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Brain Mechanisms of Genetic and Inflammatory Double-Hit Vulnerability to Developmental Social Dysfunctions

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Abbreviations

16p +/-	16p11.2 proximal deletion
ACC	Anterior Cingulate Cortex
ADHD	Attention Deficit Hyperactivity Disorder
ASD	Autism Spectrum Disorder
AUC	Area Under the Curve
CNS	Central Nervous System
CNV	Copy Number Variant
CTRL	Control
DI	Discrimination Index
dlPFC	DorsoLateral Prefrontal Cortex
DREADDs	Designer Receptors Exclusively Activated by Designer Drugs
DSM	Diagnostic and Statistic Manual of mental disorders
E/I	Excitatory Inhibitory Balance
EDT	Emotion Discrimination Test
GABA	Gamma-Aminobutyric acid
GCaMP	Genetically encoded calcium indicator
GD	Gestational Day
HMW	High Molecular Weight
IHC	Immunohistochemistry
IP	Intraperitoneal

IL	Infralimbic
LMW	Low Molecular Weight
LPS	Lipopolysaccharide
MIA	Maternal Immune Activation
MO	Medial Orbital
mPFC	Medial Prefrontal Cortex
NDD	Neurodevelopmental Disorders
PCR	Polymerase Chain Reaction
PFC	Prefrontal cortex
PL	Prelimbic
PLX	PLX6522
PND	Post Natal Day
PSTH	Peristimulus Time Histogram
PV	Parvalbumin
SCZ	Schizophrenia
SNP	Single Nucelotide Polymorphism
SOM	Somatostatin
ToM	Theory of mind
wt	Wild-type

CHAPTER 1

General Introduction and Outlines of the Thesis

General Introduction

Neurodevelopment and Neurodevelopmental disorders

The term "Neurodevelopment" refers to the process of growth and maturation of the nervous system, which it is essential for the development of normal cognition and for the social abilities of every individual. This process spans from the life in-utero through early adulthood, and although the research in this field is quickly advancing, its mechanisms are not yet fully understood. Nevertheless, a deeper understanding of the processes underlying neurodevelopment is crucial for investigating the mechanisms of behavior and for identifying, addressing, and potentially preventing developmental alterations.

Neurodevelopmental disorders (NDD) are the clinical conditions arising from atypical neurodevelopment: the term refers to neuropsychiatric alterations characterized by an early onset (in infancy, childhood, or adolescence) and functional impairments in cognitive domains such as language, behavior, motor skills, emotions and social processes [1]. NDD disorders include conditions such as autism spectrum disorder (ASD), attention deficits and hyperactivity disorder (ADHD), learning disabilities, intellectual disabilities and schizophrenia. All NDD can significantly impact individuals' lives and global everyday functioning by affecting education, employment, and the ability to attain and maintain social relationships [2]. Indeed, these challenges are not just confined to the person affected by the disorder, but they extend to families, who often need to provide support and care [2]. Moreover, the social economic burden is substantial, and it includes direct costs for medical and educational support as well as indirect costs such as loss of productivity for both carriers and caregivers [2–4]. The increasing prevalence of neurodevelopmental conditions [5] underscores the need for efficient diagnostic systems and preventive and therapeutic interventions: for this reason, advancing our understanding of neurodevelopment—both its typical progression and the mechanisms underlying abnormalities—is of high importance: such knowledge can be the highly precious for early diagnosis, targeted interventions, and ultimately to improve the outcomes.

Autism Spectrum Disorders

ASD is a neurodevelopmental condition characterized by challenges in language, social abilities and behavior[1], whose clinical presentation varies widely in severity. The diagnostic criteria, according to DSM-5, require an early onset of significant social interaction and communication deficits, paired with repetitive behavior or restricted interests or activities [1]. Social deficits are a key feature of ASD: they include qualitative and quantitative alterations in social reciprocity, in non-verbal communication and ultimately in the understanding, attainment and maintenance of social relationships [1]. In particular, ASD affected individuals often struggle to recognize and interpret emotional cues and to engage in reciprocal social behaviors [6,7].

Challenges with emotional recognition, which is the ability to identify and interpret emotional states in oneself and others, are common findings in the context of ASD [8,9]. Deficits in emotion recognition vary from the failure to recognize main emotions, like joy or fear, to struggles in distinguishing between subtle differences in facial expressions, such as sadness and frustration. This process is not limited to facial expression, but it involves also other sensory channels and contextual cues related to emotions, such as the recognition of tone of voice and of body language [10]. Taken together, these difficulties in decoding social signals can hinder the individual's ability to engage in reciprocal interactions [11], leading to reduced sociability and/or difficulties in forming and maintaining social relationships, often resulting in social isolation [12].

Sociability, defined as the drive to seek and maintain social interactions (social motivation), is another domain commonly impaired in ASD [13], with different levels of severity: some individuals with ASD may apparently display a lack of interest for social engagement, while others may desire connection but may struggle in understanding how to interact effectively [14]. One of the reasons beyond such variability is that emotional and social deficits are usually interwoven with broader cognitive and neurological challenges, such as attentional difficulties to social stimuli, impaired theory of mind (meaning the ability to understand others' thoughts and intentions), and atypical sensory processing [15]. Such heterogeneity in the symptom presentation is one of the reasons why ASD is referred to as a spectrum of disorders: while some individuals may show profound deficits in recognizing and responding to social cues, others may have milder impairments or rely on

compensatory mechanisms to navigate social settings, leading to a variety of ASD phenotypes with different level of impairments and severity [1].

Comprehensively evaluating social deficits and subtle differences in ASD phenotypes is crucial for the appropriate diagnosis and for addressing therapeutic interventions. Therefore, the diagnostic process involves a range of standardized tools, each designed to capture different aspects of the disorder, including social functioning. The Autism Diagnostic Observation Schedule (ADOS) [16] and the Autism Diagnostic Interview-Revised (ADI-R) [17] are among the most commonly used diagnostic tools. The ADOS is semi-structured, play-based assessment usually administered in youth by a clinician. It is often referred to as the "gold standard" for diagnosing ASD: it measures social interaction, communication, play, and repetitive behaviors across various modules tailored to different developmental and language levels. The ADOS captures social deficits by evaluating the ability to initiate and respond to social overtures, eye contact, shared attention, and the use of gestures. The ADI-R is a structured interview that is conducted with caregivers, to gather detailed information about the individual's developmental history and current functioning. The ADI-R explores social deficits by evaluating behaviors such as the presence of social smile, reciprocal conversation, and the ability to form peer relationships.

The insights gained from these assessments are usually designed for the diagnosis, but they can also provide useful information for the following interventions, such as social skills training or personalized strategies to foster peer connections and reduce social isolation [18] and also to study and characterize the multitude of ASD phenotypes [19] . Unfortunately, despite tailored educational and support and many years of research, to date there are no effective treatments that can significantly revert social deficits in ASD [20], and one of the possible reasons beyond that, is that the brain mechanisms underlying normal and pathological social cognition are still obscure. Understanding the mechanisms underlying such deficits, especially in the context of NDD, is essential to find appropriate strategies for prevention and ultimately to design effective targeted treatments.

The genetic behind ASD

One of the reasons beyond the difficulties in treating ASD is that its causes are multifactorial, and even though some factors have been identified as potential contributors, their role is not fully understood. Two main subgroups of ASD can be identified from a causal perspective: idiopathic (having an unknown origin) and syndromic [21]. Idiopathic ASD accounts for the majority of cases [22] and is thought to arise from a complex interplay of genetic, environmental, and epigenetic factors. The clinical phenotype of idiopathic ASD was first described by Kanner [23] with ASD manifesting alone, with social and behavioral alterations but without concurrent neurological problems. In contrast, syndromic forms of ASD, which however represent a smaller subset of cases [21], have a clinical presentation in which ASD is accompanied by a cluster of other problems, usually neurological, leading to a definition of specific syndromes, which support a further clinical investigation on a possible genetic cause. The syndromic forms of ASD are, in fact, generally linked with genetic alterations [21], which include single nucleotide polymorphisms (SNP, which are variations in a single nucleotide at a specific position in the genome, when one nucleotide is replaced by another); copy number variants (CNV, which are structural alterations in the genome resulting in the duplication or deletion of large segments of DNA); or other genetic mutations (such as frameshift), in which the insertion or deletion of a number of nucleotides leads to a disruption of the reading frame, ultimately changing the resulting protein.

Advances in genomics have identified hundreds of genes and genetic conditions associated with ASD, many of which are involved in synaptic function, neural connectivity, or early brain development [24]. Some examples include the SHANK3 [25] (0.28% of ASD) and CHD8 [26] (0.21% of ASD) genes [21]. However, despite significant progress, many questions about ASD remain unanswered, and even in the context of ASD with a genetic origin, the link between many genotypic alterations and the man phenotypes of ASD is unclear. Genetic alterations could impact neurodevelopmental processes by disrupting essential mechanisms at the molecular and cellular level [27]. Such mechanisms are, for instance, synaptogenesis [28], neuronal migration [29], and neurotransmitter signaling [30,31], whose disruption can trigger cascading effects on the developing brain's structural and functional organization [27,28]. For example, genetic alterations often affect neurotransmitter regulation, particularly the balance between excitatory and inhibitory signaling, which is believed to be one of the core features beyond ASD [32,33]: genes encoding components of

GABAergic and glutamatergic neurotransmission are frequently affected [33,34], leading to disruptions in neuronal activity and firing patterns, by altering receptor sensitivity or binding efficiency and by impairing synaptic communication and plasticity. Another key aspect of these disruptions involves cytoskeletal dynamics [35,36], which are critical, among others, for neuronal migration and axon guidance, disrupting the precise wiring of neural circuits, and ultimately leading to structural abnormalities in the brain.

Genetic mutations (SNP, CNV or other) can affect such processes by qualitatively and quantitatively changing proteins relevant for the process [21]. This effect is often a consequence of alterations of dosage sensitive genes, and/or by disruptions of the gene structure (and therefore function), but also by the induction of gain or loss of function in a protein. Indeed, genetic mutations can also lead to chromosomal rearrangements and alterations of epigenetic regulation.

The disruption of dosage-sensitive genes, which rely on finely-tuned expression levels to maintain their function [37], is believed to be a key mechanism in NDD. Deletions or even hemi-deletions can reduce gene dosage [37,38], which can lead to insufficient protein production, while duplications can cause overexpression, which on the other hand can disrupt cellular homeostasis. Similar outcomes can be also a consequence of alterations in dosage sensitive-genes, which can occur when SNPs located in regulatory regions, such as promoters or enhancers, alter transcription factor binding sites [37]. In the context of NDD, the reduction in transcriptional output can disrupt pathways essential for early brain development, including those governing neuronal excitability, cortical patterning, and synaptic architecture.

Beyond dosage sensitivity, genetic alterations often disrupt the structure of genes directly, therefore impacting qualitatively on the resulting protein function: this is true, for example, for deletions that remove coding sequences, or SNPs introducing missense or nonsense mutations [37,39]. For instance, nonsense mutations result in truncated proteins, completely abolishing their functionality. These disruptions are particularly detrimental in neurodevelopmental genes that play foundational roles, such as those involved in guiding axonal pathways [40] or establishing neuronal connectivity [37]. Moreover, genetic alterations can also lead to protein loss-of-function or gain-of-function, which in turn might result in reduced or absent protein activity, or overactive or ectopic protein activity [37]. In either case, this can result in a disruption of the balance required for normal brain development.

Chromosomal rearrangements accompanying deletions and duplications add another layer of complexity, in the context of genetic mutation relevant for NDD, by interfering with regulatory regions such as enhancers and promoters [41,42]. The disruption of these regions, which control the timing and location of gene expression, can lead to dysregulation of genes even if the latter have intact coding sequences. Of note, if the gene is already altered because of one of the mechanisms reported above, the loss of these regulatory networks can even magnify the impact of the initial alteration, leading to widespread developmental consequences.

Ultimately, genetic alterations have a profound impact on epigenetic regulation, particularly in genes involved in chromatin remodeling and histone modification, ultimately leading to transcriptional dysregulations and further destabilizing gene expression programs essential for brain development [43].

The 16p11.2 proximal deletion

The proximal deletion of the chromosomal region 16p11.2 is one of the CNV more significantly associated with ASD, accounting for approximately 1% of all ASD cases [44,45]. This deletion is so-called “typical” (being more common than the “atypical” distal deletion, which is less linked with ASD) [46]. It occurs between the breakpoints 4 and 5 of the short arm of chromosome 16 and has an estimated prevalence of about 1 in 2000 individuals [47]. The phenotypic expression of the 16p11.2 deletion is highly variable [45,47,48,48,49], even among carriers in the same family [50], underscoring the necessity for personalized diagnostic and therapeutic approaches. A notable predisposition to obesity has been documented [47,51], with significant increases in body mass index often evident from early childhood. Some carriers may present with structural brain abnormalities, epilepsy, and macrocephaly [47,51]. Moreover, carriers have a heightened risk of developing neurological and psychiatric disorders, exhibiting moderate quantitative and qualitative differences in phenotypic profiles [45,49,52,53]. Developmental delays are prevalent, particularly in motor skills and language acquisition, leading to challenges in fine and gross motor coordination as well as speech articulation [47]. Mild intellectual disability is also common, affecting learning abilities and adaptive functioning. Furthermore, studies have indicated that carriers of the 16p11.2 deletion may experience psychiatric conditions, including ADHD, anxiety disorders, and mood disorders [48,52–

[54]. Behaviorally, traits associated with ASD are frequently observed, as we have recently reviewed [55]. Approximately 0.5% of individuals with ASD who undergo genetic testing are found to have the proximal 16p11.2 microdeletion, and within the population carrying this CNV, the prevalence of ASD has been reported to range from 11% to 27% [45,56]. Beyond ASD diagnoses, specific social and cognitive alterations have been identified among the carriers of this 16p11.2 deletion: it was estimated that, excluding those diagnosed with ASD, 60% of the remaining individuals exhibited impairments in social interaction and 73% showed deficits in communication [45]. Moreover, children carrying the 16p11.2 deletion demonstrated greater impairments in social interaction and communication compared to their non-carrier siblings, including, for instance, problems with imitation, demonstrating interest, engaging in play, and conveying social cues during interactions with peers [48,57].

The mechanisms underscoring the disorders arising in the context of this 16p11.2 deletion are still undisclosed, also due to the fact that the penetrance is incomplete [58] and that none of the genes studied so far, and belonging to this region, were significantly linked with ASD [59]. Recently it was suggested that probably a rearrangement of the chromosome following the deletion could partially explain the many problems arising from this condition [60], but no clear link between the genetic and the phenotype has been established. Indeed, the proximal 16p11.2 deletion encompasses a group of genes that play critical roles in essential neurodevelopmental processes, including synaptogenesis, neuronal migration, neuronal function, and neurotransmitter regulation. Genes such as SEZ6L2 and DOC2A located in the 16p11.2 locus are fundamental for synaptogenesis [61,62]. SEZ6L2 is involved in regulating synaptic interactions and signal transmission, ensuring the proper formation and maintenance of neuronal connections, and is involved in epilepsy [63]; DOC2A contributes to synaptic transmission and neurotransmitter release in a calcium-dependent manner, particularly influencing excitatory synapses [64]. Neuronal migration, another essential step for the architectural organization of the brain, also requires some genes in the 16p11.2 deleted segment. TAOK2 (TAO Kinase 2) plays a pivotal role in brain development by guiding neuronal migration and the development of the cortical structure [65]; KCTD13 influences neuronal proliferation and migration, with mutations in this gene linked to cortical developmental abnormalities [66]. Genes like PRRT2 and INO80E are of high importance for neuronal functioning: PRRT2 modulates synaptic transmission by regulating the release of neurotransmitters [67] and has been associated with disorders such as epilepsy and paroxysmal movement disorders [68]; INO80E contributes to

chromatin remodeling, a process that underlies neuronal plasticity and gene transcription, which are essential for proper neuronal development and function [69]. In addition to these specific roles, some genes in the 16p11.2 region have complementary functions that span multiple neurodevelopmental processes.

Notably, besides genes involved in the brain functioning and development, this CNV encompasses several genes that are known for their role in the relevance for the immune system, particularly in immune cell signaling, activation, and migration, although the exact effects remain to be fully elucidated. Genes like SPN (Sialophorin, encoding for CD43), LAT (Linker for Activation of T cells), and CORO1A (Coronin 1A), have been associated with T cell activity [70–72]. Among them, the most notable is SPN, which encodes a glycoprotein expressed on the surface of T cells, B cells, and monocytes, involved in immune cell adhesion, migration, and activation [71]. It regulates T cell signaling and cytoskeletal dynamics, both of which are essential for immune synapse formation and inflammatory responses. LAT is another critical gene within this region, functioning as an adaptor protein in T cell receptor (TCR) signaling. LAT facilitates the assembly of signaling complexes downstream of the TCR, mediating T cell activation and proliferation [70]. Additionally, MAPK3 (Mitogen-Activated Protein Kinase 3) contributes to cytokine signaling and immune cell survival: as part of the MAPK/ERK pathway, MAPK3 regulates cell signaling in both immune and non-immune contexts, playing a role in responses to pathogens and inflammation [73–75]. Another key immune-related gene in the 16p11.2 locus is CORO1A. This gene is crucial for T cell homeostasis and activation, as it regulates actin cytoskeleton dynamics necessary for immune cell motility and signaling [72]. Disruptions in CORO1A can impair both adaptive and innate immune responses. Mice lacking CORO1A mice display a reduction of the peripheral T cells, probably by impacting survival and migration [76]. CORO1A also plays a role in macrophage function, particularly in preventing the fusion of phagosomes and lysosomes [77], which is essential for immune cells for engulfing and digesting pathogens and cellular debris, as it assists in the reorganization of the actin cytoskeleton necessary for phagocytosis. Other genes in the 16p11.2 locus that were liked with the activity of the immune system are CDIPT (CDP-Diacylglycerol-Inositol 3-Phosphatidyltransferase) and KCTD13. CDIPT is also involved in phosphatidylinositol synthesis, a critical component of membrane signaling in immune cells, which disruption may indirectly affect signaling dynamics in immune pathways [78]. Similarly, KCTD13, though better known for its neurodevelopmental roles, intersects with pathways that influence immune

regulation and signaling [79–81]. It has been long proved that the nervous system, and especially the brain, is not isolated and that many of its function during and after development depend on a proper interplay with the immune system. It is now well established that many neurodevelopmental processes are influenced by immune-brain interactions, and that alterations in the activity of the immune system can lead to aberrant brain phenotypes. For example overexpression of CD47, which is involved in T cell recruitment and signaling that promotes macrophage-mediated phagocytosis, has been reported to be linked with macrocephaly in the context of the proximal 16p11.2 deletion [82]. Moreover, it was reported that almost half of the carriers of this deletion are prone to frequent infections, indicating a potential disruption of some features of the immune system in the context of this condition [47]. This suggests that a deeper understanding of the brain-immune crosstalk in the context of the 16p11.2 deletion could be very promising to further disclose potential mechanisms involved in neurodevelopmental disorders arising in the context of this mutation.

The role of microglia in ASD

The neurodevelopmental processes during the first and second trimesters involve many cells, including neural progenitors (which are responsible for generating neurons and glia during neurogenesis), radial glial cells (which are needed as a scaffolding for migrating neurons), neurons, (undergoing differentiation, migration, and synapse formation) and microglia (which are the brain's resident immune cells) [83].

Microglia originate from erythromyeloid progenitor cells in the embryonic yolk sac. During early development they migrate into the central nervous system (CNS), where they are involved in many processes including phagocytosis of apoptotic cells, removal of unnecessary neurons and synapses through synaptic pruning [84], and regulation and facilitation of neuronal activity, which are crucial during the neurodevelopment [85,86]. They also influence processes such as tissue maintenance, response to damage, and defense against pathogens [84].

Not surprisingly, microglia have emerged as critical players in the neural circuits of ASD [87]. Human neuroimaging studies with PET tracers binding to microglial activation markers show increased microglial activity in individuals with ASD, indicating a reactive state [88]. Moreover,

post-mortem studies in brains from individuals with ASD reveal altered microglial morphology, density, and activation states, particularly in regions crucial for executive and social functions like the dorsolateral prefrontal cortex (dlPFC) [87,89]. Consistently, genome-wide transcriptomic have identified changes in microglial-specific gene expression: reactive microglia express genes involved in phagocytosis, migration, and synaptic interaction, suggesting pathological activation in ASD [90].

Recent evidence further suggests that microglial dysfunction contributes significantly to the structural, functional and behavioral deficits observed in ASD [91,92]. During the postnatal period, microglia is fundamental for synaptic pruning, by eliminating weak or inactive synaptic connections to refine and optimize neural circuits: this process ensures that only the most functionally active synapses are preserved, facilitating the maturation of efficient brain networks [93]. In ASD, disruptions in microglial pruning have been linked to widespread abnormalities in synaptic development and therefore in connectivity [94], but microglial dysfunction in ASD is not limited to pruning deficits. In cortical regions responsible for long-range connectivity, microglia interact with other cell types, such as astrocytes and interneurons, influencing local and global circuit dynamics and the behavioral outcomes [95]. Microglia communicate bi-directionally with neurons through many mechanisms, including direct contact at synapses and indirect signaling via soluble factors [85,86]. This communication is fundamental for microglial recruitment, as well as for synapse development, plasticity, and transmission, ultimately influencing network synchronization. In fact, a critical aspect of neural circuitry in ASD is the balance between excitatory and inhibitory (E/I) signaling [96], meaning the dynamic interaction of glutamatergic excitatory neurons and GABAergic inhibitory neurons [33]. This balance is essential for normal brain function, ensuring that neuronal activity is neither excessively excitatory nor overly suppressed. Microglia is an important mediator of the E/I balance through its interactions with both excitatory and inhibitory neurons, by regulating synapses: a study using Cx3cr1 knockout mice, which lack the fractalkine receptor essential for neuron-microglia signaling, have demonstrated profound deficits in synaptic pruning, that were paired with functional and behavioral deficits [97]. Mice exhibited an excess of immature excitatory synapses, deficits in the number of synapses per axonal input, and weakened functional connectivity between the medial prefrontal cortex (mPFC) and the hippocampus. Moreover, these abnormalities were paired with behavioral phenotypes commonly associated with ASD, including social deficits and repetitive behaviors. Another study provided

evidence of the role of microglia (a specific subset, GABA-receptive) in modulating the synapses of inhibitory neurons in particular during post-natal development [98]. This effect could suggest that the interplay between microglia and E/I balance could provide an interesting framework to further understand the mechanisms underlying abnormal social behaviors in ASD.

Cortical interneurons and ASD

The E/I balance requires the maintenance of an appropriate signal to noise ratio between excitatory and inhibitory circuits and is controlled by different mechanisms at different levels [96]. At the cellular level, besides microglia, it includes many neuronal subpopulations. Within the framework of inhibitory signaling, specific interneuron subtypes have been identified as pivotal players, particularly parvalbumin (PV) and somatostatin (SOM) expressing interneurons, which represent the 40 and 30% of total GABAergic interneurons in rodent neocortex [99]. These cells originate from the medial ganglionic eminence and undergo migration, synapse formation, and activity-dependent refinement, making them susceptible to disruptors during neurodevelopment. These two populations have distinct membrane and functional properties [100,101], therefore they are believed to play different roles in the context of social behavior [102,103].

PV expressing (PV+) interneurons are among the most studied in the context of E/I balance, and therefore in the context of ASD al well [34]. These fast-spiking inhibitory neurons are GABAergic cells responsible for regulating gamma oscillations (30–80 Hz) though their synchronized activity [104]. In fact, they frequently form contacts with each other and they are generally are located around perisomatic regions of pyramidal cells [100,101]. They receive excitatory input from pyramidal neurons via NMDA receptor-mediated signaling, and they play a crucial role in regulating neural networks by inhibiting local excitatory neurons through GABAergic mechanisms. Gamma oscillations, and therefore PV+ interneurons in the mPFC, are closely linked to cognitive functions such as attention and working memory, and especially with sociability [105]. In fact, studies in humans indicate that gamma power in the prefrontal cortex (PFC) increases during a social interaction task [106], and studies in mice demonstrated that inhibition and activation of PV+ interneurons in mPFC can decrease and increase gamma power, respectively, and reduce or increase social interaction, depending on the gamma frequencies [102,107]. PV+ interneurons

inhibition was reducing gamma power as well as the interest for a social versus a non-social target, while when synchronized activation of PV+ interneurons was augmenting gamma power, social interaction was enhanced. Of note, this was observed specifically at low (20-50 Hz), but not high (50-80 Hz) gamma frequencies [102].

Not surprisingly, it is believed that the alterations in E/I balance observed in ASD may result from changes in the number of PV+ interneurons or in their functional properties, as PV+ interneurons were previously implicated in ASD [108,109]. In fact, alterations in the function or density of PV+ interneurons have been consistently implicated in ASD, with postmortem studies [90] and animal models reporting reductions in PV+ interneuron number and density [110,111], while others were showing a reduction of PV expression [112,113]. Moreover, experimental studies have shown that PV knockout mice exhibit behavioral phenotypes resembling core ASD symptoms, such as social deficits, communication impairments, and repetitive behaviors [111].

Another type of inhibitory interneurons, the SOM expressing (SOM+) are also markedly represented in the rodent PFC [99]. Differently from PV+ interneurons, they do not form contacts with each other, as they mostly target distal dendrites of excitatory pyramidal cells where they play a key role in regulating their activity [100,101]. By modulating the E/I balance at the dendritic level, they help fine-tune neural responses: similar with PV+ interneurons, activation of SOM+ interneurons at low gamma frequency can produce a prosocial effect, but conversely, inhibition of these neurons does not affect sociability [107]. On the other hand, inhibition of SOM+ interneuron in the mPFC abolishes affective state discrimination while this is not true for PV+ interneurons [103]. Although less studied than PV+ interneurons, evidence suggests that disruptions in SOM+ interneurons may contribute to the altered synaptic plasticity and connectivity patterns observed in ASD [34,114]. In fact, changes in the transcriptional profiles of the SOM+ interneuron in the superficial layers of cortex were consistently found in post-mortem studies of ASD brains [90]. They involved transcripts of genes linked with the glutamatergic receptor function and signaling, with components of the action potential, and with mRNA splicing (which play critical roles in synaptic connectivity); these genes were mostly downregulated. On the other hand, genes involved in inflammatory response, in transport along cytoskeleton and in calcium-mediated axon guidance were upregulated in SOM+ interneurons [90]. Interestingly, similar changes in the transcripts relevant for the inflammatory response were described also in glial cells, shifting towards reactive

or activated inflammatory states in ASD, compared with controls. Moreover, in ASD, reactive microglial cells also increased expression of genes related to cell morphogenesis and integrin signaling [90]. Given that interactions between microglia and GABAergic inhibitory interneurons are crucial for the E/I balance, further investigating the relationships between cortical interneurons and microglia in the context of social behavior in ASD might open interesting perspective for a deeper understanding of mechanisms linked with ASD-relevant social impairments.

Summary

Syndromic forms of ASD provide a unique opportunity to investigate the behavioral and cellular deficits associated with the disorder in the context of neuro-immune interplay. The 16p11.2 proximal deletion is particularly well-suited for studies on inflammation and the potential neuro-immune mediators that may influence the manifestation of ASD, given that the deleted sequence includes many genes involved in the activity of the immune system. However, current knowledge about the interaction between this CNV and the ASD phenotypes remains scarce.

A critical feature of ASD is the presence of social behavioral alterations, for which effective treatments are still scarce. Gaining a deeper understanding of the cortical and cellular alterations underlying these behavioral problems is crucial for developing targeted prevention and therapeutic strategies. Characterizing social behaviors involves addressing both general sociability and more complex socio-cognitive abilities, such as emotion discrimination. Emotion discrimination, in particular, has been poorly studied in the context of the 16p11.2 deletion, despite its relevance to social behavioral deficits. Understanding alterations in these social functions, along with the circuits and cellular players involved, is fundamental to elucidate the mechanisms underlying ASD-related deficits.

Given the pivotal role of microglia in the context of the 16p11.2 deletion, it is important to assess inflammation and the specific contributions of these cells in modulating social behavior. The 16p11.2 deletion is a condition in which the vulnerability to infections could be strongly linked with its neurodevelopmental features, and the contribution of microglia to such alterations, especially in time-windows which are critical for brain development, might prove important to better understand the mechanisms underlying social deficits in ASD.

Recent evidence highlights the critical roles of specific neuronal populations, particularly PV and SOM expressing inhibitory neurons, in social behavior, including sociability and emotion discrimination. However, how they might interact with microglia/inflammatory processes relevant to social behavior in the context of 16p11.2 is still unknown.

Furthermore, while there is growing interest in the interplay between PV+ interneurons and microglia, research on the relationship between SOM+ interneurons and microglial activity is sparse. This is despite emerging post-mortem evidence in humans that both mechanisms are highly relevant to ASD. This gap is particularly striking given the potential importance of microglia in modulating neuronal function and behavior, as well as the critical but underexplored role of SOM+ interneurons in neural circuitry.

Aims and Outline

This study aims to investigate potential mechanisms underlying the neuro-immune mechanisms relevant for ASD by systematically investigating the social-behavioral characteristics of the 16p11.2 deletion mouse model and the response to immune manipulations. This work is divided into a series of interrelated steps, each building upon the previous, to provide a comprehensive framework for studying the interplay between genetic vulnerabilities, immune environmental challenges, and neural development.

In **CHAPTER 2,** I will provide a social-behavioral **characterization of the 16p11.2 adult mice.** This first step focuses on establishing a comprehensive characterization of four different social features of the 16p11.2 deletion mouse model in adulthood. The focus will be on sociability, social memory, recognition of social novelty and emotion discrimination. The characterization will take advantage of two mouse lines of 16p11.2 on two different genetic backgrounds, inbred **(C57BL/6J)** and outbred **(CD1).** By comprehensively assessing multiple aspects of social behavior, this phase will provide information about the social behavioral profile of 16p11.2 deleted mice in distinct genetic backgrounds (to control for possible interaction with genetic factor external to the micro-deleted region).

Building on this, in **CHAPTER 3** I will assess the 16p11.2 vulnerability to an **immunological challenge in adulthood**. I will evaluate the post-acute behavioral response to an immunological challenge, replicating a viral-like infection in 16p11.2 and in their wild-type siblings in a C57BL/6J background. By comparing pre- and post-infection behaviors, this part of the study will assess whether the 16p11.2 deletion confers increased sensitivity to inflammatory challenges. This step provides insights into the interaction between genetic predisposition and immunological stressors, shedding light on how these factors collectively influence behavioral outcomes.

