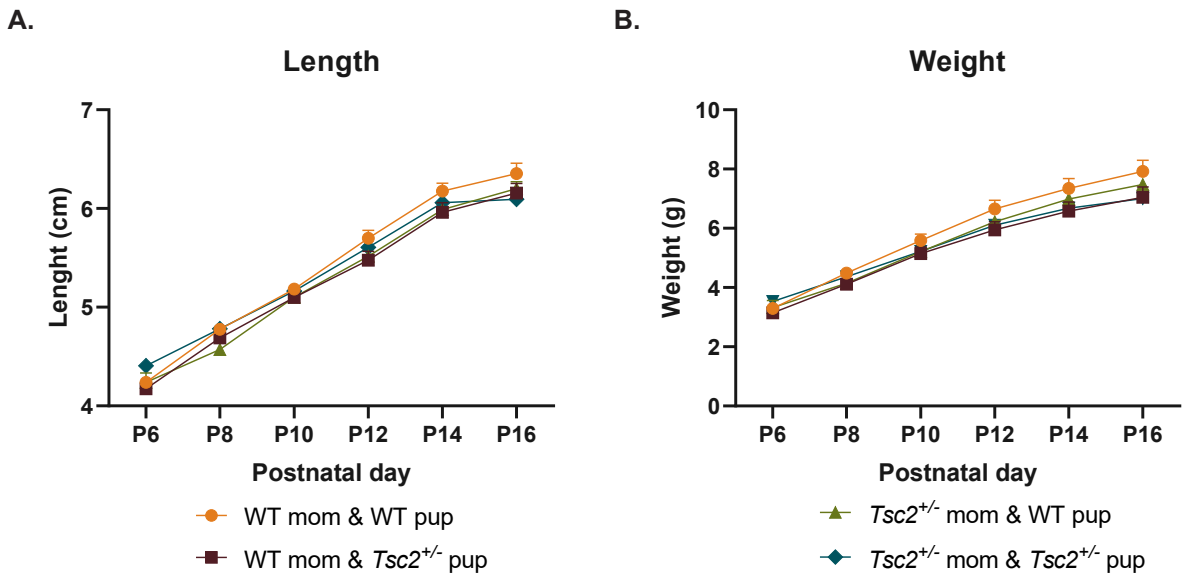


Figure 17 | Length and weight

A. Length and **B**. weight measured on PND6, 8, 10, 12, 14 and 16. Two-way ANOVA followed by Sidak's multiple comparisons test. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups) = 13; n (WT mom & *Tsc2*$^{+/-}$ pups) = 16; n (*Tsc2*$^{+/-}$ mom & WT pups) = 18; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups) = 17.

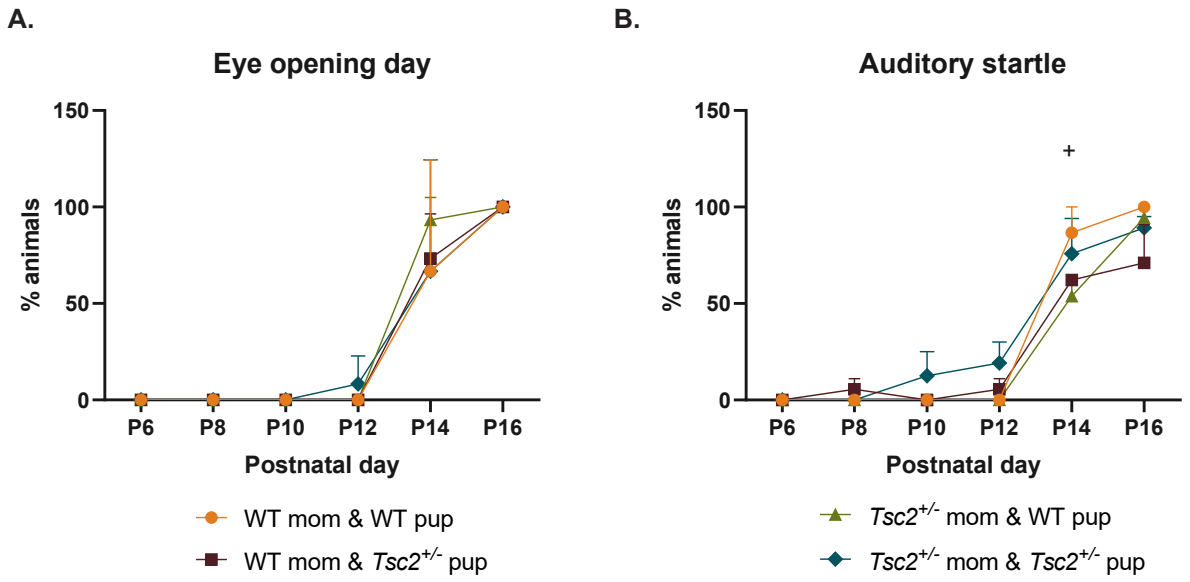


Figure 18 | Eye opening day and auditory startle response

A. Eye opening day and **B**. startle response to an auditory stimulus, registered on PND6, 8, 10, 12, 14 and 16. Two-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; +: significant difference between WT mother & WT pups and *Tsc2*$^{+/-}$ mom & WT pups experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups) = 13; n (WT mom & *Tsc2*$^{+/-}$ pups) = 16; n (*Tsc2*$^{+/-}$ mom & WT pups) = 18; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups) = 17.

On PND6 through PND14, surface righting reflex, negative geotaxis reflex, cliff aversion, locomotion, forelimb grasp and nest seeking tests were assessed to evaluate motor and vestibular system development (Figure 19).

On surface righting reflex test, we found that on PND6 WT pups born from WT mothers performed significantly better than WT pups born from *Tsc2*$^{+/-}$ mothers (WT pups: WT mother = 10.45 ± 3.166 vs *Tsc2*$^{+/-}$ mother = 30.00 ± 0.000, p<0.0001). Simultaneously, *Tsc2*$^{+/-}$ pups with *Tsc2*$^{+/-}$ mothers scored better than *Tsc2*$^{+/-}$ pups with WT mothers (*Tsc2*$^{+/-}$ pups: WT mother = 22.17 ± 2.727 vs *Tsc2*$^{+/-}$ mother = 12.16 ± 3.206, p<0.0001) (Figure 19A).

Additionally, on negative geotaxis reflex test, *Tsc2*$^{+/-}$ pups from *Tsc2*$^{+/-}$ females performed significantly better than the other experimental groups, both on PND6 (WT mother & *Tsc2*$^{+/-}$ pups = 30.00 ± 0.000 vs *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ pups = 19.96 ± 2.282, p = 0.0002; *Tsc2*$^{+/-}$ mother & WT pups = 30.00 ± 0.000 vs *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ pups =19.96 ± 2.282, p = 0.0275) and PND8 (WT mother & *Tsc2*$^{+/-}$ pups = 22.95 ± 2.297 vs *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ pups = 13.32 ± 2.306, p = 0.0005; *Tsc2*$^{+/-}$ mother & WT pups = 21.14 ± 2.665 vs *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ pups = 13.32 ± 2.306, p = 0.0056) (Figure 19B).

On cliff aversion test, WT pups with *Tsc2*$^{+/-}$ mothers scored significantly worse than the other groups on PND8 (WT mother & WT pups = 3.435 ± 0.6525 vs *Tsc2*$^{+/-}$ mother & WT pups = 5.774 ± 1.588, p = 0.0379; *Tsc2*$^{+/-}$ mother & WT pups = 5.774 ± 1.588 vs *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ pups = 2.340 ± 0.2305, p = 0.0001) (Figure 19C). These animals, from this age onwards, seem to have normalized their behavior, approaching the other experimental groups.

Regarding locomotion, we found that within pups born from heterozygote females, those carrying the *Tsc2*$^{+/-}$ genotype performed better at PND10 (*Tsc2*$^{+/-}$ mother & WT pups = 24.89 ± 2.057 vs *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ pups = 17.94 ± 2.413, p = 0.0174) and PND12 (*Tsc2*$^{+/-}$ mother & WT pups = 12.23 ± 2.346 vs *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ pups = 5.074 ± 1.535, p = 0.0131) (Figure 19D).

Similarly, among the pups born from WT females, those with a WT genotype performed better at PND10 in the forelimb grasp test (WT mother & WT pups = 2.594 ± 0.8723 vs WT mother & *Tsc2*$^{+/-}$ pups = 5.604 ± 0.7452, p = 0.0158) (Figure 19E).

Overall, there seems to be an influence of the maternal genotype in the development of motor skills and of the vestibular system, as experimental groups where both mother and offspring share the same genotype perform better than those where maternal and pup genotypes are dissonant.

Interestingly, on nest seeking test, which evaluates pro-social behavior, we found that again *Tsc2*$^{+/-}$ pups with *Tsc2*$^{+/-}$ mothers performed better than those with WT mothers, on PND8 (WT mother & *Tsc2*$^{+/-}$ pups = 116.8 ± 3.225 vs *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ pups = 76.95 ± 8.377, p = 0.0021). However, the contrary was observed regarding WT pups on PND12, as those with WT mothers scored worse than those with *Tsc2*$^{+/-}$ mothers (WT mother & WT pups = 69.25 ± 11.61 vs *Tsc2*$^{+/-}$ mother & WT pups = 28.66 ± 7.351, p = 0.0029) (Figure 19F).

This hints towards a differential development of motor and of social skills in these animals, where the interaction of maternal and offspring genotypes does not impact in the same manner the various aspects of development.

Additionally, we segregated pups by sex to understand the impact of maternal genotype on each specific biological sex.

We found that WT male pups performed better than their respective female littermates in surface righting in PND6 (male, WT mother & WT pups = 5.115 ± 1.051 vs female, WT mother & WT pups = 18.99 ± 6.741, p < 0.0001) and PND8 (male, *Tsc2*$^{+/-}$ mother & WT pups = 2.236 ± 0.4437 vs female, *Tsc2*$^{+/-}$ mother & WT pups = 9.276 ± 4.283, p = 0.0128) (Figure 20A).

Further, WT females with *Tsc2*$^{+/-}$ mothers had improved scores in negative geotaxis, compared to WT males with *Tsc2*$^{+/-}$ mothers in PND8 (male, *Tsc2*$^{+/-}$ mother & WT pups = 25.13 ± 3.132 vs female, *Tsc2*$^{+/-}$ mother & WT pups = 16.16 ± 4.081, p = 0.0484) (Figure 20B).

Additionally, *Tsc2*$^{+/-}$ males with WT mothers had increased reaction to auditory stimuli than *Tsc2*$^{+/-}$ females with WT mothers in PND14 (male, WT mother & *Tsc2*$^{+/-}$ pups = 80.53 ± 10.03 vs female, WT mother & *Tsc2*$^{+/-}$ pups = 33.33 ± 16.67, p = 0.0293) (Figure 20C). We also found several significant maternal genotype-dependent differences within each biological sex, which were in line with the results previously described here (Annexes I and II).

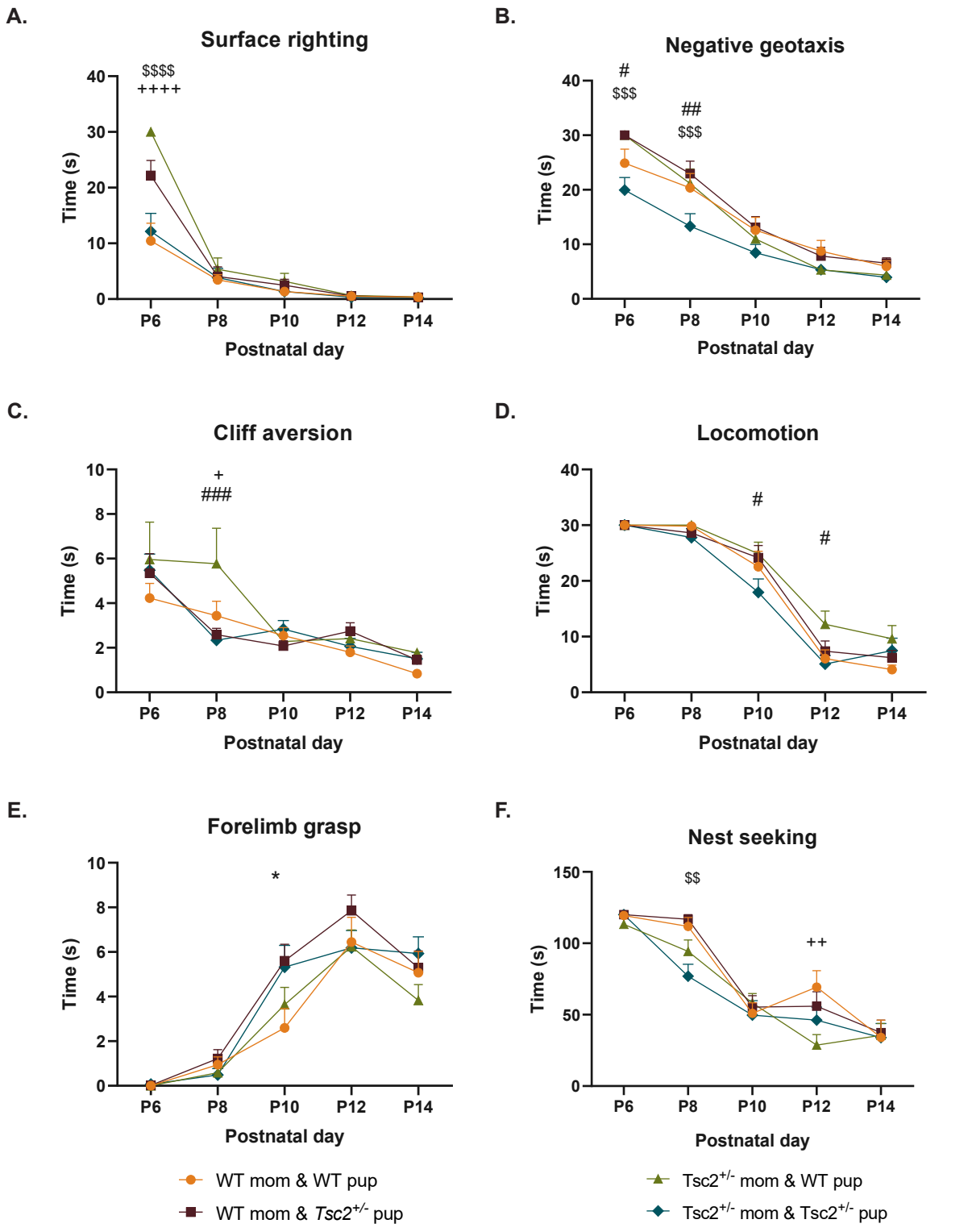


Figure 19 | Developmental milestones

A. Surface righting reflex, **B**. negative geotaxis reflex, **C**. cliff aversion, **D**. locomotion, **E**. forelimb grasp, and **F**. nest seeking tests, performed on PND6, 8, 10, 12 and 14. Two-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; *: significant difference between WT mother & WT pups and WT mom & *Tsc2*$^{+/-}$ pups experimental groups; +: significant difference between WT mother & WT pups and *Tsc2*$^{+/-}$ mom & WT pups experimental groups; #: significant difference between *Tsc2*$^{+/-}$ mother & WT pups and *Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups experimental groups; $: significant difference between WT mother & *Tsc2*$^{+/-}$ pups and *Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups) = 13; n (WT mom & *Tsc2*$^{+/-}$ pups) = 16; n (*Tsc2*$^{+/-}$ mom & WT pups) = 18; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups) = 17.

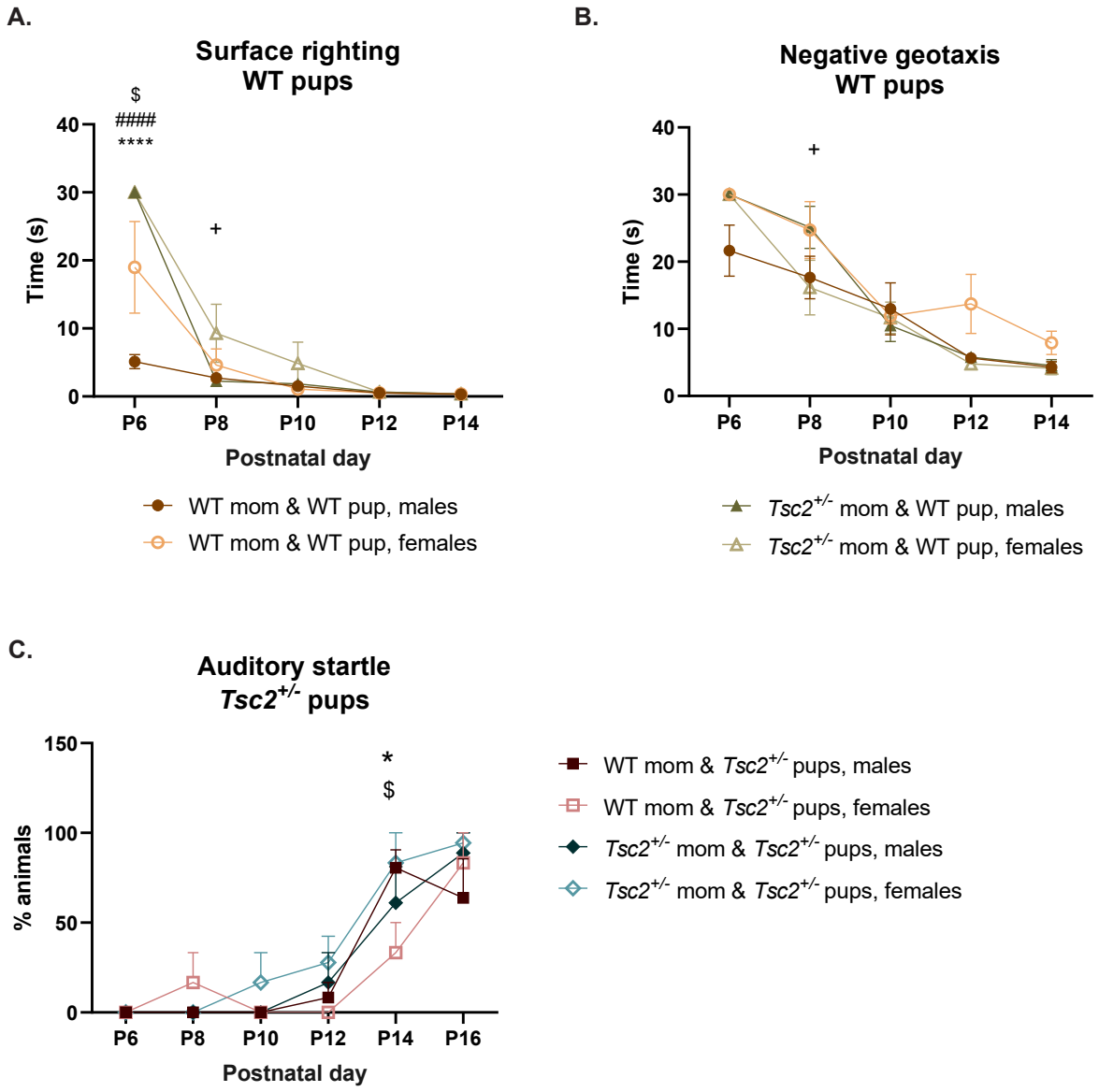


Figure 20 | Sex differences in developmental milestones

A. Surface righting reflex and **B**. negative geotaxis reflex tests of WT pups, performed on PND6, 8, 10, 12 and 14; **C**. startle response to an auditory stimulus of *Tsc2*$^{+/-}$ pups, registered on PND6, 8, 10, 12, 14 and 16. Two-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; *: significant difference between WT mom males and WT mom females experimental groups; +: significant difference between *Tsc2*$^{+/-}$ mom males and *Tsc2*$^{+/-}$ mom females experimental groups; #: significant difference between WT mom males and *Tsc2*$^{+/-}$ mom males experimental groups; $: significant difference between WT mom females and *Tsc2*$^{+/-}$ mom females experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups, males) = 8; n (WT mom & WT pups, females) = 5; n (WT mom & *Tsc2*$^{+/-}$ pups, males) = 10; n (WT mom & *Tsc2*$^{+/-}$ pups, females) = 6; n (*Tsc2*$^{+/-}$ mom & WT pups, males) = 10; n (*Tsc2*$^{+/-}$ mom & WT pups, females) = 8; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups, males) = 8; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups, females) = 9.

Finally, on PND6 until PND10, we recorded maternal separation-induced USVs, to assess communication skills. We found no effect of maternal genotype among WT pups' USV composition (Figure 21).