Following the previous findings, I will focus on adolescence. I will assess in **CHAPTER 4** if the 16p11.2 mice in C57BL/6J background display **social behavioral alterations during adolescence**. The aim is to address if a social profile relevant for ASD could be identified early in

the life of 16p11.2 animals, to find early markers of social deficits that could emerge during adolescence and naturally revert in adulthood.

Building on these findings, in **CHAPTER 5** I will assess **16p11.2 vulnerability to a prenatal immunological challenge.** Here I evaluated if maternal immune activation (MIA), induced using Poly I:C, could disrupt the social developmental profile of 16p11.2 adolescent offspring.

Following the findings in adolescence, in **CHAPTER 6** I will explore the interplay between **microglia and neuronal populations relevant for social behavior** in adolescence. This step involves the mapping the neural circuits that could be potentially implicated in behavioral abnormalities in adolescence, with a focus on the role of microglia. By first establishing these mechanisms in the C57BL/6J background, the findings will provide a solid foundation for future applications of this knowledge to the 16p11.2 model.

Finally, in **CHAPTER 7**, I will summarize the **results** of this study and address the **limitations** and the questions that remain still open, indicating the **future directions** of this work.

By systematically addressing each of these steps, this study aims to unravel the complex interplay between genetic vulnerabilities, environmental stressors, and neuro-immune interactions in the 16p11.2 deletion model. This comprehensive approach aims to provide a framework for identifying critical mechanisms underlying neurodevelopmental disorders, giving new perspective for the future development of therapeutic strategies to address their associated social deficits.

CHAPTER 2

Social Behavior in a Mouse Model of 16p11.2 Proximal Deletion

Abstract

The 16p11.2 deletion is a genetic mutation that increases the risk of developing autism in carriers, but presents with highly variable phenotypic outcomes. Limited knowledge exists on how genotype links to phenotype, and while the deleted region is strongly preserved during the evolution, and mice models have been developed to study this mutation, research on social behavior in mice with the 16p11.2 deletion remains inconclusive. Additionally, no studies to date have examined the ability to discriminate emotion in these models. Here, a battery of behavioral social tests was conducted to assess social behavior and emotional discrimination in mice on both inbred (C57BL/6J) and outbred (CD1) genetic backgrounds.

16p11.2 mutant mice did not exhibit alterations in sociability, social novelty, social memory, or the discrimination of positive and negative affective states in conspecifics compared to their wild-type littermates when tested as adults, both in the inbred and in the outbred background. This suggests that the mutation alone is insufficient to produce phenotypic alterations, consistent with observations in human carriers where not all individuals display social behavior deficits. These findings support further research aimed at identifying additional factors that may precipitate genetic vulnerability and lead to the development of phenotypes relevant to autism, potentially informing strategies for better understanding and potentially mitigating autism-related phenotypes.

Introduction

The 16p11.2 deletion locus, strongly related to a genetic vulnerability for ASD [44], provides a valuable framework for translationally studying the mechanisms underlying neurodevelopmental disorders, particularly ASD. In fact, this genomic region is highly conserved across evolution [115], and its syntenic counterpart is present in various species, including mice. Several mice models replicating the human proximal 16p11.2 deletion syndrome have been developed by inducing haploinsufficiency in the syntenic region, which is found on mouse chromosome 7 [116–119]. The first of these models was described in 2011 by Mills and Horev [119], and other models followed with variations in the size of the deleted segment and the genetic background of the mice, as summarized in Table 1.

These models were well characterized and serve as valuable tools for studying the molecular, behavioral, and neuropsychiatric impacts of the 16p11.2 deletion especially in-vivo. In fact, many of the phenotypic characteristics of these mice recapitulate those observed in human carriers of the deletion [116,117,119,120], making them a great opportunity for understanding the underlying mechanisms of neurodevelopmental disorders, as we have recently reviewed [55]. For instance, these mice models show alterations in brain structure and connectivity, especially involving the mPFC, which mirrors those observed in human carriers of the deletion [121,122].

Table 1: Mice models of the 16p11.2 deletion

Model	**Genomic Sequence**	**Background**
Mills [119]	Slx1b-Sept1 (593 kb - 29 genes)	Mixed C57BL/6N and 129S5/SvEvBrd
Dolmetsch [117]	Coro1a-Spn (475 kb - 27 genes)	Mixed 129S5/SvEvBrd × C57BL/6J and CD1
Herault [116]	Sult1a-Spn (1.1 Mb - 38 genes)	C57BL/6N and C57BL/6N × C3B

In the context of behavioral deficit relevant for ASD, behavioral assays such as the three-chamber social interaction test, social preference tests, repetitive behavior and ultrasonic vocalization analysis have been used to assess these behaviors in mice, with mixed outcomes [55]. While

repetitive behaviors, a core feature of ASD, have been solidly observed in all the mouse models [116,117,119,120], communication deficits, such as delayed verbal production in humans, are inconsistently reflected in deficits in ultrasonic vocalizations in mice [118,120,123]. Importantly, the results about social deficits are quite inconsistent across models: no deficits have been observed in sociability [64,117,120,124,125], some deficits in social novelty recognition have been reported in the Dolmetsch model [116,126] (but not consistently in other models [117,124]), and emotion discrimination was never tested. Such heterogeneity could depend on many factors: first, the tests were performed using different paradigms, in some cases using free social interaction, in other cases with the three chamber test, and using mice of different ages [55]. Second, the mice models were tested in different backgrounds, mostly mixed, and the length of the deleted sequence was different in each mouse model (see Table 1). Third, it is possible that different environmental conditions depending on housekeeping and husbandry of mice could have influenced the outcomes [127]. Both the latter factors are particularly important in the context of the so called "second-hit" hypothesis. In fact, the CNV alone could not be sufficient to produce significant alterations in the behavior of these mice, and is likely that a second player (a "second-hit", which can be genetic or environmental) could be needed to disrupt the neurodevelopmental steps to produce the ASD-relevant phenotype. Therefore, all the above-mentioned confounders need to be properly addressed by 1) evaluating the mouse model with a comprehensive social battery, and focusing on specific phases of the mouse life; 2) assessing both an inbred background, with reduced inter-subject variability, and an outbred background, with higher genetic variability; 3) reducing the environmental confounders by using standardized and clean housing conditions. Moreover, for the first time, we have the possibility to study emotion discrimination in rodents, which is a core social ability often impaired in ASD, thanks to a behavioral assay that was only recently developed and validated [103,128].

With this first chapter, I will therefore provide a comprehensive assessment of the social behavior of 16p11.2 deleted mice in C57BL/6J background, and in CD1 background, by specifically assessing sociability and recognition of social novelty both in a free-interaction context and with the three chamber test, and emotion discrimination of positive (relief) and negative (stress) affective states of conspecifics.

Methods

Mice

Adult males and females 16p11.2 deleted mice (Mills' model, all born from breeding in the facility) were used. Their genotype was determined by Polymerase Chain Reaction (PCR) of ear snip tissue. The 16p11.2 deletion was only transmitted through the paternal line, in order to avoid potential confounders due to aberrant maternal care: for this reason, mothers were always C57BL/6J (for the inbred experiment) or CD1 (for the outbred experiment). The mutation was always kept in heterozygosis, given that homozygosis is not compatible with life both in humans and in mice. Animals were housed two to four per cage in a climate-controlled (22 ± 2 C) and specific pathogen-free animal facility, with ad libitum access to food and water throughout, a standard environmental enrichment (material for nest and cardboard house), and with a 12-hour light/dark cycle (7pm/7am schedule). All the animals were kept in a mixed housing condition, with 16p +/- (deleted) mice and their wild type siblings housed together. To avoid litter effects, no more than 2 males and 2 females (one per genotype) were used for behavioral testing. All procedures were approved by the Italian Ministry of Health (permit n. 639/2020-PR, n. 392/2021-PR, n. 15/2025-PR and their following integrations) and strictly adhere to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Routine veterinary care and animals' maintenance was provided by dedicated and trained personnel.

Behavioral assays

Experiments were run during the light phase (between 10am-5pm). All mice were handled on alternate days during the week preceding the first behavioral testing. Experimenters were blind to mouse genotypes during testing. Both male and female mice were used, with an age range between 3 and 5 months. Female mice were visually checked for estrus cycle immediately after the test and no correlation was found between estrus status and performance in the test. The experiments reported in this work were repeated independently two to four times, using mice from at least three different generations. Specific randomization in the organization of the experimental conditions is described in the results and figure legends. All the behavioral experiments described in this work

were conducting always in the following order, unless otherwise specified habituation dishabituation, sociability/social novelty, emotion discrimination relief, emotion discrimination stress.

Social Habituation/Dishabituation

The test, described in [129,130] consisted of five repeated social interaction sessions between the experimental subject and an unfamiliar stimulus mouse followed by the exposure of the experimental subject to a new unfamiliar mouse. Briefly, mice were tested in 2150E Tecniplast cages (35.5 x 23.5 x 19 cm) lightly illuminated (5±1 lux) and video-recorded using a UniBrain Fire-i™ Digital Camera. The video camera was mounted facing the front of the cage to record the session for subsequent scoring of social investigation parameters. All the equipment was kept in a sound-attenuating chamber (TSE Multi Conditioning Systems). Adult mice were individually placed in the testing cage and left to habituate for 1 hour. Single housing manipulation was not carried out to avoid any instauration of home-cage territory and aggressive behaviors. Testing began 5 minutes after the habituation in the apparatus when a stimulus mouse of the same sex and age was introduced into the testing cage for a 1-min interaction. At the end of the 1-min inter-trial, we removed the stimulus animal and placed it in an individual holding cage. We repeated this sequence for four trials with 3-min inter-trial intervals. In a fifth trial (Dishabituation), we introduced a new unfamiliar stimulus mouse in the testing cage. Videos of behaviors were recorded and subsequently scored offline (ANY-maze, Stoelting Co.). We measured the duration of the following behavioral responses performed by the tested mice, considering as social interaction anogenital sniffing (direct contact with the anogenital area), body sniffing (sniffing or snout contact with the flank area), head sniffing (sniffing or snout contact with the head/neck/mouth area) and following (time spent in following the stimulus mouse). The “sociability” score was obtained by summing all time spent in social interaction during the 1 minutes interaction sessions, from session 1 to session 4. The “novelty score” was obtained by this formula ((S1-S4)+(S5-S4))/2 where “S” are the social interaction sessions, to measure the ability of each mouse to distinguish between a familiar and an unfamiliar mouse.

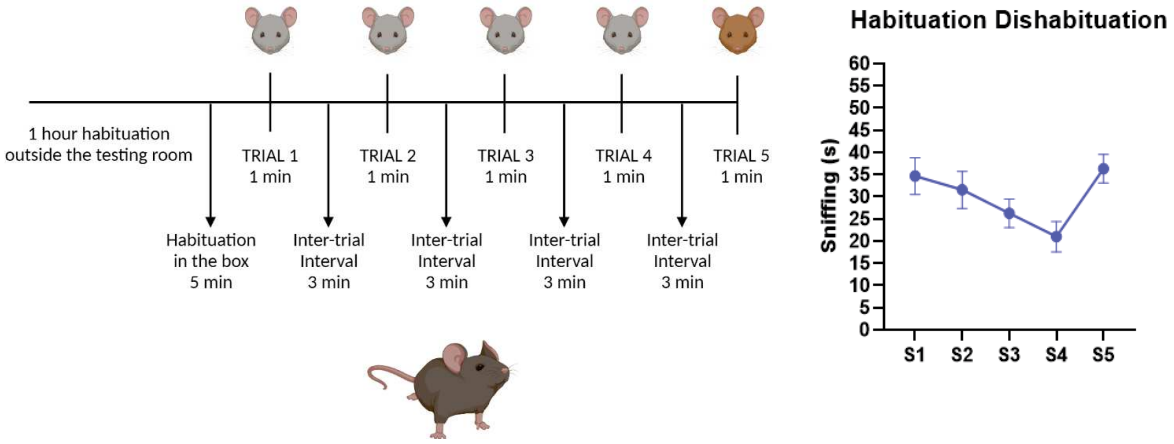

Figure 1: Habituation Dishabituation task.

During this task, the observer mouse can interact with an unfamiliar stimulus for 5 Trials, lasting 1 minute each, in a context of free movement. From Trial 1 to Trial 4, (the Habituation phase) the observer is free to interact with the same unfamiliar stimulus. The observer is expected to lose interest for social interaction across the four trials, as it becomes more familiar with the stimulus with the subsequent trials. A reduction in social sniffing is therefore expected from Trial 1 to Trial 4, which indicates social memory. During Trial 5 (Dishabituation) a new mouse, unfamiliar, is put in the testing cage and the observer mouse is expected to express interest in social novelty by increasing the sniffing for the novel stimulus. The increase in social interaction indicates recognition of social novelty.
A) Experimental protocol, including four 1-minute Trials with a first unfamiliar stimulus (symbolically represented as a grey mouse) and a last 1-minute Trial with a new unfamiliar stimulus (represented as the brown mouse). Each Trial is separated from the others by a 3-minutes lasting inter-trial interval in which the observer is alone in the testing box.
B) Representation of the sniffing behavior of the observer across the Trials during a typical session of the Habituation Dishabituation task. A reduction is expected from Trial 1 to Trial 4 (Habituation) followed by a last increase during Trial 5 when a novel stimulus is presented (Dishabituation).

Emotion Discrimination Test

Emotion discrimination test (EDT) was performed following the protocol described in [103] and [128] and represented in Figure 2. Habituation of the mice (observers and stimuli) to the testing setting occurred on three consecutive days before the first experiment; each habituation session lasted 10 minutes. Test observer mice were habituated inside a 3D printed square apparatus (34 x25x19 cm) containing a separator (150 cm) and a two quarter circle (4cm ray) on two opposite sides. Each quarter-circle is formed with metal rods (0.8cm) that allow sufficient space for social contact between the observers (freely moving in the apparatus) and the demonstrators (freely moving in the quarter-circle). The quarter circles are separated by a 3D-printed separator is located to block the reciprocal view of the stimuli animals while leaving the observer mice free to move between the two sides of the cage. For scoring, a virtual square (11 x 11 cm) is placed between each quarter-circle and the separator to define a zone associated with each stimulus. Demonstrator mice –

matched by age, background and sex to the observers – were habituated inside the same 3D printed cage (34 x25x19 cm), into quarter-circle space three consecutive times, ten minutes each. During both habituation and behavioral testing, the cages were placed inside soundproof cubicles (TSE Multi Conditioning Systems) homogeneously and dimly lit (6 ± 1 lux) to minimize gradients in light, temperature, sound and other environmental conditions that could produce a side preference. Digital cameras (imaging Source DMK 22AUC03 monochrome, Ugo Basile) were placed on top of the cage to record the three consecutive two-minute epochs, using the Anymaze program (Stoelting, Ireland). The experimental cages were replaced after each subject with clean copies to avoid scent carryover. Similarly, the rest of the apparatus was wiped down with water and dried with paper towels for each new subject.

Before the test, mice were habituated to the experimental setting as reported above. One hour prior to behavioral testing, observer mice were placed in a room adjacent to the testing room. Fifteen minutes before the experiment, the observer was put alone in the testing cage for pre-test habituation. Immediately before the test, the demonstrator (stimuli) mice were gently moved into the testing cubicles. The 6-minute experiment began after placing one emotionally 'neutral' and one 'emotionally altered' demonstrator in the EDT apparatus. The order of insertion and the side of insertion of the neutral and the emotionally-altered demonstrators was randomly assigned. Demonstrators were test-naive and used only once. In some cases, we re-used the same demonstrator for maximum two/ three times, with always at least one week between each consecutive test. No differences were observed in the performance of the observer mice depending on the demonstrators' previous experience. Neutral demonstrators did not undergo any kind of manipulation and on the testing day: they were brought inside their home cages in the experimental room one hour before the experiment began. Relief demonstrators were water deprived 23 hours before the experiment. One hour before the test, ad libitum access to water was reestablished, and mice were brought inside the experimental room in their home cages. Food was available ad libitum all the time and some extra pellets were put inside the home cage during the 1-hour water reinsertion. Stress demonstrators were taken inside the experimental room for an hour prior to the beginning of the test. Fifteen minutes before the beginning of the test, mice were put in a restrainer and after removal they were immediately put inside the testing cage. The test lasts 6 minutes in total, but is divided in 3 trials of 2 minutes each, to better assess the dynamics of discrimination, usually occurring during the first two minutes of the test. Behavioral scoring was manually

performed a posteriori from videos by trained experimenters, blind to the manipulations of both the observers and demonstrators. An experimenter was considered trained after an inter-rater reliability with expert experimenters (r score) of 0.954. A sniffing event was defined when the observer touched with the nose the demonstrators' wire cup or when the observer's nose directly touched the demonstrator. The Discrimination Index (DI) was calculated to facilitate the comparison between groups, by dividing the difference between the sniffing for the Stressed or the Relief mouse and the Neutral by the total exploration with the following formula: DI = ((Emotionally altered) – Neutral)/(Emotionally altered + Neutral)).

Sociability / Social Novelty

The test apparatus and procedures were the same as described in the EDT, but in this case the stimuli were not manipulated. A schematic representation of the task is reported in Figure 3. In the first phase of the test, the "Sociability" phase, lasting 6 minutes after the 15 minutes habituation, a neutral Mouse stimulus (unfamiliar for the observer) was put in the apparatus together with an Object stimulus (unfamiliar for the observer). During this "Sociability" phase, the observer could choose to interact with a Salient stimulus (Mouse) or with a Non-salient (Object). After this stage, the Object was removed and substituted with a new neutral Unfamiliar mouse, and the 6-min test of "Social Novelty" started immediately. During this stage, the observer could interact with the Unfamiliar mouse (Salient stimulus) or with the Familiar mouse (Non-salient stimulus). As for the emotion discrimination, each part of the task is divided in 3 trials lasting 2 minutes each.

The DI was calculated to facilitate the comparison between groups, by dividing the difference between the sniffing for the Salient stimulus (Mouse or the Unfamiliar mouse) and the Non-salient stimulus (Object or the Familiar mouse) by the total exploration of stimuli with the following formula: DI = ((Salient – Non-salient)/(Salient + Non-salient)).

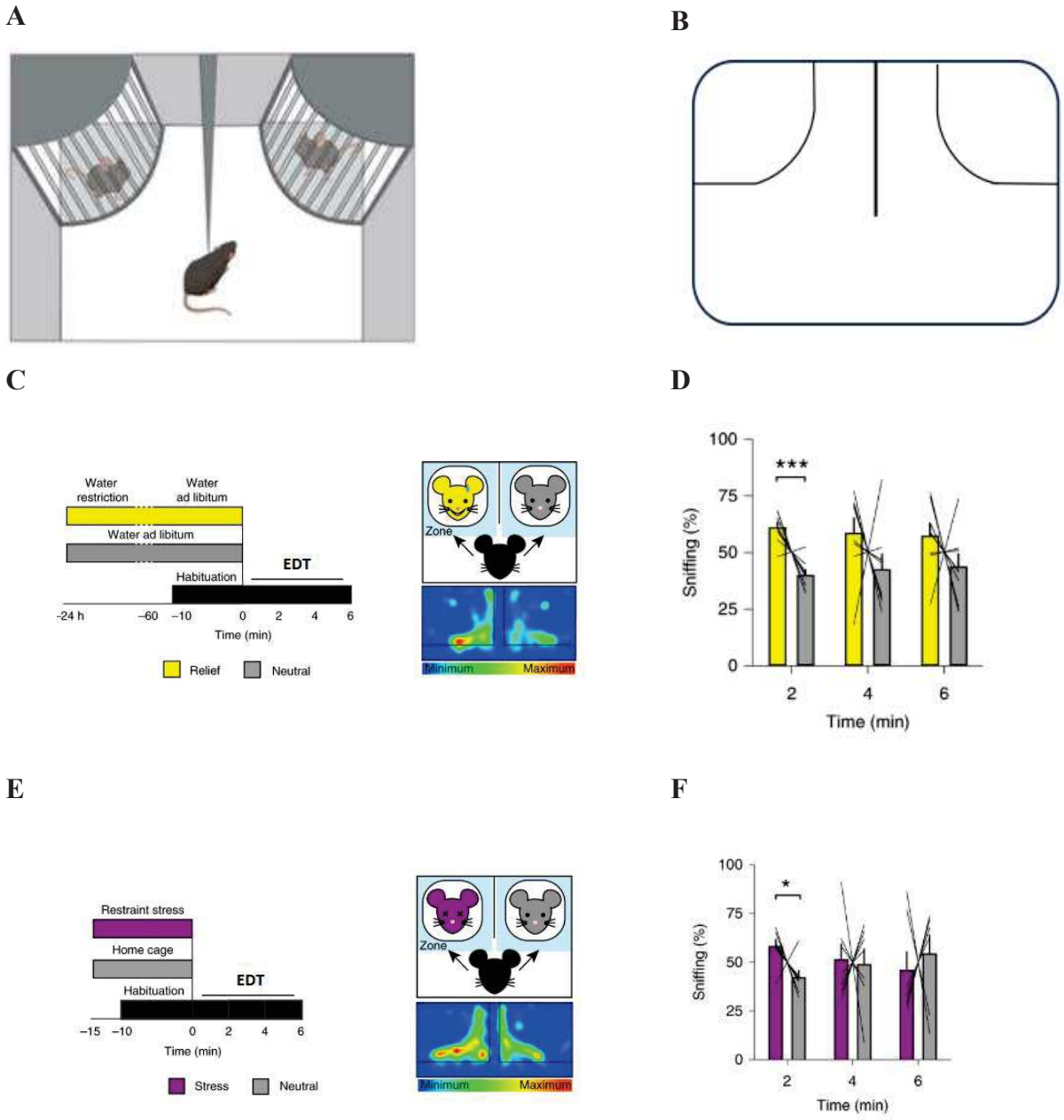

Figure 2: The Emotion Discrimination Task.

A) Schematic representation of a test inside the EDT apparatus.
B) View from above of the EDT apparatus.
C) Protocol for the Emotion Discrimination - Relief.
D) Representation of the results obtained with the validation of the Emotion Discrimination – Relief. Discrimination generally occurs during the first 2 minutes of the experiments, which represent the first Trial.
E) Protocol for the Emotion Discrimination – Stress.
F) Representation of the results obtained with the validation of the Emotion Discrimination – Stress. Discrimination generally occurs during the first 2 minutes of the experiments, which represent the first Trial.
*Figure **C,D,E,F** are Adapted from Scheggia D., Managò F., et al. (2020) Nature Neuroscience, with permission* [103].

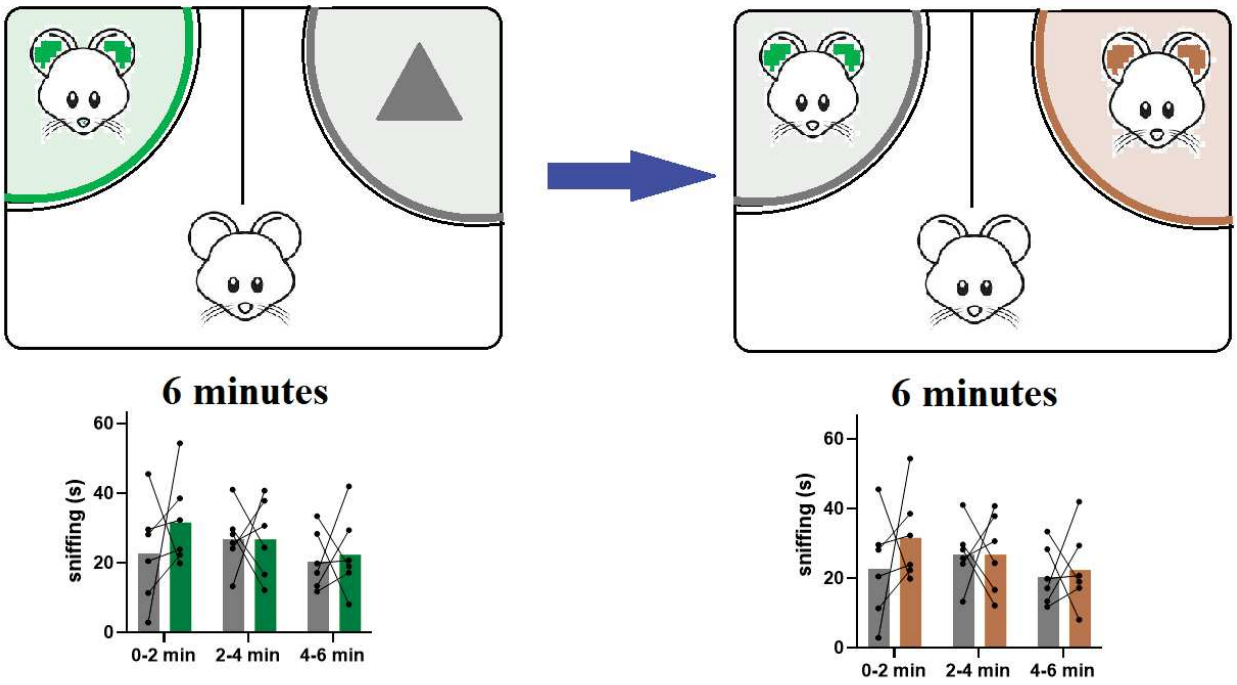

Figure 3: Sociability - Social Novelty Task.

The test is divided in two parts, the Sociability and the Social Novelty stages.
The Sociability phase is represented on the left side, the Social Novelty part on the right side. The triangle represents an Object. The green mouse is the first Mouse presented during the Sociability phase. The brown mouse is the second mouse presented, which is Unfamiliar during the Social Novelty stage.
During the 6 minutes of the Sociability stage, the observer can explore the Mouse or the Object.
In the following 6 minutes of the Social Novelty phase, the mouse that was in the apparatus since the beginning of the Sociability phase becomes the Familiar one, while the Object is replaced with a second (Unfamiliar) mouse.
Each stage (Sociability and Social Novelty) is divided in three Trials lasting 2 minutes each. For each Trial, the interaction time with the stimuli is calculated by manually scoring the time the observer spends sniffing each stimulus.

Statistics

Statistical analyses were performed using R Studio 4.0.3 with a custom-made code (for emotion discrimination, sociability, social novelty) and Graphpad Prism 8 (for habituation dishabituation). Results are expressed as mean ± SEM throughout the manuscript and in all the graphs, and the relevant statistics are reported in the figure legends and not in the main text to facilitate readability. For the analyses of emotion discrimination, each observer behavior towards the two different demonstrator mice was calculated as sniffing time (seconds). Following the normality (Shapiro-Wilk) and homoscedasticity (Levene) tests, parametric or the equivalent non-parametric tests were

used to analyze the data, followed by the appropriate post-hoc and multiple comparison correction. In case of models with more complex factorial design (including the factor "Genotype" and more than one repeated variable, i.e the trials and the emotional/social condition) an RM-ANOVA or a linear mixed effect model (LME) was used, while the f1.ld.f2 test in R was used as non-parametric test from the nparLD R package [131]. For the Total Exploration time, for which there is only one repeated variable (the Trials) a RM-ANOVA or a Friedman test were used to assess specific effects, followed by Dunn or Tukey post-hoc tests to measure between factors effect, or followed by post-hoc Paired t-test or Wilcoxon test to measure within-factors effect. The specific test used and the relevant statistics are reported in the figure legends. For the habituation-dishabituation task, a 2-way ANOVA was performed with Graphpad Prism by including the Genotype as factor and Trials as repeated measures, followed by the relevant post-hoc (Sidak's multiple comparison test). To compare Sociability and the Novelty Index, an independent t-test was performed between the two Genotype levels. The accepted value for significance was $p < 0.05$ after multiple comparison correction: p value is always expressed as adjusted, unless otherwise specified. The sample sizes are reported in the figure legends. All analyses were performed with blinding of the experimental conditions.

Results

16p +/- mice on C57BL/6J background display normal social memory, sociability, and recognition of social novelty.

I first performed the Habituation-Dishabituation test in 16p +/- adult mice, a test designed to allow an exploration of social behavior in the context of free social interaction. I did not observe any significant difference between 16p +/- and wild-type littermates in sociability, social memory and recognition of social novelty. Social interaction from Session 1 to 4 was reduced over time, indicating preserved social memory in both groups (Figure 4 A). 16p+/- and wild-type displayed similar levels of sociability, since total social interaction (sum of the time spent sniffing stimuli from Session 1 to Session 4, Figure 4 B) was not different between groups. Both groups displayed similar levels of interest for social novelty, as observed from time spent interacting with novel mice during Session 1 and 5 (Figure 4A) and similar novelty score (Figure 4 C).

Sociability and social novelty were also assessed with an equivalent of the three-chamber test, the Object vs Mouse test followed by the Familiar vs Unfamiliar test. During the 6 minutes of the Sociability stage, animals could spend time sniffing a non-social unfamiliar stimulus (Object) or an unfamiliar mouse (Mouse). Both the wild-type and the 16p +/- mice significantly preferred the social stimulus, especially during the first 2 minutes of the test (Figure 5 A). There was no effect of the Genotype in the preference for the social stimulus, indicating a similar behavior between the two groups. A significant interaction between Genotype, Trial and the Variable was observed, with 16p +/- preferring the Mouse over the Object during Trial 1 and 2 but not in Trial 3, while wild-type were significantly preferring the Mouse over the Object during Trial 1 and 3 but not in Trial 2 (Figure 5 A). This pattern is made clearer by the Discrimination Index (DI) representation in Figure 5 C, where there was a significant difference in the DI between groups during Trial 2, with a higher DI in 16p +/- compared with wild-type.

Social novelty was assessed immediately after sociability during the same testing session, during the Social Novelty stage, where an Unfamiliar stimulus was put in replacement of the Object, to further assess the behavior of the observer mouse during the following 6 minutes of the test. No

effect of Genotype was observed, with both groups significantly preferring the Unfamiliar mouse especially during the first two minutes of the test (Figure 6 A, C).

The overall exploration of both the stimuli over the Sociability (Figure 5 B) and the Social Novelty stages was not different between the two groups (Figure 6 B) indicating no effect of the Genotype in the exploratory behavior.

16p +/- mice on C57BL/6J background can discriminate affective states of conspecifics.

The EDT was performed to assess the ability of 16p +/- mice to discriminate between a positive affective state (Relief) and a neutral one. Both 16p +/- and wild-type animals were able to discriminate the positive emotion, as evident by the main effect of the Variable and by the DI (Figure 7 C). There was a clear sniffing preference for the Relief mouse, which was significant during the first two minutes of the test, although in the 16p +/- group such significance was lost at multiple comparisons. No differences were observed between the two groups, as there was not main effect of Genotype or significant interactions (Figure 7 A).