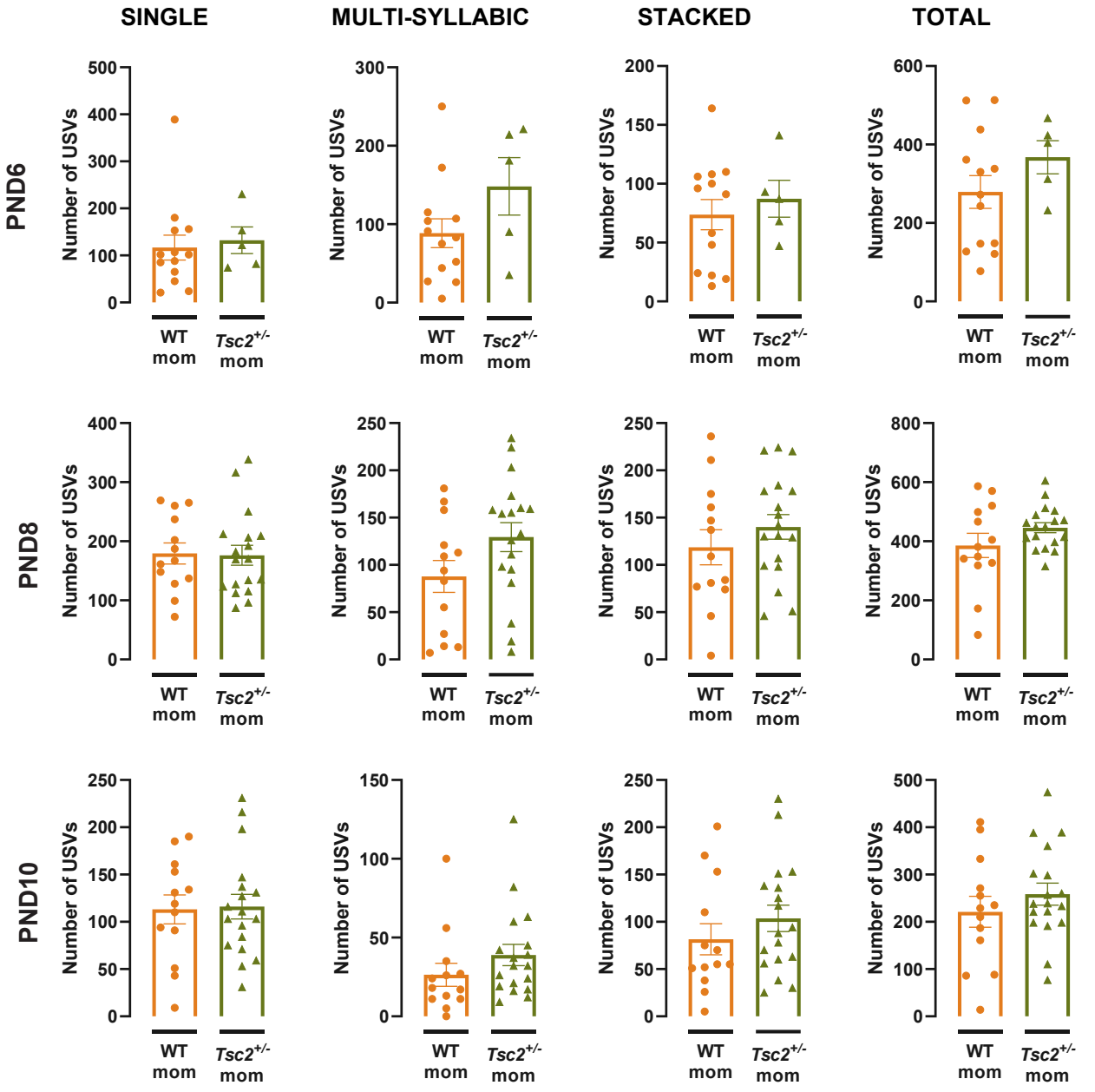


Figure 21 | Composition of ultrasonic vocalizations of WT pups

Number of single, multi-syllabic and stacked USVs (Young et al, 2010), and total number of USVs produced by WT pups born from WT or *Tsc2*$^{+/-}$ dams, on PND6, 8 and 10. Unpaired t-test; $p<0.05$. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups) = 13; n (*Tsc2*$^{+/-}$ mom & WT pups, PND6) = 5; n (*Tsc2*$^{+/-}$ mom & WT pups, PND8 and PND10) = 18.

However, we found some interesting sex differences that show that maternal genotype affects communicative skills specifically in female WT pups, where those with a WT mother produce USVs in a reduced number and with less complexity (Annex III). Moreover, the WT pups with WT mothers vocalized with significantly increased power on PND8 (WT mother & WT pups = -85.93 ± 0.7597 vs *Tsc2*$^{+/-}$ mother & WT pups = -81.23 ± 1.204, p = 0.0107) and on PND10 (WT mother & WT pups = -87.60 ± 0.7404 vs *Tsc2*$^{+/-}$ mother & WT pups = -82.43 ± 1.254, p = 0.0043) (Figure 22E).

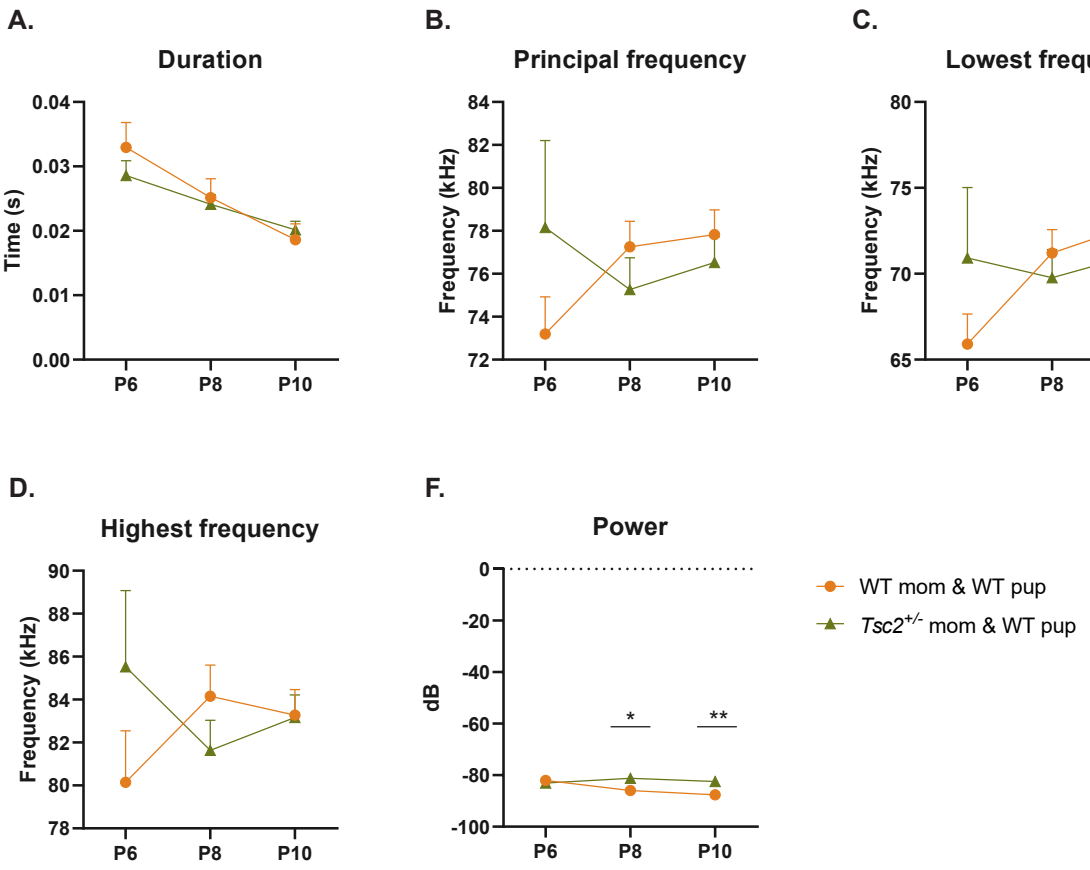


Figure 22 | Characteristics of ultrasonic vocalizations of WT pups

A. Duration, **B**. principal frequency, **C**. lowest frequency, **D**. highest frequency, and **E**. power (sound intensity) of USVs produced by WT pups born from WT or *Tsc2*$^{+/-}$ dams, on PND6, 8 and 10. Mixed effects analysis followed by Tukey's multiple comparisons test; p<0.05; *: significant difference between WT mother & WT pups and *Tsc2*$^{+/-}$ mom & WT pups experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups) = 13; n (*Tsc2*$^{+/-}$ mom & WT pups, PND6) = 5; n (*Tsc2*$^{+/-}$ mom & WT pups, PND8 and PND10) = 18.

On the other hand, *Tsc2*$^{+/-}$ pups offspring displayed differences in early communication skills. Indeed, on PND6, *Tsc2*$^{+/-}$ pups with a heterozygote mother produced significantly more multi-syllabic (WT mother & *Tsc2*$^{+/-}$ pups = 42.81 ± 10.04 vs *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ pups = 84.53 ± 13.90, p = 0.0222) and stacked (WT mother & *Tsc2*$^{+/-}$ pups = 71.25 ± 17.45 vs *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ pups = 137.4 ± 19.66, p = 0.0177) vocalizations, which is reflected in an increase in number of total maternal separation-induced USVs (WT mother & *Tsc2*$^{+/-}$ pups = 216.9 ± 31.37 vs *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ pups = 362.2 ± 30.64, p = 0.0024) (Figure 23). Overall, *Tsc2*$^{+/-}$ pups exhibited a vocal repertoire with increased complexity.

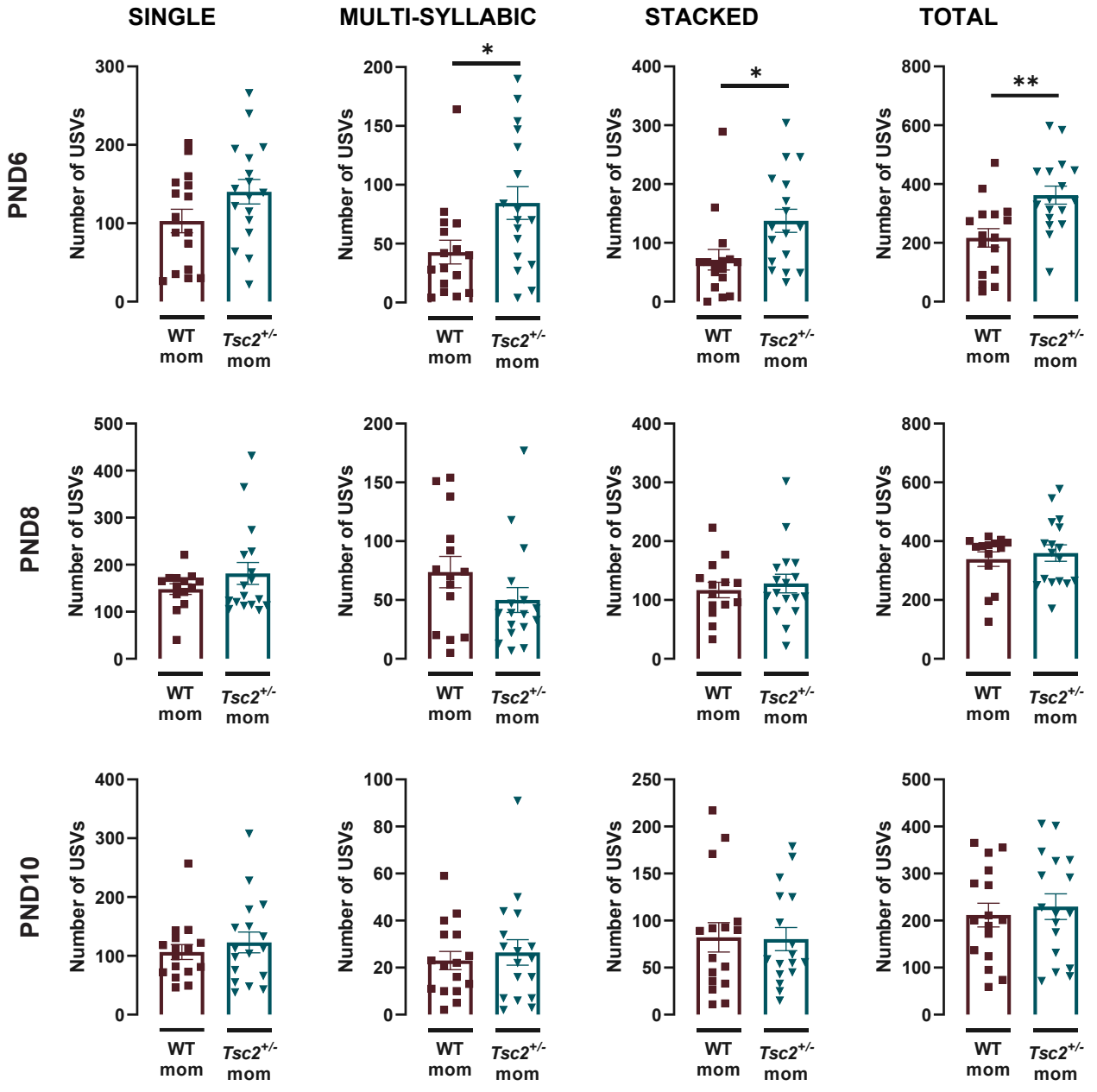


Figure 23 | Composition of ultrasonic vocalizations of *Tsc2*$^{+/-}$ pups

Number of single, multi-syllabic and stacked USVs (Young et al, 2010), and total number of USVs produced by *Tsc2*$^{+/-}$ pups born from WT or *Tsc2*$^{+/-}$ dams, on PND6, 8 and 10. *(continues on next page)*

(continuation of previous page) Unpaired t-test; p<0.05. *: significant difference between WT mother & *Tsc2*$^{+/-}$ pups and *Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups experimental groups. Data represented as mean ± SEM; n (WT mom & *Tsc2*$^{+/-}$ pups) = 16; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$pups) = 17.

Furthermore, these *Tsc2*$^{+/-}$ pups with *Tsc2*$^{+/-}$ mothers also produced longer USVs in the same timepoint (WT mother & *Tsc2*$^{+/-}$ pups = 0.02264 ± 0.002202 vs *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ pups = 0.03006 ± 0.003227, p = 0.0392) (Figure 24A). This evidence suggests different profiles of communication in *Tsc2*$^{+/-}$ pups, which are dependent on the maternal genotype.

No significant differences were found regarding principal, lowest and highest frequency of the USVs between any of the experimental groups (Figures 22B-D and 24B-D), and there were no sex differences concerning any of the USV parameters studied here, nor in USVs composition of mutant pups (Annexes IV - VI).

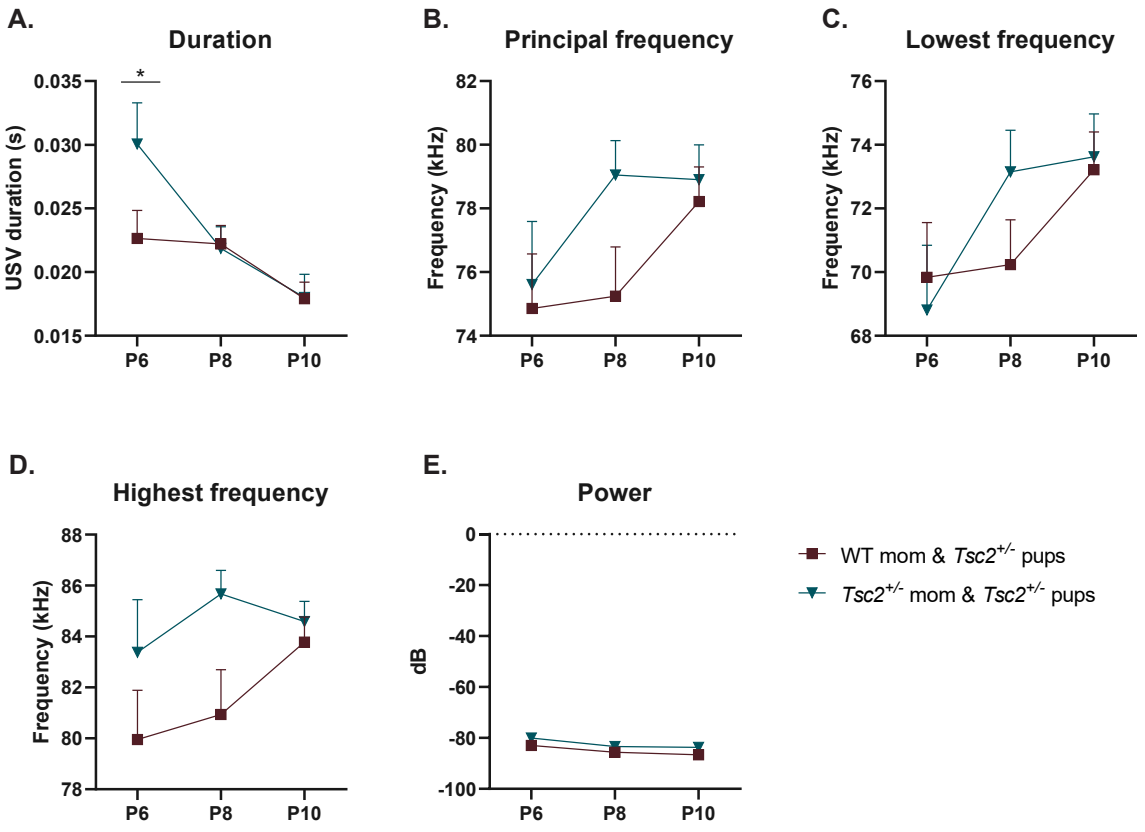


Figure 24 | Characteristics of ultrasonic vocalizations of *Tsc2*$^{+/-}$ pups

A. Duration, **B**. principal frequency, **C**. lowest frequency, **D**. highest frequency, and **E**. power (sound intensity) of USVs produced by *Tsc2*$^{+/-}$ pups born from WT or *Tsc2*$^{+/-}$ dams, on PND6, 8 and 10. Mixed effects analysis followed by Tukey's multiple comparisons test; p<0.05; *: significant difference between WT mother & *Tsc2*$^{+/-}$ pups and *Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups experimental groups. Data represented as mean ± SEM; n (WT mom & *Tsc2*$^{+/-}$ pups) = 16; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups) = 17.

4.1.2. Social odor discrimination test reveals maternal x offspring genotype interaction that modulates social behavior

To investigate whether the interaction with a heterozygous mother during infancy affected the offspring's social behavior later in life, we performed a social odor discrimination test at PND25. During this test, each animal was presented with a non-social aroma of almond extract, and with a social odor of age- and sex-matched animals. Importantly, before doing these experiments, a preliminary study was done to understand with which non-social and social odors the animals interacted the most (Figure 9).

WT pups with either *Tsc2*$^{+/-}$ or WT mothers exhibited a very similar behavior during the non-social odor phase. The highest engagement with the non-social odor swab happened in the first presentation and the animals showed a steady decrease in interest. Interestingly, during the social odor presentation the engagement profiles of the two groups differ. While WT mice with WT mothers show a roughly constant sniff time over the three odor presentations, WT mice with *Tsc2*$^{+/-}$ mothers show a significantly increased interest in the second social odor presentation (WT animal & WT mother = 1.262 ± 0.5757 vs WT animal & *Tsc2*$^{+/-}$ mother = 8.043 ± 2.434, p = 0.0049) (Figure 25A). This seems to indicate that WT animals born from WT mothers have decreased sociability compared to WT animals born from *Tsc2*$^{+/-}$ dams.

Regarding *Tsc2*$^{+/-}$ offspring, no significant differences were found during either social or non-social odor presentations (Figure 25B). Indeed, even though *Tsc2*$^{+/-}$ animals born from *Tsc2*$^{+/-}$ females seemed to display more interest in the first two presentations of the non-social odor, compared to those born from WT females, this was not statistically significant. This increased engagement of the *Tsc2*$^{+/-}$ animals with *Tsc2*$^{+/-}$ mothers continued through social odor presentation, however, still with no significant differences from the other experimental group. Interestingly, while the *Tsc2*$^{+/-}$ pups & *Tsc2*$^{+/-}$ mother group had a continuous decrease in interest in the social odor, this was not the case for the *Tsc2*$^{+/-}$ mice with WT mothers, as these show an increase in engagement time with the swab in the last social odor presentation. These results may suggest a more socially anxious behavior in the group of *Tsc2*$^{+/-}$ animals with WT mothers, however, we cannot firmly claim this as statistical significance was not reached.

Overall, there seems to be an interaction between both maternal and offspring genotypes which impact social behavior during juvenile age.

No sex differences were found in any of the experimental groups in this test (Annex VII). USVs recorded during this test revealed a very low number of calls emitted during the test, with no differences found between any of the experimental groups (Annex VIII).

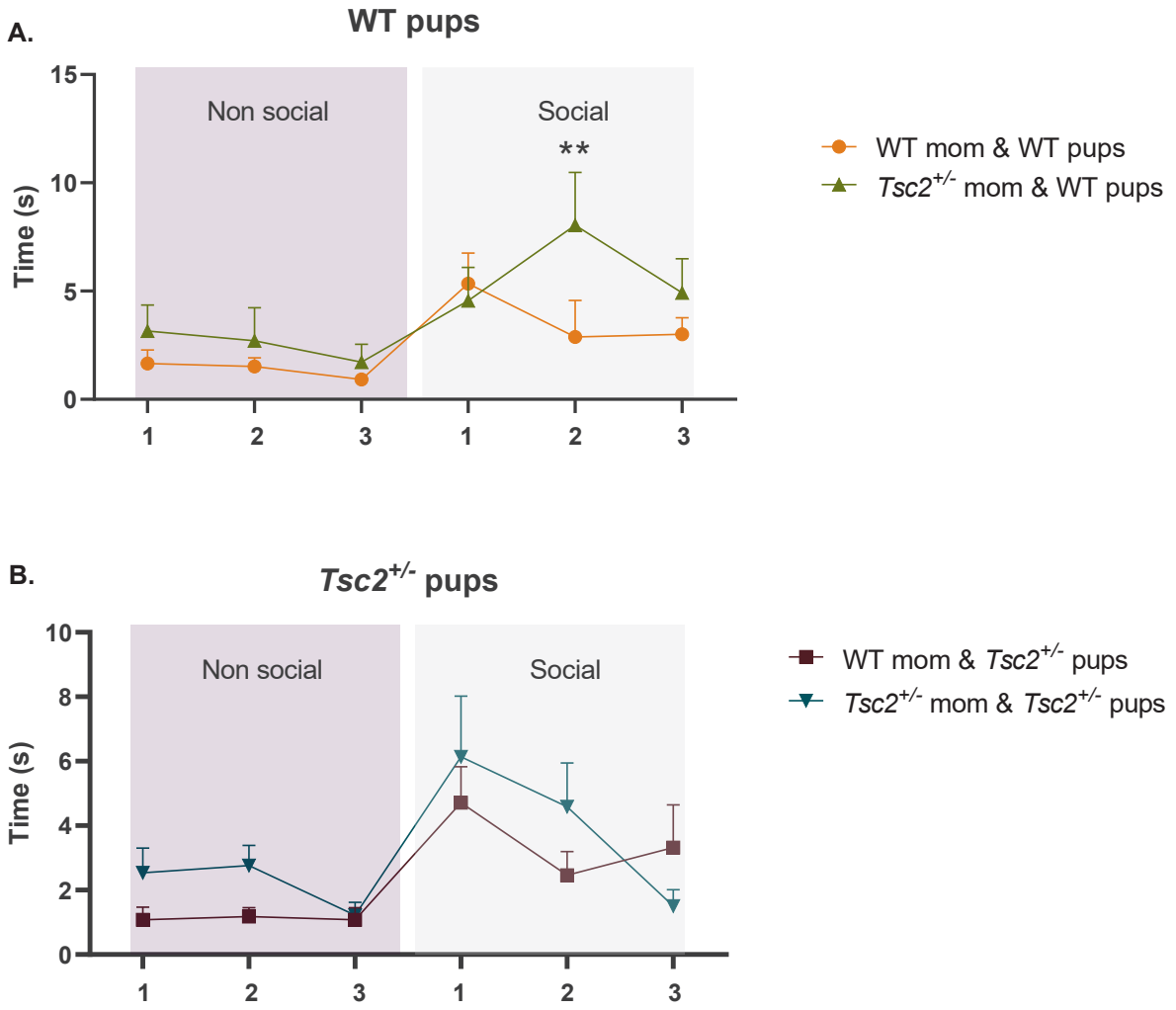


Figure 25 | Social odor discrimination test

Time spent engaging with non-social and social odors by **A**. WT offspring born from WT or *Tsc2*$^{+/-}$ dams, and by **B**. *Tsc2*$^{+/-}$ offspring born from WT or *Tsc2*$^{+/-}$ dams. Mann-Whitney test; $p<0.05$; *: significant difference between WT mother & WT pups and *Tsc2*$^{+/-}$ mom & WT pups experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups) = 13; n (WT mom & *Tsc2*$^{+/-}$ pups) = 16; n (*Tsc2*$^{+/-}$ mom & WT pups) = 16; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups) = 17.

4.1.3. Maternal genotype specifically influences repetitive behavior of heterozygous female offspring

Next, we explored the influence of maternal genotype in repetitive/stereotyped behavior of offspring in a later age by performing marble burying test at PND30. Here, each animal was let explore an arena filled with sawdust and marbles.

Assessing genotype effects, there were no significant differences between WT mice in either of the timepoints of the test (Figure 26A). Nevertheless, independently from maternal genotype, the males buried significantly more marbles than the females during the last 10 minutes of the test (10-minute timepoint: male, *Tsc2*$^{+/-}$ mother = 3.600 ± 0.5812 vs female, *Tsc2*$^{+/-}$ mother = 1.500 ± 0.2673, p = 0.0329; 20-minute timepoint: male, WT mother = 4.875 ± 0.9149 vs female, WT mother = 1.800 ± 0.8602, p = 0.0048; male, *Tsc2*$^{+/-}$ mother = 5.700 ± 0.6333 vs female, *Tsc2*$^{+/-}$ mother = 3.500 ± 0.4629, p = 0.0222) (Figure 26B).

Regarding *Tsc2*$^{+/-}$ mice offspring, we found that maternal genotype influenced the number of buried marbles at the 20-minutes timepoint of the test. Indeed, *Tsc2*$^{+/-}$ animals born from *Tsc2*$^{+/-}$ females buried significantly more marbles than those born from WT females (WT mother = 4.500 ± 0.6708 vs Tsc2+/- mother = 6.294 ± 0.6517, p = 0.0326) (Figure 26C), indicating a more repetitive, stereotyped behavior. Interestingly, if we discriminate the animals by sex, we find that the different number of marbles buried by the groups of *Tsc2*$^{+/-}$ mice is caused specifically by the females. Indeed, while the males buried a quite similar number of marbles during the test, the female mice born from *Tsc2*$^{+/-}$ females buried significantly more than the *Tsc2*$^{+/-}$ females with WT mothers at the 20-minutes timepoint (female, WT mother = 3.333 ± 0.5578 vs female, *Tsc2*$^{+/-}$ mother = 6.889 ± 1.047, p = 0.0024) (Figure 26D).

Overall, in WT mice, even though maternal genotype does not impact stereotyped behavior, there is an increase in stereotyped behavior in males, compared to the females of the same experimental group. Whereas in *Tsc2*$^{+/-}$ animals, maternal genotype impacts repetitive behavior specifically in the females, where those born from *Tsc2*$^{+/-}$ mothers display a more severe phenotype than those with a WT mother.

Finally, USVs were recorded during this test to find a possible link between genotype, behavior and communication. However, USVs produced were in low number and no significant differences were found between the groups (Annex IX).

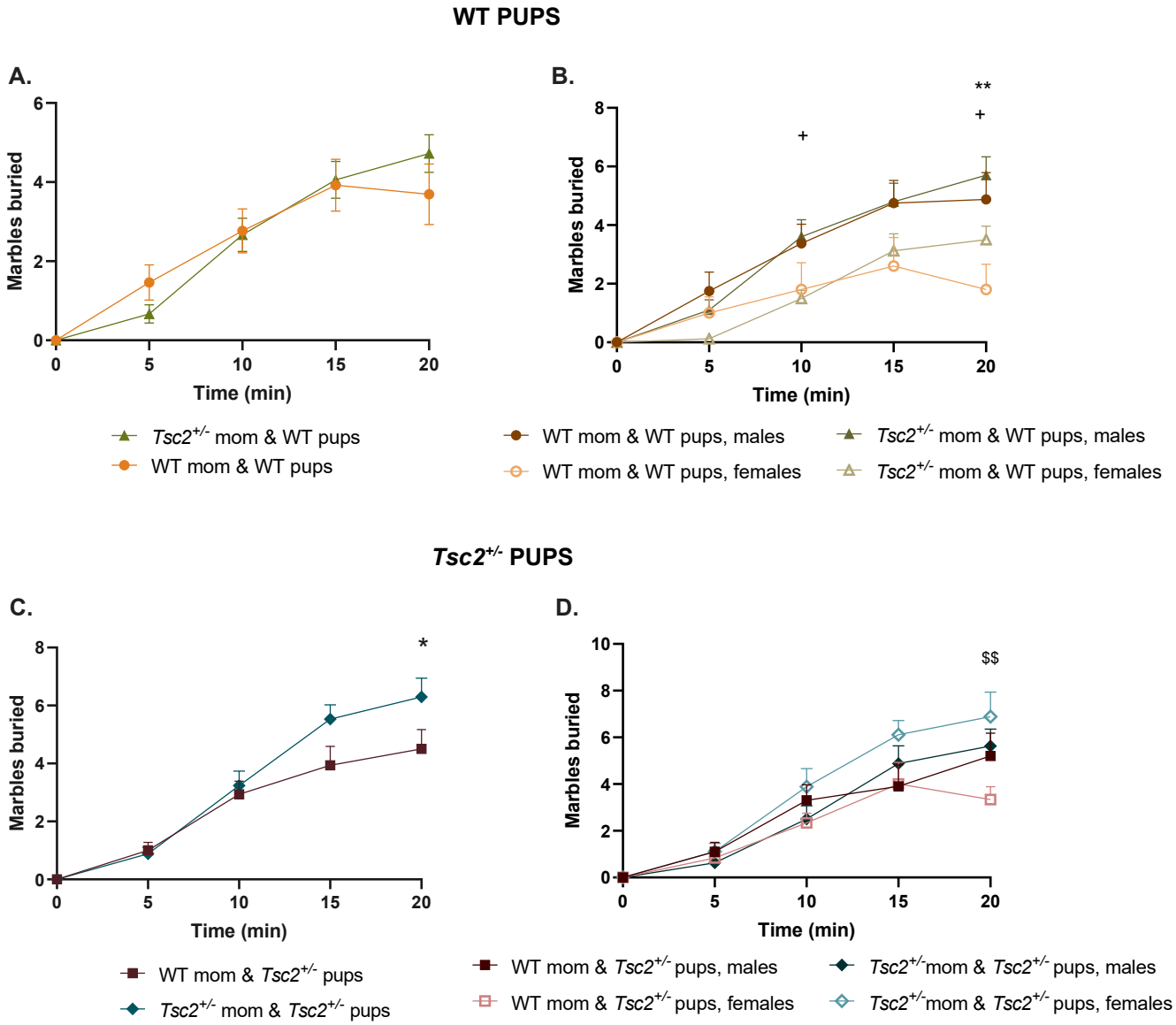


Figure 26 | Marble burying test

A. Number of buried marbles over time by WT offspring born from WT or *Tsc2*$^{+/-}$ dams, further segregated by sex (**B**). Mixed effects analysis followed by Sidak's multiple comparisons test; $p<0.05$; *: significant difference between WT mom males and WT mom females experimental groups; +: significant difference between *Tsc2*$^{+/-}$ mom males and *Tsc2*$^{+/-}$ mom females experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups) = 13 (8 males + 5 females); n (*Tsc2*$^{+/-}$ mom & WT pups) = 18 (10 males + 8 females).

C. Number of buried marbles over time by *Tsc2*$^{+/-}$ offspring born from WT or *Tsc2*$^{+/-}$ dams, further segregated by sex (**D**). Mixed effects analysis followed by Sidak's multiple comparisons test; $p<0.05$; *: significant difference between WT mom & *Tsc2*$^{+/-}$ pups and *Tsc2*$^{+/-}$ mom and *Tsc2*$^{+/-}$ pups experimental groups; $: significant difference between WT mom females and *Tsc2*$^{+/-}$ mom females experimental groups. Data represented as mean ± SEM; n (WT mom & *Tsc2*$^{+/-}$ pups) = 16 (10 males + 6 females); n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups) = 17 (8 males + 9 females).

4.1.4. Maternal genotype does not mediate offspring's cognitive ability

To study the influence of maternal genotype in the cognitive capacity, specifically in the offspring's short-term memory, we performed a Y-maze test at PND35.

We report no genotype- or sex-dependent differences regarding distance covered and mean speed in the novel arm of the Y-maze, nor concerning time ratio (time spent in novel arm / time spent in all arms) (Figure 27).

Further, USVs were recorded during this test and analyzed to investigate whether there this task elicited differing communication profiles. However, the USVs produced were in low number and no significant differences were found between any of the experimental groups (Annex X).

In sum, we did not find evidence suggesting an influence of the maternal genotype in the cognitive performance of the adolescent offspring.

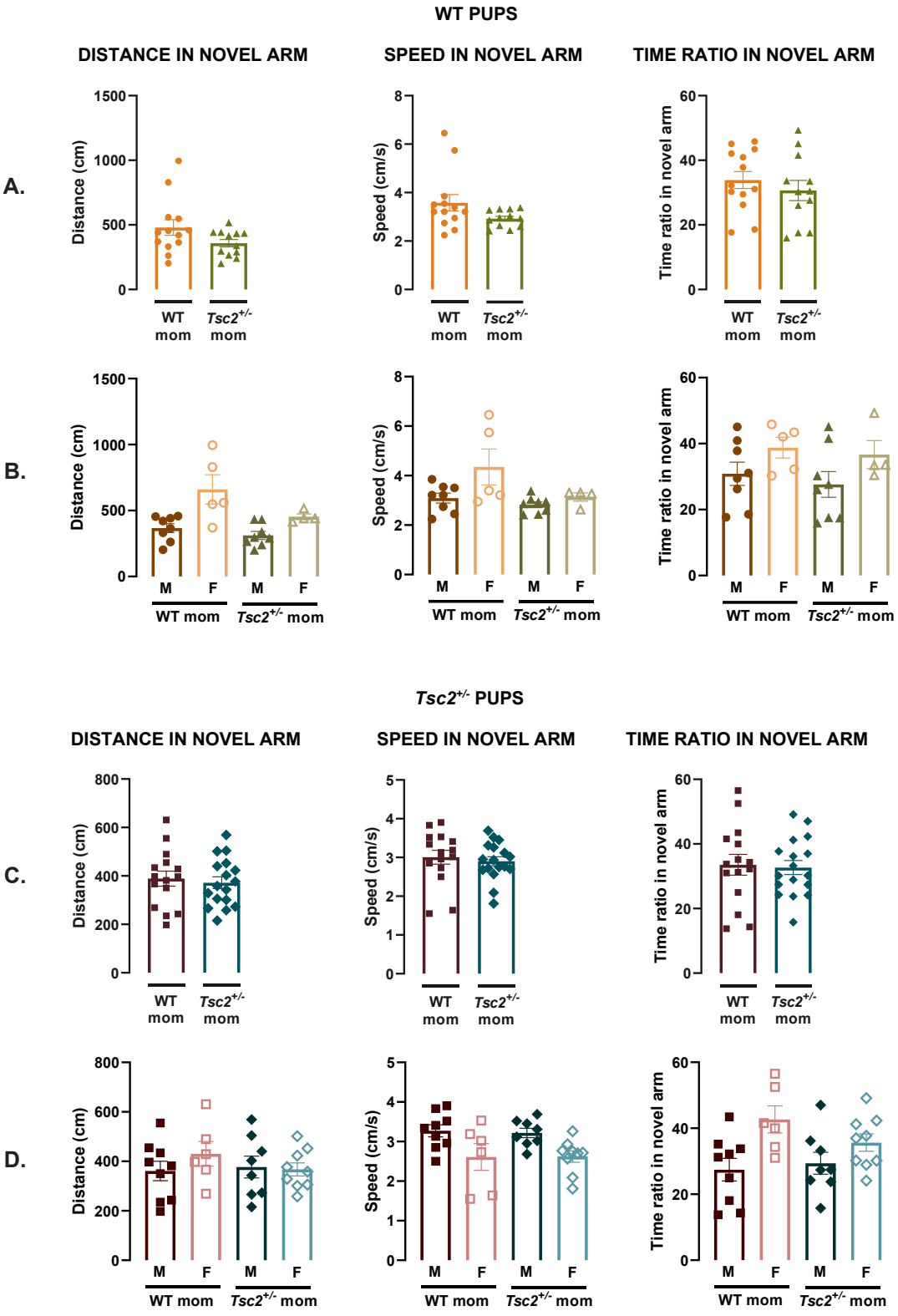


Figure 27 | Y-maze test

A. Distance covered, speed and time ratio in the novel arm of the Y-maze, by WT offspring born from WT or *Tsc2*$^{+/-}$ dams, further segregated by sex (**B**). **C**. Distance covered, speed and time ratio in the novel arm of the Y-maze, by *Tsc2*$^{+/-}$ offspring born from WT or *Tsc2*$^{+/-}$ dams, further segregated by sex (**D**). Kruskal.Wallis test followed by Dunn's multiple comparisons test; $p<0.05$. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups) = 13 (8 males + 5 females); n (*Tsc2*$^{+/-}$ mom & WT pups) = 12 (8 males + 4 females); n (WT mom & *Tsc2*$^{+/-}$ pups) = 16 (10 males + 6 females); n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups) = 17 (8 males + 9 females).

4.2.

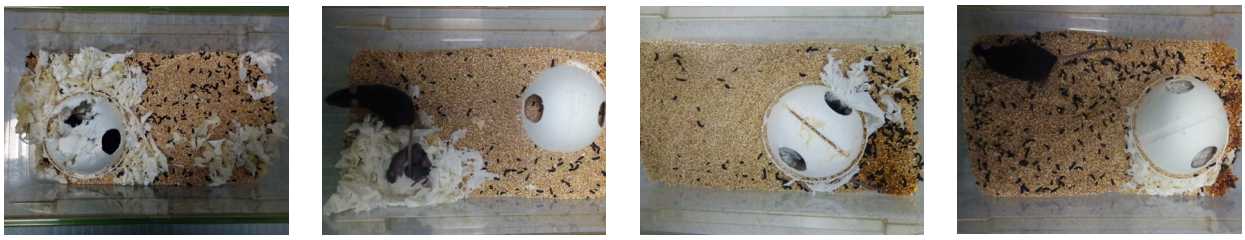
MATERNAL CARE AND BEHAVIOR

In this work, we also investigated maternal instinct and behavior, and whether this was influenced by maternal and/or offspring genotype. For that, we analyzed nesting and pup retrieval behavior over time of each dam used in this work, and performed the reunion test and the marble burying test. Due to the reduced number of dams per experimental group, this part of this work functions as a preliminary study.

4.2.1. Nesting behavior seems to be mediated by a maternal x offspring genotype interaction

As nesting skills are a measure of maternal instinct and behavior, we scored the state of the nests from PND6 until PND16. The reduced number of litters per experimental group hindered reaching statistical significance, however, some interesting observations can be made. While looking for an effect of offspring genotype, within the groups of WT mothers, those with WT pups showed poorer nests than those with *Tsc2*$^{+/-}$ pups consistently throughout the time of study (Figure 28A). Importantly, when we searched for an effect of maternal genotype, we found that within *Tsc2*$^{+/-}$ pups (Figure 28D), those litters with *Tsc2*$^{+/-}$ mothers had lower nesting scores than those with WT mothers, also in all studied timepoints.

Overall, there seems to exist an interaction between maternal and offspring genotypes mediating nesting behavior.