Both groups were able to significantly discriminate the Stress mouse in the EDT for negative affective states, in particular during the first 2 minutes of the test, as indicated by the DI (Figure 8 C). No differences were observed between the two groups, as there was no main effect of Genotype or interaction of Genotype with other factors (Figure 8 A). Finally, 16p +/- and their wild type littermates did not display significant differences in the total time spent exploring the stimuli during the three trials (for Relief + Neutral see Figure 7 B, for Stress + Neutral see Figure 8 B) indicating that the exploration of the stimuli was similar between groups.

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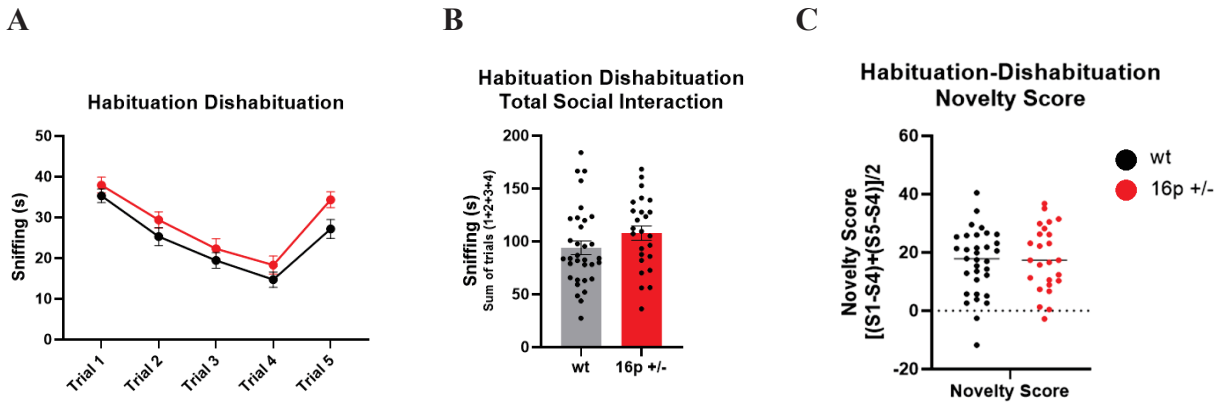


Figure 4: Habituation Dishabituation task in wild-type and 16p11.2 adult littermate mice with a C57BL/6J background.

N(wt) = 33 (15 males, 18 females). N(16p +/-) = 25 (14 males, 11 females).

A) Habituation Dishabituation. Social interaction for each Trial.

A two-way repeated measures ANOVA was conducted (Genotype and Trial as factors). The main effect of Genotype was not significant ($F_{(1, 56)} = 3.200$, $p = 0.0790$). The interaction between Trial and Genotype was not statistically significant ($F_{(4, 224)} = 0.7644$, $p = 0.5494$). There was a main effect of Trial ($F_{(3.096, 173.4)} = 57.72$, $p < 0.0001$), indicating a significant reduction of sniffing across trials (indicating social memory).

Pairwise comparisons using Sidak's multiple comparisons test were performed in wild-type and in 16p +/-.

In the wild-type group the sniffing behavior significantly decreased from Trial 1 to Trial 4 (Mean Difference = -20.58, $p < 0.0001$) indicating normal social memory. A significant increase of the sniffing time between Trial 4 and Trial 5 (Mean Difference = 12.45, $p < 0.0001$) indicates a recognition of social novelty.

A similar pattern was observed in the 16p +/-, with a significant decrease of sniffing from Trial 1 to Trial 4 (Mean Difference = -19.66, $p < 0.0001$) and a significant increase between Trial 4 and Trial 5 (Mean Difference = 16.07, $p < 0.0001$).

B) Total Social Interaction (Trial 1- Trial 4)

The unpaired t-test comparing total social interaction between wild-type and 16p +/- revealed no significant difference between the two groups ($p = 0.1528$, $t_{(56)} = 1.450$).

C) Novelty Score

The unpaired t-test comparing the total novelty score between wild-type and 16p +/- revealed no significant difference between the two groups ($p = 0.7266$, $t_{(56)} = 0.3514$). The Novelty Score is calculated as the average difference between Trial 1 and Trial 4 (S1-S4) and Trial 5 and Trial 4 (S5-S4).

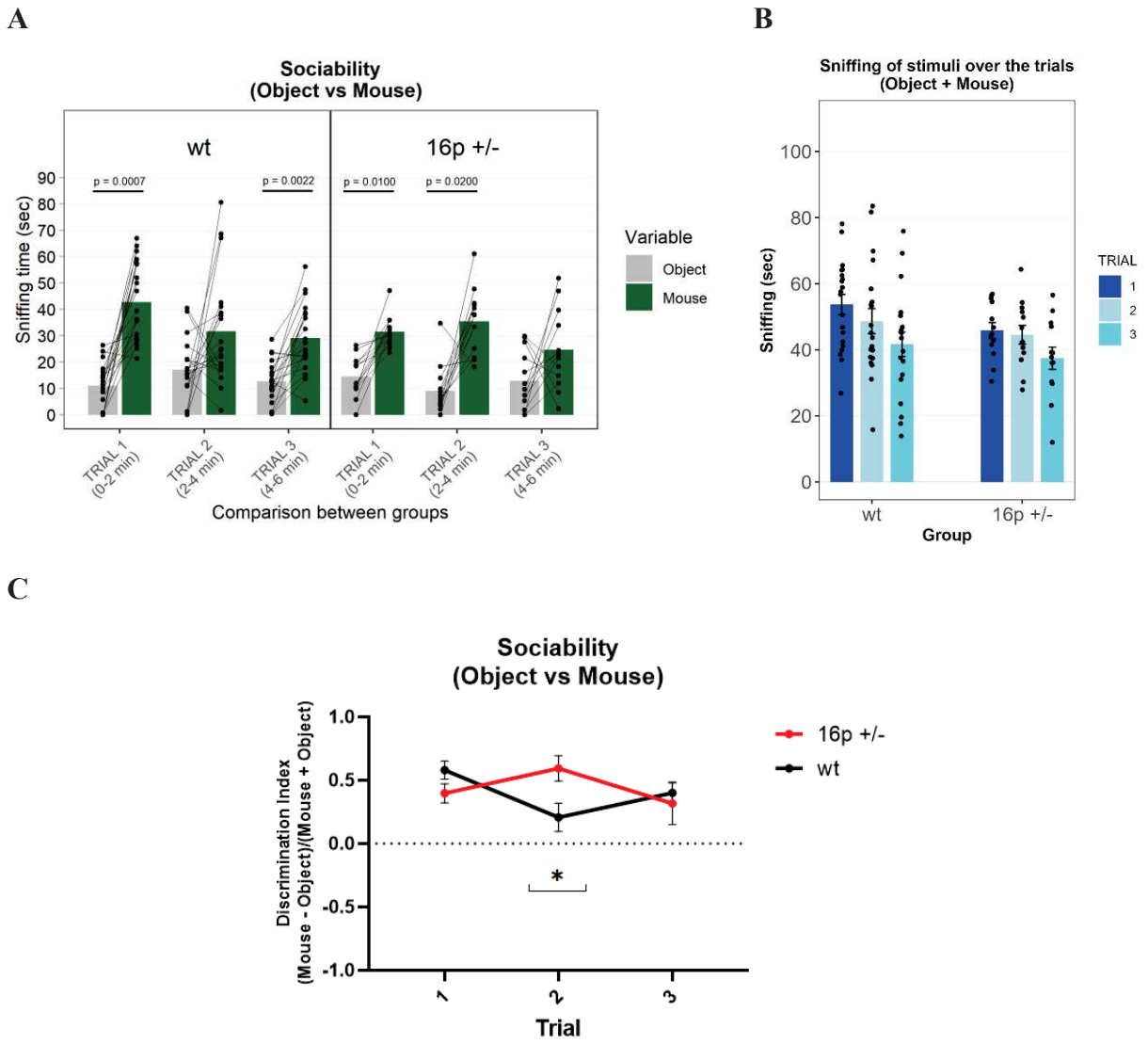


Figure 5: Sociability in wild-type and 16p11.2 adult littermate mice with a C57BL/6J background (Object vs Mouse).

N(wt) = 19 (10 males, 9 females). N(16p +/-) = 12 (8 males, 4 females).

A) Sociability Test. Comparison between wild-type and 16p +/-

The graph represents the time spent sniffing the Object or the Mouse during the 6 minutes of the test, divided in 3 Trials of 2 minutes each. Statistics were calculated using a non-parametric test (f1.ld.f2) which relies on a non-parametric ANOVA. The test included Genotype as a factor (2 levels), and Trial and Variable (Object or Mouse) as repeated measures (3 and 2 levels respectively). The test was followed by post-hoc analysis with Dunn's test adjusted with Bonferroni's method.

Genotype showed no significant effect ($F = 1.134$, $df = 1$, $p = 0.2868$). Similarly, the interaction between Genotype and Trial was not significant ($F = 0.287$, $df = 1.929$, $p = 0.7424$). The Genotype by Variable interaction ($F = 0.020$, $df = 1$, $p = 0.8872$) was also non-significant: Genotype did not differentially affect responses to the two variables (Object and Mouse). There was a **3-way interaction between Genotype, Trial, and Variable** ($F = 5.052$, $df = 1.743$, $p = 0.0090$).

There was a main effect of Trial ($F = 6.689$, $df = 1.929$, $p = 0.0014$), indicating that responses varied significantly across trials, independently from Genotype. Variable (Object vs. Mouse) had a significant effect ($F = 84.062$, $df = 1$, $p < 0.0001$), suggesting clear differences in the sniffing the social and non-social stimulus independent from Genotype. The interaction between Variable and Trial was not significant ($F = 2.404$, $df = 1.743$, $p = 0.0979$).

Post hoc analyses (Dunn test with Bonferroni correction) were conducted to assess pairwise differences, particularly focusing on the significant main effect of Variable (Object vs Mouse).
In the wild-type group, significant differences were found in Trial 1 ($p = 0.0007$) and Trial 3 ($p = 0.0022$), while Trial 2 showed a trend but did not reach significance after Bonferroni adjustment ($p = 0.3563$).
In the 16p +/- group, significant differences between Object and Mouse were observed in Trial 1 ($p = 0.0100$) and Trial 2 ($p = 0.0200$), but not in Trial 3 ($p = 0.5610$).
To further explore the significant three-way interaction between Genotype, Trial, and Variable, the Dunn test with Bonferroni correction is applied also to compare the sniffing time for each Variable and Trial between the Genotypes.
For the Mouse, a significant difference between 16p +/- and wild-type is observed in Trial 1 ($p = 0.0427$), with higher sniffing times in the wild-type group. No significant differences are found in Trial 2 and Trial 3 ($p = 0.2103$ and $p = 0.3198$, respectively).
For the Object, no significant difference between 16p +/- and wild-type is observed in Trial 1 ($p = 0.2767$) and Trial 3 ($p = 0.7965$). However, a significant difference is found in Trial 2 ($p = 0.0175$), with higher sniffing time in the wild-type group.
Wild-type: Trial 1 (Object = 11.05 ± 1.82; Mouse = 42.64 ± 3.30); Trial 2 (Object = 17.05 ± 2.46; Mouse = 31.58 ± 4.60); Trial 3 (Object = 12.66 ± 1.76; Mouse = 28.99 ± 2.86).
16p +/-: Trial 1 (Object = 14.42 ± 2.09; Mouse = 31.43 ± 1.65); Trial 2 (Object = 9.06 ± 2.53; Mouse = 35.45 ± 3.34); Trial 3 (Object = 12.87 ± 3.04; Mouse = 24.58 ± 4.10).
For each group, results are reported as Mean ± SEM.

B) Total exploration of stimuli.

The analysis of the total time spent sniffing both the stimuli (Object + Mouse) with a 2-way RM ANOVA revealed a significant main effect of Trial ($F = 15.893$, $p = 0.0004$) but the main effect of Genotype was not significant ($F = 2.118$, $p = 0.1560$). The interaction between Genotype and Trial was also not significant ($F = 0.448$, $p = 0.5085$).

C) Discrimination Index (DI)

The DI was calculated in order to better represent the preference for the social stimulus across Trials and to facilitate the visual comparison between groups. A positive DI indicates preference for the Mouse. A two-way ANOVA was performed to compare the DI between groups, including Genotype as factor and Trial as repeated measure. **There was a significant interaction between Trial and Genotype** ($F_{(2, 62)} = 4.472$, $p = 0.0153$). The main effect of Trial was not significant ($F_{(1.857, 57.57)} = 0.8449$, $p = 0.4273$), nor was the main effect of Genotype ($F_{(1, 31)} = 0.2002$, $p = 0.6577$). Sidak's multiple comparisons test indicated that the difference between 16p +/- and wild-type was not significant in Trial 1 ($t_{(28.66)} = 1.758$, $p = 0.2448$), and in Trial 3 ($t_{(17.58)} = 0.4445$, $p = 0.9614$). However, in the Trial 2 the difference was significant ($t_{(30.57)} = 2.578$, $p = 0.0443$).

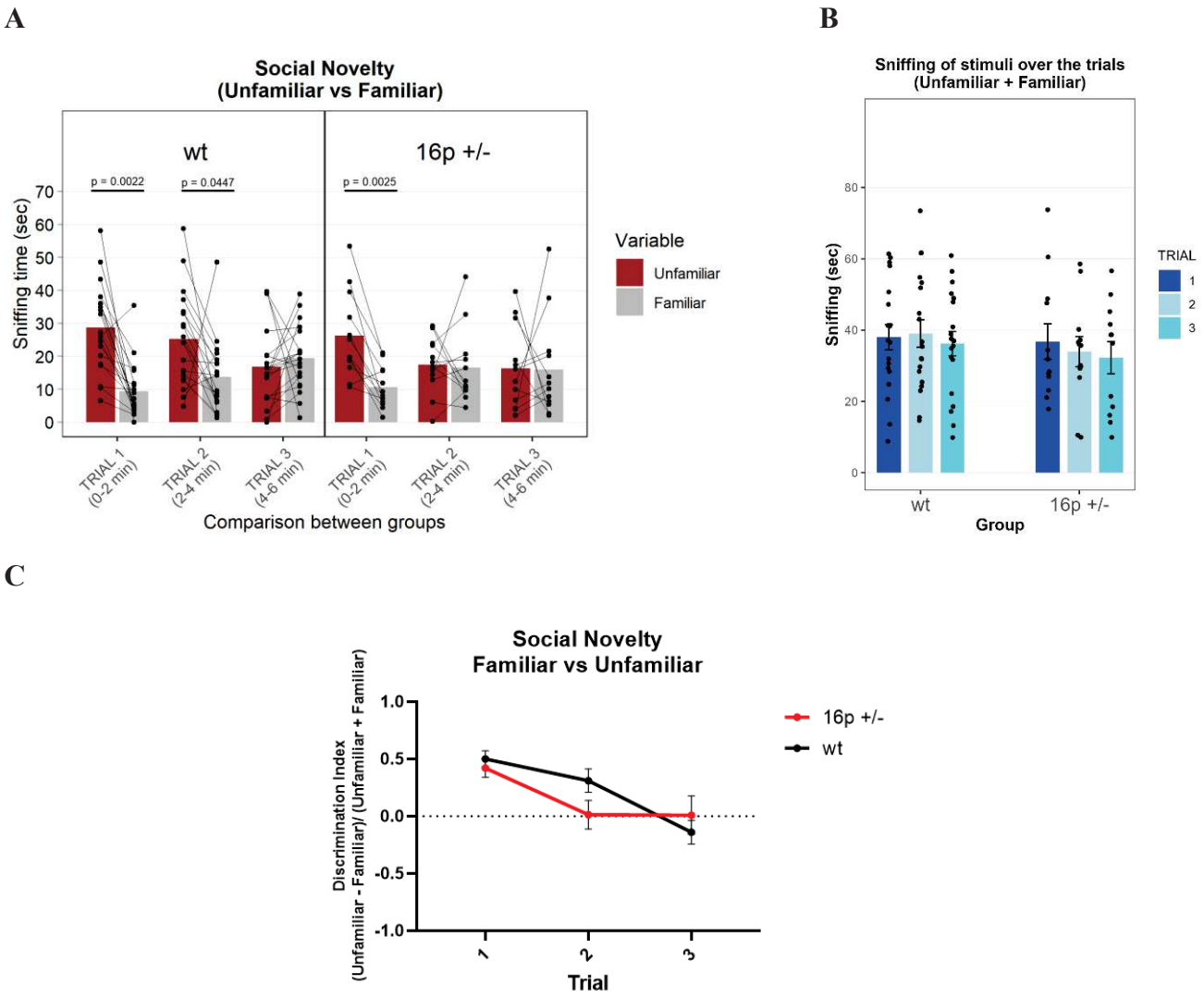


Figure 6: Social Novelty in wild-type and 16p11.2 adult littermate mice with a C57BL/6J background (Familiar vs Unfamiliar)

N(wt) = 19 (10 males, 9 females). N(16p +/-) = 12 (8 males, 4 females).

A) Social Novelty Test. Comparison between wild-type and 16p +/-

The graph represents the time spent sniffing the Familiar or the Unfamiliar mouse during the 6 minutes of the test, which was divided in 3 trials of 2 minutes each. Statistics were calculated using a non-parametric test (f1.ld.f2) which relies on a non-parametric ANOVA. The test included Genotype as a factor (2 levels), and Trial and Variable (Familiar or Unfamiliar) as repeated measures (3 and 2 levels respectively).

The main effect of Genotype was not significant ($F = 0.355$, $p = 0.5511$), nor the Genotype-related interactions, including Genotype by Trial ($F = 1.162$, $p = 0.3116$), Genotype by Variable ($F = 0.412$, $p = 0.5211$), and the three-way interaction between Genotype, Trial, and Variable ($F = 1.413$, $p = 0.2434$).

There was a significant main effect of the Variable ($F = 18.428$, $p < 0.0001$). The main effect of Trial was not significant ($F = 0.614$, $p = 0.5359$), suggesting no overall changes in responses across trials. The interaction between Variable and Trial was significant ($F = 10.748$, $p < 0.0001$), suggesting that the differences between responses to Familiar and Unfamiliar stimuli varied across trials.

Post hoc tests (Dunn test with Bonferroni correction) were conducted to further explore these effects within each Trial and Genotype. In the wild-type group, there was a significant preference for the Unfamiliar stimulus in Trial 1 ($p = 0.0022$) and in Trial 2 ($p = 0.0447$) indicating a clear preference for social novelty. In Trial 3, however, no significant difference was observed $p = 0.8512$). In the 16p +/- group, a significant difference was found in Trial 1, where sniffing

of the Unfamiliar stimuli was significantly higher than the Familiar stimuli ($p = 0.0025$). No significant differences were observed in Trial 2 ($p > 0.9999$) or Trial 3 ($p > 0.9999$).

B) Total exploration of stimuli.

The analysis of the total time spent sniffing both the stimuli (Familiar + Unfamiliar) with a 2-way RM ANOVA did not reveal any significant main effects or interactions. The main effect of Genotype was not significant ($F = 0.47$, $p = 0.4990$), as well as the one of the Trial ($F = 1.079$, $p = 0.3080$) and the interaction between Genotype and Trial ($F = 0.233$, $p = 0.6330$).

C) Discrimination Index (DI)

The DI was calculated in order to better represent the preference for the novel stimulus across Trials and to facilitate the visual comparison between groups. A positive DI indicates preference for the Unfamiliar mouse. At the 2-way ANOVA, no significant effect of Genotype is detected ($F_{(1, 29)} = 0.7322$, $p = 0.3992$) and no interaction is observed between Trial and Genotype ($F_{(2, 58)} = 2.034$, $p = 0.1401$). There was a significant main effect of Trial ($F_{(1.712, 49.66)} = 11.40$, $p = 0.0002$).

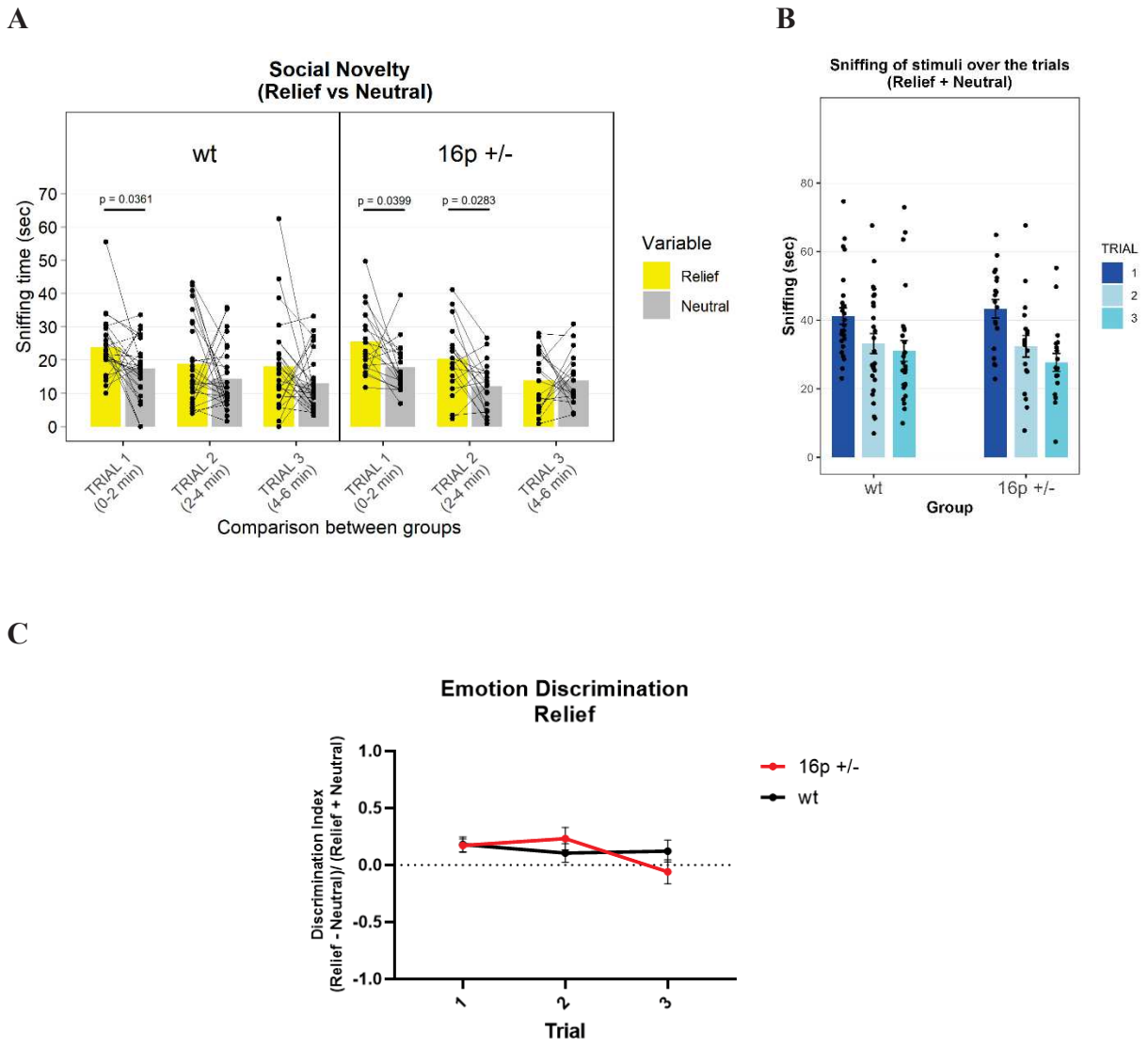


Figure 7: Emotion Discrimination (Relief) in wild-type and 16p11.2 adult littermate mice with a C57BL/6J background.

N(wt) = 27 (13 males, 14 females). N(16p +/-) = 19 (13 males, 5 females).

A) Emotion Discrimination Task.

The graph represents the time spent sniffing the Relief or the Neutral stimuli during the 6 minutes of the test, which was divided in 3 trials of 2 minutes each. Statistics were calculated using a non-parametric test (f1.ld.f2) using a non-parametric ANOVA. The test included Genotype as a factor (2 levels), and Trial and Variable (Relief or Neutral) as repeated measures (3 and 2 levels respectively). The main effect of Genotype was not significant ($F = 0.014$, $p = 0.9062$). Furthermore, no significant interactions were observed between Genotype and Trial ($F = 0.325$, $p = 0.7095$), or between Genotype and Variable ($F = 0.019$, $p = 0.8892$). The three-way interaction between Genotype, Trial, and Variable was also not significant ($F = 1.473$, $p = 0.2300$). There was a main effect of Trial ($F = 33.248$, $p < 0.0001$), and of Variable ($F = 11.935$, $p = 0.0006$) but no significant interactions between Variable and Trial ($F = 1.611$, $p = 0.2013$).

The Dunn post hoc analysis with Bonferroni correction was then performed to further explore the main effect of the Variable. For the wild-type group, in Trial 1, a significant difference is observed between the time spent sniffing the Relief and the Neutral($p = 0.0362$) but not in Trial 2 ($p = 0.4382$); and in Trial 3 ($p = 0.5240$).

In the 16p +/- group, a significant difference is observed in Trial 1 ($p = 0.0399$) and in Trial 2 ($p = 0.0283$) while in Trial 3, no significant difference is found ($p = 0.9839$).

B) Total exploration of the stimuli

The Kruskal-Wallis test was performed to assess the group differences for the total time spent exploring the stimuli, but no significant effects were observed ($H = 0.006$, $p = 0.9362$).

C) Discrimination Index (DI)

The DI was calculated with the following formula DI = ((Relief – Neutral)/(Relief + Neutral)) was calculated in order to better represent the preference for the relief across Trials and to facilitate the visual comparison between groups. A positive DI indicates preference for the Relief demonstrator. The Kruskall-Wallis test was performed to compare DI between wild-type and 16p +/- and no differences were observed between groups ($H = 0.0903$; $p = 0.7638$).

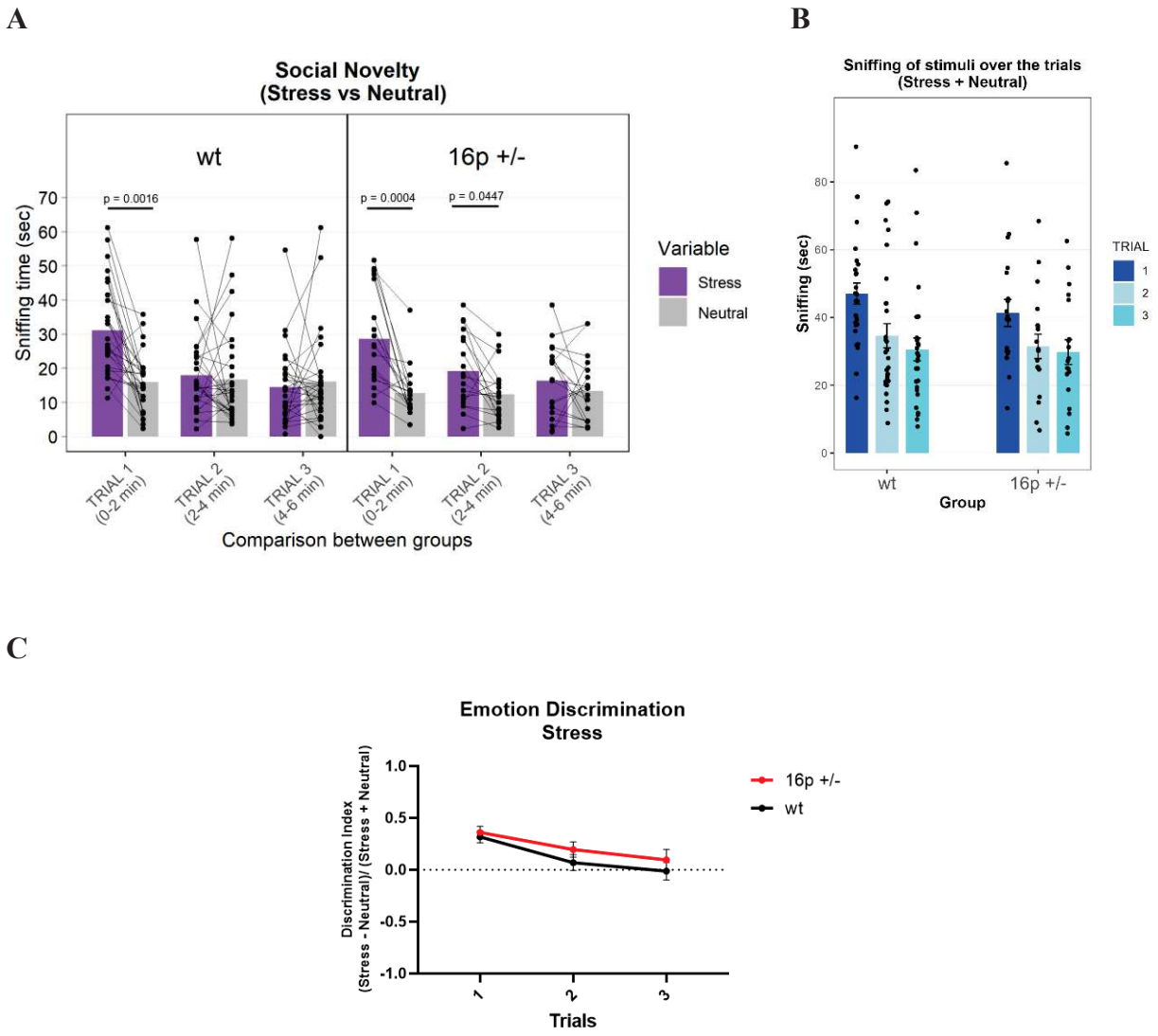


Figure 8: Emotion Discrimination (Stress) in wild-type and 16p11.2 adult littermate mice with a C57BL/6J background.

N(wt) = 28 (14 males, 14 females). N(16p +/-) = 19 (13 males, 6 females).

A) Emotion Discrimination Task.

The graph represents the time spent sniffing the Stress or the Neutral stimuli during the 6 minutes of the test, which was divided in 3 trials lasting 2 minutes each. Statistics were calculated using a non-parametric test (f1.ld.f2) using a non-parametric ANOVA with Genotype as a factor (2 levels), and Trial and Variable (Stress or Neutral) as repeated measures (3 and 2 levels respectively). The main effect of Genotype was not significant ($F = 0.311$, $df = 1$, $p = 0.5771$), as well as the interactions between Genotype and Trial ($F = 1.453$, $p = 0.2341$), Genotype and Variable ($F = 2.358$, $p = 0.1246$), and the three-way interaction between Genotype, Trial, and Variable ($F = 0.086$, $p = 0.9100$). The main effect of Variable was significant ($F = 28.346$, $p < 0.0001$), showing that there was a clear preference for the altered demonstrator. The main effect of Trial was significant ($F = 17.015$, $p < 0.0001$), indicating that responses varied significantly across Trials. The interaction between Variable and Trial was also significant ($F = 9.917$, $p < 0.0001$). At the Dunn post hoc analysis, corrected with the Bonferroni method, a significant difference between the time spent sniffing the Stress and the Neutral demonstrator was observed in the wild-type group in Trial 1 with a preference for the Stress demonstrator ($p = 0.0004$). In Trial 2 and in Trial 3 no significant difference was found ($p > 0.9999$ in both Trials). In the 16p +/- group, there was a significant preference for the Stress stimulus in Trial 1 ($p = 0.0016$) and in Trial 2 ($p = 0.0447$) but not in Trial 3 ($p > 0.9999$).