Score 1 Score 2 Score 3 Score 4

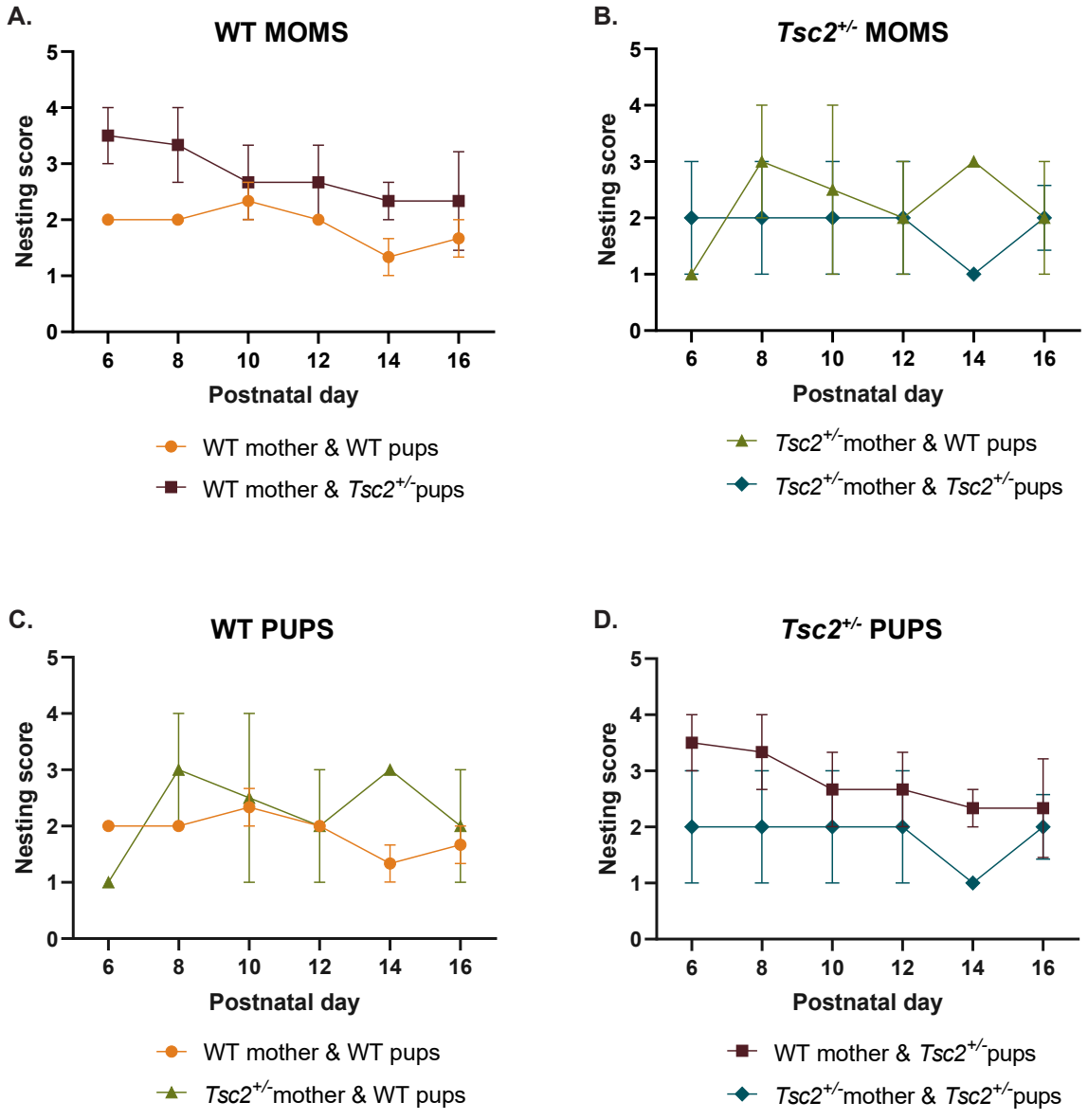


Figure 28 | Nesting behavior

Nesting scores of **A**. WT dams with WT or *Tsc2*$^{+/-}$ litters, and of **B**. *Tsc2*$^{+/-}$ dams with WT or *Tsc2*$^{+/-}$ litters. Nesting scores were also compared within WT and *Tsc2*$^{+/-}$ litters (**C** and **D**, respectively). Mixed effects analysis followed by Sidak's multiple comparisons test; $p<0.05$. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n = 3 litters for each experimental group.

4.2.2. Retrieval of pups is less effective in litters where mother and pups share the same genotype

To investigate how maternal and/or offspring genotype influences maternal care, we observed the retrieval of each pup by its dam into the nest after separation, from PND6 until PND16.

We found that within WT mothers, those with a WT litter took significantly more time to start retrieval of each pup on PND6, compared to WT mothers with a *Tsc2*$^{+/-}$ litter (WT mother & WT litter = 46.52 ± 18.09 vs WT mother & *Tsc2*$^{+/-}$ litter = 17.77 ± 3.892, p = 0.0007) (Figure 29A). Concerning *Tsc2*$^{+/-}$ mothers, those with *Tsc2*$^{+/-}$ litters retrieved significantly less pups than any other experimental group on PND12 (*Tsc2*$^{+/-}$ mother & WT litter = 88.89 ± 11.11 vs *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ litter = 42.50 ± 25.29, p = 0.0101; WT mother & *Tsc2*$^{+/-}$ litter = 86.67 ± 6.667 vs *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ litter = 42.50 ± 25.29, p = 0.0159) (Figure R29B).

When pups born from WT mothers were segregated by sex, we found that specifically the WT female pups were retrieved at a significant later time than *Tsc2*$^{+/-}$ female pups (female, WT mother & WT pup = 63.96 ± 49.54 vs female, WT mother & *Tsc2*$^{+/-}$ pup = 11.31 ± 3.489, p = 0.0021) (Figure 29C).

Regarding those born from heterozygous dams, we found that specifically the male heterozygous pups were retrieved in a percentage which was dependent on offspring genotype. Indeed, male *Tsc2*$^{+/-}$ pups born from *Tsc2*$^{+/-}$ dams were retrieved in a significantly lower proportion than male WT pups born from *Tsc2*$^{+/-}$ dams (male, *Tsc2*$^{+/-}$ mother & WT pup = 100.0 ± 0.000 vs male, *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ pup = 44.43 ± 29.40, p = 0.0454) (Figure 29F).

Interestingly, when we observed the effect of maternal genotype, we found that the female *Tsc2*$^{+/-}$ pups born from WT females were retrieved in a significantly higher proportion than female *Tsc2*$^{+/-}$ pups born from *Tsc2*$^{+/-}$ females (female, WT mother & *Tsc2*$^{+/-}$ pup = 100.0 ± 0.000 vs female, *Tsc2*$^{+/-}$ mother & *Tsc2*$^{+/-}$ pup = 44.43 ± 24.21, p = 0.0016) (Annex XI). Overall, this evidence shows that litters with *Tsc2*$^{+/-}$ mothers and *Tsc2*$^{+/-}$ pups have a decreased retrieval percentage.

In summary, in experimental groups where mother and offspring share the same genotype, the dams display a less effective retrieval behavior.

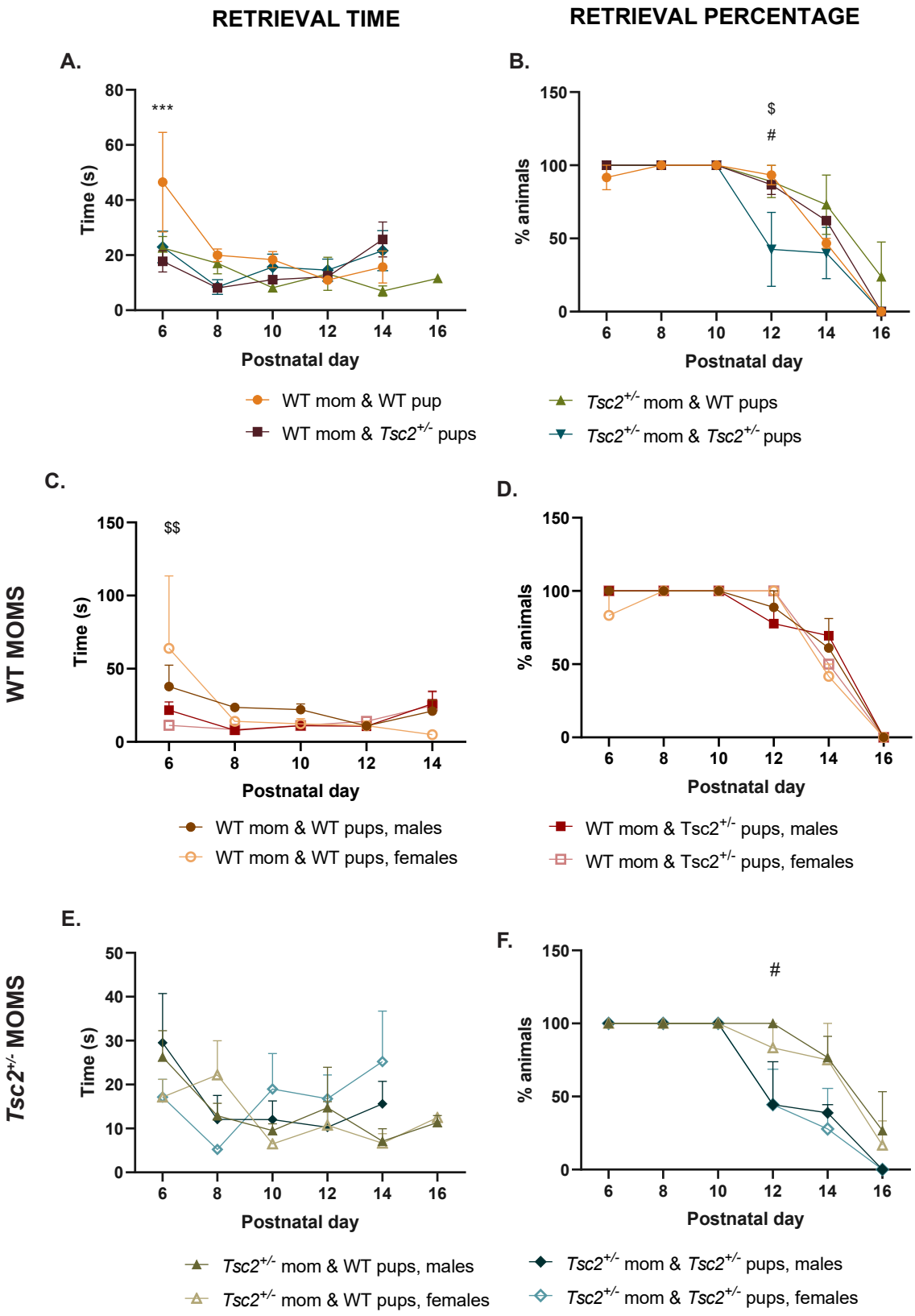


Figure 29 | Retrieval behavior

A. Retrieval time, and **B**. retrieval percentage of pups by their dams, registered on PND6, 8, 10, 12, 14 and 16. Two-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; *: significant difference between WT mother & WT pups and WT mom & *Tsc2*$^{+/-}$ pups experimental groups; #: significant difference between *Tsc2*$^{+/-}$ mother & WT pups and *Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups experimental groups; $: significant difference between WT mother & *Tsc2*$^{+/-}$ pups and *Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups experimental groups.*(continues on next page)*

(continuation of previous page) Retrieval time and retrieval percentage were further segregated by sex within WT dams (**C** and **D**) and *Tsc2*$^{+/-}$ dams (**E** and **F**). Two-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$ #: significant difference between WT mom males and *Tsc2*$^{+/-}$ mom males experimental groups; $: significant difference between WT mom females and *Tsc2*$^{+/-}$ mom females experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups) = 13 (8 males + 5 females); n (WT mom & *Tsc2*$^{+/-}$ pups) = 16 (10 males + 6 females); n (*Tsc2*$^{+/-}$ mom & WT pups) = 18 (10 males + 8 females); n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups) = 17 (8 males + 9 females).

4.2.3. Reunion test suggests a link between pup USVs and maternal behavior

To analyze maternal instinct, we performed the reunion test at PND9, to investigate whether maternal and/or offspring genotype affected the dams' behavior.

During the first part of the test (habituation phase, with both dam and pups on the same side of the partitioned arena), we found no significant differences between any of the groups regarding time spent huddling the pups, exploring the arena or grooming. However, *Tsc2*$^{+/-}$ dams with a *Tsc2*$^{+/-}$ offspring did spend slightly less time huddling their pups. Further, it is interesting to note that *Tsc2*$^{+/-}$ mothers, either with WT or *Tsc2*$^{+/-}$ pups, spent more time grooming than WT mothers, which is in accordance to the increased repetitive behavior found in this animal model (Figure 30A).

Next, in the separation phase of the reunion test (where the dam is placed in the novel side of the partitioned arena), although again no significant differences were found, there is a tendency of the mothers with WT pups to spend more time by the wall next to which the pups were placed and less time exploring (Figure 30B).

Finally, in the reunion phase of this test, no statistical significance was found regarding the behaviors studied. Again, *Tsc2*$^{+/-}$ mothers showed an increased tendency towards repetitive, stereotyped behaviors, and *Tsc2*$^{+/-}$ mothers with WT pups seemed to prefer to spend less time in the side of the arena where the litter was placed, compared to the females of the other experimental groups (Figure 30C).

USVs were recorded in all phases of the reunion test. As adult mice vocalize in an extremely reduced number, we considered that all USVs were produced by the pups, and none by the dams. Number of USVs were normalized for number of pups in each litter. We found no significant differences concerning total number or complexity of the calls per pup (Figure 31). Nevertheless, it is interesting to see that the groups with WT pups vocalized more during the separation phase of the test, which may explain the increased time spent by the mothers at the wall next to the litter. Finally, in the reunion phase of the test, we report an increased number of USVs produced by WT pups with WT mothers.

In summary, though statistical significance was not reached in this study, there seems to exist a relation between pup communication and maternal instincts during the separation part of the reunion test.

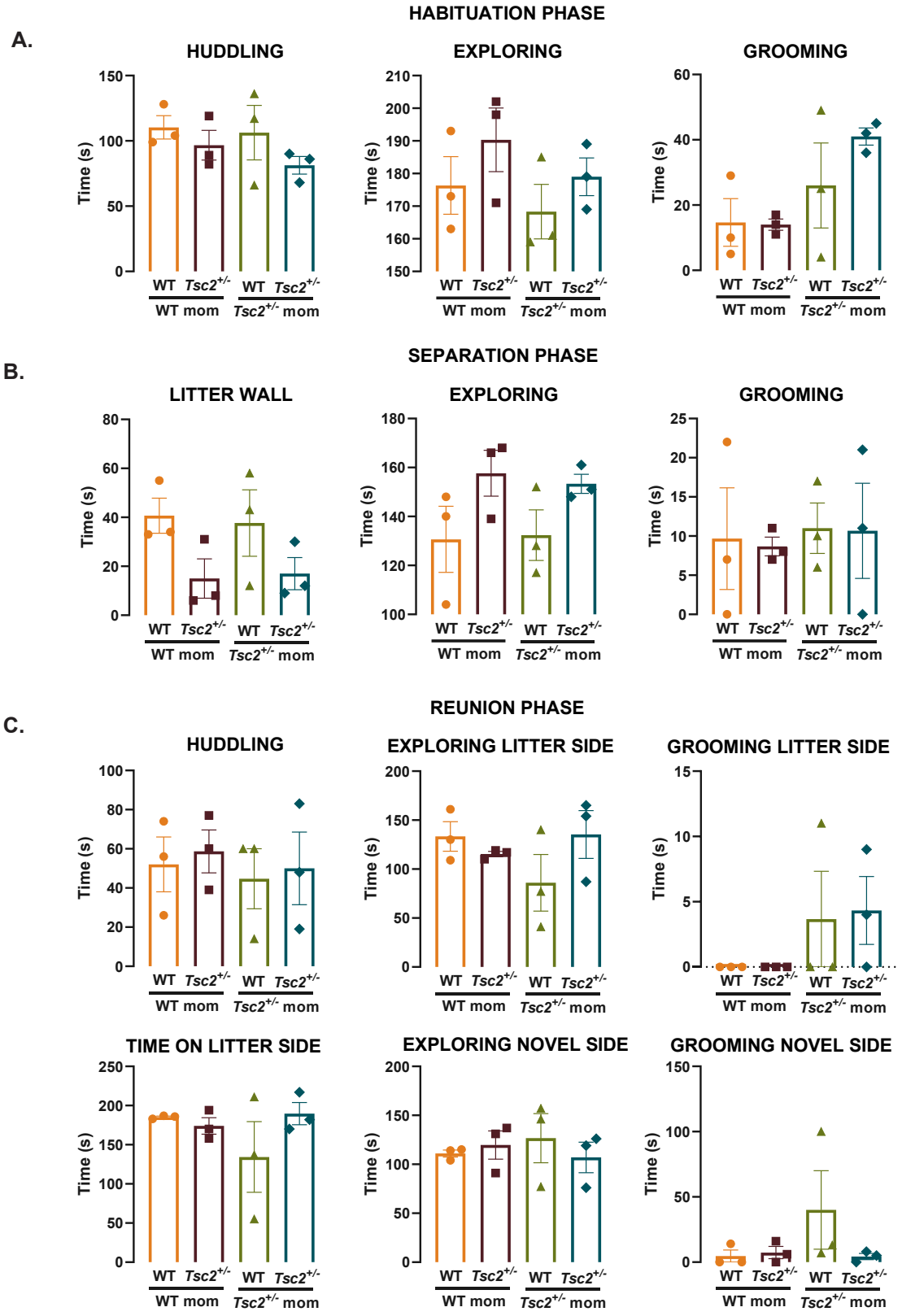


Figure 30 | Reunion test behavior

A. Time spent by the dams huddling the litter, exploring the compartment and self-grooming during the habituation phase of the test. **B**. Time spent by the dams next to the wall closer to the litter, exploring the novel compartment and self-grooming during the separation phase of the test. **C**. Time spent by the dams huddling, exploring and self-grooming and total time in the litter side of the arena, and exploring and self-grooming in the novel side of the arena, during the reunion phase of the test. Kruskal-Wallis test followed by Dunn's multiple comparisons test; $p<0.05$. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n = 3 litters for each experimental group. experimental groups.

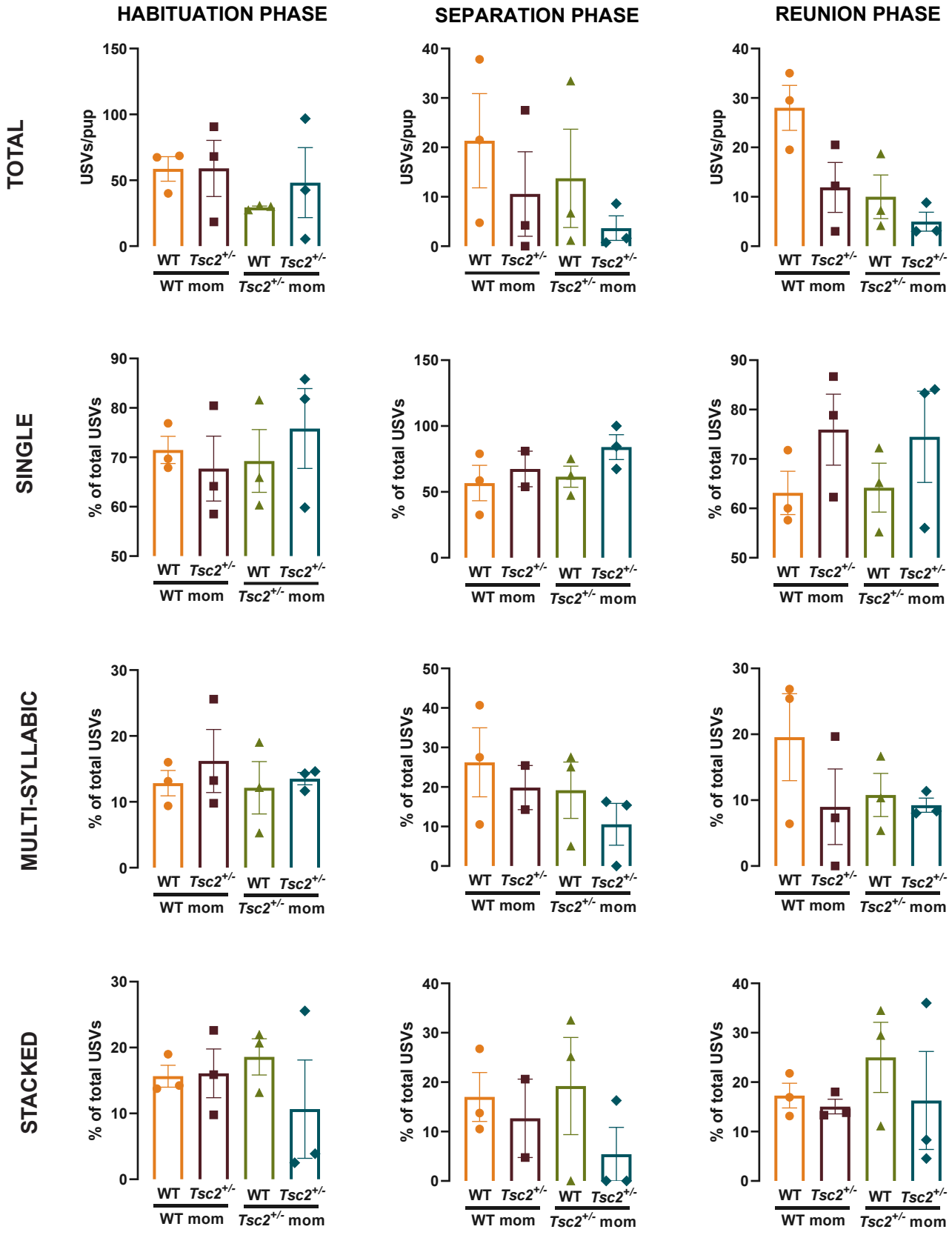


Figure 31 | Ultrasonic vocalizations produced in reunion test

Total number of USVs and number of single, multi-syllabic and stacked USVs (Young et al, 2010) produced in each phase of the reunion test. Kruskal-Wallis test followed by Dunn's multiple comparisons test; $p<0.05$. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n = 3 litters for each experimental group.

4.2.4. Offspring genotype does not influence maternal repetitive behavior

To investigate whether interaction with a heterozygous litter would influence the repetitive behavior of the mother, we performed the marble burying test with each mother at PND21, age of weaning.

No significant differences were found during neither of the timepoints of the test between the experimental groups, indicating that maternal repetitive behavior is not altered by its litter's genotype (Figure 32A, B). Further, the USVs produced were in low number and no significant differences were found between any of the experimental groups. However, there seems to be a tendency of WT mothers which had *Tsc2*$^{+/-}$ litters to vocalize more than those which had WT litters (Figure 32C).

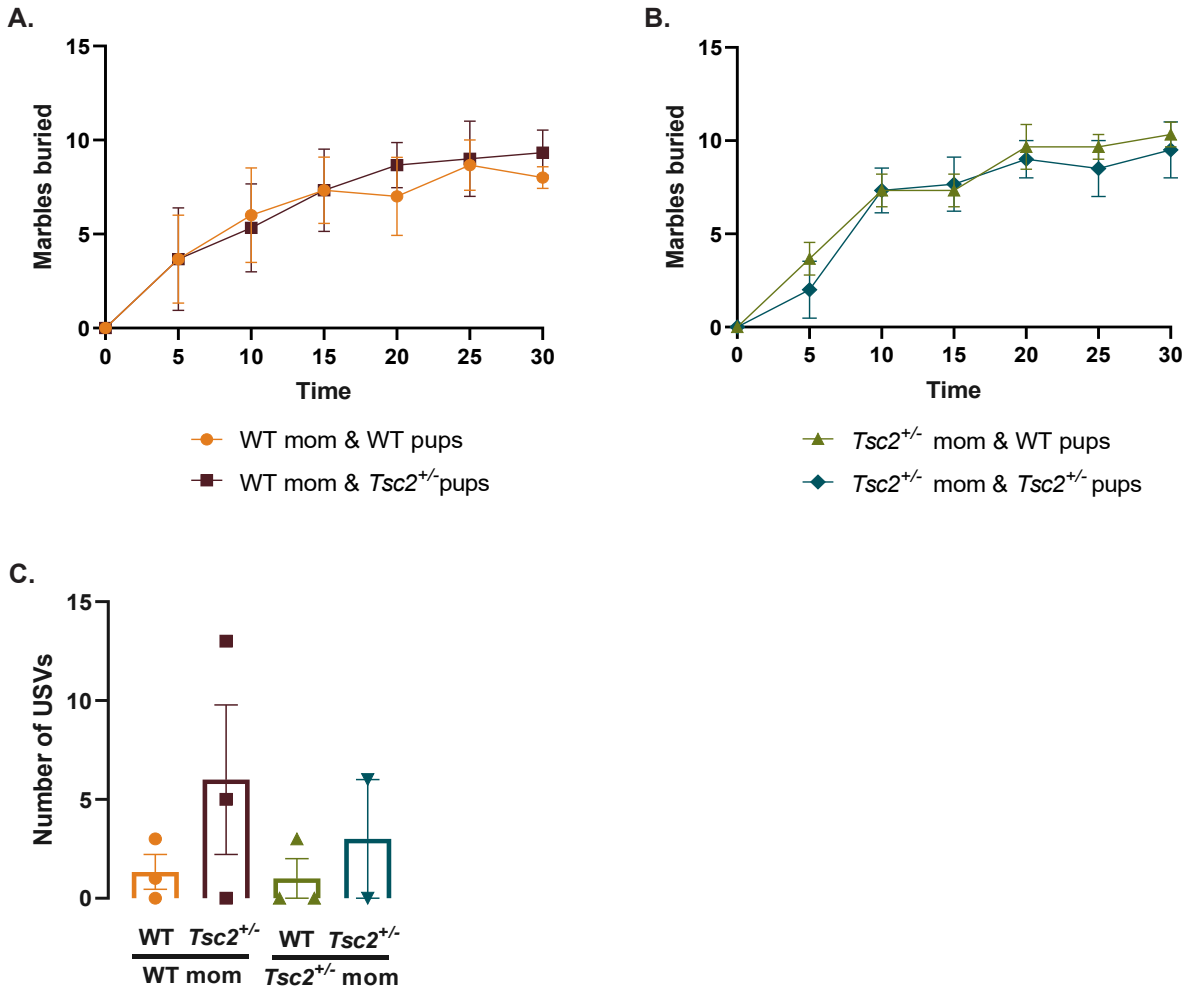


Figure 32 | Dams' marble burying test

A. Number of buried marbles over time by WT dams with WT or *Tsc2*$^{+/-}$ litters and by **B**. *Tsc2*$^{+/-}$ dams with WT or *Tsc2*$^{+/-}$ litters. Mixed effects analysis followed by Sidak's multiple comparisons test; $p<0.05$. No significant differences found between any of the experimental groups. C. Total number of USVs produced by WT and *Tsc2*$^{+/-}$ dams during the marble burying test. Kruskal-Wallis test followed by Dunn's multiple comparisons test; $p<0.05$. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n = 3 for each experimental group.

4.3. SEROTONIN AND OXYTOCIN LEVELS

4.3.1. Maternal x offspring genotypes' interaction mediates offspring plasma serotonin levels

As serotonin and oxytocin are important hormones involved in behavior and are implicated in the mechanisms underlying ASD phenotypes, we analyzed plasma serotonin and oxytocin levels at PND38 through ELISA, as well as plasma, hypothalamus and amygdala oxytocin levels of the mothers used in this study.

Regarding plasma serotonin levels, we found no significant genotype or sex differences among WT offspring (Figure 33A, B). However, there was a decrease in *Tsc2*$^{+/-}$ mice born from *Tsc2*$^{+/-}$ mothers, compared to *Tsc2*$^{+/-}$ mice born from WT mothers (*Tsc2*$^{+/-}$ animal & WT mother = 9.021 ± 0.1142 vs *Tsc2*$^{+/-}$ animal & *Tsc2*$^{+/-}$ mother = 8.392 ± 0.2384, p = 0.0245) (Figure 33C). No sex differences were found in this analysis (Figure 33D). No differences were found regarding plasma oxytocin levels of the offspring (Figure 34).

Concerning oxytocin levels in plasma, amygdala and hypothalamus of the mothers, we found no statistical differences between any of the experimental groups in study (Figure 35). However, the number of individuals per group is reduced and hinders the possibility of obtaining results with sufficient statistical power.

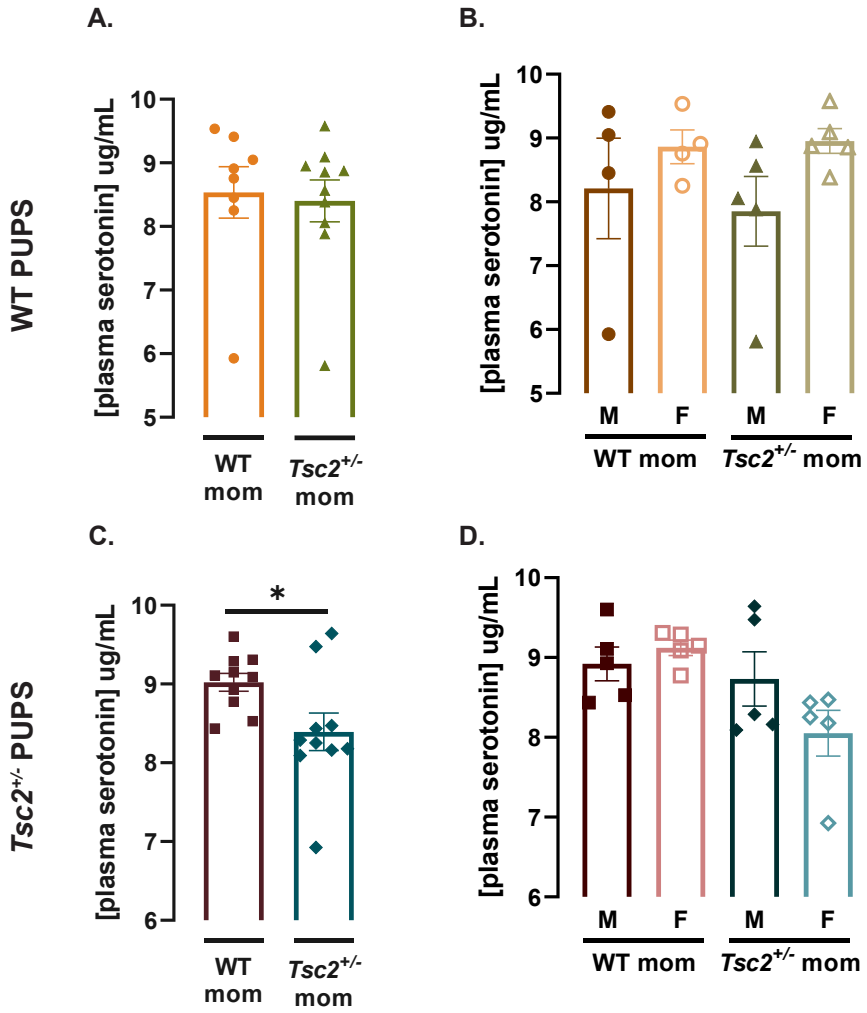


Figure 33 | Offspring plasma serotonin

A. Levels of plasma serotonin in WT offspring born from WT or *Tsc2*$^{+/-}$ dams. Mann-Whitney test; $p<0.05$. No significant differences found between any of the experimental groups. **B**. Levels of plasma serotonin in WT male and female offspring born from WT or *Tsc2*$^{+/-}$ dams. Kruskal-Wallis test followed by Dunn's multiple comparisons test; $p<0.05$. No significant differences found between any of the experimental groups. **C**. Levels of plasma serotonin in *Tsc2*$^{+/-}$ offspring born from WT or *Tsc2*$^{+/-}$ dams. Mann-Whitney test; $p<0.05$. *: significant difference between WT mother & *Tsc2*$^{+/-}$ pups and *Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups experimental groups. D. Levels of plasma serotonin in *Tsc2*$^{+/-}$ male and female offspring born from WT or *Tsc2*$^{+/-}$ dams. Kruskal-Wallis test followed by Dunn's multiple comparisons test; $p<0.05$. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups) = 8 (4 males + 4 females); n (WT mom & *Tsc2*$^{+/-}$ pups) = 10 (5 males + 5 females); n (*Tsc2*$^{+/-}$ mom & WT pups) = 10 (5 males + 5 females); n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups) = 10 (5 males + 5 females).

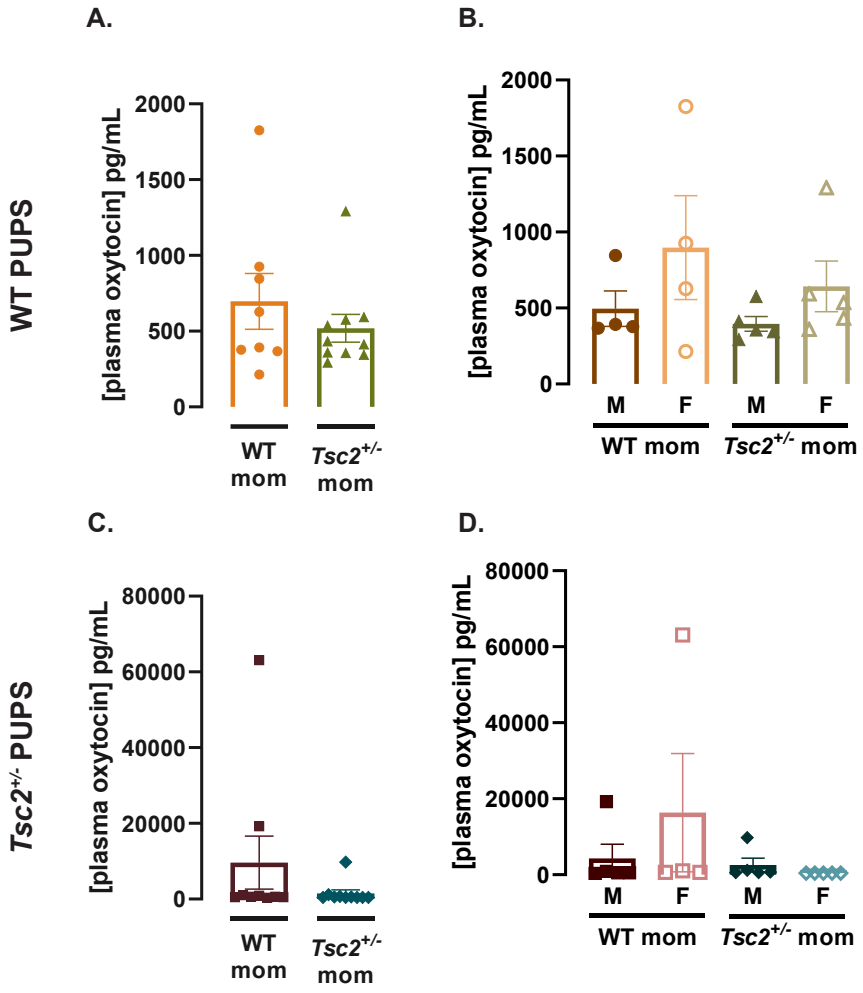


Figure 34 | Offspring plasma oxytocin

A. Levels of plasma oxytocin in WT offspring born from WT or *Tsc2*$^{+/-}$ dams. Mann-Whitney test; $p<0.05$. No significant differences found between any of the experimental groups. **B**. Levels of plasma oxytocin in WT male and female offspring born from WT or *Tsc2*$^{+/-}$ dams. Kruskal-Wallis test followed by Dunn's multiple comparisons test; $p<0.05$. No significant differences found between any of the experimental groups. **C**. Levels of plasma oxytocin in *Tsc2*$^{+/-}$ offspring born from WT or *Tsc2*$^{+/-}$ dams. Mann-Whitney test; $p<0.05$. No significant differences found between any of the experimental groups. **D**. Levels of plasma oxytocin in *Tsc2*$^{+/-}$ male and female offspring born from WT or *Tsc2*$^{+/-}$ dams. Kruskal-Wallis test followed by Dunn's multiple comparisons test; $p<0.05$. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups) = 8 (4 males + 4 females); n (WT mom & *Tsc2*$^{+/-}$ pups) = 9 (5 males + 4 females); n (*Tsc2*$^{+/-}$ mom & WT pups) = 10 (5 males + 5 females); n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups) = 10 (5 males + 5 females).

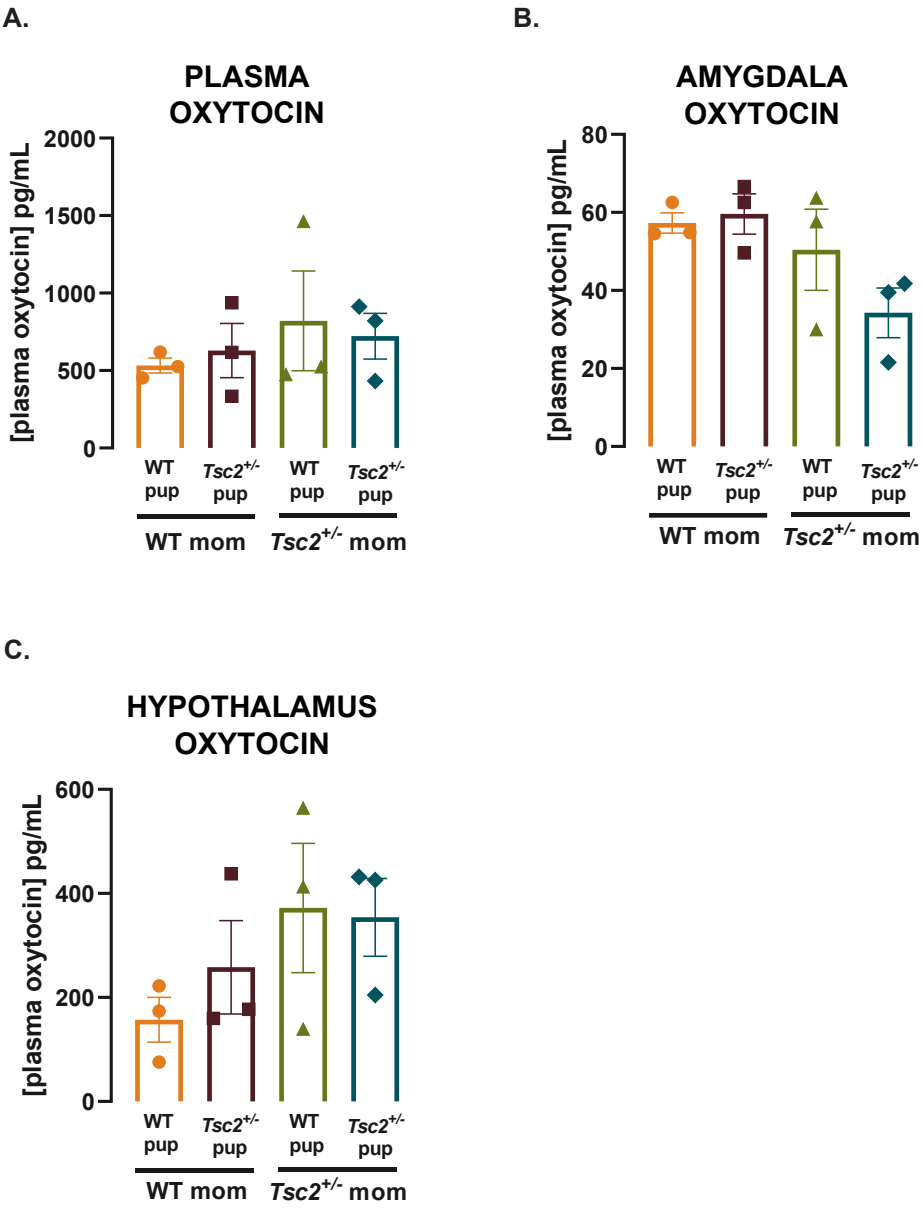


Figure 35 | Dams' oxytocin

Levels of oxytocin in **A**. plasma, **B**. amygdala, and **C**. hypothalamus of the dams used in this study. Kruskal-Wallis test followed by Dunn's multiple comparisons test; $p<0.05$. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n = 3 for each experimental group.

CHAPTER 5

DISCUSSION

DISCUSSION

Here, we performed a longitudinal study in a mouse model of ASD to investigate the impact of the maternal genotype on offspring's early development and later social and repetitive behaviors and cognitive skills. Furthermore, we investigated maternal care behavior to understand whether it was modulated by offspring's genotype and state of development.

We found a mother-offspring interaction, in which sharing the same genotype favors the early development of the pup's motor skills and vestibular system. Importantly, we found that regarding pro-social and communicative skills, the litters in which both mother and pups were mutant performed better, but not the litters where both mother and pups were wild-type. This interesting finding suggests that the social interaction between mother and offspring is complex and affects the various milestones of development differently.

The few studies with our mouse model of ASD that have studied the influence of the dam's genotype in offspring have reported interesting results. Greene-Colozzi et al. (2014) report that *Tsc2* haploinsufficiency in either dam or offspring results in a decrease in separation-elicited USVs, as well as in a delay in achieving developmental milestones. This is against our own data, which show that mutant pups born from mutant dams have a faster milestone development and produce more USVs. Interestingly, Young et al. (2010) found elevated vocalization rates in pups born from $Tsc2^{+/-}$ mothers and suggest a deficient dam-pup interaction in the experimental groups where genotypes were not shared, which is in accordance to our claim.

We must, however, point out that these three investigations were performed under different study designs. Indeed, while Young evaluated only one timepoint, both our study and Greene-Colozzi's assessed development over several days. Moreover, we formed litters with the same genotype across all offspring, unlike the other two studies, and having littermates of a different genotype may mask some behavioral outcomes. Nonetheless, our data underlies maternal genotype as a key factor in the developmental profiles of the offspring, and shows the need for more studies to take it into consideration.

Mother-child relationship in ASD has been studied since autism was first described in the 20th century. In his 11-case studies paper, Kanner (1968) claimed that the children's "desire for aloneness and sameness" could be potentiated by a lack of interest from their families and by shallow early parental relationships. Later, Bettelheim strongly supported such view, stating that a cold, distant mother was the cause of the disorder, giving rise to the so-called "refrigerator-mother theory" or Bettelheim's theory of autism (Rosmalen et al., 2020). This theory was later debunked, firstly with a 1977 twin study that showed great genetic predisposition for the development of ASD (Folstein & Rutter, 1977); and then with subsequent investigations reporting the complex etiology of the syndrome, encompassing genetic, epigenetic and environmental factors (Amaral, 2017; Chaste & Leboyer, 2022). In fact, more recent human studies on mother-child relationship find that warmth and praise from the parental figures have a positive impact in the behavioral symptoms of the patients (Smith et al., 2008; Woodman et al., 2015; Bishop-Fitzpatrick et al., 2016; Woodman et al., 2016), and not that the lack of affection could be the source of the disorder, which is in line with our results.