B) Total exploration of the stimuli

The Kruskal-Wallis test was performed to assess the group differences for the total time spent exploring the stimuli, but no significant effects were observed ($H= 0.5846$, $p = 0.4445$).

C) Discrimination Index (DI)

DI = ((Stress – Neutral)/(Stress + Neutral)). The DI was calculated in order to better represent the preference for the Stress across Trials and to facilitate the visual comparison between groups. A positive DI indicates preference for the Stress demonstrator. No significant interaction between Trial and Genotype is observed ($F_{(2, 90)} = 0.1711$, $p = 0.8430$). A significant main effect of Trial is found ($F_{(1.981, 89.16)} = 8.489$, $p = 0.0004$), while the main effect of Genotype is not significant ($F_{(1, 45)} = 1.780$, $p = 0.1889$).

16p +/- mice on CD1 background display normal sociability, social memory and recognition of social novelty.

We then compared if 16p +/- mice could display more clear social deficits in the context of an outbred background (CD1), generally characterized by higher genetic variability and a wider repertoire of social behaviors. We did not observe any significant difference between 16p +/- and wild-type in any of the social domains assessed during the habituation and dishabituation test in 16p +/- and wild-type mice on CD1 background (Figure 9 A). 16p+/- and wild-type displayed similar levels of sociability, described by comparable total social interaction time measured from trial 1 to trial 4 (Figure 9 B). Both groups displayed similar levels of social memory, as social interaction was significantly reducing over the trials, being significantly different from Trial 1 to Trial 4 (Figure 9 A). The recognition for social novelty was preserved as well in both groups, as evident from the time spent interacting with novel mice at Trials 1 and 5 (Figure 9 A) and with the Novelty Score, was not different between groups (Figure 9 C).

Discrimination of affective state is not clear in CD1 background.

I then investigated if in the context of an outbred background, such as CD1, 16p +/- mice displayed abnormalities in the ability to discriminate emotions compared with the wild-type siblings. In the EDT for positive affective states (Relief) both the wild-type and the 16p +/- were not able to discriminate the altered demonstrator from the neutral one, in none of the trials (Figure 10 A and Figure 10 B). Regarding the negative affective states (Stress), independently from the Genotype, discrimination was not occurring during the test overall, but only during specific trials, as indicated by the significant effect of the interaction between Trial and the Variable (Figure 11 A). However, no significant difference between the sniffing of the Stressed and the Neutral demonstrator was found at the post-hoc tests, both in wild types and in 16p +/-. Moreover, there was no main effect of Genotype or any interaction of the Genotype with other factors, indicating that there is no effect of the 16p +/- genotype in influencing the ability to discriminate emotions. The total time spent exploring both the stimuli was not different between groups in the EDT for negative affective states (Figure 11 B), but was significantly higher in the 16p +/- mice compared with wild-type

only during the first two minutes of the EDT for positive affective states (Figure 10 C). The DI further confirms no difference between groups in the ability to discriminate positive or negative affective states, despite a trend in the EDT for positive emotions towards higher ability in the 16p +/- groups during Trial 1, which was not confirmed by the post-hoc tests (Figure 10 D).

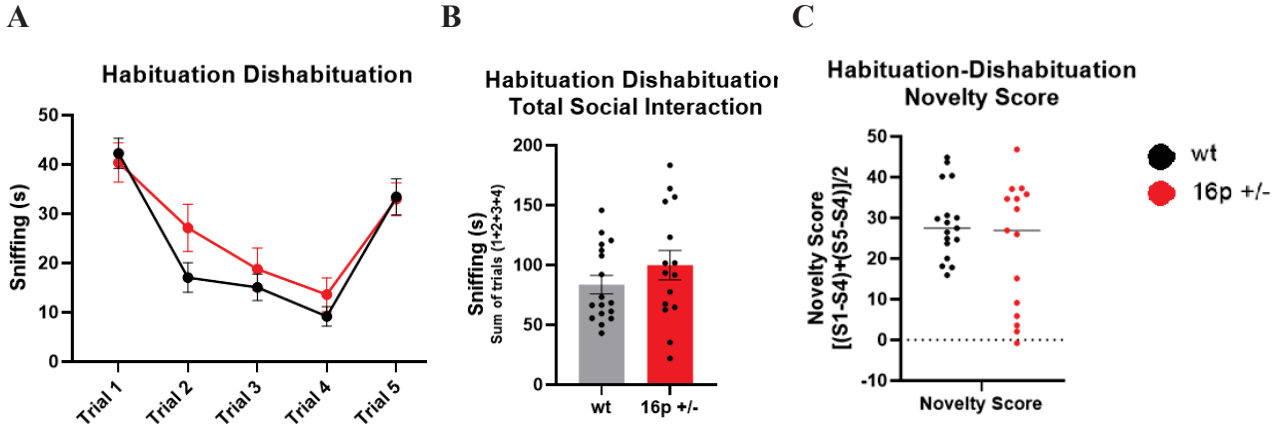


Figure 9: Habituation Dishabituation task in wild-type and 16p11.2 adult littermate mice with a CD1 background.

N (wt) = 17 (8 males, 9 females); N(16p +/-): 15 (8 males, 7 females).

A) Habituation Dishabituation. Social interaction for each Trial.

A two-way repeated measures ANOVA including Trial and Genotype as factors was performed. The main effect of Genotype was not significant ($F = 0.832$, $df = 1, 30$, $p = 0.3690$), as well as the interaction between Trial and Genotype was not significant ($F = 1.494$, $df = 4, 120$, $p = 0.208$). The main effect of Trial was highly significant ($F = 40.35$, $df = 3.277, 98.32$, $p < 0.0001$).

The post hoc Sidak's multiple comparisons indicated that in the wild-type group, responses in Trial 1 were significantly higher compared to Trial 2 ($t = 7.91$, $p < 0.0001$), Trial 3 ($t = 6.21$, $p = 0.0001$), and Trial 4 ($t = 10.10$, $p < 0.0001$) indicating normal social memory. The difference between Trial 1 and Trial 5 was not significant ($t = 1.65$, $p = 0.7138$) while Trial 4 showed significantly lower responses compared to Trial 5 ($t = 6.71$, $p < 0.0001$) indicating normal social novelty recognition.

Similarly, in the 16p +/- group, responses in Trial 1 were significantly higher compared to Trial 3 ($t = 4.19$, $p = 0.0091$) and Trial 4 ($t = 5.54$, $p = 0.0007$), but the difference between Trial 1 and Trial 2 did not reach significance ($t = 3.18$, $p = 0.0652$). The comparison between Trial 1 and Trial 5 did not reach significance ($t = 2.30$, $p = 0.3191$) and Trial 4 responses were also significantly lower than those in Trial 5 ($t = 5.07$, $p = 0.0017$) indicating normal recognition of social novelty.

B) Total sociability

Sociability was measured as the sum of the sniffing time from Trial 1 to Trial 4, was not different between groups, as compared with the unpaired t-test ($t = 1.143$, $df = 30$, $p = 0.2620$).

C) Novelty Score

The unpaired t-test comparing Total Novelty Scores between the wild-type and 16p +/- groups revealed no significant difference ($t = 1.253$, $df = 30$, $p = 0.2200$).

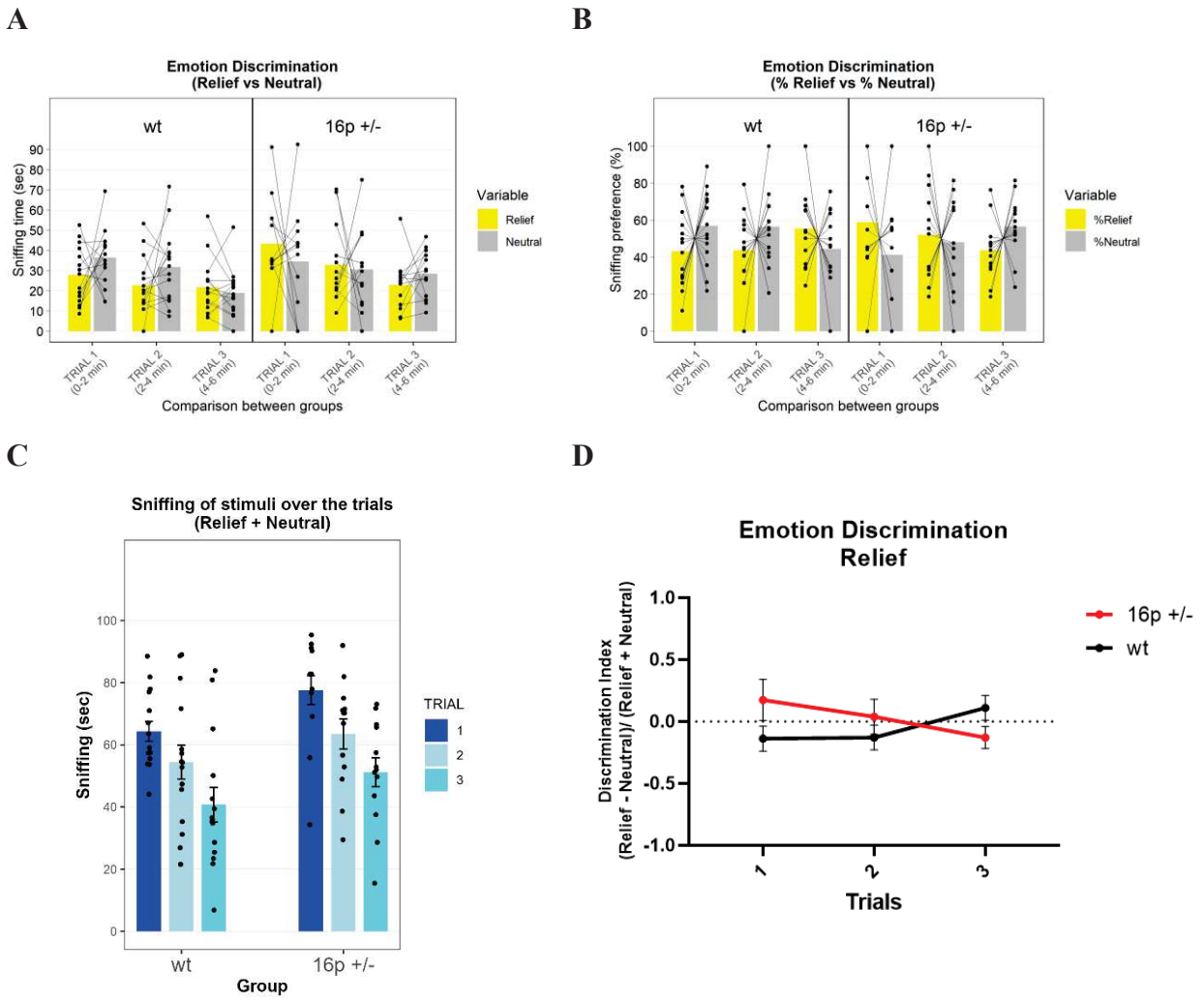


Figure 10: Emotion Discrimination (Relief) in wild-type and 16p11.2 adult littermate mice with a CD1 background.

N (wt) = 15 (6 males, 9 females); N(16p +/-): 13 (6 males, 7 females).

A) <u>Emotion Discrimination Task.</u>

The graph represents the time spent sniffing the Relief or the Neutral stimuli during the 6 minutes of the test, which was divided in 3 trials lasting 2 minutes each. Statistics were calculated using a non-parametric test (f1.ld.f2), a non-parametric ANOVA with Genotype as a factor (2 levels), and Trial and Variable (Relief or Neutral) as repeated measures (3 and 2 levels respectively). There was a significant main effect of Trials ($F = 21.774$, $df = 1.584$, $p < 0.0001$), but no significant effects for Genotype ($F = 2.612$, $df = 1$, $p = 0.106$), Variable ($F = 0.608$, $df = 1$, $p = 0.436$), or of their interactions including the Genotype by Trial interaction ($F = 0.372$, $df = 1.584$, $p = 0.640$), the Variable by Trial interaction ($F = 0.095$, $df = 1.893$, $p = 0.900$), the Genotype by Variable interaction ($F = 1.076$, $df = 1$, $p = 0.300$), and the three-way interaction between Genotype, Trial, and Variable ($F = 2.125$, $df = 1.893$, $p = 0.122$).

B) <u>Emotion Discrimination Task. Preference expressed in percentage.</u>

This graph represents the percentage of preference for the Relief or the Neutral stimuli during the three 2-minute trials of the EDT Relief test. Preference was calculated, for each trial, with the following formula: %Relief = (Time sniffing Relief stimulus during the trial)/(Total sniffing of the stimuli during the trial); %Neutral = (Time sniffing Neutral stimulus during the trial)/(Total sniffing of the stimuli during the trial). Given that the total exploration time in the 16p +/- group was significantly higher than in the wild-type group, the percentage allows to evaluate preference while controlling for the differences in exploratory behavior. The Kruskall-Wallis test found no significant differences between the groups ($H_{(1)} = 0.3193$, $p = 0.5720$).

C) Total exploration of the stimuli.

The Kruskal-Wallis test for the total exploration time revealed a significant effect ($H = 5.375$, $df = 1$, $p = 0.020$). The post hoc Dunn's test with Bonferroni correction comparing the total exploration time between 16p +/- and wild-type groups revealed a significant difference in Trial 1 but not in Trials 2 and 3. In Trial 1, the 16p +/- group showed significantly higher total exploration compared to the wild-type group ($z = 2.511$, $p = 0.012$, adjusted $p = 0.012$). In Trial 2 ($z = 1.175$, $p = 0.240$, adjusted $p = 0.240$) and in Trial 3 ($z = 1.820$, $p = 0.069$, adjusted $p = 0.069$) no significant difference was observed between the two groups.

D) Discrimination Index.

The DI was calculated in order to better represent the preference for the Relief across Trials and to facilitate the visual comparison between groups. A positive DI indicates preference for the Relief demonstrator. DI = ((Relief – Neutral)/(Relief + Neutral)). A significant interaction between Trial and Genotype is observed ($F_{(2, 52)} = 3.398$, $p = 0.0410$). However, no significant main effects are found for Trial ($F_{(1.890, 49.13)} = 0.1652$, $p = 0.8365$) or Genotype ($F_{(1, 26)} = 0.5651$, $p = 0.4590$). The post-hoc analysis with Sidak's multiple comparisons revealed no differences between groups in all the trials: Trial 1 ($p = 0.3261$), Trial 2 ($p = 0.7123$), Trial 3 ($p = 0.2339$).

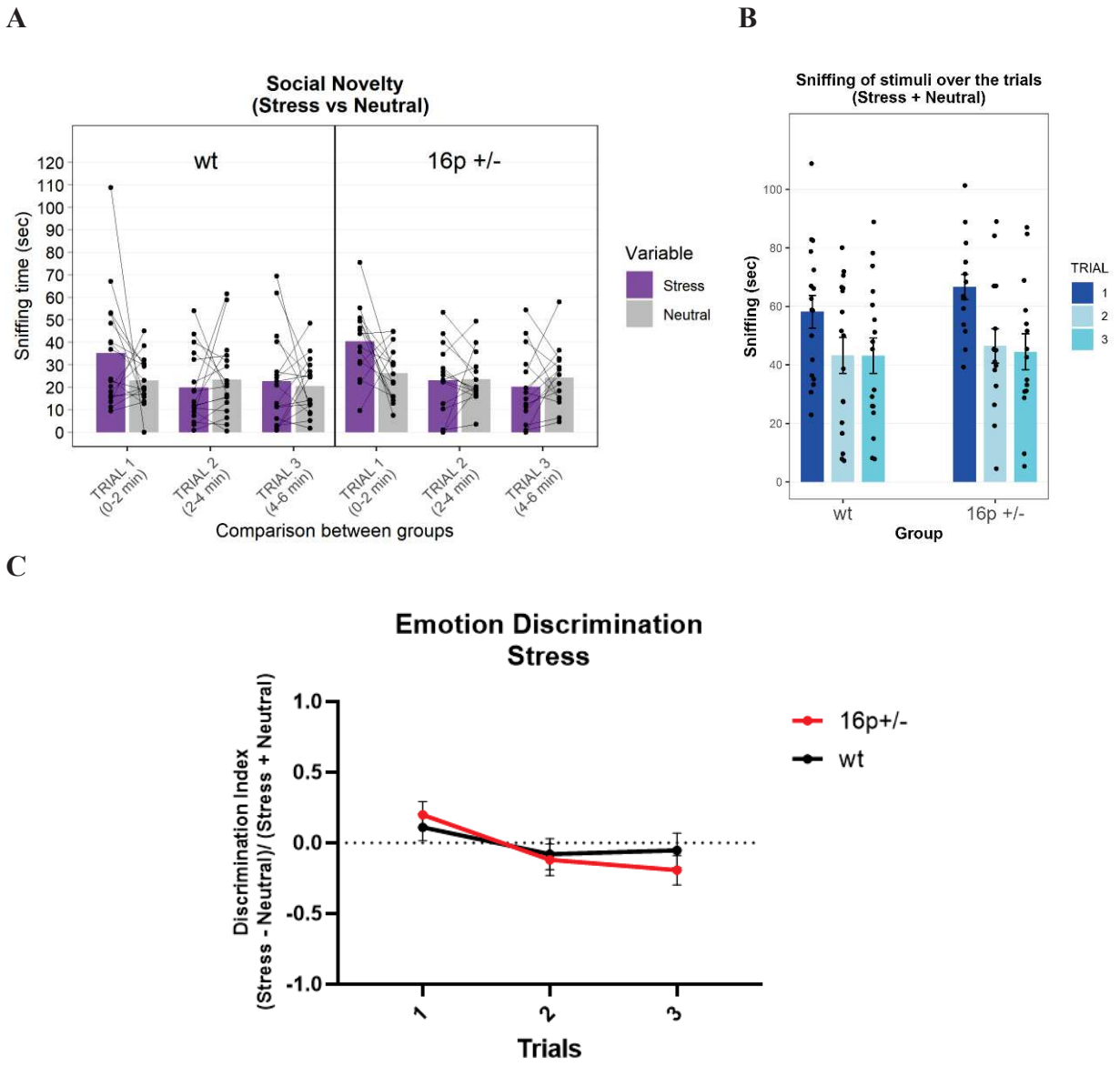


Figure 11: Emotion Discrimination (Stress) in wild-type and 16p11.2 adult littermate mice with a CD1 background.

N (wt) = 17 (8 males, 9 females); N(16p +/-): 15 (8 males, 7 females).

A) <u>Emotion Discrimination Task.</u>

The graph represents the time spent sniffing the Stress or the Neutral stimuli during the 6 minutes of the test, which was divided in 3 trials lasting 2 minutes each. Statistics were calculated using a non-parametric test (f1.ld.f2) using a non-parametric ANOVA with Genotype as a factor (2 levels), and Trial and Variable (Stress or Neutral) as repeated measures (3 and 2 levels respectively). The main effect of Trial was significant ($F = 17.814$, $df = 1.871$, $p < 0.0001$), indicating that responses varied significantly across trials. While the main effect of Variable was not significant ($F = 0.225$, $df = 1$, $p = 0.6355$), the interaction between Variable and Trial was significant ($F = 4.980$, $df = 1.992$, $p = 0.0069$), suggesting that the differences in sniffing between Stress and Neutral stimuli depended on the Trial. There was no main effect of Genotype ($F = 0.952$, $df = 1$, $p = 0.3291$), or significant interactions involving Genotype, including Genotype by Trial ($F = 0.917$, $df = 1.871$, $p = 0.3941$), Genotype by Variable ($F = 0.024$, $df = 1$, $p = 0.8788$), and the three-way interaction between Genotype, Trial, and Variable ($F = 0.864$, $df = 1.992$, $p = 0.4211$). The post hoc analysis with Dunn multiple comparison test, followed by Bonferroni correction, revealed no significant differences. Specifically, in the wild-type group, the sniffing of Stress and Neutral was not different between groups in Trial 1 ($p > 0.9999$), in Trial 2 ($p > 0.9999$) and Trial 3 ($p > 0.9999$). In the 16p +/- group, no differences were

observed between the Sniffing of Stress and Neutral: in Trial 1 there was a trend ($p = 0.0381$), but this was not significant after adjustment (adjusted $p = 0.2290$). In Trial 2 and Trial 3 no significant difference were found ($p > 0.9999$ in both).

B) Total exploration of the stimuli.

The ANOVA for the total exploration time of the stimuli (Stress + Neutral) revealed a significant main effect of Trial ($F = 41.081$, $df = 1$, $p < 0.0001$) but the main effect of Genotype was not significant ($F = 0.335$, $df = 1$, $p = 0.567$) as well as the interaction between Genotype and Trial ($F = 1.567$, $df = 1$, $p = 0.220$), suggesting that Genotype did not influence total exploration overall or during specific trials.

C) Discrimination Index.

The DI was calculated in order to better represent the preference for the Stress across Trials and to facilitate the visual comparison between groups. DI = ((Stress – Neutral)/(Stress + Neutral)). A positive DI indicates preference for the Stress demonstrator. A 2-way RM-ANOVA indicates a significant main effect of Trial ($F_{(1.707, 51.21)} = 5.328$, $p = 0.0109$), but no significant main effect of Genotype ($F_{(1, 30)} = 0.08444$, $p = 0.7734$) and no significant Trial × Genotype interaction ($F_{(2, 60)} = 0.7402$, $p = 0.4813$).

Discussion

The experiments conducted in this Chapter allowed for a comprehensive characterization of 16p11.2 deletion mice across various social domains, including sociability, social novelty, social memory, and emotion discrimination for both positive and negative emotions. No significant differences were observed between 16p +/- mutant and wild-type animals in any of these tests.

The results for sociability are consistent with previous findings, as sociability has generally shown no impairments in adult 16p +/- mice under baseline conditions. Both the Mills and Dolmetsch models have reported normal sociability scores in the three-chamber social interaction test and in reciprocal social interactions during early postnatal development [117,120,123,124]. Social novelty has followed a similar trend across different 16p11.2 mouse lines. The Mills and the Dolmetsch models seem to exhibit normal social novelty scores in three-chamber paradigms [117,124], but studies on the Herault model, which uses a C57BL/6N x C3B background, have reported a lack of preference for social novelty, suggesting that the discrepancies may be attributable to the different genetic backgrounds [116,126]. However, I could not find social novelty abnormalities in the CD1 background, indicating that abnormalities in the recognition of social novelty could be background-specific.

This study is also the first to assess emotion discrimination in the context of this deletion, and in mice with CD1 background: therefore, no prior reports in the literature are available to compare with our findings. Of note, we found that wild-type animals from the CD1 strain failed to discriminate between animals in altered emotional states and those in neutral conditions. Further investigation is required, and the test should be repeated in pure CD1 animals to determine whether this impairment is due to mixed housing conditions (as these animals were raised alongside 16p +/- siblings) therefore masking social deficits in 16p +/- animals, or if the strain itself is inherently unsuitable for this task.

Another possible explanation for the lack of significant findings is that this mouse model may not fully recapitulate the human phenotype, as is the case for other features of the condition [119,120]. For example, while obesity is a common feature of the 16p11.2 deletion in humans, mice with the same deletion usually have reduced body weight [120]. Similarly, motor delays frequently observed in humans are not consistently mirrored in mouse models, where motor activity seems to be normal

or even heightened during earlier phases of life [120]. It must be noted that previous studies have shown that sociability deficits may emerge in the 16p11.2 deletion Mills model under certain conditions. For example, Mitchell et al. demonstrated sociability deficits following the stress of an injection[125], and Wang et al. observed similar deficits after injections of a GqDREADD [64]. These findings support the hypothesis that the genotype alone may produce a vulnerability to social deficits, but the manifestation of these deficits may require a second genetic or environmental "hit," probably of inflammatory origin. For this reason, in CHAPTER 3, I will further explore the second-hit hypothesis in adult 16p +/- mice by investigating the effects of an immunological challenge to determine whether this factor can induce social deficits in adulthood that are relevant to ASD.

CHAPTER 3

Social behavior in a mouse model of 16p11.2 deletion after recovery from a viral-like challenge in adulthood

Abstract

The 16p11.2 proximal deletion is a genetic mutation associated with neurodevelopmental disorders, yet it is insufficient on its own to cause social behavioral deficits in mouse models, regardless of genetic background. Human carriers of this deletion often experience frequent infections, and several genes within the deleted region are involved in immune system function. Similarly, 16p11.2 mutant mice may be particularly susceptible to immune challenges and a transient microglial activation could result in social deficits after recovery from the acute phase.

To test this hypothesis, adult 16p11.2 mutant mice and their wild-type littermates were subjected to a viral-like immune challenge with a low dose (40 mg/kg) of polyinosinic-polycytidylic acid, low molecular weight (Poly I:C LMW). Behavioral assessments were conducted before the challenge and after a recovery period of at least 10 days, to analyze sociability, novelty recognition, social memory, and emotion recognition (negative emotions).

Results showed no genotype-specific alterations in any of the tested domains. However, immune challenge-related effects independent of genotype were observed in sociability, with a reduction in exploration of the social stimulus compared to baseline, and in emotion discrimination, that was impaired in the post-challenge phase. These findings suggest that an immune challenge can have lasting effects on social behavior even after recovery. Nevertheless, these effects do not appear to be amplified in 16p11.2 mutant mice, which do not exhibit differential vulnerability compared to wild-type mice.

Overall, these results demonstrate that while immune challenges can modulate social behavior, the 16p11.2 deletion does not confer an increased susceptibility to such effects, further supporting the conclusion that additional factors are required to elicit behavioral deficits in this genetic model.

Introduction

During life, the immune system is continuously involved in facing challenges, varying from environmental stressors such as toxins or psychosocial stress, to immune activation induced by pathogens including viruses and bacteria. These immune challenges produce important effects on many physiological systems, including the central nervous system where they influence mood, cognition, and behavior even in healthy individuals [132]. The recent COVID-19 pandemic has highlighted the need to understand the systemic and neural consequences of viral infections: in fact SARS-CoV-2, has been associated with a range of neurological symptoms [133]. Studying the effect of immunological challenges on the CNS is particularly crucial in the context of a genetic heightened vulnerability for neuro-psychological disorders.

Experimental studies have been pivotal in elucidating how systemic immune activation impacts the brain, providing various tools allowing the induction of different types of immunological challenges. A widely used tool in this context is Polyinosinic:polycytidylic acid (Poly I:C), a synthetic double-stranded RNA (dsRNA) analogue that mimics the effects of a viral infection [134]. Poly I:C triggers innate immune responses by engaging toll-like receptor 3 (TLR3) [134,135]. The following signaling cascade leads to the upregulation of the production of type I interferons (IFN-I), pro-inflammatory cytokines such as IL-6 and TNF-α, and to dendritic cell maturation [134]. This response usually peaks around 2-3 hours after the administration with a full recovery after 24-36 hours [134]. These mechanisms mirror the physiological responses elicited during actual viral infections, making Poly I:C a widely-used tool for studying the broader effects of systemic inflammation.

The efficacy of Poly I:C in inducing immune responses and behavioral changes depends on its molecular weight. High molecular weight (HMW) Poly I:C elicits significant systemic and CNS cytokine responses compared to low molecular weight (LMW) Poly I:C [134,136]. Studies have shown that HMW Poly I:C at doses such as 12 mg/kg induces pronounced sickness behaviors in animals, including hypo locomotion, reduced engagement in exploratory activities, and significant febrile responses, paralleled with elevated levels of cytokines such as IFN-β, IL-6, and TNF-α in both peripheral tissues and the brain [134]. In contrast, LMW Poly I:C, even at higher doses (e.g., 40–80 mg/kg), achieves comparable sickness behaviors but induces less pronounced cytokine elevations,

resulting in a milder inflammatory response [134]. LMW Poly I:C, therefore, could be a reliable tool in contexts where there could be an important vulnerability to immunological challenges.

The interaction between genetic predispositions and immune challenges, often referred to as a "double hit," is an interesting hypothesis that could explain while some neuropsychiatric conditions occur, and many studies have been conducted by evaluating the combination of a genetic vulnerability and an infection-like challenge [135]. However, most studies have focused on critical time windows of development, such as the intrauterine life, with protocols such as the one of maternal immune activation (MIA) during pregnancy [137]. Fewer studies have evaluated the effects of immune challenges in adulthood in the context of genetic mutations. One recent study [138] investigated the impact of immune challenges, both with Poly I:C and lipopolysaccharide (LPS), on a 15q13.3 deletion mouse model, finding that the hippocampal expression of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) was significantly higher compared to wild-type mice, providing an initial evidence supporting the double challenge hypothesis. In the context of the 16p11.2 deletion, no studies to date have investigated the effects of an immune challenge on carriers of this genetic alteration. Given that approximately half of individuals with the 16p11.2 deletion report frequent lifetime infections [47], supporting the idea of a link between their genetic profile and altered immune function, this effect of double challenges in the context of this mutation is worth investigating, also after considering our negative findings reported in CHAPTER 2. To address this, the aim of this chapter is to evaluate the behavioral responses of 16p11.2 deletion carriers to a mild immune challenge induced by a low molecular weight (LMW) Poly I:C at a dose of 40 mg/kg. This specific dose has been selected based on a prior study [134], where it produced a mild inflammatory response in C57BL/6J, which is suitable in the context of 16p11.2 deletion where we hypothesize higher susceptibility to infections. Behavioral testing was conducted at least 10 days post-challenge to ensure that the acute phase of the inflammation has resolved, providing an opportunity to investigate mid-term social behavioral effects [139]. To further support the choice of this time-window, a morphology assessment of microglia was performed in order to compare indicators of microglial activation between a group treated with saline, the acute microglial response after 24 hours post-injection, and the post-acute microglial response after 10 days following the injection.

Methods

Unless otherwise specified and further described here, all the behavioral tests, the housing conditions used in this chapter are the same as described in CHAPTER 2, section Methods.

Mice

Adult males and females 16p11.2 deleted mice (Mills' model, all born from breeding in the facility) in C57BL/6J background were used, with an age range between 3 and 4 months. The 16p11.2 mutation was only transmitted through the paternal lineage, in order to avoid potential confounders due to aberrant maternal care: for this reason, mothers were always C57BL/6J. Animals were housed two to four per cage in a climate-controlled (22 ± 2 C) and specific pathogen-free animal facility, with ad libitum access to food and water throughout, a standard environmental enrichment (material for nest and cardboard house), and with a 12-hour light/dark cycle (7pm/7am schedule). All the animals were kept in a mixed housing condition, with 16p +/- (deleted) mice and their wild type siblings housed together. To avoid litter effects, no more than 2 males and 2 females (one per genotype) were used for behavioral testing.

Immune challenge

To induce immune activation, LMW Poly I:C dissolved in a sterile, pyrogen-free solution and administered via intraperitoneal injection was used. The LMW was purchased from Invivogen (catalog code: tlrl-picw): compared to other commercially available compounds, the Invivogen product contains low levels of endotoxins and exhibits consistent molecular weight across batches [140]. Poly I:C does not cause severe distress in animals, as its effects are short-lived (with cytokine and thermal response peaking within 3 hours post-injection and complete resolution of sickness behavior within 36 hours of injection [134,136,141,142]. Sickness behavior was checked in all the animals 2 hours after administration and 24 hours later, together with body weight.

The dose of 40 mg/kg was used, based on a previous study that identified 40 mg/kg as the minimum dose required to induce mild changes in locomotor activity, signs of sickness behavior indicative of inflammatory cascade activation, and alterations in inflammatory mediators such as TNF-α and IL-6 [134]. The injection was always administered between 9 and 11 AM, following the assessment

of body weight (injection volume 10 mL/kg). Behavioral assessments were conducted 10 days post-injection, a timeframe chosen to ensure resolution of acute microglial inflammation [139]. To further confirm the choice of this time window, a microglial morphology assessment was performed.

Behavioral assays

To minimize the number of animals required, a within-subject design was employed, as represented in Figure 12. Baseline behavioral testing was conducted when animals were 3 months-old, after handing as described before. The social battery described in CHAPTER 2 was applied (habituation dishabituation task, sociability and social novelty task, emotion discrimination for stress). After a resting period of at least one week post-baseline testing, Poly I:C was administered, followed by 10 days or rest post-injection. After 10 days post-injection, the animals underwent the same behavioral battery as performed at baseline, but the all the stimuli were novel, to prevent the potential confounder of familiarity between observers and stimuli.

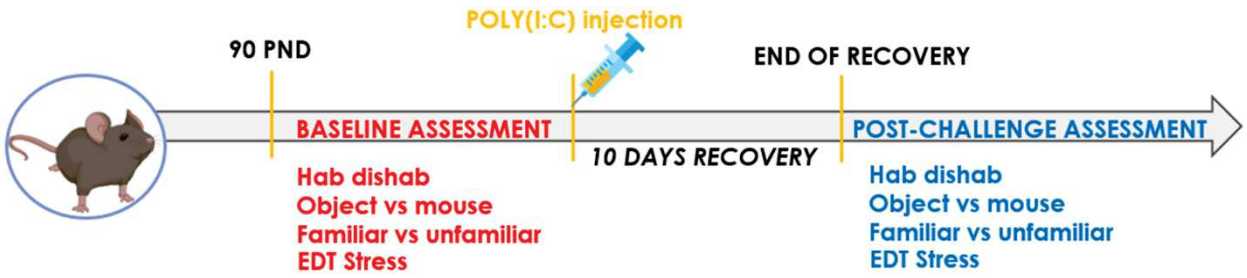


Figure 12: Timeline of the behavioral assessments.

Histology

To assess the effects of the Poly I:C injection on microglial morphology, wild type mice underwent an injection of 40 mg/kg Poly I:C or of saline, and were then sacrificed after 24 hours or after 10 days following the injection. Mice were deeply anesthetized (urethane 20%) and transcardially perfused with 4% paraformaldehyde in PBS at pH 7.4. Brains were dissected, fixed in 4% PFA overnight at 4°C, and cryoprotected in 30% sucrose in PBS for 36 hours at 4°C. On the first day, coronal sections of the brain were cut with a thickness of 40 µm using a HM450 microtome (Thermo Fisher Scientific). The sections were washed three times in PBS for 10 minutes each.

Blocking was performed to reduce non-specific binding by incubating the sections in a solution containing 10% normal goat serum (NGS) and 0.3% Triton X-100 in PBS, for 4 hours at room temperature (RT). Sections were then incubated overnight at 4°C in a primary antibody solution with PBS with 1% NGS and 0.5% Triton X-100, containing the anti-Iba1 antibody (Rabbit, FUJIFILM Wako Pure Chemical Corporation, cat n. 019-19741) at a dilution of 1:500. On the second day, the sections were washed three times for 10 minutes each in PBS containing 0.5% Triton X-100 and then incubated for two hours at RT in a secondary antibody solution (PBS with 1% NGS and 0.5% Triton X-100, anti-Rabbit antibody conjugated to Alexa Fluor 488 (1:1000, Molecular Probes, A-11008). Sections were then washed three times for 5 minutes each in PBS containing 0.5% Triton X-100, followed by a 5-minute wash in PBS, and a 15-minute wash in PBS with DAPI (1:1000). Finally, the sections are rinsed three additional times in PBS for 10 minutes, mounted on slides and covered with cover slips. Z-stacks were then acquired using a 40x-oil magnification with a confocal microscope (Leica CTR6500) using a 504 nm, focusing on the mPFC. For each mouse, 3 different acquisitions in different areas of the mPFC were acquired (Z stacks of 21 slices, 1.01 micron thickness, with a 1024*1024 resolution and a total thickness of 20.124 micron).

Statistics

All the statistics test were performed similarly to what was described in the previous CHAPTER 2. For habituation-dishabituation task, a 3-way ANOVA was performed with Graphpad Prism by considering the trials and the challenge as repeated measures, followed by Tukey's multiple comparisons tests. Further details about each tests are reported in the Figure legends.

For the Object vs Mouse, Familiar vs Unfamiliar and Emotion Discrimination task, a custom script was written in R as described in the previous chapter, allowing automatized detection of data normality and homogeneity and the consequent choice of the following parametric or non-parametric tests and post-hoc, followed by Bonferroni correction. For analysis including 2 factors (Challenge and Genotype) and 2 repeated measures (Trial, Variable) a linear mixed effect model (lme) was used as parametric test, in order to properly evaluate the effects of the factors (Genotype, Challenge, Trial, Variable) and of their interactions followed by the appropriate post-hoc analysis. For non parametric data, the f2.ld.f1 function of R was used from the nparLD package[131],

considering Genotype and Challenge as f2, and the interaction between trial and variable as f1. All the testing were followed by post-hoc analysis, described in detail in the caption of the figures. For the assessment of the differences in the total exploration time, the aov (analysis of variance) function of R was used for parametric analysis, or a mixed effects model with correct random effects for non-parametric data. Both tests were considering Genotype, Challenge and Trials as factors. For the analysis of microglia morphology, a semi-automatized method was used. The method is fully described in [143] and provides a plugin for FIJI for a semi-automatized microglial detection and morphology quantification, including a skeleton and a Sholl analysis, followed by a statistic analysis that identifies the morphology predictors of inflammation (Inflammation Index package for R).

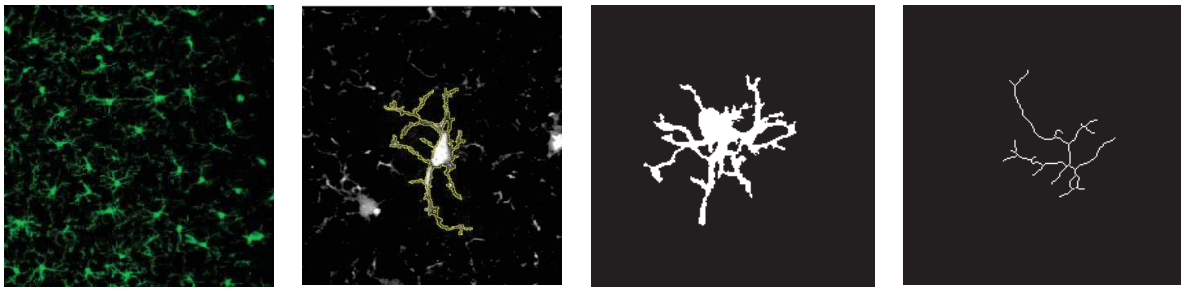


Figure 13: Representative pictures of steps of the semi-automatized microglial morphology analysis.

After preprocessing and quality assessment of the Z-stack, the cells are detected and a mask is automatically generated for each cell. Following a manual quality assessment, each mask is quantified and the skeleton and the Sholl analysis are automatically performed.

Results

Microglial morphology in wild type mice indicates a reactive state after 24 hours following an immunological challenge, but returns to normality after 10 days.

A preliminary assessment of microglial morphology was performed in a batch of C57BL/6J mice in order to evaluate if microglia morphology would change after 24 hours and after 10 days following an intraperitoneal injection of 40 mg/kg LMW Poly I:C. In Figure 15, a representative image of the microglia in all the conditions is provided.

Parameters were calculated after performing a semi-automated Skeleton and the Sholl analysis, with the Microglia Morphology plugin for FIJI described in [143] and the attached R-package which automatically extracts the best predictors of the differences between the two groups, following a principal component analysis (PCA).

As expected, the analysis revealed significant differences in microglial morphology 24 hours after Poly I:C administration compared to saline-treated controls. In particular, following a in the Poly I:C – 24 hours group compared with saline, the number of Branches (the number of branches in the mask skeleton) and of Quadruple-points (the number of junctions with 4 branches) were identified (based on their contribution to explaining variance within the dataset) as the most robust indicators of microglial response, with a mask-size of 300 μm^2.

Besides the indicators mentioned above, a Kruskall-Wallis test, followed by a Dunn multiple comparison adjustment, was performed to assess the differences in the parameters between the Poly I:C – 24 hours group and the one treated with saline, with a mask-size of 300 μm^2.

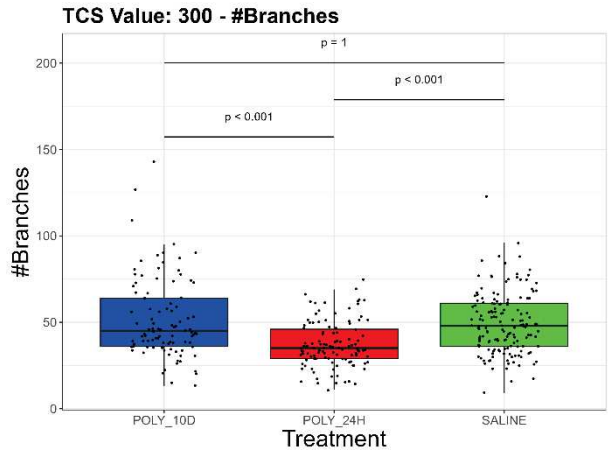
Table 2 and Figure 14 recapitulate all the significant findings. These findings indicate a morphological simplification and loss of complexity in microglia following the challenge, with a reduction in microglial branching indicating an activated or reactive phenotype.
The analysis performed on observations at 10 days post-injection (Poly I:C - 10 days group), indicated that the microglial status underwent a general recovery, with no significant differences

from the saline-treated group for most parameters (Figure 14). The only significance was observed for the variable Primary Branches, indicating the number of branches originating directly from the cell soma (Kruskal-Wallis test, $H = 12.15$, p-value = 0.0023; Dunn's test: $Z = 2.47$, adjusted p-value = 0.0401; mean for Poly I:C - 10 days group: 4.52, SEM = 0.14; mean for the saline group: 4.08, SEM = 0.11). The results confirm that the 10-days window is appropriate for inducing a transient microglial activation and for studying the behavioral effects without the confounder of the acute effects of treatment.

Table 2: Significant differences between microglial morphology parameters between saline-treated and the Poly I:C treated animals.

After the automated skeleton and Sholl analysis, all the parameters relevant for microglial morphology were compared between the two groups. Given that parameters were not satisfying normality and homogeneity assumptions, a Kruskall-Wallis test was performed followed by Dunn post-hoc test. The Bonferroni adjusted p-value is reported for post-hoc. The meaning of each Variable is described in the "Info" column.

Variable	**Kruskal-Wallis H**	**Dunn (post-hoc)**		**Poly IC 24 h**		**Saline**		**Info**
		Z	**Adj. p-value**	**Mean**	**SEM**	**Mean**	**SEM**	**(from Clarke et al, 2021)**[143]
Branches	41.71	-5.93	0.00	37.11	1.24	49.54	1.43	the number of branches in the mask skeleton
End-point voxels	37.21	-5.64	0.00	19.68	0.59	25.07	0.65	the number of endpoints voxels in the mask skeleton
Junctions	37.43	-5.78	0.00	17.22	0.59	23.01	0.68	the number of junctions in the mask skeleton
Junction voxels	35.60	-5.47	0.00	35.64	1.36	47.65	1.50	the number of junction voxels in the mask skeleton
Quadruple points	46.22	-6.03	0.00	1.98	0.17	3.58	0.19	the number of junctions with 4 branches
Triple points	21.88	-4.65	0.00	14.84	0.50	18.81	0.56	the number of junctions with 3 branches
Average Branch Length	73.79	7.10	0.00	4.65	0.09	3.86	0.04	the average length of all branches in the mask skeleton
Centroid Value	9.90	-2.75	0.02	3.88	0.09	4.24	0.09	the ordinate of the geometric center of a linear plot of number of branches against radius
Maximum Branch Length	15.31	3.47	0.00	16.75	0.55	14.25	0.29	the maximum length of a branch in the skeleton
Maximum Number of Intersections	17.42	-3.36	0.00	7.84	0.19	8.75	0.17	the highest number of processes at any given radius
Mean of Intersections	9.89	-2.75	0.02	3.88	0.09	4.24	0.09	the mean number of processes across all sampled radii
Perimeter	8.98	-2.98	0.01	285.81	7.46	316.17	7.31	the perimeter around the cell mask
Skeleton Area	9.53	-3.12	0.01	52.20	1.37	58.17	1.37	the area occupied by the cell skeleton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p < 0.001
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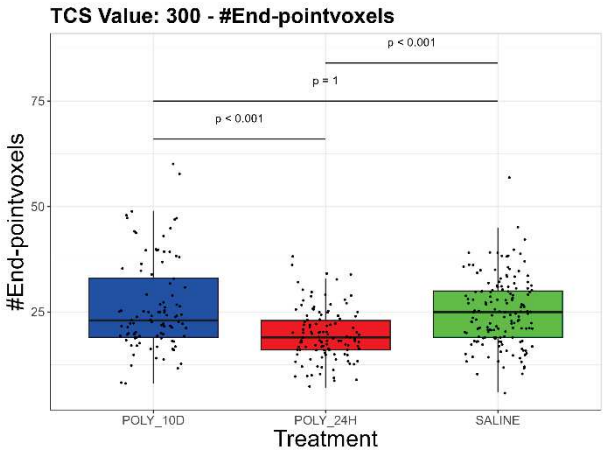
150
100
50
0
POLY_10D
POLY_24H
SALINE
Treatment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#End-pointvoxels
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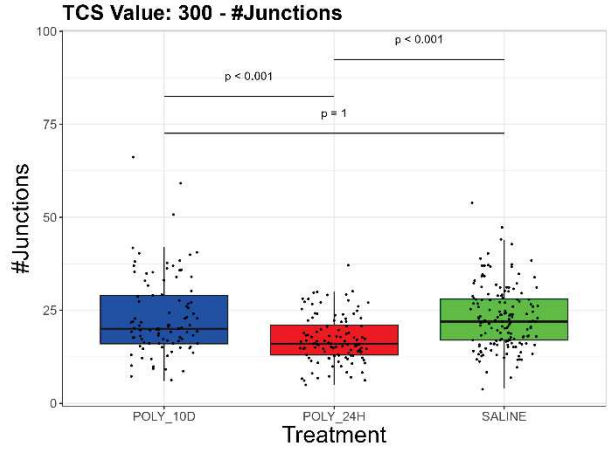
50
25
POLY_10D
POLY_24H
SALINE
Treatment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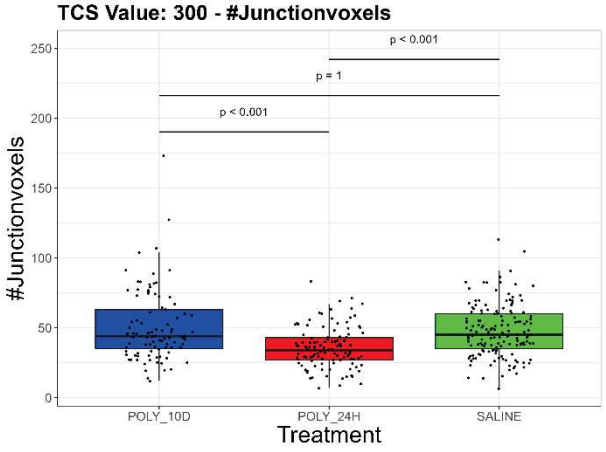
#Junctions
100
75
50
25
0
POLY_10D
POLY_24H
SALINE
Treatment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#Junctionvoxels
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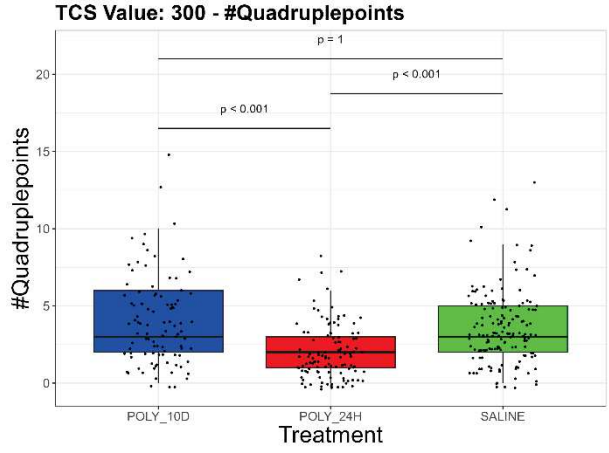
200
150
100
50
0
POLY_10D
POLY_24H
SALINE
Treatment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#Quadruplepoints
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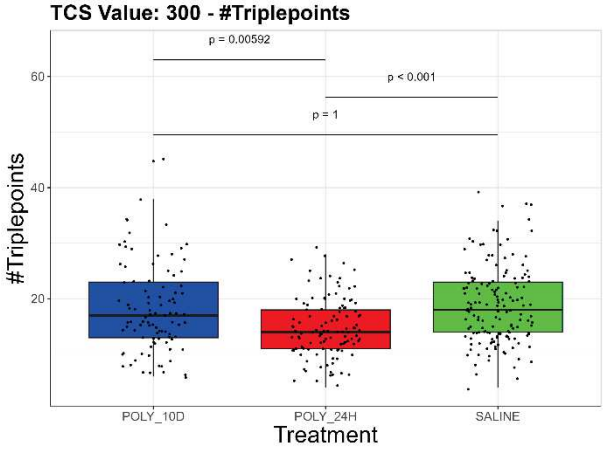
15
10
5
0
POLY_10D
POLY_24H
SALINE
Treatment


TCS Value: 300 - #Triplepoints
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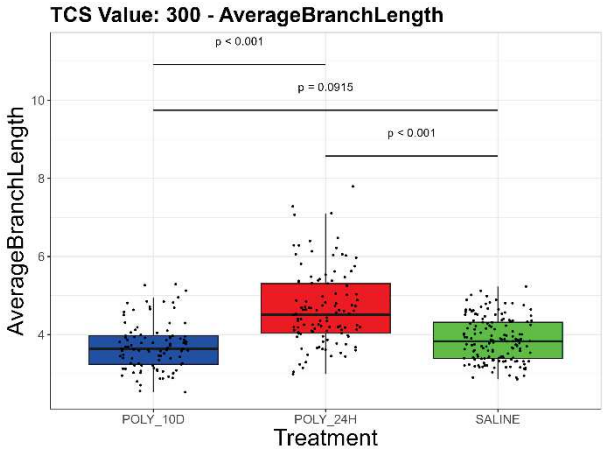
#Triplepoints
60
40
20
POLY_10D
POLY_24H
SALINE
Treatment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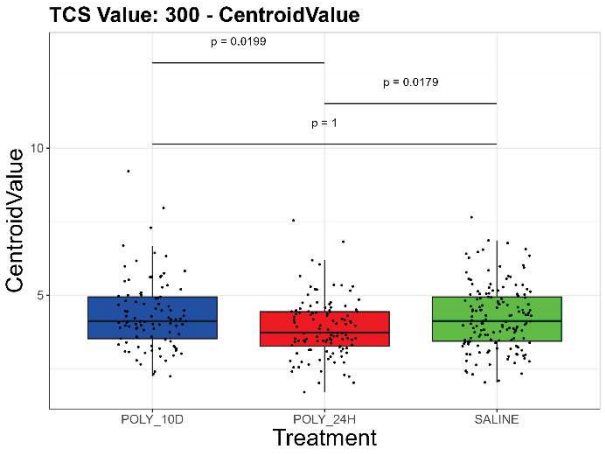
8
6
4
POLY_10D
POLY_24H
SALINE
Treatment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p = 1
CentroidValue
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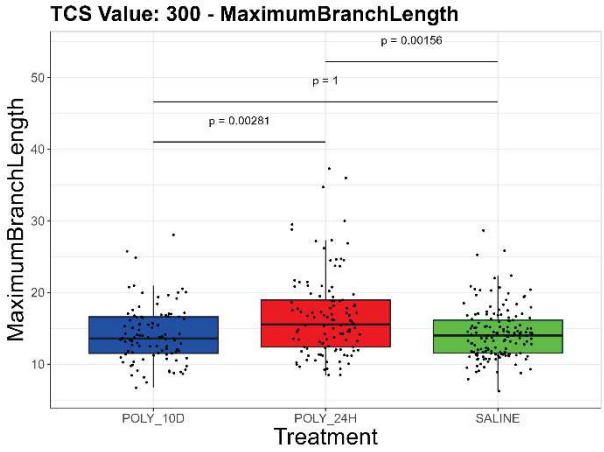
5
POLY_10D
POLY_24H
SALINE
Treatment


TCS Value: 300 - MaximumBranchLength
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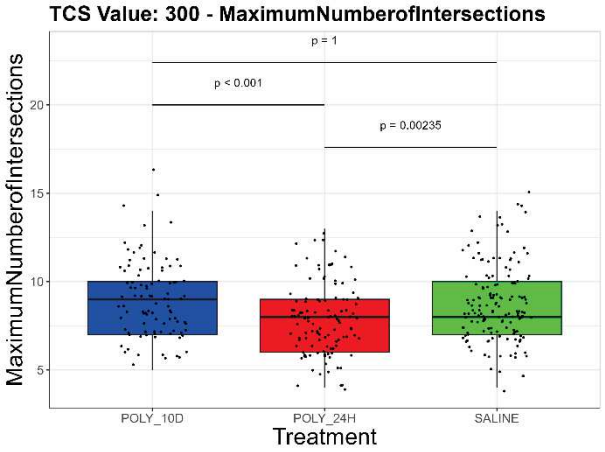
p = 1
p = 0.00281
MaximumBranchLength
50
40
30
20
10
POLY_10D
POLY_24H
SALINE
Treatment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MaximumNumberofIntersections
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15
10
5
POLY_10D
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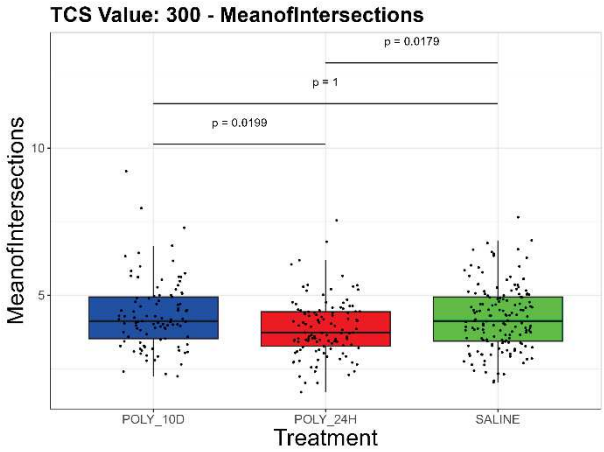
SALINE
Treatment


TCS Value: 300 - MeanofIntersections
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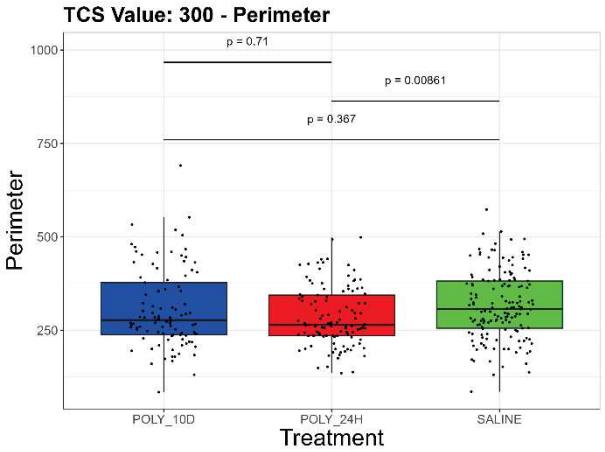
p = 1
p = 0.0199
MeanofIntersections
10
5
POLY_10D
POLY_24H
SALINE
Treatment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Perimeter
1000
750
500
250
POLY_10D
POLY_24H
SALINE
Treatment

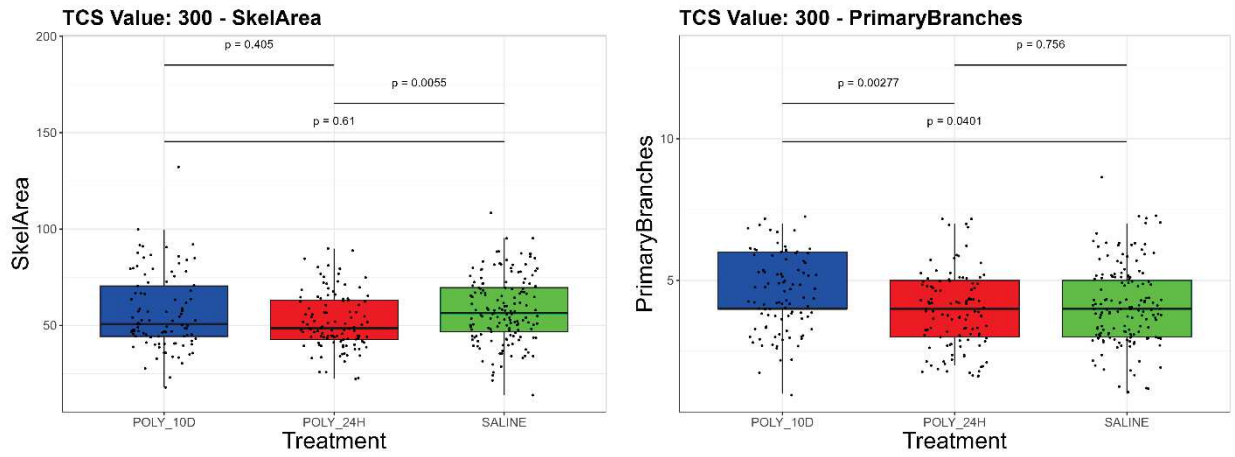


Figure 14: Significant microglial morphology differences between saline and after 24 hours or 10 days following an immunological challenge with 40mg/kg LMW Poly I:C.

Each dots represents a single microglial cell. Adjusted p-values are reported in the graphs. Only the significant comparisons between saline (**GREEN**) and treatment with Poly I:C – 24 hours (**RED**) or with Poly I:C – 10 days (**BLUE**) are represented in the graphs.
Saline: N = 4 animals, 3 acquisitions for each animal.
Poly I:C – 24h: N =2, 3 acquisitions for each animal.
Poly I:C – 10 days: N = 3, 3 acquisitions for each animal.

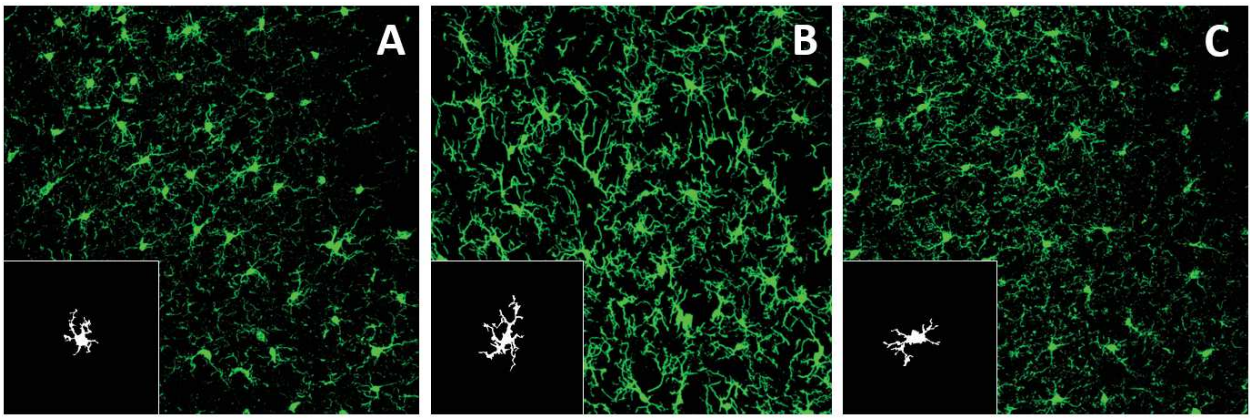


Figure 15: Representative images of microglia after the immunological challenges and in the controls

IBA-1 staining. The green images represent the Z-projection of the Z-stacks acquired in the mPFC. The images in the inset report the masks of some representative cells.
A) Microglia after 10 days following the Poly I:C injection. Corresponding to the **BLUE** boxplot in Figure 14.
B) Microglia after 24 hours following Poly I:C injection. Corresponding to the **RED** boxplot in Figure 14.
C) Microglia after saline. Corresponding to the **GREEN** boxplot in Figure 14.

16p +/- mice do not differ from their wild type siblings in sociability, social memory and social novelty after an immunological challenge.

I first tested whether the 16p +/- mice could display abnormalities in sociability, following remission after a viral-like challenge with Poly I:C. I first applied the habituation-dishabituation task, in which mice were free to interact for trials lasting 1 minute each.

At baseline, before the challenge, I replicated the results observed in 16p +/- without challenges, and no differences in these social domains were observed between 16p +/- and wild-type (Figure 16 A, B, C). Following the immunological challenge, no significant effects were observed in 16p +/- or in wild-type in sociability, in social memory and in recognition of social novelty.