Indeed, and much against the refrigerator-mother theory, we found that it is not solely the maternal genotype/behavior what may be mediating offspring's development, but rather an interaction between the genotypes of both. Importantly, we found that in all tests, pups of all experimental groups reached the same level of development by the last timepoint studied. This indicates that all pups developed adequately, only showing differing developmental profiles over time, and not a worse outcome. It is quite interesting to note that the litters that presented a faster developmental profile were the ones where pups shared their genotype with the dams. This begs the question whether this means that dam and pup have a better relationship when genotypes are shared, and so dams can tend to the pup's needs better, resulting in faster development in pups.

In this sense, we also investigated maternal care, to understand how it related to pup development. Strikingly, we found that the nesting quality of shared-genotype litters had a consistently lower score. Additionally, pup retrieval was less efficient in these same litters. Finally, in the reunion test there was a tendency of mutant dams with mutant pups to spend less time huddling their pups during the first part of the test, preferring to explore the arena. This data suggests that in litters where genotype is shared, there was a relaxation in maternal care, which curiously did not affect pup development at all. The effect of maternal care has been previously studied, with reports of exposure to chronic unpredictable mild stress (CUMS) leading to increased maternal care and alterations in the offspring's hippocampal white matter (Wong et al., 2019). Further, postnatal touch stimulation caused altered hormone secretion and gene expression in hippocampus, midbrain and frontal cortex of the offspring (Jutapakdeegul et al., 2003), which could reverse behavioral and molecular deficits (Chatterjee et

al., 2007), underlying the importance of a maternal relationship for an adequate pup development. Contrariwise, Young et al. (2010) did not find a relation between altered communication profiles and maternal care, claiming that pup vocalizations were directly dependent on the genotype of both dam and pup.

Our results seem to indicate that maternal behavior was less effective as a response to the more developed state of the litter, in other words, there was no need for more attention and care. The fact that we observed this development state-maternal care relation in a manner that was dependent on the genotypes of both dams and litters suggests a synergistic effect, where sharing the genotype may create a better relationship among the litter, which in turn facilitates development, which finally promotes a relaxation in maternal measures. To fully understand if this is the case, it would be important to observe maternal behavior in even earlier days, as we only started tracking at postnatal day 6, and during longer periods of time in the home cage. Indeed, Liu et al. (1997) observed maternal care during the first 10 days of the litters' lives, finding that this is a critical period that dictates stress response in adulthood, through modulation of the HPA axis.

Precisely because we wanted to investigate the effect of maternal genotype and care in a later age, we assessed the offspring's social and repetitive behavior and cognitive skills during adolescent age, from postnatal day 25 until 35.

The importance of maternal relationship for proper social ability during juvenile age has already been shown in a model of mice with hearing deficits, which hindered adequate maternal behavior and resulted in social abnormalities in the offspring (Wu et al., 2009). Accordingly, providing extra maternal care through "double mothering" from birth increased social motivation in *Oprm1*$^{-/-}$ mice, which lack the μ-opioid receptor gene, leading to deficient sociability (Garbugino et al., 2016).

In our study, we found that WT pups born from WT mothers had decreased preference to engage with a social odor, while *Tsc2*$^{+/-}$ pups born from *Tsc2*$^{+/-}$ mothers showed a tendency to be more social, even though it was not statistically significant. Importantly, this is in line with the results found in the nest seeking test and USVs recordings performed during early infancy. As such, these results reinforce the notion that although same-genotype experimental groups have an increase in their motor and vestibular early development, their social skills are strikingly opposite and long-lasting until at least adolescent age.

It is interesting to note that these two contrasting experimental groups both showed reduced maternal care measures; this highlights the difference between "maternal care measures" and "maternal relationship", which do not necessarily go hand-in-hand. Indeed, WT offspring born from mutant dams had a tendency to display increased social anxiety, underlying our claim that sharing a mutant genotype may create a better relationship between mother and pup and prove beneficial for the

development of social skills.

Concerning repetitive behavior, we found that maternal heterozygosity leads to increased stereotyped behavior in mutant offspring, specifically in females. This is as expected, as our mouse model has previously been shown to display repetitive behavior (Reith et al., 2013), including previous work by our group where we also showed a sex-specific increase in restrictive behaviors in females (Ferreira et al., 2022).

In this work, we also performed a repetitive behavior-eliciting task in the dams after weaning of their litters, to investigate whether their behavior was affected, and we found no differences between any experimental groups. This may be because restrictive behaviors are attributed to CNS insult, pharmacological agents or restricted environment/housing, as well as to genetic mutations which increase susceptibility to these behaviors (Gandhi & Lee, 2021). As such, it would be unlikely to find altered repetitive behavior in the dams, apart from their naturally occurring behavior according to their genotype.

Finally, we found no effect of maternal genotype in short-term memory in this work. Reshetnikov et al. (2020) found that postnatal maternal separation impacted long-term spatial and recognition memory in the female adult offspring. On the other hand, Sun et al. (2021) found that although maternal separation and early weaning led to deficiencies in working memory, there were no differences found in spatial reference memory, nor in associative learning. It seems that even within the maternal separation animal models there is no consensus regarding the impact of maternal care in the cognitive skills of the adult offspring. Moreover, while the aforementioned works were performed when the offspring were 3 months old, in adulthood, we performed cognitive tests at postnatal day 35, still a juvenile age. As such, differences found in our study would be very subtle at best.

The roles of serotonin and oxytocin have long been studied in the etiology of ASD manifestations, with a particular emphasis on social skills and repetitive behavior.

Hyperserotonemia was associated with altered social and communicative skills and with repetitive behavior (Veenstra-VanderWeele et al., 2012; Tanaka et al., 2018). Here, we found that while the WT offspring showed no maternal genotype-dependent significant differences in plasma serotonin levels, heterozygous offspring with heterozygous mothers had a decrease in plasma serotonin compared to those born to WT dams. Although this may seem unexpected at first, as this is a mouse model of ASD and hyperserotonemia is a biomarker of the disease, these results are in line with our findings of increased sociability in the double mutant experimental group.

However, interestingly, several studies found that enhancement of serotonin signaling rescued social deficits and decreased stereotypies (Nakai et al., 2017; Cai et al., 2019; Walsh et al., 2021). Overall, it seems that the role of serotonin in the

manifestation of ASD-like traits is not yet clearly understood, hindered by the fact that different animal models, study paradigms and timepoints are used. Importantly, we measured systemic levels of oxytocin and serotonin in plasma, which may not be linearly correlated to their levels in the CNS and pathway activity.

On the other hand, we found no differences in plasma oxytocin levels, despite studies reporting an interaction between the serotonergic and the oxytocinergic pathways (Nagano et al., 2018). Indeed, enhancement of oxytocin signaling and oxytocin administration were found to improve behavioral deficits (Peñagarikano et al., 2015; Cherepanov et al., 2021; Kitagawa et al., 2021; Choe et al., 2022). As such, we cannot firmly conclude about the effect of maternal genotype on oxytocin and serotonin levels of the offspring, and their consequences on behavior.

A review by Sanson and Bosch (2022) claims that abnormal oxytocin signaling leads to impaired maternal care, which in turn causes deficits in the oxytocinergic system of the offspring. Moreover, a thought-provoking study found that oxytocin administration in pups improved retrieval efficiency by their mother, in a mouse model that typically shows delays in retrieval (Da Prato et al., 2022). In our work, we also measured oxytocin levels in plasma, hypothalamus and amygdala of the dams. Even though this represented only a preliminary study with a reduced number of individuals per experimental group, with no significative changes found, the evidence in the literature gives exciting insight regarding the important role played by oxytocin in maternal behavior, and leaves us wondering whether the WT dams with WT offspring and the mutant dams with mutant offspring would show disfunctions in their oxytocinergic signaling at some point during their litters' neonatal period.

CHAPTER 6

CONCLUDING REMARKS AND FUTURE DIRECTIONS

CONCLUDING REMARKS AND FUTURE DIRECTIONS

This work has shed light on the intricate relationship between mother and offspring, and how their genotypes affect each other regarding development and behavior.

During the first days of life of each litter, the dynamics between mother and pups, and their respective maternal behavior and state of development, modulate the offspring's developmental profile and later behavioral outcomes. In fact, we found that pups that share their genotype with their mother display an earlier motor and vestibular development, while their mothers have a lower score in maternal care measures, suggesting that maternal behaviors are, at least partly, determined by the litter's own necessities. Interestingly, only mutant x mutant, and not WT x WT, litters showed improved social and communicative skills, hinting that while these two experimental groups may have shared a similar level of maternal care, they did not experience the same social maternal relationship.

These are thought-provoking notions that deserve further research. In future studies, a longer, more detailed observation of maternal care and maternal relationship with the litter ought to be performed during the very first days after birthing. This will provide important insight on the bonding experience between mother and pups, and will allow to more definitely conclude about its influence on the litter's developmental profile and later behaviors. It would also be interesting to study the offspring's outcomes even later in life, into adulthood, and even whether their own parental behaviors are affected by their early experiences.

Moreover, a more complete study on the influence of serotonin and oxytocin on development, social behavior and maternal instinct would be a significant addition to the study of mother-offspring relationship. The drawing of a blood and brain serotonergic and oxytocinergic profile over the mother and offspring's lives would allow to achieve a more comprehensive picture on this matter.

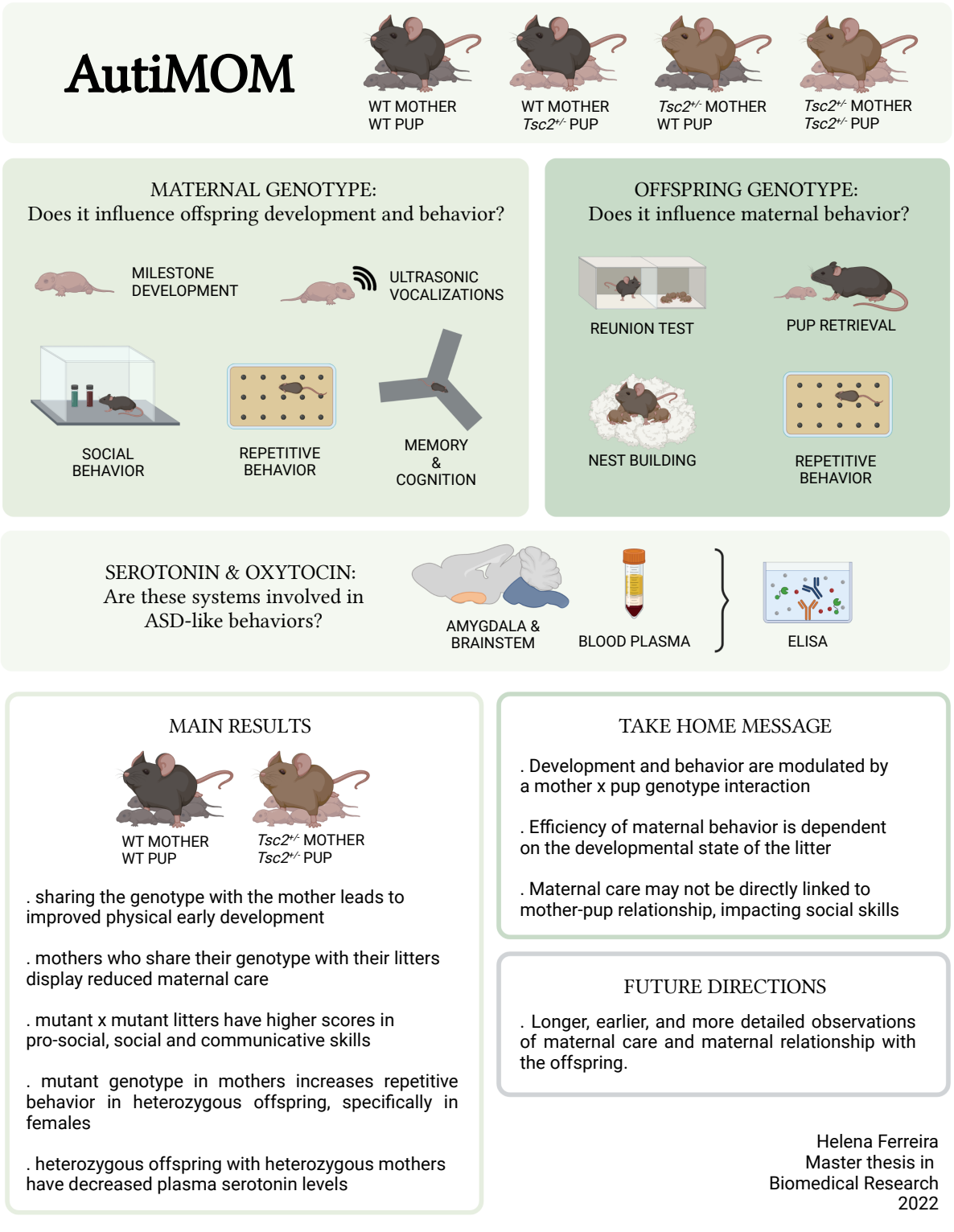


Figure 36 | Graphical abstract

Graphical abstract representing the main research questions, methods and conclusions of this work. Created with BioRender.com

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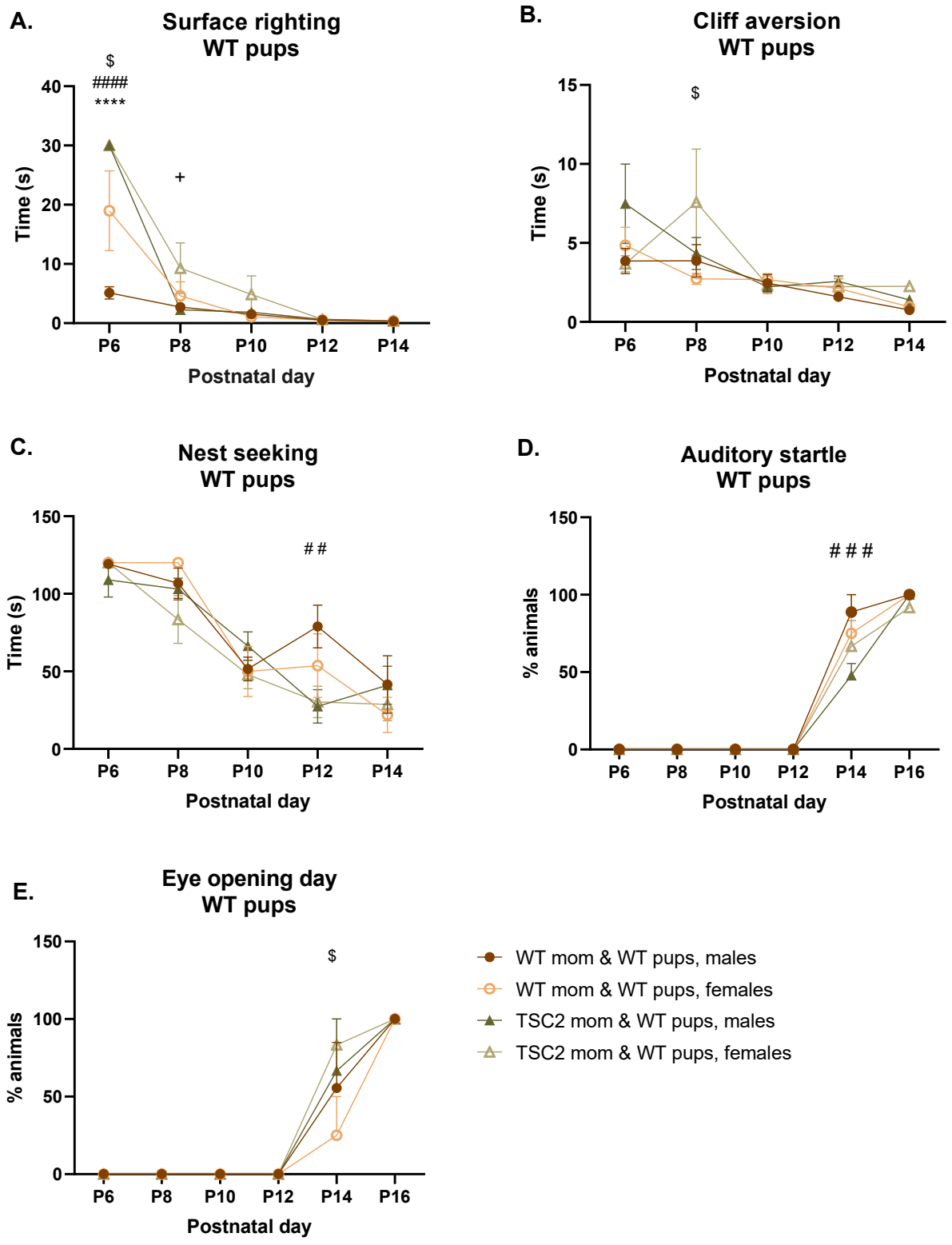
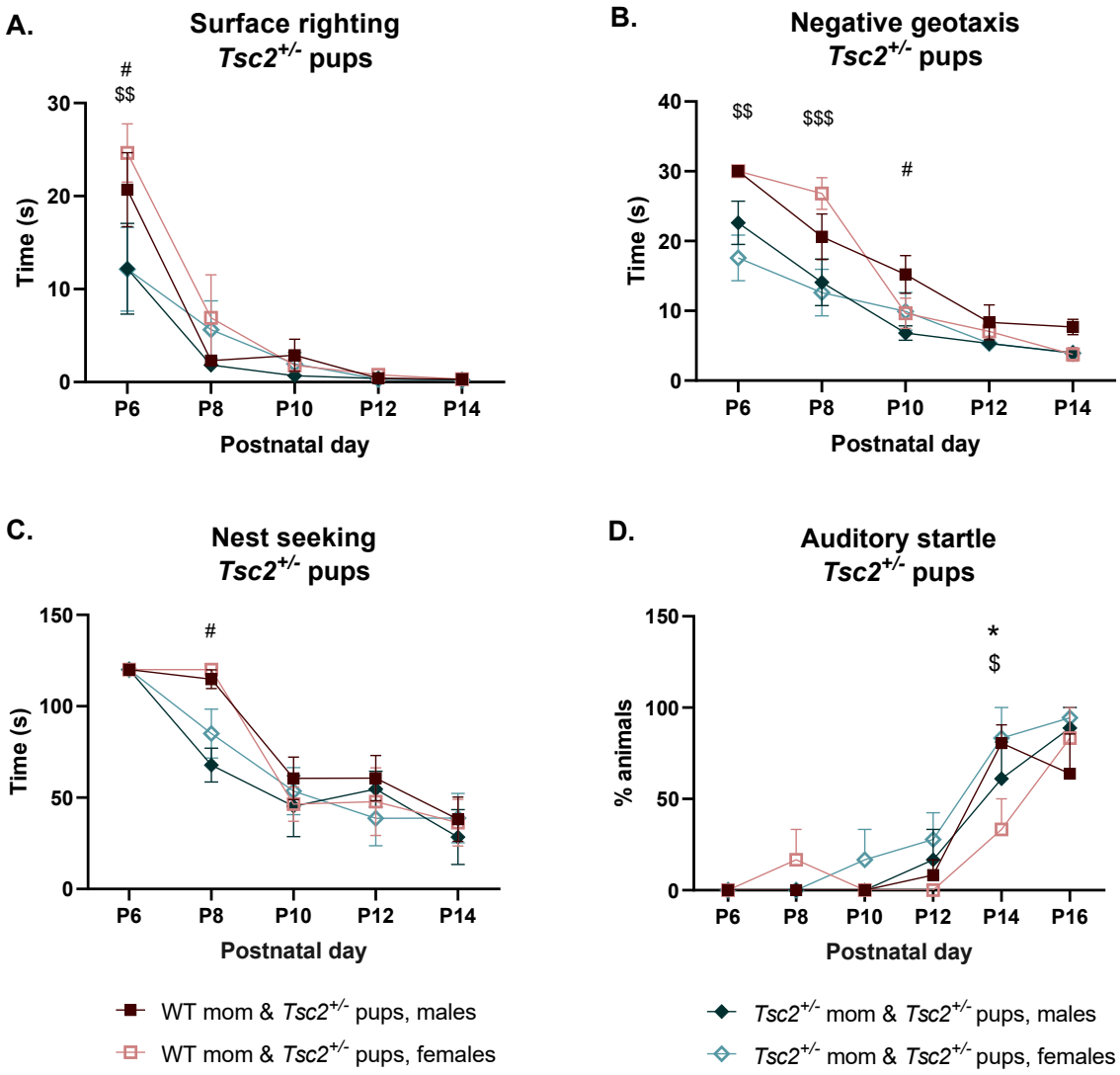
ANNEXES