Moreover, there were no differences between the two groups in social memory (Figure 16 A, B, C) recognition of social novelty (Figure 16 D) and sociability (Figure 16 E) following the challenge with Poly I:C.

The same domains were assessed with the Object vs Mouse and the Familiar vs Unfamiliar tests. Again, the sociability measured with the Object vs Mouse test was not significantly affected by the genotype or by interactions between challenge and genotype, indicating that the combination of the two factors is not sufficient to produce deficits in adult 16p +/- mice (Figure 17 A, B). However, there was a significant effect of the immunological challenge in modulating the response to the task, independently from the genotype, with a significant reduction of the time spent sniffing the Mouse over the trials both in 16p +/- and in wild type mice.

Social Novelty discrimination (Figure 18) was not affected by the genotype or by the immunological challenge or by their interaction, indicating that the combination of the 16p +/- deletion and the immunological challenge, as well as the double challenge, is not producing effects on recognition of social novelty. The total exploration time was not affected by genotype or by the immunological challenges in none of the two experiments. However, an interaction between the immunological challenge and the trials was observed during the social novelty test, indicating a reduction of the total exploration over the trials that was more pronounced after the immunological challenge but was independent from the genotype. This could indicate that mice were less willing to keep exploring stimuli after the immunological challenge, and that this effect mirrors the significant reduction in the exploration of the mouse observed over the trials.

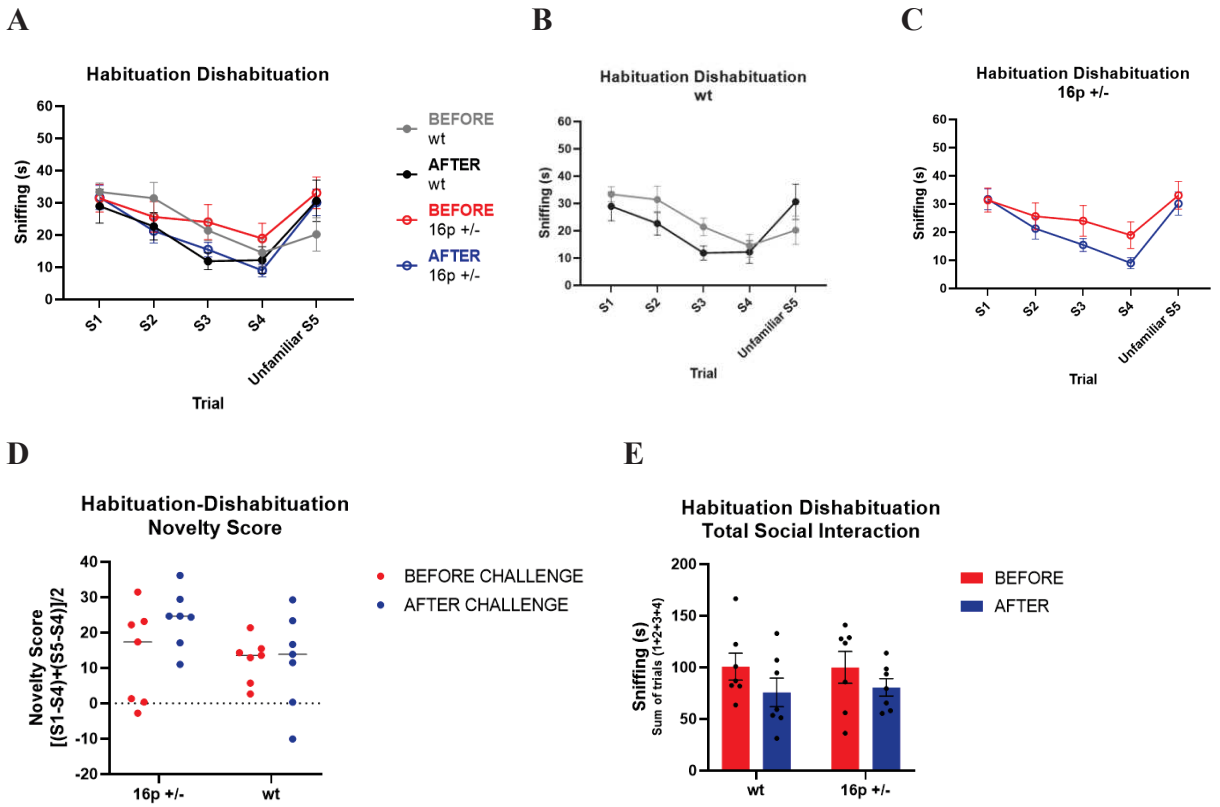


Figure 16: Habituation Dishabituation task before and after an immunological challenge.

N(wt)= 7 (4 males, 3 females) and N(16p +/-) = 7 (3 males, 4 females).

The habituation dishabituation task was repeated twice on the same animals, before and 10 days after an immunological challenge with Poly I:C. Therefore, a mixed effect analysis was performed considering Trials and Challenge as repeated measures.

A) Habituation Dishabituation.

The main effect of Trial was significant ($F_{(2.934, 38.14)} = 20.60$, $p < 0.0001$) while neither Challenge ($F_{(1, 42)} = 1.267$, $p = 0.2667$) nor Genotype ($F_{(1.000, 13.00)} = 0.1639$, $p = 0.6922$) showed significant main effects. The interaction between **Trial and Challenge only approached significance** ($F_{(4, 42)} = 2.461$, $p = 0.0599$). Other interactions, including Trial and Genotype ($F_{(2.010, 21.10)} = 2.181$, $p = 0.1375$), Challenge and Genotype ($F_{(1, 42)} = 0.1184$, $p = 0.7325$), and the three-way interaction of Trial, Challenge, and Genotype ($F_{(4, 42)} = 2.425$, $p = 0.0629$), were not statistically significant.

Independently from Challenge and Genotype, the post hoc analysis for Trials using Tukey's multiple comparisons showed a significant difference between S1 and S2 (mean difference = 6.098, 95% Confidence Interval (CI) [0.7175, 11.48], $p = 0.0205$), between S1 and S3 (mean difference = 13.15, 95% CI [7.081, 19.23], $p < 0.0001$), and between S1 and S4 (mean difference = 17.69, 95% CI [11.24, 24.15], $p < 0.0001$), indicating normal social memory. The comparison between S1 and the unfamiliar S5 condition was not significant (mean difference = 2.838, 95% CI [-3.722, 9.397], $p = 0.7150$), indicating normal recognition of social novelty, as supported also by the significant difference between S4 and the unfamiliar S5 condition (mean difference = -14.86, 95% CI [-21.57, -8.148], $p < 0.0001$).

To evaluate the Trial x Challenge interaction, the data for Genotype were collapsed and a 2-way ANOVA was performed including Challenge and Trial as repeated measures. Again, no effects were found for any of the factors, with the interaction Trial x Challenge only approaching significance ($p=0.0539$).

B) Habitaution Dishabituation: wild-types.

Regarding the single Genotypes, the Tukey test indicates that in wild-type social memory was preserved, as indicated by the significant difference at the comparison between S1 and S4 (mean difference = 18.86, 95% CI [7.312, 30.42], $p = 0.0006$). However, sniffing at S1 and with the unfamiliar S5 condition showed a significant difference (mean difference = 13.16, 95% CI [1.606, 24.71], $p = 0.0201$), indicating that wild-type mice failed to recognize the novel mouse at Trial 5, as confirmed also by the absence of significance between S4 and S5 (mean difference = -5.706, 95% CI [-17.26, 5.846], $p = 0.5998$). After the challenge, the difference between S1 and S4 was still significant (mean

difference = 16.75, 95% CI [5.200, 28.30], p = 0.0022), indicating unchanged social memory, while comparison between S1 and the S5 condition (mean difference = -1.656, 95% CI [-13.21, 9.896], p = 0.9929) was not significant. The significant difference between S4 and the S5 condition (mean difference = -18.41, 95% CI [-29.96, -6.856], p = 0.0008), indicates normal recognition of novelty.

C) Habituation Dishabituation: 16p +/-

Similarly, the 16p +/- had normal social memory (difference between S1 and S4) both before (mean difference = 12.46, 95% CI [0.2836, 24.64], p = 0.0416), and after the challenge (mean difference = 22.70, 95% CI [10.52, 34.88], p < 0.0001). Recognition of social novelty was normal, as evident by the comparison between S4 and the unfamiliar S5 condition before (mean difference = -14.17, 95% CI [-26.34, -1.990], p = 0.0131) and after the challenge (mean difference = -21.15, 95% CI [-33.32, -8.968], p < 0.0001).

D) Novelty Score

The novelty score was not significantly different between groups. There was no main effect of Genotype ($F_{(1, 12)}$ = 3.781, p = 0.0757), Challenge ($F_{(1, 12)}$ = 1.219, p = 0.2912), or interactions between Genotype and Challenge ($F_{(1, 12)}$ = 2.663, p = 0.1286).

E) Total Social Interaction

Total social interaction was calculated as the sum of the social interactions from Trial 1 to Trial 4.

The main effect of Genotype was not significant ($F_{(1, 12)}$ = 0.04151, p = 0.8420), with mean social interaction (SI) scores very similar between wild-type (mean = 88.24) and 16p +/- (mean = 90.32). The main effect of Challenge was also not significant ($F_{(1, 12)}$ = 2.135, p = 0.1697). **The mean SI scores were higher before the challenge (mean = 100.4) compared to after the challenge (mean = 78.21), but the difference of 22.15 (95% CI [-10.88, 55.17]) did not reach statistical significance.** The interaction between Genotype and Challenge was not significant ($F_{(1, 12)}$ = 0.03457, p = 0.8556).

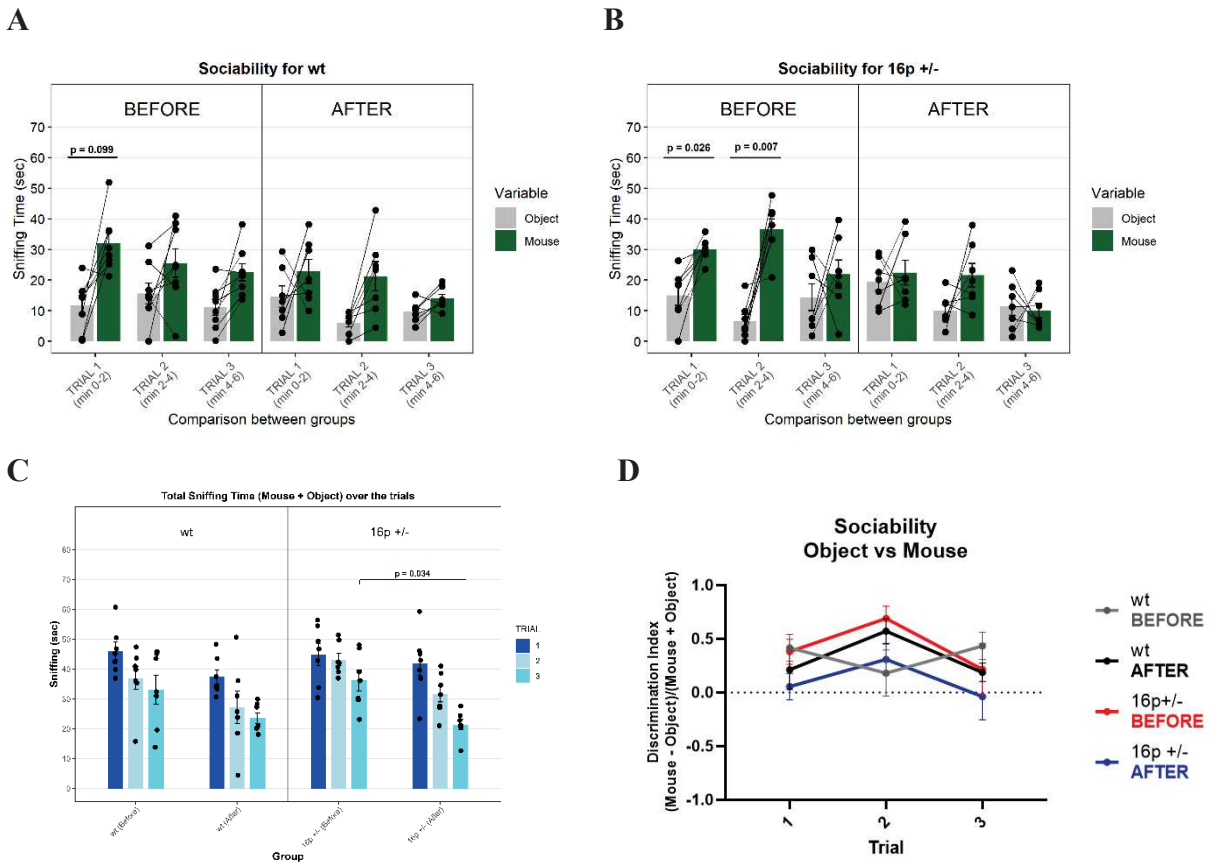


Figure 17: Sociability (Object vs Mouse) test before and after challenge.

N(wt)= 7 (4 males, 3 females) and N(16p +/-) = 7 (3 males, 4 females).

A repeated measures ANOVA was conducted with Genotype and Challenge as between-subject factors, and Trial and Variable (Object vs. Mouse) as within-subject repeated measures. The analysis revealed significant main effects of Trial ($F_{(2, 120)} = 9.25$, $p = 0.0002$) and Variable ($F_{(1, 120)} = 71.73$, $p < 0.0001$), as well as a significant interaction between Trial and Variable ($F_{(2, 120)} = 5.84$, $p = 0.0038$). **Challenge had a significant main effect** ($F_{(1, 24)} = 12.89$, $p = 0.0015$) while there was no significant main effect of Genotype ($F_{(1, 24)} = 0.87$, $p = 0.3591$). None of the interactions involving Genotype were significant, including: Genotype by Challenge ($F_{(1, 24)} = 0.007$, $p = 0.9345$), Genotype by Trial ($F_{(2, 120)} = 0.30$, $p = 0.7430$), Genotype by Variable ($F_{(1, 120)} = 0.06$, $p = 0.8049$), Genotype by Challenge by Trial ($F_{(2, 120)} = 0.37$, $p = 0.6883$), Genotype by Challenge by Variable ($F_{(1, 120)} = 2.56$, $p = 0.1121$), Genotype by Trial by Variable ($F_{(2, 120)} = 2.70$, $p = 0.0711$), and the four-way interaction of Genotype by Challenge by Trial by Variable ($F_{(2, 120)} = 2.01$, $p = 0.1378$).

The **interaction between Challenge and Variable** was significant ($F_{(1, 120)} = 11.35$, $p = 0.0010$), while the Challenge by Trial ($F_{(2, 120)} = 0.52$, $p = 0.5979$) and Challenge by Trial by Variable ($F_{(2, 120)} = 0.27$, $p = 0.7621$) interactions were not. Independently from the Genotype, post-hoc Tukey tests revealed no significant differences for the sniffing of the Object across trials when comparing sniffing times before and after the immunological challenge: (Trial 1: mean difference = -2.89, $p = 0.354$; Trial 2: mean difference = 1.91, $p = 0.433$; Trial 3: mean difference = 1.86, $p = 0.541$). However, **for the sniffing of the Mouse, a significant reduction after the challenge was observed in Trial 1** (mean difference = 8.72, $p = 0.015$) **and Trial 3** (mean difference = 10.30, $p = 0.002$), while Trial 2 did not show a significant difference (mean difference = 8.78, $p = 0.061$).

A) Sniffing time for the Mouse and the Object in the wild-type animals.

Each dot represents one animal. Given the main effect of the Variable, in the post-hoc analysis using paired t-tests, comparisons between the variables Object and Mouse were conducted for both the 16p +/- and wild-type groups across the two Challenge conditions for each trial.

For the wild-type animals, before the challenge, animals were not able to significantly discriminate between the social and non-social stimuli during Trial 1 ($t_{(6)} = -3.30$, $p = 0.016$, adjusted $p = 0.099$), but discrimination occurred only at

a trend level. In Trial 2, no significant difference was found ($t_{(6)} = -1.45$, adjusted $p > 0.9999$). Similarly, in Trial 3, no significant difference was observed ($t_{(6)} = -3.23$, adjusted $p = 0.107$).
After the challenge, no significant discrimination was observed in any trial, including Trial 1 ($t_{(6)} = -1.17$, adjusted $p > 0.9999$), Trial 2 ($t_{(6)} = -3.41$, adjusted $p = 0.086$), and Trial 3 ($t_{(6)} = -2.20$, adjusted $p = 0.422$).

B) Sniffing time for the Mouse and the Object in the 16p +/- animals.

Before the challenge, a significant difference between the sniffing of Mouse and Object was observed in Trial 1 ($t_{(6)} = -4.46$, adjusted $p = 0.026$), with animals showing a clear preference for the social stimulus (Mouse) (29.94 ± 1.41) over the Object (14.95 ± 3.26). This preference remained significant in Trial 2 ($t_{(6)} = -5.72$, adjusted $p = 0.007$), where sniffing for the Mouse (36.71 ± 3.27) was higher than for the Object (6.51 ± 2.30). In Trial 3, no significant difference was found ($t_{(6)} = -0.90$, adjusted $p > 0.9999$). After the challenge, no significant differences were observed in any trial, including Trial 1 ($t_{(6)} = -0.52$, adjusted $p > 0.9999$), Trial 2 ($t_{(6)} = -2.07$, adjusted $p = 0.503$), and Trial 3 ($t_{(6)} = 0.29$, adjusted $p > 0.9999$). Results are reported as Mean ± SEM.

C) Total exploration time.

A repeated measures ANOVA was conducted to evaluate the effects of Genotype, Challenge, and Trial on Total exploration Time. No significant main effects or interactions were observed for Genotype ($F_{(1)} = 2.09$, $p = 0.153$), Challenge ($F_{(1)} = 0.30$, $p = 0.584$), Trial ($F_{(2)} = 0.07$, $p = 0.932$), Genotype × Challenge ($F_{(1)} = 0.02$, $p = 0.892$), Genotype × Trial ($F_{(2)} = 0.58$, $p = 0.562$), or the three-way interaction Genotype × Challenge × Trial ($F_{(2)} = 0.73$, $p = 0.487$). However, a significant **interaction was found between Challenge and Trial** ($F_{(2)} = 4.51$, $p = 0.015$), indicating that the effect of Challenge on Total Time varied across trials. Post-hoc paired t-tests were conducted to compare Total Time before and after the Challenge for each Trial and Genotype. In the wild-type animals, no significant differences in Total Time were observed before and after the Challenge across any of the trials. In Trial 1 ($t_{(6)} = 2.95$, $p = 0.153$), Trial 2 ($t_{(6)} = 1.39$, $p > 0.9999$), and Trial 3 ($t_{(6)} = 1.99$, $p = 0.559$), the comparisons were not significant. In the 16p +/- animals, there was no significant difference between Total Time before and after the Challenge in Trial 1 ($t_{(6)} = 0.48$, $p > 0.9999$) or Trial 2 ($t_{(6)} = 3.54$, $p = 0.073$). However, **in Trial 3, a significant reduction in Total Time was observed after the Challenge** ($t_{(6)} = 4.19$, $p = 0.034$), with animals spending less time exploring stimuli after the Challenge (21.40 ± 1.74) compared to before the Challenge (36.23 ± 3.50).

D) Discrimination Index.

The discrimination index was provided to further clarify the differences between groups. The 2-way ANOVA revealed significant effects for **Treatment ($p = 0.0266$) and the Genotype × Treatment interaction** ($p = 0.0459$) but further post-hoc analysis did not reveal significant differences between groups.

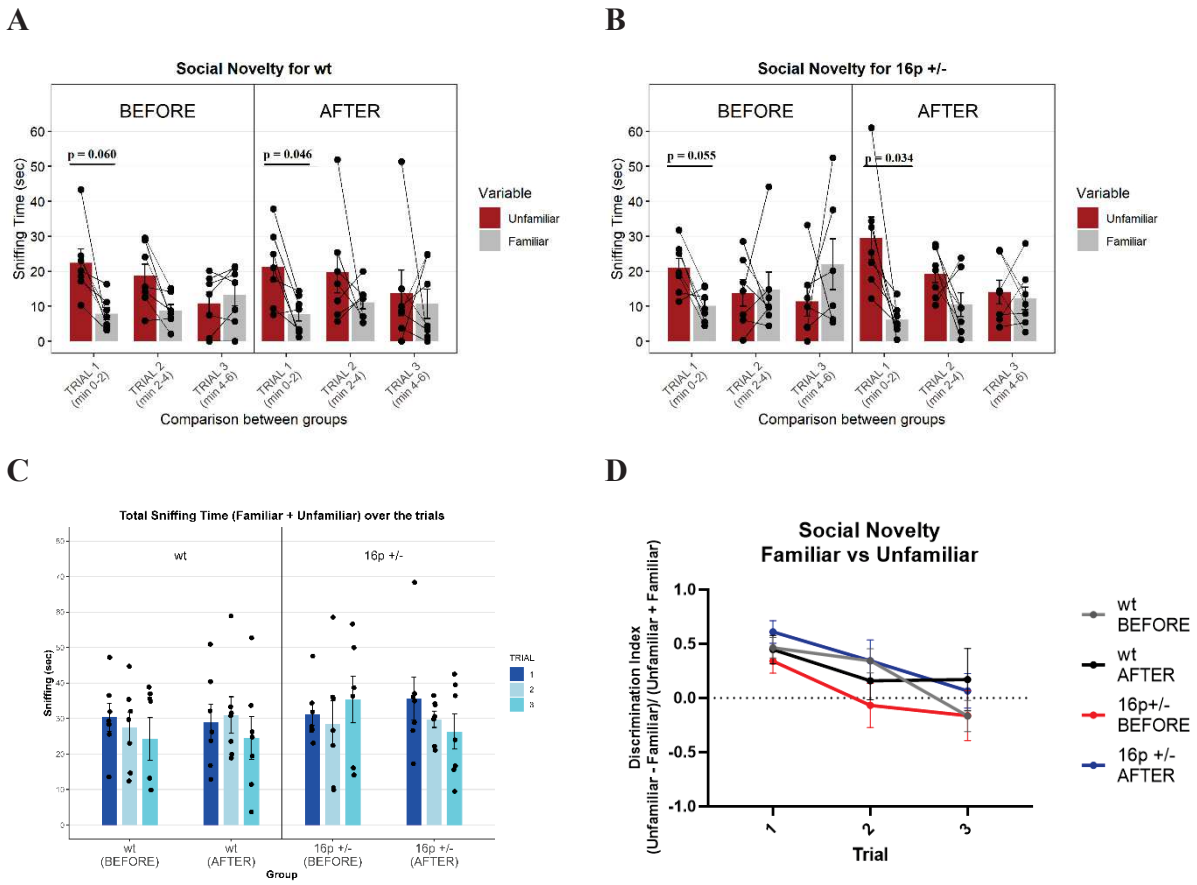


Figure 18: Social Novelty (Familiar vs Unfamiliar) test before and after challenge.

N(wt)= 7 (4 males, 3 females) and N(16p +/-) = 7 (3 males, 4 females).

A non-parametric ANOVA (f2.ld.f1) was conducted to evaluate the effects of Genotype, Challenge, Trial x Variable. The analysis revealed no significant main effects for Genotype ($F_{(1)} = 0.46$, $p = 0.497$) or Challenge ($F_{(1)} = 0.07$, $p = 0.799$). Additionally, no significant interactions were observed for Genotype × Challenge ($F_{(1)} = 0.07$, $p = 0.795$), Genotype × Trial × Variable ($F_{(3.98)} = 0.15$, $p = 0.961$), Challenge × Trial × Variable ($F_{(3.98)} = 0.68$, $p = 0.607$), or the three-way interaction of Genotype × Challenge × Trial × Variable ($F_{(3.98)} = 0.82$, $p = 0.512$). However, a significant interaction was found between Trial and Variable ($F_{(3.98)} = 10.78$, $p < 0.001$).

A) Familiar vs Unfamiliar in wild-type.

For the wild-types, paired t-tests revealed that for wild-type animals, at baseline, no significant differences were observed between sniffing of Unfamiliar and Familiar stimuli in Trial 1 ($p = 0.060$), Trial 2 ($p = 0.061$), and Trial 3 ($p = 0.607$). After the challenge, a significant difference was found in Trial 1 ($p = 0.046$), while Trials 2 and 3 did not show any significant differences: Trial 2 ($p = 0.823$) and Trial 3 ($p > 0.9999$).

B) Familiar vs Unfamiliar in 16p +/-

For the 16p +/- animals, at baseline, no significant differences were found between sniffing of Unfamiliar and Familiar stimuli in Trial 1 ($p = 0.055$), Trial 2 ($p > 0.9999$), and Trial 3 ($p > 0.9999$). After the challenge, a significant difference was observed in Trial 1 ($p = 0.034$). No significant differences were detected in Trial 2 ($p = 0.494$) and Trial 3 ($p > 0.9999$).

C) Total exploration.

A repeated measures ANOVA was conducted to examine the effects of Genotype, Challenge, and Trial on the total exploration time. The analysis revealed no significant main effects for Trial ($F_{(2)} = 0.82$, $p = 0.443$), Genotype ($F_{(1)} = 0.99$, $p = 0.323$) or Challenge ($F_{(1)} = 0.01$, $p = 0.919$), for Genotype × Challenge ($F_{(1)} = 0.08$, $p = 0.785$), Genotype × Trial ($F_{(2)} = 0.30$, $p = 0.740$), Challenge × Trial ($F_{(2)} = 0.27$, $p = 0.763$), or the three-way interaction of Genotype × Challenge × Trial ($F_{(2)} = 0.55$, $p = 0.581$). In the wild-type group, no significant differences were found in the total

exploration before and after the challenge, for each Trial (Trial 1: $p = 0.060$; Trial 2: $p = 0.061$; Trial 3: $p = 0.607$). In the 16p +/- group, no significant differences were observed between the total exploration before and after the challenge (Trial 1: $p = 0.055$; Trial 2: $p > 0.9999$; Trial 3: $p > 0.9999$).

D) Discrimination Index.

The DI was provided to further clarify the differences between groups. A positive DI indicates a preference for the Unfamiliar mouse. No significant main effects or interactions of Genotype or Challenge were observed at the 3-way ANOVA (with Trial, Genotype and Challenge as factors) where only the Trial had a main effect ($F_{(1.626, 19.51)} = 8.050$, $p = 0.0044$). Genotype ($p = 0.6522$), Treatment ($p = 0.1077$), Trial x Genotype ($p = 0.8566$), Trial x Treatment ($p = 0.7235$), Genotype x Treatment ($p = 0.2114$), and Trial x Genotype x Treatment ($p = 0.3392$) had no significant effects.

Emotion discrimination of negative affective states is impaired by an immunological challenge independently from the 16p +/- deletion.

The EDT was performed to assess the ability to discriminate negative affective states in conspecifics. Significant effects of the Variable (Stress vs Neutral) and interactions of the Variable with the Trials were found, indicating that animals are able to discriminate the affective states of conspecifics and that this ability changes during the test, with discrimination occurring mostly the first 2 minutes of the task (Figure 19 A, B). However, there were no significant main effects of the Genotype and of the Challenge, or of their interactions with the other factors, indicating that the ability does not depend on these factors or on their combination. The interaction between Challenge by Variable by Trial was approaching significance, suggesting that there could be some mild differences induced by the Challenge. This was further supported by the analysis of the DI indicating an interaction between Challenge and Trial (Figure 19 D) indicating a higher DI at baseline compared with the DI after the Challenge in Trial 1, further suggesting that the Challenge impairs the discrimination during the first 2 minutes of the test. These results suggest that the immunological challenge could mildly impair the ability to discriminate negative affective states of conspecifics in mice, independently from Genotype, therefore without inducing genotype-specific effects in 16p +/- adult mice compared with wild-type littermates. The total exploration time of the stimuli was not significantly affected by Genotype or Challenge or by their interaction, indicating that the immunological challenge doesn't affect the exploratory behavior both in wild type and in 16p +/- mice during the EDT.

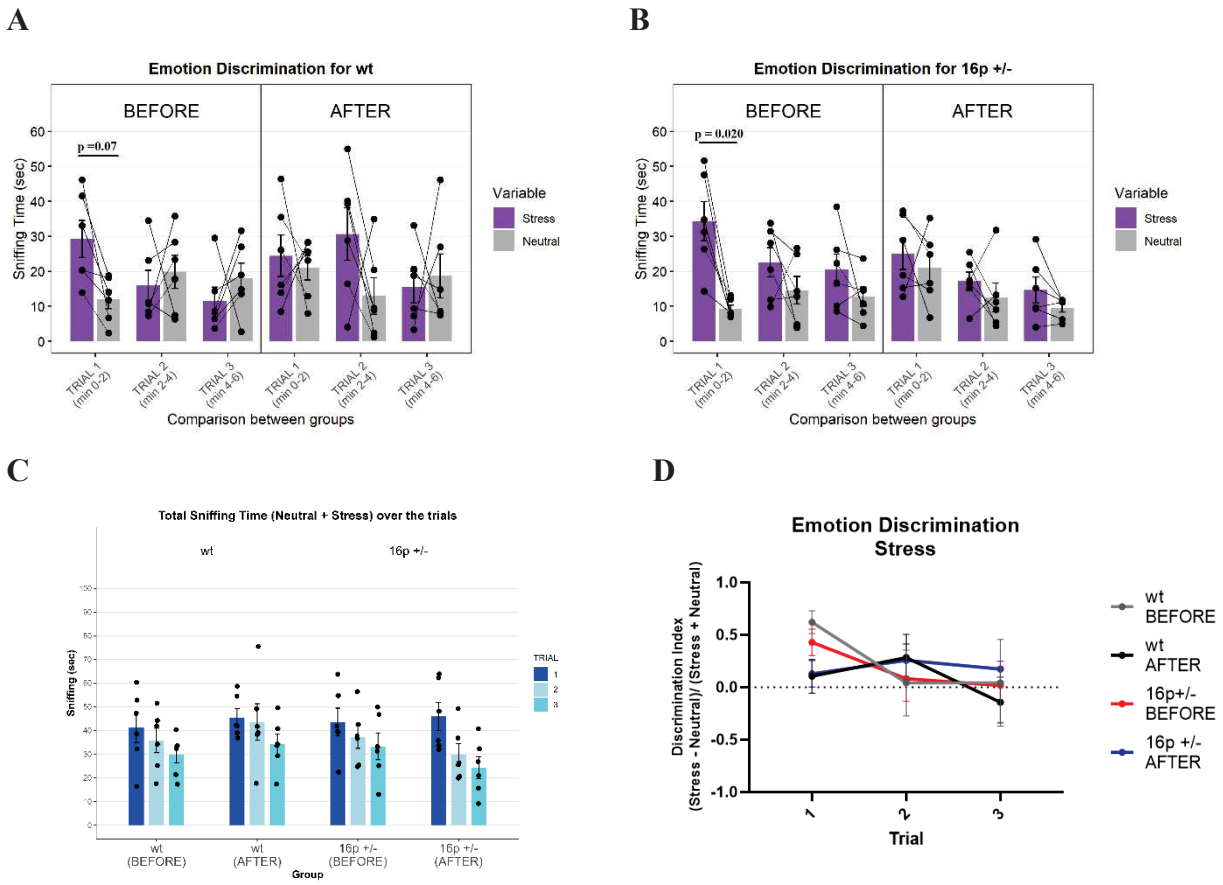


Figure 19: Emotion Discrimination (Stress vs Neutral) before and after an immunological challenge *N(wt)= 6 (3 males, 3 females) and N(16p +/-) = 6 (4 males, 2 females)*.

A non-parametric ANOVA (f2.ld.f1) was conducted to evaluate the effects of Genotype, Challenge, Trial, and Variable (Stress vs Neutral). A significant interaction was observed between Trial and Variable ($F_{(3.60)}$ = 5.18, p = 0.001), indicating that the discrimination of Stress changes over trials. **The interaction Challenge × Trial × Variable (F(3.60) = 2.10, p = 0.085) was approaching significance**. The analysis revealed no significant main effects for Genotype ($F_{(1)}$ = 0.13, p = 0.722) or Challenge ($F_{(1)}$ = 0.02, p = 0.886). Additionally, no significant interactions were observed for Genotype × Challenge ($F_{(1)}$ = 0.94, p = 0.333), Genotype × Trial × Variable ($F_{(3.60)}$ = 1.02, p = 0.393), or the three-way interaction of Genotype × Challenge × Trial × Variable ($F_{(3.60)}$ = 0.91, p = 0.449). To further clarify the effects observed at the 3-way interaction between Challenge, Trial and Variable, paired t-tests were conducted to explore differences between the exploration of Stress and Neutral stimuli for each Trial, within each Genotype.

A) Emotion Discrimination: Stress. Wild type.

For wild-type animals, before the challenge, no significant differences were observed between the sniffing of the Stress and Neutral stimuli across all trials, with only a trend after Bonferroni correction for multiple comparisons in Trial 1 (p = 0.0700), and no significance for Trial 2 (p > 0.9999), and Trial 3 (p > 0.9999). After the challenge, in the wild-type group, no significant differences were found between stress and neutral conditions: Trial 1 (p > 0.9999), Trial 2 (p = 0.469), and Trial 3 (p > 0.9999). The post-hoc analysis using Dunn's test with Bonferroni correction, comparing the sniffing time of the Stressed and of the Neutral before and after the challenge, found no significant differences in any of the Trials or conditions (all adjusted p-values > 0.9999).

B) Emotion Discrimination: Stress. 16p +/-.

16p +/- animals before the challenge, were significantly preferring the Stressed stimulus in Trial 1 (p = 0.020), but no significant differences were observed in Trial 2 (p = 0.818), and Trial 3 (p = 0.559). After the challenge, the preference for the stressed stimuli was not significant anymore in Trial 1 (p > 0.9999), Trial 2 (p > 0.9999), and Trial 3 (p = 0.420). The post-hoc analysis using Dunn's test with Bonferroni correction, comparing the sniffing time of the Stressed with the Neutral before and after the challenge, found no significant differences in any of the trials and conditions (all adjusted p-values = 1).

C) Total exploration of stimuli.

A repeated measures ANOVA was conducted to evaluate the effects of Genotype, Challenge, Trial, and their interactions on Total exploration Time. The analysis revealed no significant main effects for Trial ($F_{(2)} = 0.30$, $p = 0.744$), Genotype ($F_{(1)} = 0.89$, $p = 0.349$) or Challenge ($F_{(1)} = 0.01$, $p = 0.931$). Additionally, there were no significant interactions for Genotype × Challenge ($F_{(1)} = 2.58$, $p = 0.114$), Genotype × Trial ($F_{(2)} = 0.44$, $p = 0.646$), Challenge × Trial ($F_{(2)} = 0.07$, $p = 0.931$), or the three-way interaction of Genotype × Challenge × Trial ($F_{(2)} = 0.41$, $p = 0.667$).

D) Discrimination Index.

The mixed-effects model analysis for the DI showed that the effect of Trial was not statistically significant ($F_{(1.775, 21.30)} = 2.974$, $p = 0.078$), nor was Genotype ($F_{(1, 12)} = 0.322$, $p = 0.581$) or Challenge ($F_{(1.000, 12.00)} = 0.0022$, $p = 0.963$). The **interaction between Trial and Challenge was significant** ($F_{(1.975, 11.85)} = 5.157$, $p = 0.025$). There was no significant interaction between Trial and Genotype ($F_{(2, 12)} = 0.610$, $p = 0.559$), Genotype and Challenge ($F_{(1, 12)} = 0.0202$, $p = 0.889$), or the three-way interaction of Trial, Genotype, and Challenge ($F_{(2, 12)} = 0.467$, $p = 0.638$). At the following mixed analysis after collapsing the Genotypes, in order to evaluate only **Trial and Challenge**, the interaction was significant ($F_{(2, 44)} = 3.179$, $p = 0.0513$), while the other main effects were not for Trial ($F_{(1.912, 42.06)} = 3.044$, $p = 0.0604$) and Challenge ($F_{(1, 22)} = 0.2242$, $p = 0.6405$). The Sidak's multiple comparisons test indicated that **in Trial 1, the difference between the DI before the challenge (mean = 0.1176) and after the challenge (mean = 0.4867) was statistically significant** (mean difference = -0.3691, 95% CI = -0.7244 to -0.01392, $p = 0.0401$), while there was no difference in Trial 2 (mean difference = 0.2008, 95% CI = -0.3554 to 0.7569, $p = 0.7324$) and Trial 3 (mean difference = -0.009304, 95% CI = -0.5277 to 0.5091, $p > 0.9999$).

Discussion

In contrast with my hypothesis, the behavioral assessment did not show any differences in social novelty, social memory and emotion discrimination in 16p +/- or in wild-type mice following a mild immunological challenge in adulthood. In particular, the result do not support the double-hit hypothesis in adulthood, as indicated by the absence of significant differences between the 16p +/- animals and their wild type siblings.

The performance of both the groups at baseline, which was in line with the one described in CHAPTER 2, was generally not different from the one observed after the challenge. This was observed despite preliminary evidence that the 40 mg/kg Poly I:C administration is able to temporarily change the brain inflammatory status, as indicated by the microglial morphology analysis in wild-type animals.

However, and in line with this, a mild effect of the Challenge (which was independent from the Genotype) was observed on sociability, suggesting that this domain might be particularly susceptible from immunological interferences. This was evident as a trend in the habituation-dishabituation task and more clearly in the Object vs. Mouse task, where there was a significant reduction in the sniffing time for the Mouse (but not for the Object). This effect could be related to an impairment in olfactory abilities caused by Poly I:C administration, which is known to induce a IL-17 mediated olfactory cell apoptosis following intranasal administration [144,145]. This is supported also by evidence from the behavior during the EDT that was impaired after the immunological challenge: in fact, in this task, emotion discrimination strongly depends on both visual and olfactory cues [103]. However, given that the in our case Poly I:C was administered IP, and that the tests were performed after a 10 days-time-window which would have allowed for at least a partial recovery, probably this is not the mechanism affecting sociability. Different mechanisms could be responsible for this sociability alteration, which needs to be addressed with further control experiments, such as by using only social odors instead of mice [103,128], and after increasing the sample size. Moreover, a further assessment of microglial morphology in 16p +/- mice and in their wild type siblings should confirm the trajectory of the microglial response that were observed in the experiments with C57BL/6J animals reported before. This approach must be

combined with a peripheral cytokine levels assessment, in order to further understand how these changes correlate with behavioral effects.

Overall, the absence of significant results supporting the double-hit hypothesis, might indicate that the immune challenge administered in adulthood, when the brain is already fully developed, is not sufficient to induce significant behavioral deficits, and probably this is because the circuits and the cellular population relevant for social domains are already mature. Critical developmental windows, such as adolescence or pregnancy, may be more sensitive to such manipulations.

One intriguing hypothesis is that the social abilities might not be affected in adulthood due to the fact that 16p +/- animals are housed with the wild-type siblings during their whole life, and that this condition might serve as a social training and might reduce social abnormalities which could be detected in earlier stages of life. In fact, the confounding effect of mixed housing condition in determining the social behavioral outcome in 16p +/- mice has been established before [127]. Previous assessments were conducted in early stages of the mouse life by assessing ultrasonic vocalization and motor skills in young 16p11.2 mice [120], but to my knowledge a full assessment of social behavior in 16p11.2 adolescent mice was never performed. Therefore, as a next step, in CHAPTER 4 I will investigate whether this strain presents behavioral alterations during earlier life stages, where potential effects may be more evident than in adulthood and more relevant for NND where social alterations are usually present since the earliest infancy.

CHAPTER 4

Social behavior in a mouse model of 16p11.2 deletion during adolescence

Abstract

Adult 16p11.2 deleted mice in C57BL/6J background do not display social impairments in sociability, social novelty, social memory and emotion discrimination. Adolescence is a critical period for social development, being characterized by ongoing brain maturation especially for PFC. The emotion discrimination test for positive and negative affective states was never applied in adolescent mice, and no previous comprehensive evaluation of the social behavior of 16p11.2 mice was performed in adolescence. To fill these gaps, the full social battery assessment was applied in adolescent 16p11.2 mice and in their wild-type littermates.

Results indicate that sociability, social memory, recognition of social novelty and discrimination of negative affective states are abilities that can be effectively assessed using the social battery in adolescence. However, both wild-type and 16p11.2 deleted mice showed impairments in discriminating positive affective states, thus suggesting that this paradigm might not be suitable for the testing of emotions in adolescence, or that the ability to discriminate positive emotions develops later in the life of mice.

During adolescence, 16p11.2 deleted mice exhibit no major impairments in social behavior across domains such as sociability, social memory, recognition of social novelty, and discrimination of negative affective states. However, only mild effects emerged, hinting at a subtle phenotype. Specifically, in tasks involving choices between social (mouse) and non-social (object) stimuli, 16p11.2 mice displayed a tendency toward increased exploration of non-social stimuli compared with the wild-type littermates. Moreover, they were spending more time with the familiar mouse compared with their wild-type littermates. In conclusion, while no significant impairments in social behavior were observed during adolescence in 16p11.2 deleted mice, subtle differences emerged, emphasizing the importance of adolescence as a window to study nuanced behavioral phenotypes for an assessment of the interaction between genetic vulnerability and environmental factors.

Introduction

Adolescence is a critical period of development in social animals, including mice, and it is characterized by profound behavioral and neurobiological transformations that lay the foundation for adult social interactions and cognitive competencies [146]. Social activities in adolescence represent an essential learning phase during which individuals develop and refine social rules, cooperative behaviors, and communication strategies [147]. Such interactions are vital for survival, as they prepare individuals to navigate complex social structures in adulthood, and are paired with important changes in the brain structure and connectivity [148].

From a behavioral perspective, adolescence is an ideal time to detect late manifestations of NDD: while neurodevelopmental disruptions may originate in prenatal or early postnatal stages, their effects on behavior and cognition often surface more clearly during adolescence or young adulthood, as it is usually the case for schizophrenia [149,150]. This is because the maturation of neural circuits relevant for complex social and cognitive functions reaches a critical threshold during this time [151]. Processes such as experience-mediated synaptic pruning and the refinement of excitatory-inhibitory balance are at their peak during this period [152,153], particularly in regions like the PFC where they are foundational for the correct development of executive functions [154] and for higher-order social functions such as social cognition, emotional regulation, and social decision-making [13,155,156]. If these processes are disrupted during adolescence, whether due to already-present genetic factors, environmental stressors, or inflammatory insults, the resulting alterations in circuit connectivity and function can have enduring consequences on behavior and cognitive abilities [157–159]. Moreover, people affected by less- severe forms of NDD, and relying on compensatory strategies during childhood, often struggle in interacting with peers during adolescence [160], when the social demands are increasing [12,14]. Social impairments, atypical interactions with peers, or reduced sociability during this period can serve as indicators of an undiagnosed NDD but also act as a further stressor when they lead to social isolation or social defeat [161].

Studying adolescence in mice provides a unique opportunity to explore how environmental factors, such as immune challenges or stress, interact with genetic vulnerabilities to influence developmental trajectories. Understanding how genetic and environmental factors converge during adolescence can thus provide valuable information for designing strategies to mitigate the long-

term effects of neurodevelopmental disorders. Given these premises, in this Chapter I will assess the social behavior of 16p11.2 mice during mid adolescence (PND 30-45) [162]. The aim is to ascertain if social behavioral impairments relevant for ASD are present in this early phase of life, when mice are still learning the social structures: in particular, emotion recognition is remarkably important during adolescence in humans, where it serves as a fundamental for a successful learning of complex social interactions [163], and this ability was never assessed before in adolescent wild-type mice nor in models relevant for NDD.

Methods

Unless further specified here, the behavioral tests used, and the statistical analysis, have already been described in CHAPTER 2.

Mice

Adolescent males and females 16p11.2 deleted mice (Mills' model, all born from breeding in the facility) were used, with an age range between 30 and 49 days of age. The 16p11.2 mutation was only transmitted through the paternal lineage, in order to avoid potential confounders due to aberrant maternal care: for this reason, mothers were always C57BL/6J. Animals were housed two to four per cage in a climate-controlled (22 ± 2 C) and specific pathogen-free animal facility, with ad libitum access to food and water throughout, a standard environmental enrichment (material for nest and cardboard house), and with a 12-hour light/dark cycle (7pm/7am schedule). All the animals were kept in a mixed housing condition, with 16p +/- (deleted) mice and their wild type siblings housed together. To avoid litter effects, no more than 2 males and 2 females (one per genotype) were used for behavioral testing. All the animals were ear-tagged for genotype assessment not earlier than P21, weaned at P28 and separated in same-sex cages of 2-4 siblings.

Behavioral protocol

All the animals underwent the same testing battery described in CHAPTER 2 for the adult 16p +/- mice. However, given that adolescence is a short time-window, the tests were performed more closely to each other compared with adulthood, and habituation to the testing apparatus lasted only 2 days rather than 3. After weaning at PND 28, animals were left undisturbed for 2 days in order to let them adapt to the new housing conditions. Following that, they were handled for 3 consecutive days in order to habituate to the experimenter and to reduce anxiety levels. Handling was performed both for the observers (the 16p +/- and the wild-type mice) and for the stimuli (C57BL/6J mice age matched for sex and age with the observers). Following handling, animals underwent the habituation-dishabituation task, around PND 34. Then, they were habituated to the emotion discrimination apparatus for 2 sessions, in 2 different days, lasting 10 minutes each. Observers and stimuli were habituated in different moments in order to prevent observer to get

familiar with the stimuli odors, and cages were cleaned with 70% ethanol between an animal and the following one. Then, the Object vs Mouse and Familiar vs Unfamiliar test was performed, followed by the Familiar vs Unfamiliar test. After 2-3 days of recovery, the emotion discrimination – Relief was assessed. Mice were left then undisturbed for extra 3 days, and then they underwent the emotion discrimination test – Stress.

Results

16p +/- adolescent mice display normal sociability, social memory and social novelty recognition.

The first test that was performed was the habituation-dishabituation task. This task allows an exploration of social behavior in a context of free social interaction.

Both wild-type and 16p +/- mice displayed normal sociability, as indicated by the total exploration time from Trial 1 to Trial 4 (Figure 20 B), normal social memory, indicated by the significant decline in the sniffing time from Trial 1 to 4 (Figure 20 A), and normal recognition of social novelty, as indicated by the novelty index (Figure 20 C), which resumes the difference in sniffing between the unfamiliar mice (Trial 1 and Trial 5) and the familiar mouse (Trial 4) (Figure 20 A). Notably, there were no differences between adolescent 16p +/- and wild-type during this test, indicating that in the context of free social interaction 16p +/- do not display abnormalities in any of these domains, compared with their cagemates.

Sociability and Social Novelty recognition were also assessed with the Object vs Mouse (Figure 21) and with the Familiar vs Unfamiliar test (Figure 22), respectively. During such tests, choices are more restricted as the observer is expected to express a preference for a social (Mouse) versus a non-social stimulus (Object) or for a Familiar versus a non-Familiar social stimulus. During the Sociability stage of the test (Figure 21), the observers were able to discriminate between the social and the non-social stimulus, by significantly preferring the first, as indicated by the main effect observed for the factor “Variable” (Figure 21 A). The interaction between Variable and Trial indicates that the preference was varying across Trials, with a significant discrimination occurring mostly during the first 2 minutes of the test (Figure 21 A and C). Interestingly, there was a significant main effect of the Genotype, indicating some group differences between 16p +/- and wild-type. The effect was further explored at the post hoc: however, none of the post-hoc tests was significant, and only a trend was found for the exploration of the Object, with higher interaction times in the 16p +/- compared with wild-type during Trial 3. To better understand the main effect of Genotype, more analysis were performed by excluding the effect of the Trial in the model, by analyzing pulled data in which all the interaction with the Object or with the Mouse were summed

for all the Trials, in order to quantify the total exploration for the Object and for the Mouse during the 6 minutes (Figure 21 D). Again, the preference for the Mouse was significant in both groups, but no group differences were observed in the total time spent sniffing the Object or the Mouse. Moreover, the total exploration time was not different between the two groups, indicating no alterations in the exploratory behavior in 16p +/- during this task, compared with wild-type (Figure 21 B).

Following the Sociability phase, the Object was replaced with an Unfamiliar mouse and the test continued for 6 more minutes, for the next Social Novelty stage (Figure 22). Again, animals were significantly preferring the Unfamiliar mouse, indicating normal recognition of Social Novelty (Figure 22 A, B, D). The interaction between Trial and Variable and the following post-hoc analysis indicated that preference was significant during the first two-minutes of the test. A main effect of Genotype was observed, suggesting a different type of behavior between the two groups. To further investigate this effect, post-hoc tests were performed, but there were no differences between groups in the time spent sniffing the Familiar or the Unfamiliar Mouse during each trial. Given that the analysis on the total exploration time revealed a significant effect for the Genotype (Figure 22 C), with 16p +/- exploring more the stimuli compared with wild-type, the percentage of preference for each type of stimulus was calculated (i.e: %Unfamiliar = Unfamiliar (s)/Total exploration(s) *100) for each Trial (Figure 22 B). This was done in order to control from the increased explorative behavior observed in the 16p +/- mice (Figure 22 C). No significant effects of Genotype were observed, while the effects of the Variable and its interaction with the Trial were still significant. These results indicate overall a significant preference for the Unfamiliar mouse, independent from the Genotype, especially during the first Trial, and normal recognition of the Social Novelty in 16p +/- adolescent mice, in line with the results observed during the habituation dishabituation task.

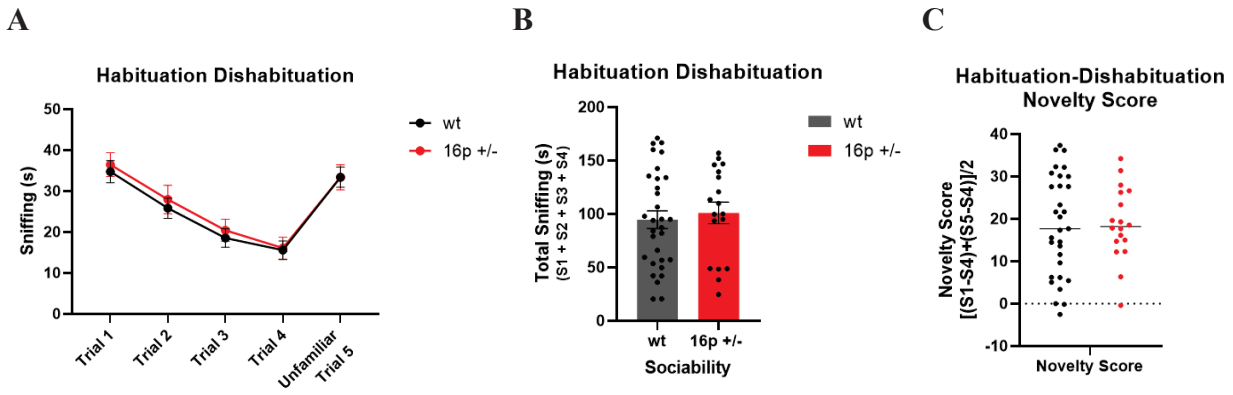


Figure 20: Habituation Dishabituation task in 16p +/- and wild-type adolescent mice.

N(wt)= 31 (16 males, 15 females) and N(16p +/-) = 18 (13 males, 5 females).

A) Habituation Dishabituation.

Sniffing time across trials was analyzed using a Two-way RM ANOVA. There was a significant effect of trial ($F_{(3.263, 153.4)} = 43.42$, $p < 0.0001$). There was no main effect of Genotype ($F_{(1, 47)} = 0.1535$, $p = 0.6970$), and no interaction between Trial and Genotype ($F_{(4, 188)} = 0.1330$, $P = 0.9701$). The Sidak's multiple comparisons test was used to analyze simple effects between Trials, within Genotypes.

In the wild-type group, there was a significant difference between Trial 1 and Trial 4 ($p < 0.0001$, $t_{(30)} = 7.542$) and between Trial 4 and Trial 5 ($p < 0.0001$, $t_{(30)} = 7.693$), indicting normal social memory and social novelty recognition, respectively. No significant difference was found between Trial 1 and Trial 5 ($p = 0.9997$, $t_{(30)} = 0.5902$) where observer were sniffing the novel stimuli for the first time, further confirming the normal recognition of social novelty. Similarly, in the 16p +/- group, normal social memory was preserved, as indicated by the significant difference between Trial 1 and Trial 4 ($p < 0.0001$, $t_{(17)} = 7.270$). Recognition of social novelty was normal as well, as indicated the significant difference between Trial 4 and Trial 5 ($p = 0.0001$, $t_{(17)} = 6.015$) and by the absence of significant difference between Trial 1 and Trial 5 ($p = 0.9971$, $t_{(17)} = 0.7859$).

B) Total Sociability.

Total sociability was measured as the total time spent sniffing the stimulus from Trial 1 to Trial 4. No significant differences were observed between 16p +/- and wild-type. In particular, the unpaired t-test indicates no effect of the Genotype ($p = 0.6384$, $t_{(47)} = 0.4730$). The mean social interaction was slightly higher in the 16p+/- group (101.0) compared to the wild-type group (94.74) but not significant.

C) Novelty Score

The novelty score (average of the difference between Trial 1 and 4, and between Trial 1 and 5, indicated in the graph as S1, S4 and S1 and S4, respectively) indicates that both groups were able to discriminate social novelty. The paired t-test comparing the Novelty Score between the wild-type group and the 16p+/- group revealed no significant difference ($p = 0.4753$, $t_{(17)} = 0.7301$), indicating no effect of the Genotype.

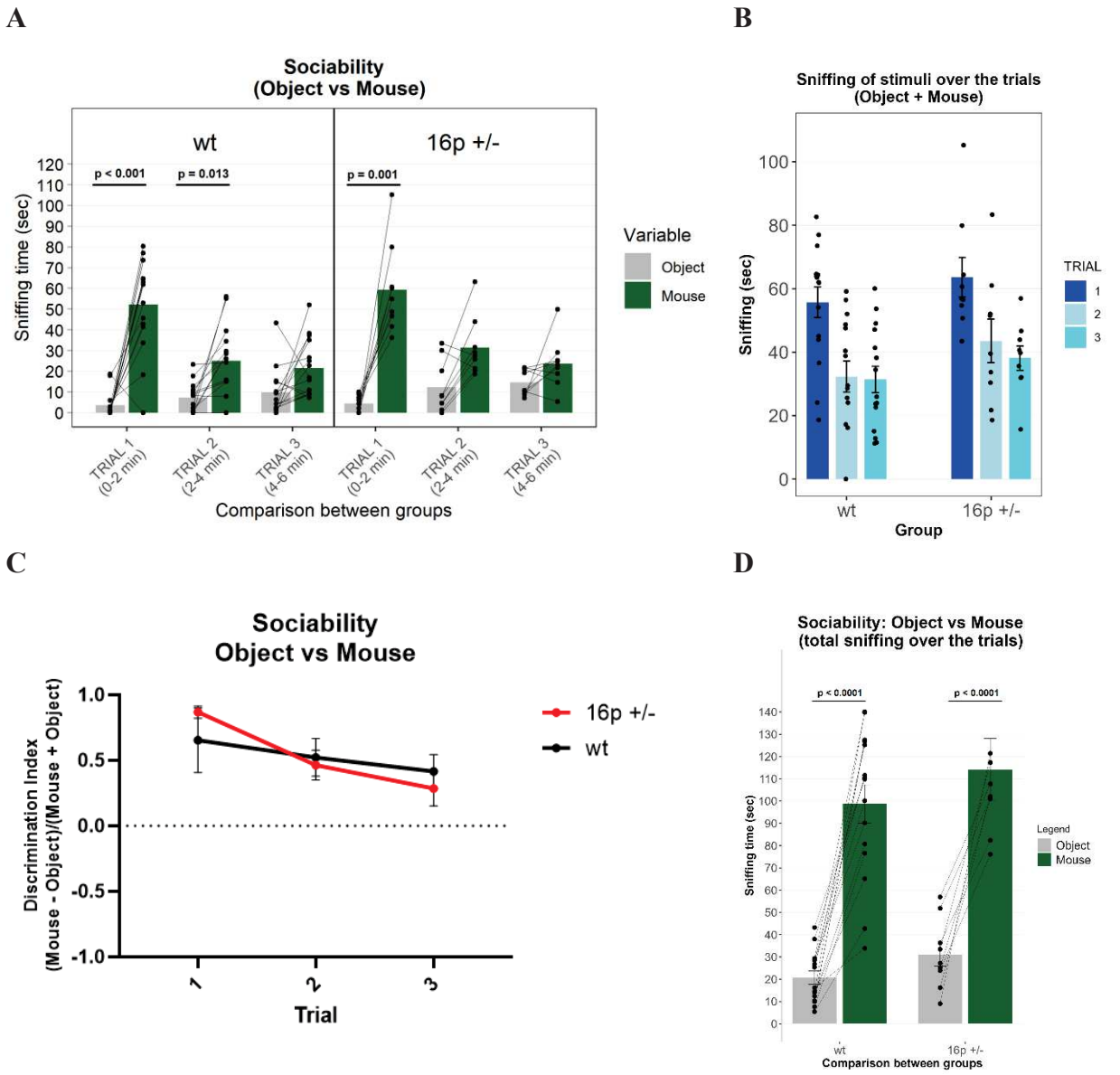


Figure 21: Sociability (Object vs Mouse) in 16p +/- and wild-type adolescent mice

N(wt)= 15 (8 males, 7 females); N(16p +/-) = 9 (6 males, 3 females).

A) Sociability (Object vs Mouse)

The graph represents the exploration of the Object and of the Mouse for each 2-min trial of the task. The non-parametric test (f1.ld.f2) was performed including Genotype as factor, and Trial and Variable (Object vs. Mouse) as repeated measures. There was a significant main effect of the Variable (Object vs Mouse, $F = 151.8015$, $p < 0.0001$), while Trial was not significant ($F = 0.6643$, $p = 0.4910$). There was a significant interaction between Variable and Trial ($F = 18.664$, $p < 0.0001$). **There was a significant main effect of Genotype ($F = 6.079$, $p = 0.0137$)** but no significant interactions: Genotype by Trial ($F = 0.3889$, $p = 0.644$); Genotype by Variable ($F = 0.0231$, $p = 0.879$), Genotype by Trial by Variable ($F = 0.4084$, $p = 0.647$).

The post hoc comparisons (Dunn post-hoc with Bonferroni adjustment) were conducted to examine differences between Genotype groups (wild-type and 16p+/-) across Variables (Object and Mouse) and Trial levels.

In the wild-type group, preference for the Mouse was significant in Trial 1 ($p < 0.0001$) and in Trial 2 ($p = 0.0126$), but not in Trial 3 ($p = 0.2031$). In the 16p+/- group, preference for the Mouse was significant in Trial 1 ($p = 0.0009$), but not in Trial 2 ($p = 0.0589$), and in Trial 3 ($p = 0.6843$).

For the Object variable, no significant difference was observed in Trial 1 between 16p+/- and wild-type ($p = 0.4083$) and in Trial 2 ($p = 0.3871$). In Trial 3, there was a significant difference between 16p+/- and wild-type in the time spent exploring the Object ($p = 0.0458$). For the Mouse variable, the comparisons did not yield significant results in Trial 1 ($p = 0.4436$), Trial 2 ($p = 0.3871$), Trial 3 ($p = 0.7194$).

B) Total exploration of stimuli..

A repeated-measures ANOVA was conducted to evaluate differences in the total exploration time of the stimuli, including Genotype and Trial as factors. No significant effect of Genotype was observed ($F_{(1, 22)} = 2.318$, $p = 0.142$). A significant main effect of Trial was found ($F_{(1, 22)} = 34.516$, $p < 0.001$). The interaction between Genotype and Trial was not significant ($F_{(1, 22)} = 0.017$, $p = 0.897$). The post hoc analysis using Sidak's multiple comparisons test evaluated differences between trials within each Genotype (16p +/- and wild-type).

For the wild-type group, significant differences were found both between Trial 1 and Trial 2 ($t_{(14)} = 4.01$, $p = 0.0039$) and between Trial 1 and Trial 3 ($t_{(14)} = 4.11$, $p = 0.0032$). No significant difference was observed between Trial 2 and Trial 3 ($t_{(14)} = 0.19$, $p = 0.9969$).

For the 16p +/- group, no significant difference was found between Trial 1 and Trial 2 ($t_{(8)} = 2.87$, $p = 0.0613$), while a significant difference emerged between Trial 1 and Trial 3 ($t_{(8)} = 4.80$, $p = 0.0041$). No significant difference was observed between Trial 2 and Trial 3 ($t_{(8)} = 1.17$, $p = 0.6204$).

C) Discrimination Index.

The DI is reported to facilitate the comparison between groups. A positive DI (indicating preference for Mouse) was observed for both the groups. There was a significant effect of Trial ($F_{(1.940, 53.34)} = 4.705$, $p = 0.0139$). However, there was no significant effect of Genotype ($F_{(1, 55)} = 0.006$, $p = 0.9376$) or the interaction between Trial and Genotype ($F(2, 55) = 0.900$, $p = 0.4125$).

D) Exploration of Object and of Mouse during the entire test.