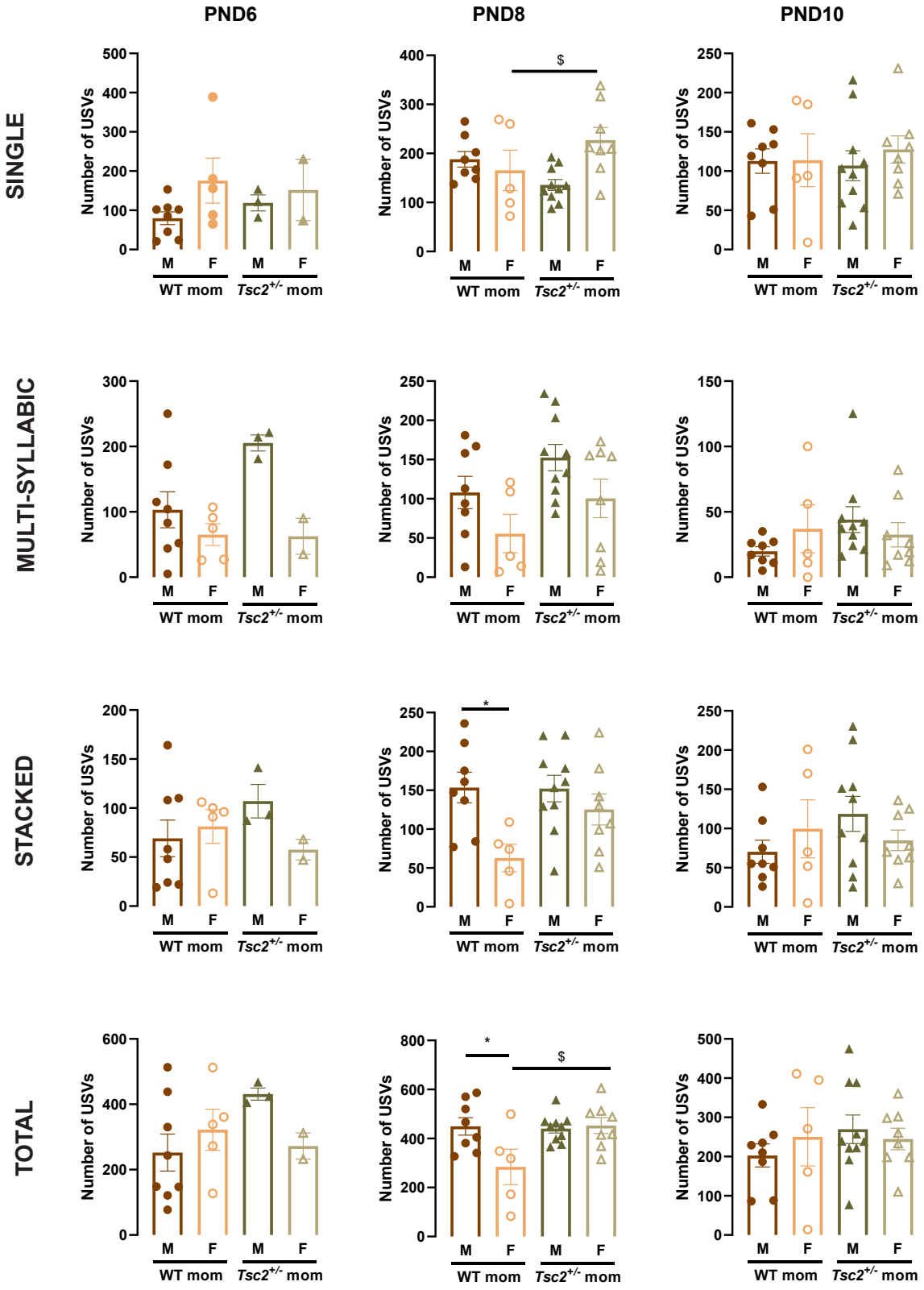
Annex I | Sex x genotype differences in developmental milestones of WT pups

A. Surface righting reflex, **B**. cliff aversion, and **C**. nest seeking tests of WT pups performed on PND6, 8, 10, 12, and 14. **D**. Startle response to an auditory stimulus and **E**. eye opening day of WT pups, registered on PND6, 8, 10, 12, 14 and 16. Two-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; *: significant difference between WT mom males and WT mom females experimental groups; #: significant difference between WT mom males and *Tsc2*$^{+/-}$ mom males experimental groups; $: significant difference between WT mom females and *Tsc2*$^{+/-}$ mom females experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups, males) = 8; n (WT mom & WT pups, females) = 5; n (*Tsc2*$^{+/-}$ mom & WT pups, males) = 10; n (*Tsc2*$^{+/-}$ mom & WT pups, females) = 8.



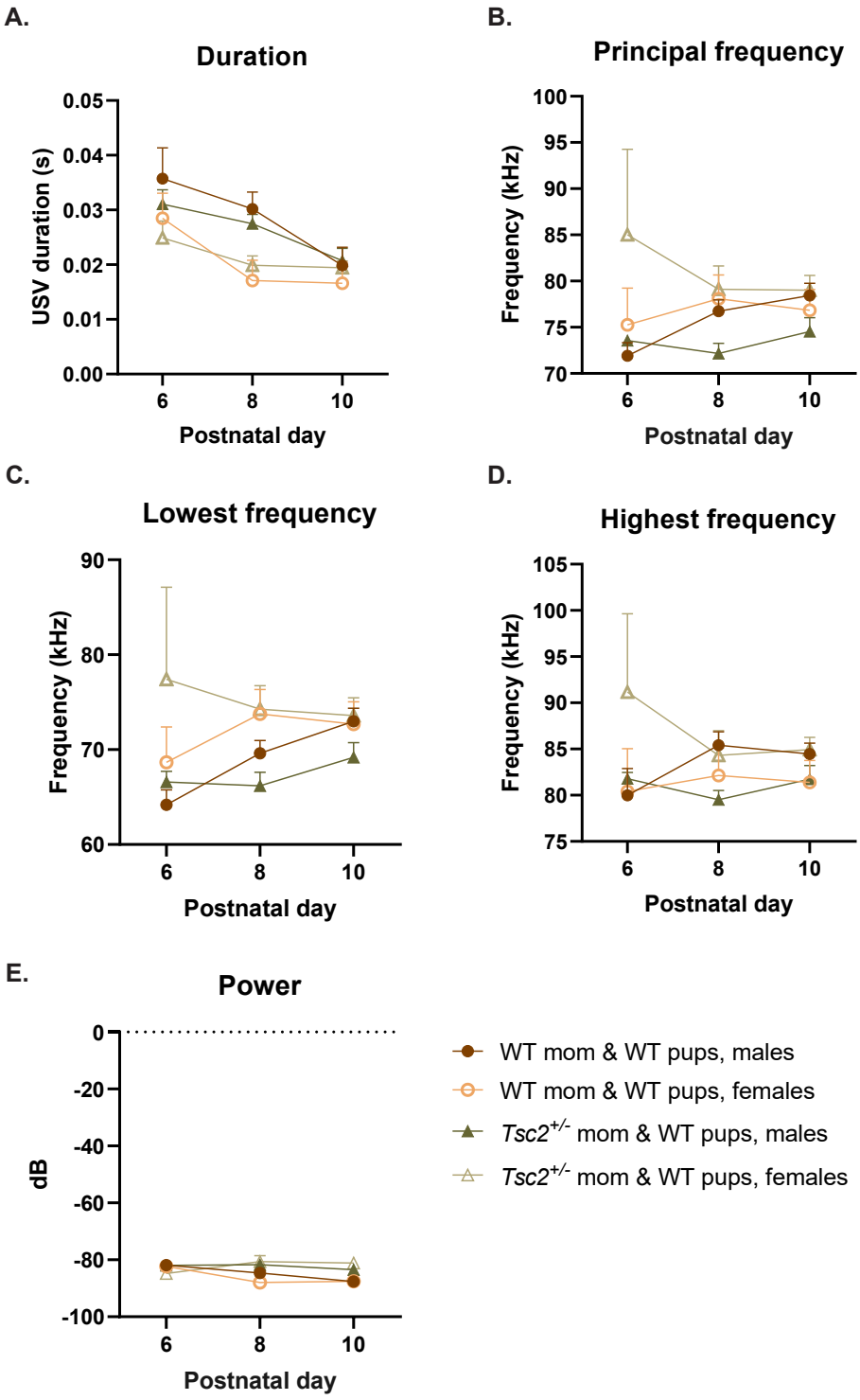
Annex II | Sex x genotype differences in developmental milestones of *Tsc2*$^{+/-}$ pups

A. Surface righting reflex, **B**. negative geotaxis reflex, and **C**. nest seeking tests of *Tsc2*$^{+/-}$ pups, performed on PND6, 8, 10, 12 and 14; **D**. startle response to an auditory stimulus of *Tsc2*$^{+/-}$ pups, registered on PND6, 8, 10, 12, 14 and 16. Two-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; *: significant difference between WT mom males and WT mom females experimental groups; #: significant difference between WT mom males and *Tsc2*$^{+/-}$ mom males experimental groups; $: significant difference between WT mom females and *Tsc2*$^{+/-}$ mom females experimental groups. Data represented as mean ± SEM; n (WT mom & *Tsc2*$^{+/-}$ pups, males) = 10; n (WT mom & *Tsc2*$^{+/-}$ pups, females) = 6; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups, males) = 8; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups, females) = 9.



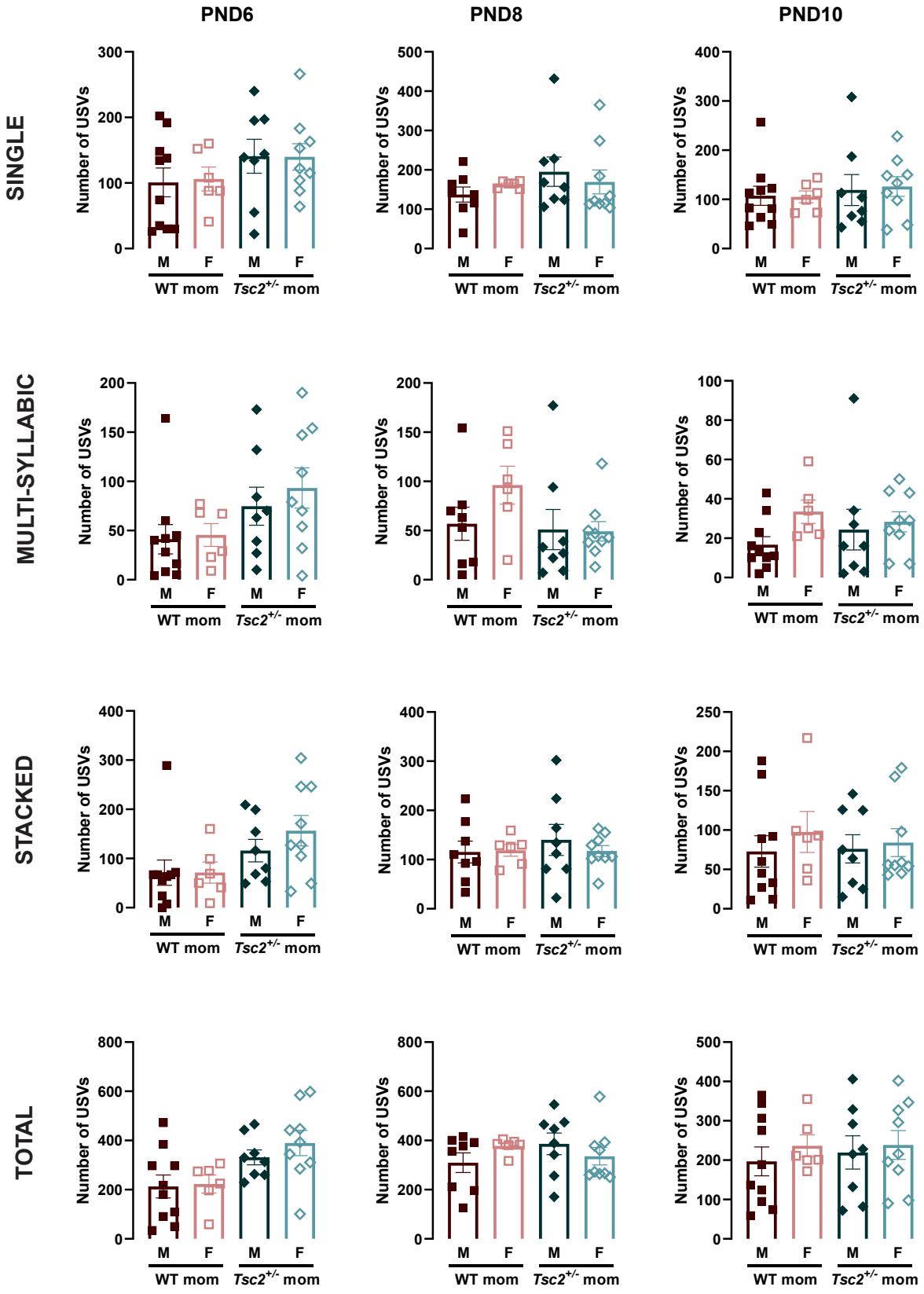
Annex III | Sex differences in composition of ultrasonic vocalizations of WT pups

Number of single, multi-syllabic and stacked USVs (Young et al, 2010), and total number of USVs produced by male and female WT pups born from WT or *Tsc2*$^{+/-}$ dams, on PND6, 8 and 10. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test; $p<0.05$. *: significant difference between WT mom males and WT mom females experimental groups; $: significant difference between WT mom females and *Tsc2*$^{+/-}$ mom females experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups, males) = 8; n (WT mom & WT pups, females) = 5; n (*Tsc2*$^{+/-}$ mom & WT pups, males, PND6) = 3; n (*Tsc2*$^{+/-}$ mom & WT pups, females, PND6) = 2; n (*Tsc2*$^{+/-}$ mom & WT pups, males, PND8 and PND10) = 10; n (*Tsc2*$^{+/-}$ mom & WT pups, females, PND8 and PND10) = 8.



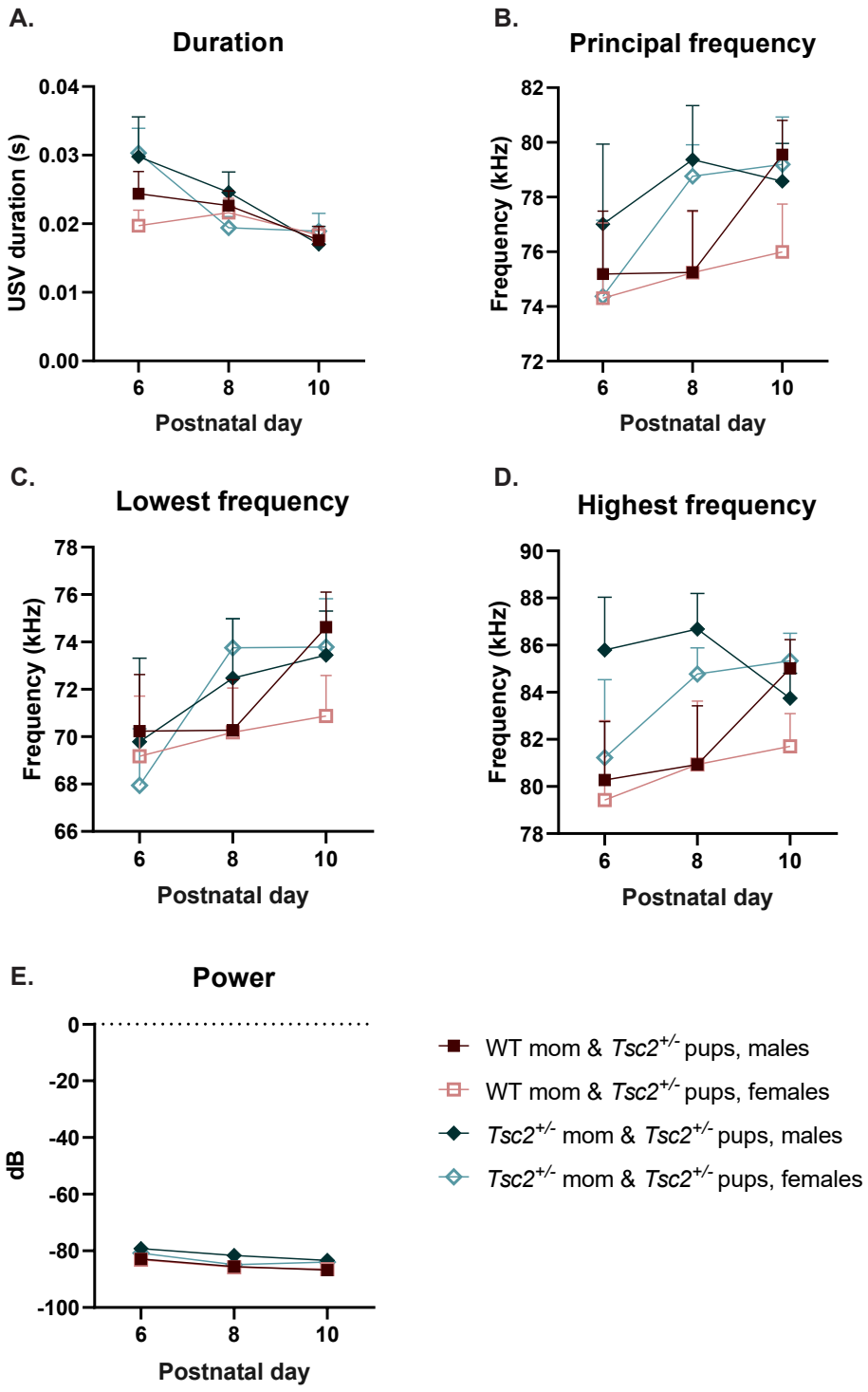
Annex IV | Sex differences in characteristics of ultrasonic vocalizations of WT pups

A. Duration, **B**. principal frequency, **C**. lowest frequency, **D**. highest frequency, and **E**. power (sound intensity) of USVs produced by male and female WT pups born from WT or *Tsc2*$^{+/-}$ dams, on PND6, 8 and 10. Mixed effects analysis followed by Tukey's multiple comparisons test; $p<0.05$. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups, males) = 8; n (WT mom & WT pups, females) = 5; n (*Tsc2*$^{+/-}$ mom & WT pups, males, PND6) = 3; n (*Tsc2*$^{+/-}$ mom & WT pups, females, PND6) = 2; n (*Tsc2*$^{+/-}$ mom & WT pups, males, PND8 and PND10) = 10; n (*Tsc2*$^{+/-}$ mom & WT pups, females, PND8 and PND10) = 8.



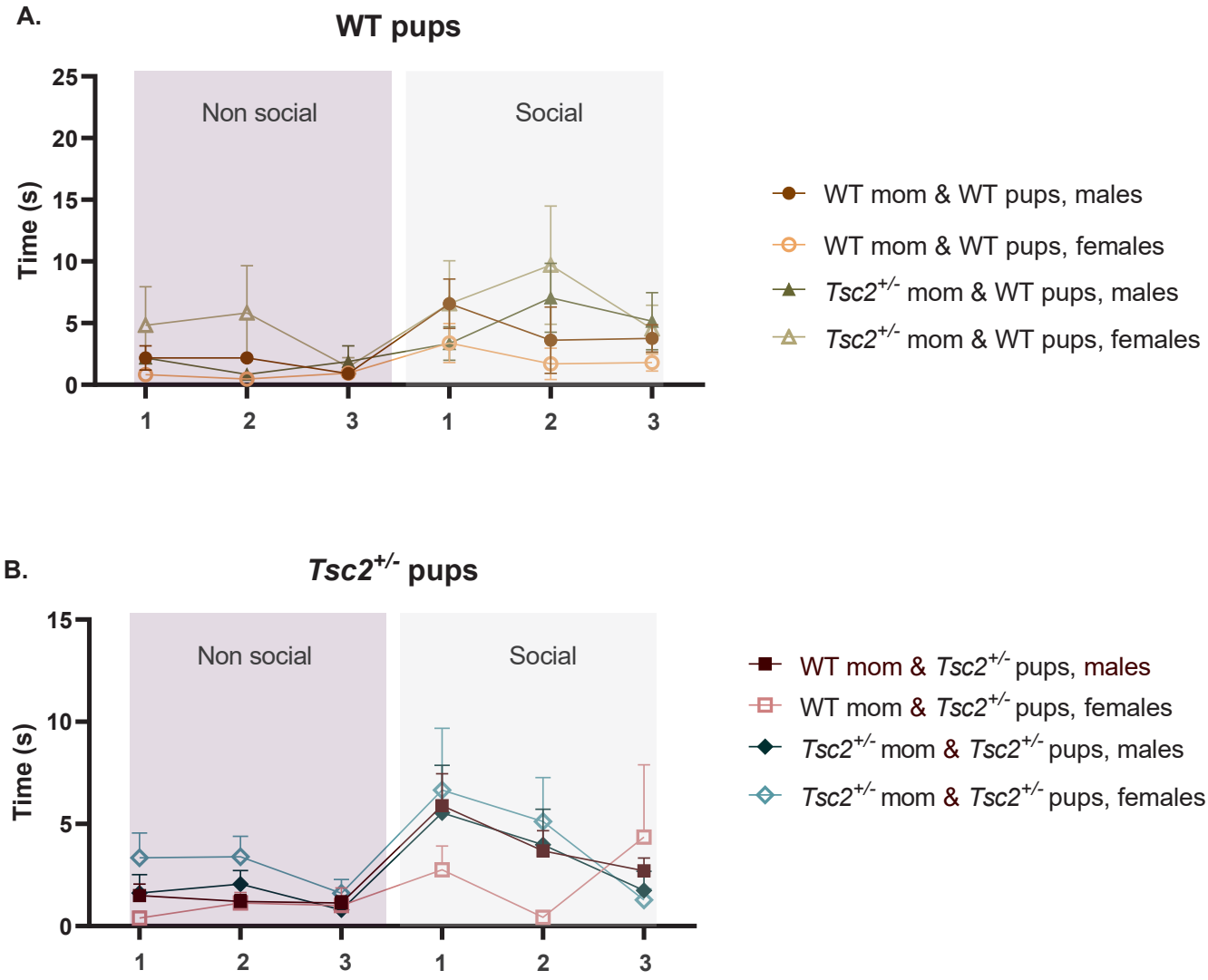
Annex V | Sex differences in composition of ultrasonic vocalizations of *Tsc2*$^{+/-}$ pups

Number of single, multi-syllabic and stacked USVs (Young et al, 2010), and total number of USVs produced by male and female *Tsc2*$^{+/-}$ pups born from WT or *Tsc2*$^{+/-}$ dams, on PND6, 8 and 10. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test; $p<0.05$. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (WT mom & *Tsc2*$^{+/-}$ pups, males) = 10; n (WT mom & *Tsc2*$^{+/-}$ pups, females) = 6; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups, males) = 8; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups, females) = 9.



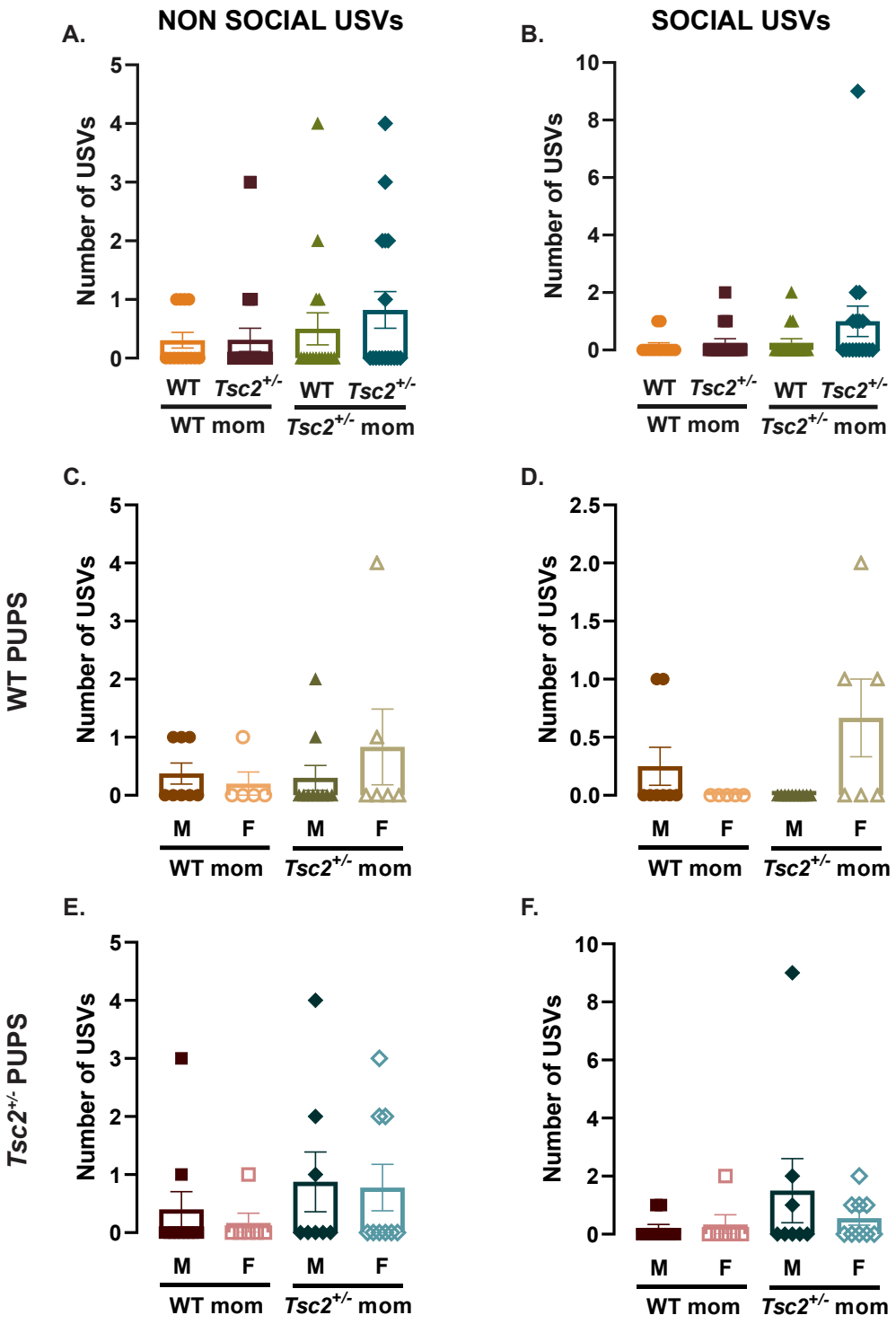
Annex VI | Sex differences in characteristics of ultrasonic vocalizations of *Tsc2*$^{+/-}$ pups

A. Duration, **B**. principal frequency, **C**. lowest frequency, **D**. highest frequency, and **E**. power (sound intensity) of USVs produced by male and female *Tsc2*$^{+/-}$ pups born from WT or *Tsc2*$^{+/-}$ dams, on PND6, 8 and 10. Mixed effects analysis followed by Tukey's multiple comparisons test; $p<0.05$. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (WT mom & *Tsc2*$^{+/-}$ pups, males) = 10; n (WT mom & *Tsc2*$^{+/-}$ pups, females) = 6; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups, males) = 8; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups, females) = 9.



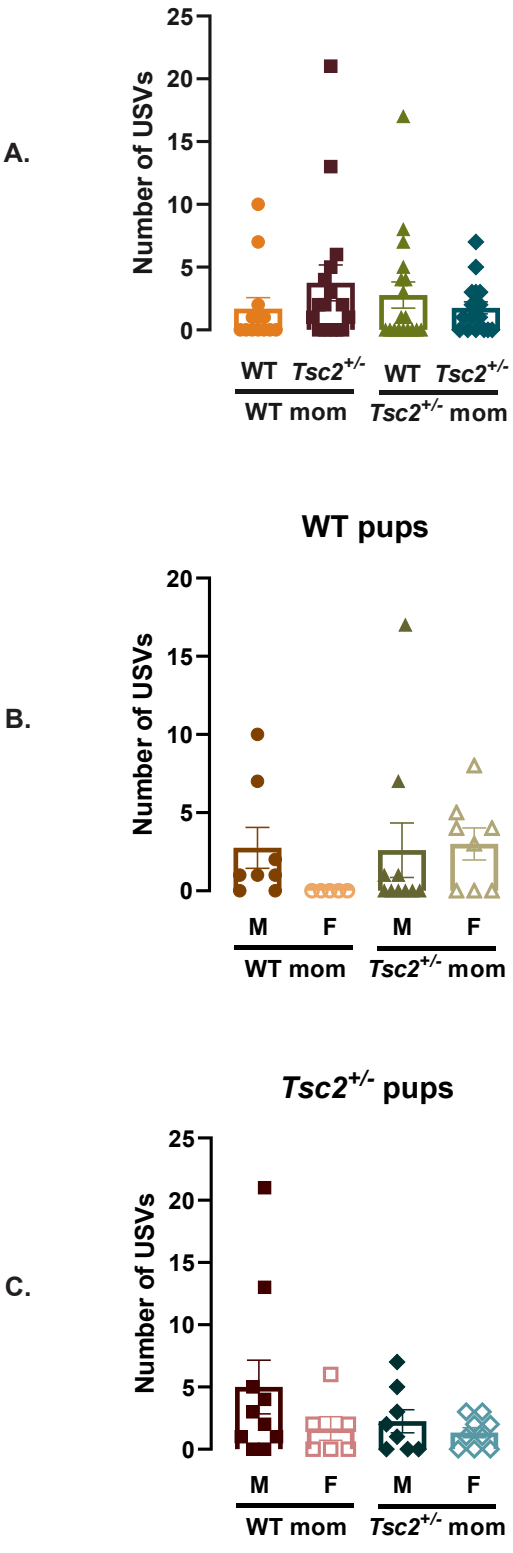
Annex VII | Sex differences in social odor discrimination test

Time spent engaging with non-social and social odors by A. WT male and female offspring born from WT or *Tsc2*$^{+/-}$ dams, and by B. *Tsc2*$^{+/-}$ male and female offspring born from WT or *Tsc2*$^{+/-}$ dams. Kruskal-Wallis test followed by Dunn's multiple comparisons test. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups, males) = 8; n (WT mom & WT pups, females) = 5; n (*Tsc2*$^{+/-}$ mom & WT pups, males) = 10; n (*Tsc2*$^{+/-}$ mom & WT pups, females) = 6; n (WT mom & *Tsc2*$^{+/-}$ pups, males) = 10; n (WT mom & *Tsc2*$^{+/-}$ pups, males) = 6; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups, males) = 8; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups, males) = 9.



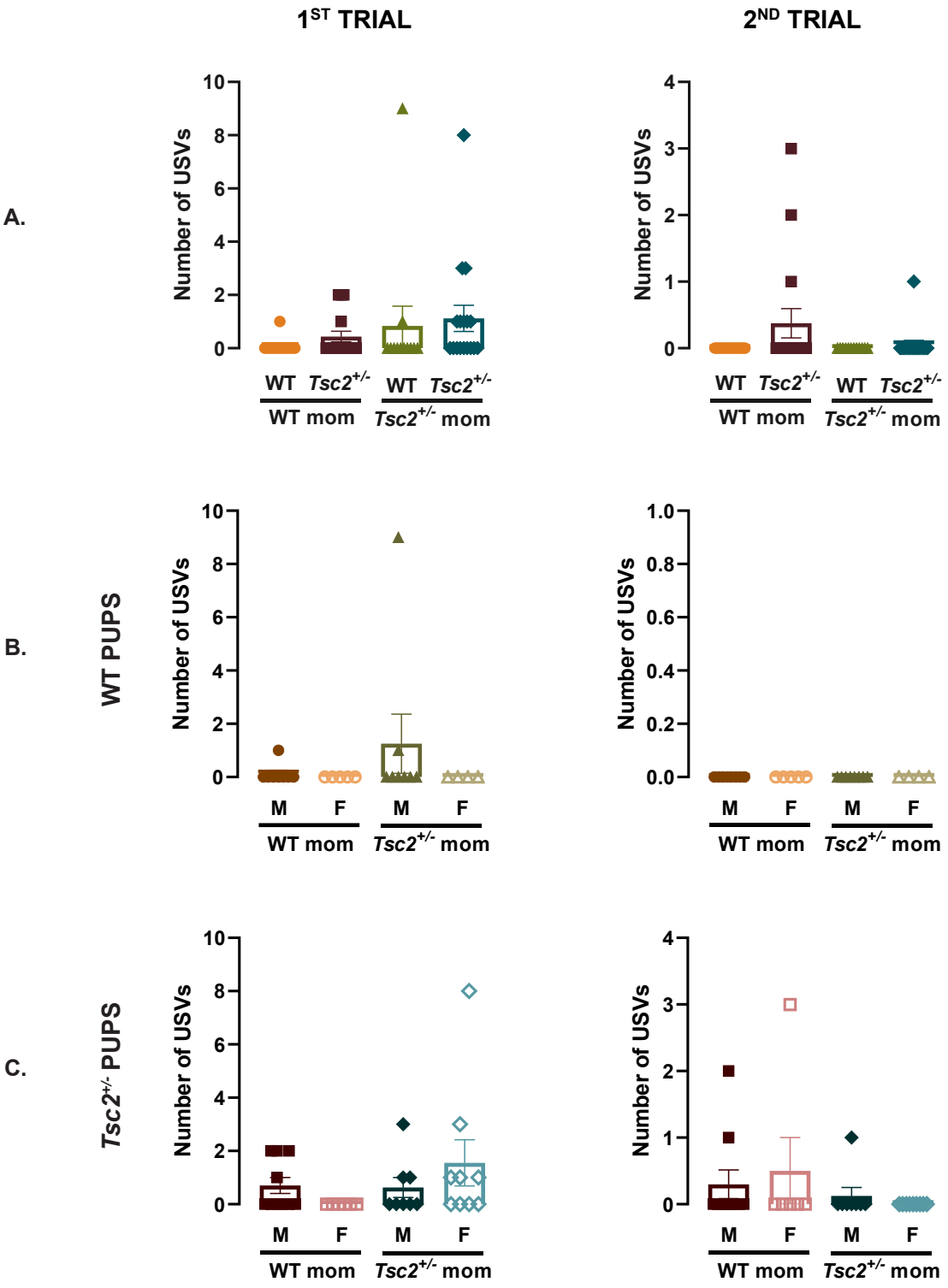
Annex VIII | Ultrasonic vocalizations produced in social odor discrimination test

Total number of USVs produced during the presentation of **A**. non-social and **B**. social odors. These were further discriminated by sex within WT pups born from WT or *Tsc2*$^{+/-}$ dams (**C** and **D**), and within *Tsc2*$^{+/-}$ pups born from WT or *Tsc2*$^{+/-}$ dams (**E** and **F**). Kruskal-Wallis test followed by Dunn's multiple comparisons test. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups) = 13 (8 males + 5 females); n (WT mom & *Tsc2*$^{+/-}$ pups) = 16 (10 males + 6 females); n (*Tsc2*$^{+/-}$ mom & WT pups) = 16 (10 males + 6 females); n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups) = 17 (8 males + 9 females).



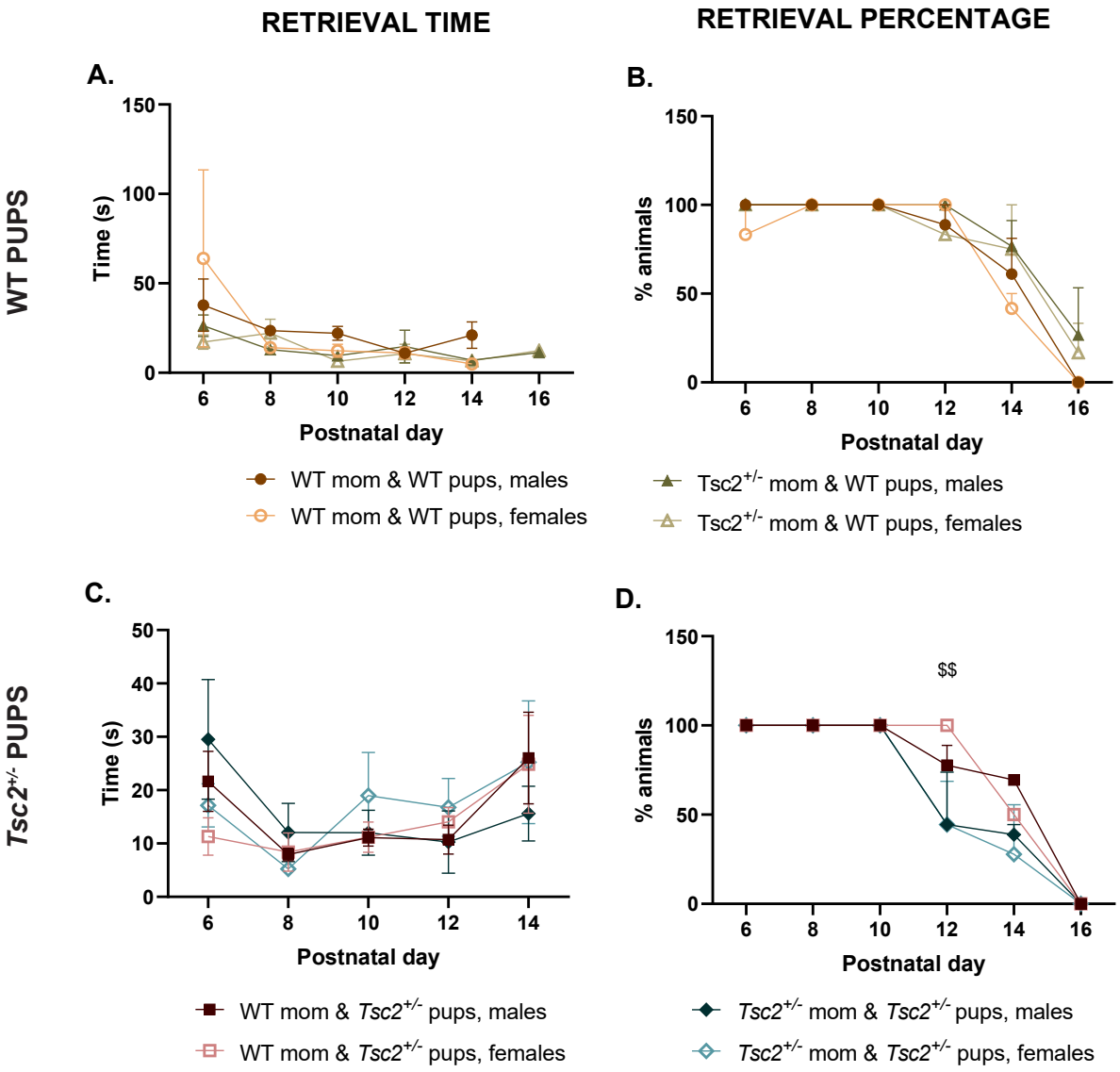
Annex IX | Ultrasonic vocalizations produced in marble burying test

A. Total number of USVs produced during marble burying test. These were further discriminated by sex within WT pups born from WT or *Tsc2*$^{+/-}$ dams (**B**), and within *Tsc2*$^{+/-}$ pups born from WT or *Tsc2*$^{+/-}$ dams (**C**). Kruskal-Wallis test followed by Dunn's multiple comparisons test. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups) = 13 (8 males + 5 females); n (*Tsc2*$^{+/-}$ mom & WT pups) = 18 (10 males + 8 females); n (WT mom & *Tsc2*$^{+/-}$ pups) = 16 (10 males + 6 females); n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups) = 17 (8 males + 9 females).



Annex X | Ultrasonic vocalizations produced in Y-maze test

A. Total number of USVs produced during each trial of the Y-maze test. These were further discriminated by sex within WT pups born from WT or *Tsc2*$^{+/-}$ dams (**B**), and within *Tsc2*$^{+/-}$ pups born from WT or *Tsc2*$^{+/-}$ dams (**C**). Kruskal-Wallis test followed by Dunn's multiple comparisons test. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups) = 13 (8 males + 5 females); n (*Tsc2*$^{+/-}$ mom & WT pups) = 12 (8 males + 4 females); n (WT mom & *Tsc2*$^{+/-}$ pups) = 16 (10 males + 6 females); n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups) = 17 (8 males + 9 females).



Annex XI | Maternal genotype on retrieval behavior

A. Retrieval time, and **B**. retrieval percentage of WT pups by their WT or *Tsc2*$^{+/-}$ dams, registered on PND6, 8, 10, 12, 14 and 16. **C**. Retrieval time, and **D**. retrieval percentage of *Tsc2*$^{+/-}$ pups by their WT or *Tsc2*$^{+/-}$ dams, registered on PND6, 8, 10, 12, 14 and 16. Two-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; $: significant difference between WT mom females and *Tsc2*$^{+/-}$ mom females experimental groups. Data represented as mean ± SEM; n (WT mom & WT pups, males) = 8; n (WT mom & WT pups, females) = 5; n (WT mom & *Tsc2*$^{+/-}$ pups, males) = 10; n (WT mom & *Tsc2*$^{+/-}$ pups, females) = 6; n (*Tsc2*$^{+/-}$ mom & WT pups, males) = 10; n (*Tsc2*$^{+/-}$ mom & WT pups, females) = 8; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups, males) = 8; n (*Tsc2*$^{+/-}$ mom & *Tsc2*$^{+/-}$ pups, males) = 9.