To further understand if the effect of Genotype was more evident independently of the Trials, the total amount of exploration during the experiment was calculated for the Object and the Mouse, for each group, and a Friedman test followed by Wilcoxon post-hoc was applied. The Friedman test was significant ($Q_{(1)} = 24$, $p < 0.001$).

At the Wilcoxon test for the Variable, there was a significant preference for the Mouse both in the 16p +/- group ($W = 81$, $p < 0.001$), and in the wild-type group ($W = 222$, $p < 0.001$).

The Mann-Whitney U test to compare the sniffing time for the stimuli between the Genotypes, indicated no significant effect for the overall exploration of Mouse between 16p +/- and wild-type ($p = 0.861$, mean 114.19 for 16p +/- and 98.70 for wild-type). For Object, the test ($p = 0.075$) revealed only a trend, with mean interaction times of 31.11 for 16p +/- and 20.72 for wild-type.

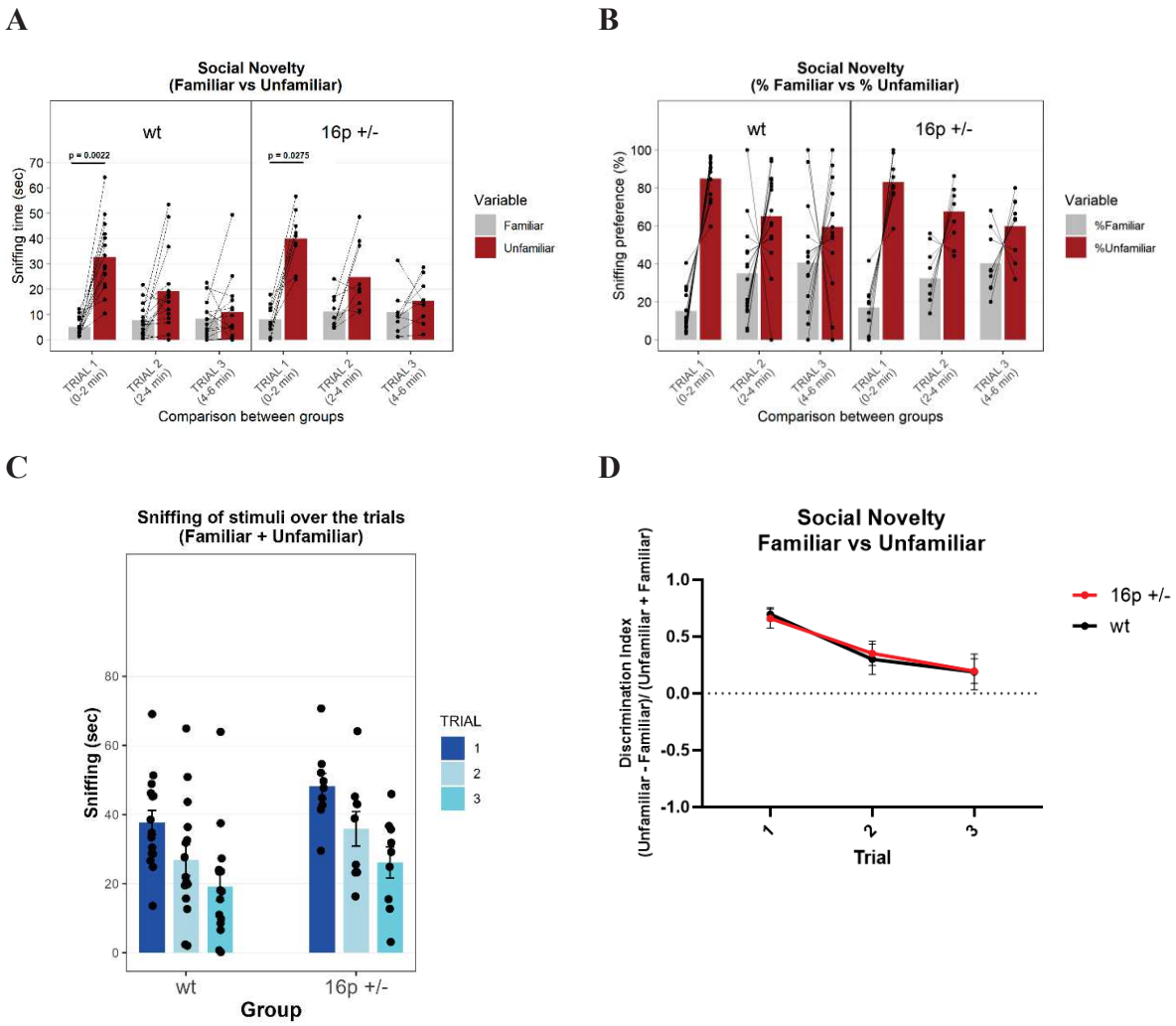


Figure 22: Social Novelty (Familiar vs Unfamiliar) in adolescent 16p +/- and wild-type littermates.

N(wt)= 15 (8 males, 7 females) and N(16p +/-) = 9 (6 males, 3 females).

A) Social Novelty (Familiar vs Unfamiliar). The graph reports the sniffing time.

A non-parametric test was performed (f1.ld.f2) was performed to evaluate the role of Genotype, Trial and Variable (Familiar vs Unfamiliar). There were **significant main effects for Genotype ($F_{(1)} = 5.21$, $p = 0.022$), Trial ($F_{(1.79)} = 7.08$, $p = 0.001$), and Variable ($F_{(1)} = 78.90$, $p < 0.001$).** Additionally, there was a significant interaction between Variable and Trial ($F_{(1.74)} = 18.69$, $p < 0.001$). No significant interactions were observed between Genotype and Trial ($F_{(1.79)} = 0.13$, $p = 0.858$), Genotype and Variable ($F_{(1)} = 0.14$, $p = 0.705$), or Genotype, Trial, and Variable ($F_{(1.74)} = 0.41$, $p = 0.635$). Given the main effect of Trial, Variable, and their interaction, a Dunn test with Bonferroni correction was performed within each group to confirm the ability to discriminate social novelty for each trial.

Discrimination was significant for wild-type during the first 2 minutes of the test, in which animals were preferring the Unfamiliar mouse, while Trials 2 and 3 were not significant: Trial 1 ($p = 0.0022$), Trial 2 ($p = 0.0553$), Trial 3 ($p = 0.9608$).

16p+/- were able to discriminate as well during the first 2 minutes of the test, significantly preferring the Unfamiliar mouse, while Trials 2 and 3 did not reveal significant differences: Trial 1 ($p = 0.0275$), Trial 2 ($p = 0.0989$), Trial 3 ($p = 0.8590$).

The post hoc analyses (Dunn with Bonferroni correction) comparing the sniffing time for the Familiar and for the Unfamiliar stimuli between 16p +/- and wild-type variables did not reveal significant differences. For the Unfamiliar

condition: Trial 1 ($p = 0.161$), Trial 2 ($p = 0.222$), Trial 3 ($p = 0.101$). For the Familiar condition: Trial 1 ($p = 0.222$), Trial 2 ($p = 0.200$), Trial 3 ($p = 0.531$).

B) Social Novelty (Familiar vs Unfamiliar). The graph reports the percentage of preference.

To further exclude that the increase in the total exploration observed in 16p+/- could affect the results of the test, this graph represents the exploration of the Familiar and of the Unfamilar mouse expressed as percentage of the total exploration for each Trial. The repeated measures ANOVA (Genotype as factor and Variable and Trial as repeated measures) revealed a main effect for the Variable (%Familiar vs %Unfamiliar): $F_{(1, 110)} = 128.56$, $p < 0.001$. No main effect for the Trial ($F_{(2, 110)} = 3.73 \times 10^{-30}$, $p > 0.9999$) was observed, but there was a significant interaction between Trial and Variable ($F_{(2, 110)} = 17.61$, $p < 0.001$).

There was no significant main effects for Genotype ($F_{(1, 22)} = 5.12 \times 10^{-30}$, $p > 0.9999$) and for the interactions between Genotype and Trial ($F_{(2, 110)} = 1.95 \times 10^{-30}$, $p > 0.9999$), Genotype and Variable ($F_{(1, 110)} = 0.008$, $p = 0.929$), or Genotype, Trial, and Variable ($F_{(2, 110)} = 0.124$, $p = 0.883$).

C) Total exploration of stimuli.

For the total exploration time, a Kruskal-Wallis test was performed to assess the differences between Genotypes (16p +/- and wild-type). There was a significant main effect of Genotype with a Kruskal-Wallis ($H = 4.96$, $df = 1$, $p = 0.0259$). Post-hoc analysis using Dunn's test and Bonferroni correction indicated a significant difference between 16p +/- and wild-type ($p = 0.0259$), **with 16p +/- spending significantly more time exploring the stimuli**, overall. To further evaluate the differences between groups by considering each Trial, analysis using Dunn's test and Bonferroni correction was performed, revealing no significance in any of the comparisons between groups. (Trial 1, mean difference = -10.44, $p = 0.0526$; Trial 2, mean difference = -9.00, $p = 0.1611$; Trial 3, mean difference = -1.52, $p = 0.1284$).

D) Discrimination Index.

The DI is reported to facilitate the comparison between groups. A positive DI (indicating preference for Unfamiliar) was observed for all the groups. At the 2-way RM-ANOVA there was a significant main effect of Trial ($F_{(1.864, 41.00)} = 8.456$, $p = 0.0011$). No significant main effect of Genotype ($F_{(1, 22)} = 0.003612$, $p = 0.9526$) or interaction between Trial and Genotype ($F_{(2, 44)} = 0.06552$, $p = 0.9367$) were observed.

Adolescent 16p +/- mice display normal recognition of negative affective states. Adolescent 16p +/- and their wild-type littermates are not able to discriminate positive affective states.

The EDT for positive affective states was first performed. No main effects of the Variable, or of the Genotype, or interactions were observed, indicating that overall nor the 16p +/- or the wild-type mice were able to discriminate the relieved state in conspecifics (Figure 23 A, C).

During the EDT for the negative affective states, animals were instead able to discriminate between a stressed and a neutral conspecific, as indicated by the significant effect of the Variable. Moreover, discrimination occurred especially during the first 2 minutes of the test, when it was significant in both 16p +/- and in wild-type. However, no significant effect of the Genotype or of its interaction with other variables was observed, indicating no difference between 16p +/- and wild-type in the ability to discriminate negative emotions (Figure 24 A, C). The total exploration for the stimuli was not affected by the Genotype as well, both in the positive and negative affective states recognition tests (Figure 23 B and Figure 24 B).

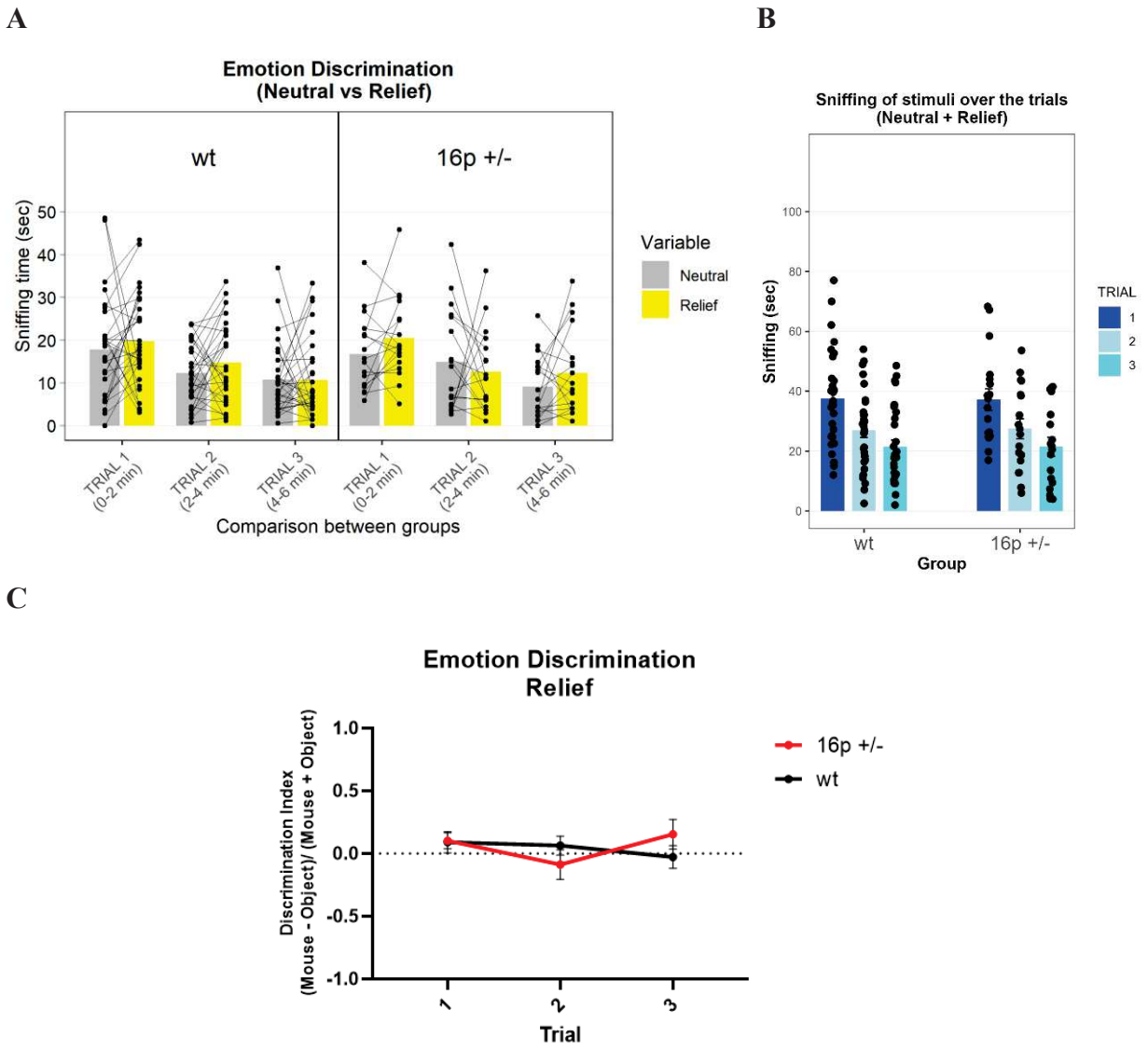


Figure 23. Emotion Discrimination (Relief) in 16p +/- vs wild-type adolescent mice

N(wt)= 30 (15 males, 15 females) and N(16p +/-) = 18 (13 males, 5 females).

A) **Emotion Discrimination: Relief**

The graph represents the sniffing time for the Relief and for the Neutral stimuli during the emotion discrimination task. A non-parametric analysis (f1.ld.f2) was performed including Genotype as a main factor and Trial and Variable as repeated measures. There was a significant main effect of Trial ($F_{1, 893}$ = 35.086, $p < 0.001$), but no main effects of Genotype ($F_{(1)}$ = 0.001, p = 0.974) and Variable ($F_{(1)}$ = 1.220, p = 0.269). There were no significant interactions, including Genotype by Trial ($F_{(1, 893)}$ = 0.275, p = 0.748), Variable by Trial ($F_{(1, 942)}$ = 0.552, p = 0.571), Genotype by Variable ($F_{(1)}$ = 0.015, p = 0.902), and Genotype by Trial by Variable ($F_{(1, 942)}$ = 1.232, p = 0.291).

B) Total exploration time.

The Kruskal-Wallis test for Total time did not reveal a significant main effect ($H_{(1)}$ = 0.012, p = 0.911), therefore, no post-hoc were performed. This indicates that the exploration time was not different between the two Genotypes.

C) Discrimination Index.

The DI is here reported to facilitate the comparisons between groups. The value near zero indicated no preference for neither the relief nor the neutral mouse.

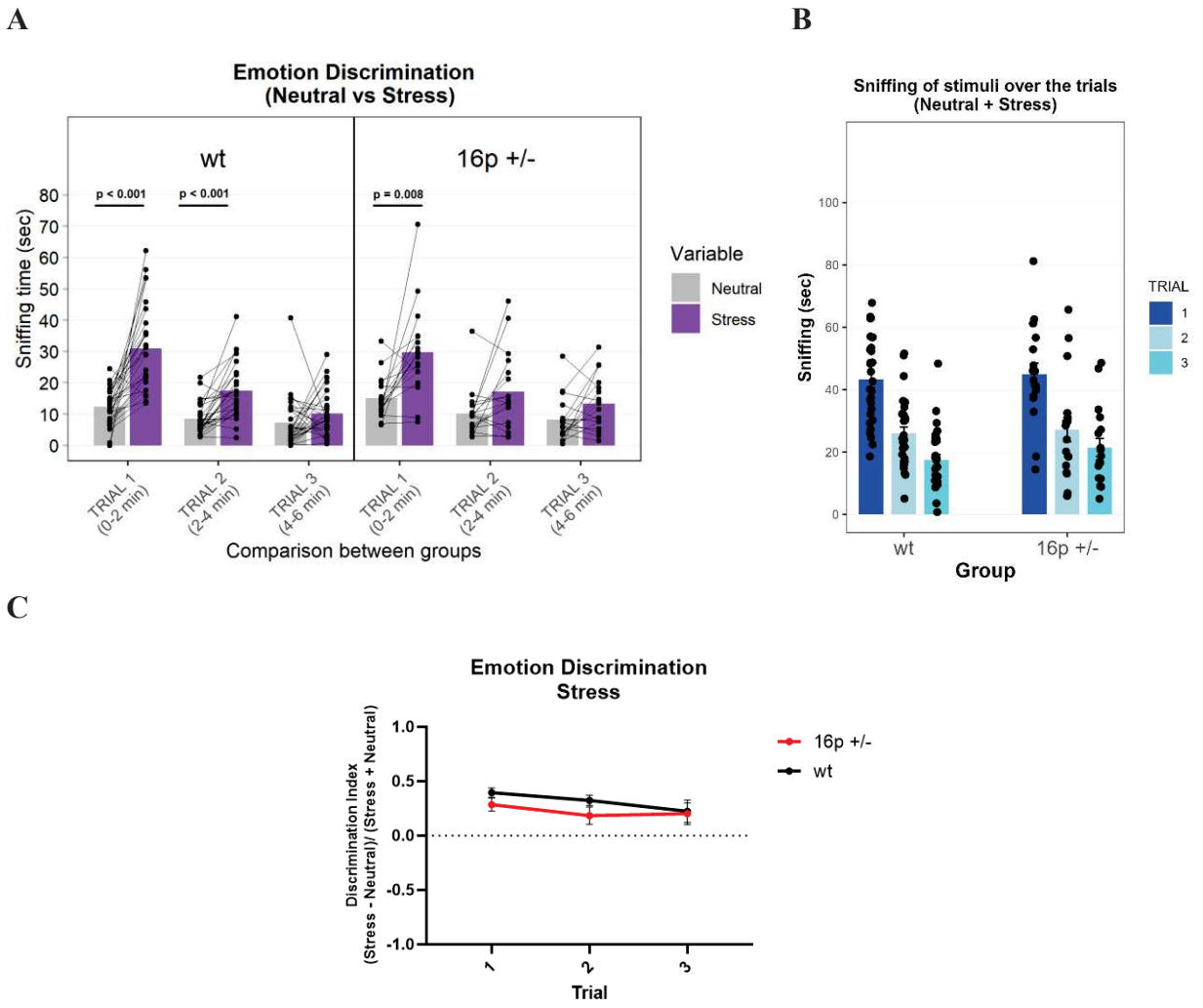


Figure 24: Emotion Discrimination (Stress) in 16p +/- and wild-type adolescent mice

N(wt)= 30 (15 males, 15 females); N(16p +/-) = 18 (13 males, 5 females).

A) <u>Emotion Discrimination: Stress</u>

The graph represents the sniffing time for the Stress and for the Neutral stimuli during the EDT. A non-parametric analysis (f1.ld.f2) was performed including Genotype as a main factor and Trial and Variable as repeated measures. There was a significant effect of Trial ($F_{(1, 982)} = 57.191$, $p < 0.001$) and Variable ($F_{(1, 1)} = 65.930$, $p < 0.001$) and of their interaction ($F_{(1, 696)} = 3.755$, $p = 0.030$). However, there were no significant effects for Genotype ($F_{(1, 1)} = 0.594$, $p = 0.441$) or its interactions: Genotype and Trial ($F_{(1, 982)} = 0.893$, $p = 0.408$), Genotype and Variable ($F_{(1, 1)} = 0.819$, $p = 0.366$), and Genotype, Trial, and Variable ($F_{(1, 696)} = 1.596$, $p = 0.206$).

Given the main effect of the Variable and of the Trial, and of their interaction (suggesting that discrimination between Stress and Neutral occurs and varies across Trials), post-hoc comparisons were performed for each Genotype group in order to further understand if each group was able to discriminate stress, and if this ability was Trial-related.

For the wild-type group, there was a significant preference for the Stressed mouse in Trial 1 ($p < 0.001$) and in Trial 2 ($p < 0.001$). In Trial 3, no significant difference was found ($p > 0.9999$).

For the 16p +/- group, there was a significant preference for Stress in Trial 1 ($p = 0.008$) but not in Trial 2 ($p = 0.197$) and in Trial 3 ($p = 0.199$).

<u>B) Total exploration time.</u>

The total exploration time was compared between groups using a Kruskal-Wallis test. No significant differences between groups were observed ($H_{(1)} = 0.328$, $p = 0.567$) therefore no post-hoc followed the analysis.

C) Discrimination Index.

The DI is here reported to facilitate the comparisons between groups. The positive value indicates a preference for the stressed stimuli. No significant main effect of Trial was found ($F_{(1.552, 71.40)} = 1.604$, $p = 0.2117$) nor of Genotype ($F_{(1, 46)} = 1.618$, $p = 0.2098$) nor of their interaction ($F_{(2, 92)} = 0.3593$, $p = 0.6991$).

Discussion

The results observed in this chapter indicate that in 16p11.2 deleted mice there are no major impairments in social behavior during adolescence in the domains of sociability, social memory, recognition of social novelty, and discrimination of negative affective states, confirming that the deletion alone is not linked with significant social deficits.

Despite the absence of clear significant results, some mild effects emerged, hinting at a subtle phenotype that may not have been fully revealed during these tests. Specifically, while performance in the habituation-dishabituation task was relatively similar between wild-type and 16p11.2 deleted mice, a tendency was observed in the latter toward increased exploration of non-social stimuli (e.g., an object) when given a choice between a social (mouse) and non-social (object) stimulus during the Object vs Mouse task. This effect was not observed in another study assessing sociability in adolescent 16p+/- mice (PND 30) [120], however in that study they used a mixed B6129SF1/J background, while here C57BL/6J was used. This highlights the potential impact of background in changing the behavioral outcomes, as previously suggested in the context of this model [55]. Interestingly, in children with ASD an heightened interest for objects is a typical feature of the pattern of restricted interests they usually display, and this characteristic was strongly related with the impairments in social communication [164]. Another feature that was previously reported in ASD, is the over attribution of salience to sensorial distractors that compete with socially relevant stimuli [165]. Both these mechanisms could be implicated in the results observed in this experiment, suggesting the presence of an under threshold ASD-like feature.

Interestingly, the same study mentioned before [120] described a deficit in novel object recognition in 16p +/- male mice at PND 70 but not at PND 42, and in females at PND 70. Here we did not observe impairment in recognition of social novelty. Such differences could depend on the fact that social novelty and general recognition of novelty for inanimate objects rely on different circuits [166], or on the different background of the experimental mice.

Interestingly, an increased total exploration of the stimuli, independent from their salience, was observed exclusively in the social novelty task, but not in the habituation dishabituation test (where mice could freely interact), and not in the other “discrimination” tasks performed in the same

apparatus. The Familiar vs Unfamiliar test differs from other tasks (such as sociability and emotion discrimination) because it is performed immediately after the 6-min lasting Sociability stage (Object vs Mouse). Moreover, this tests shifts from a less "socially stimulating" situation into one in which two social stimuli are presented together. It is possible that this effect could reflect an increased sensitivity to environmental stimuli, which is a critical feature of ASD, where affected individuals often benefit from a familiar environment with reduced levels of sensory stimulation [167]. It is therefore possible that the increased exploration of mice might reflect a general increase in the motor activity, probably reflecting anxiety. In a previous study in adolescence no increase in anxiety was seen in these animals compared with wild-type littermates, however the test was performed with the open field and not in presence of a "social stimulation" [120]. The locomotor activity during the Familiar vs Unfamiliar task will be assessed to further confirm an increase on locomotion in this specific phase of the test.

The ability to discriminate positive affective states was impaired in both 16p11.2 deleted and wild-type mice. This may reflect an intrinsic inability of adolescent mice to discriminate positive emotions in conspecifics, issues with the sensitivity of the test (e.g., linked to specific odors), or limitations in the suitability of this task for assessing positive emotion discrimination during adolescence. These possibilities will require further investigation. However, there were no Genotype - specific social impairments in the ability to discriminate affective states in conspecifics, suggesting that this ability is probably not compromised in 16p +/- adolescent mice.

In conclusion, while there are no major impairments in the social behavior of 16p11.2 deleted mice compared with their wild-type siblings during adolescence, subtle differences emerged, indicating that this phase of life, characterized by a developing brain, could provide some insight into the behavioral phenotypes relevant for ASD. These findings underscore the importance of further research to refine our understanding of social and emotional processing in this model, and provide a robust base for a further understanding of the effects of a double challenge in adolescent 16p +/- mice, which I will assess in the next Chapter.

CHAPTER 5

A viral-like prenatal immune challenge selectively alters sociability in 16p11.2 adolescent mice.

Abstract

Neurodevelopmental disorders are complex conditions influenced by both genetic and environmental factors. Critical periods such as prenatal life, early postnatal development, and adolescence are marked by heightened vulnerability to environmental stressors that can disrupt neurodevelopmental trajectories. Among these, maternal immune activation (MIA) has emerged as a key environmental factor, with prenatal infections and inflammatory responses linked to an increased risk of conditions like ASD and schizophrenia. Using a reproducible MIA model based on low molecular weight (LMW) Poly I:C, we investigated the impact of a combined genetic (16p11.2 deletion) and prenatal immunological challenge on social behavior in adolescent mice.

Our findings indicate that MIA unmasked social deficits associated with the 16p11.2 deletion in adolescence, supporting a "double-hit" hypothesis. Behavioral deficits in sociability were observed across paradigms, including the habituation-dishabituation test and the Object vs. Mouse test, highlighting reduced social interest in 16p +/- MIA mice. However, a preference for the social stimulus was still preserved in 16p +/- MIA mice, but the increased sniffing of the objects suggested an interest for less complex interactions. Social novelty recognition and emotion discrimination were not significantly affected, pointing to specific vulnerabilities in sociability rather than generalized social deficits. Notably, our MIA alone dosage regimen did not induce significant behavioral changes in wild-type mice, underscoring the synergistic interaction between genetic liability and prenatal inflammation in the context of the 16p11.2 deletion.

These results emphasize the importance of studying environmental factors like MIA in the context of genetic vulnerabilities. The observed behavioral alterations suggest that prenatal inflammatory insults can precipitate ASD-like traits in genetically susceptible individuals. Future studies focusing on microglial activation and inflammatory markers will provide further insights into the cellular and molecular mechanisms underlying these findings, offering potential targets for early interventions in individuals at risk for NDDs.

Introduction

Besides genetic alterations, the prevalence of NDD has been robustly linked with exposition to environmental factors affecting critical windows for brain maturation. Prenatal life, early post-natal life and adolescence are time windows characterized by high vulnerability for environmental stressors. Processes such cellular proliferation, differentiation, migration, synaptogenesis and pruning occur during this time frame and environmental insults during these critical windows can disrupt neurodevelopmental trajectories, leading to long-lasting structural and functional brain alterations [34,168].

It has been demonstrated that environmental disturbances during prenatal life—including maternal stress, fever, infections, and exposure to toxins—can significantly impact fetal development and increase the risk of NDD in offspring, including ASD and schizophrenia [169]. For instance, maternal infections and fever during the first and second trimesters of pregnancy have been consistently linked to an elevated risk of ASD [170,171]. Cases of ASD following congenital infections with cytomegalovirus, Zika virus, perinatal herpes simplex virus, and congenital rubella, have been consistently reported underscoring the vulnerability of the developing brain to inflammatory and infectious insults [172]. The COVID-19 pandemic has further reignited interest in the relationship between maternal infections and neurodevelopmental disorders. While direct evidence linking maternal SARS-CoV-2 infection to specific neurodevelopmental outcomes is still emerging [173], concerns have been raised about the indirect effects of maternal inflammation and cytokine storms triggered by the virus [174,175]. In fact, the mechanisms underlying these effects are thought to be mediated by the maternal immune response to infections rather than the infectious agents themselves [172]. Preliminary findings suggest that heightened levels of cytokines such as interleukin-6 (IL-6) and interleukin-17a (IL-17a), commonly observed during viral infection [137], could disrupt fetal brain development by interfering with critical processes like synaptogenesis and neuronal differentiation, particularly during early and mid-gestation [172]. In fact, the different stages of pregnancy involve distinct neurodevelopmental processes: during the first trimester, neurogenesis initiates around the fourth week of gestation, involving the proliferation of neural progenitor cells. After that, newly formed neurons begin migrating to their designated positions in the developing brain. During the second trimester, neurons organize into specific layers within the

cortex, establishing the foundation for functional neural circuits and formation of synapses between neurons commences, facilitating neural communication [34,83]. Disruptions during these periods, such as those induced by maternal infections, can interfere with these processes, leading to abnormal brain organization and function[34,168].

In order to study how the immune system interacts with the brain development, maternal immune activation (MIA) models were developed. MIA refers to the activation of the maternal immune system during pregnancy due to infections, autoimmune conditions, or other inflammatory processes, with mechanisms independent from the specific pathogen inducing the inflammation [172]. The release of pro-inflammatory cytokines and chemokines during MIA can cross the placental barrier, influencing fetal brain development by disrupting neurogenesis, cell migration, synaptogenesis, and myelination. These disruptions can lead to long-term structural and functional abnormalities in brain regions critical for social behavior and cognition [135,168,176,177].

MIA models take advantage from a variety of agents inducing an immune response [135], including polyinosinic-polycytidylic acid Poly I:C, lipopolysaccharide (LPS), influenza virus, turpentine and IL-6, which are administered to a pregnant female. Each immunogen addresses different aspect of the immune response, with relative advantages and disadvantages. For instance both LPS and Poly I:C mimic an acute and short-lasting infection, but while the first replicates the effects of a bacterial-like infection, the second is a synthetic analog of double-stranded RNA, used to mimic viral infections [178]. Other agents, such as influenza virus, can still replicate the effect of the viral infection by inducing a broader response compared to Poly I:C, but proved less control on the duration of the response [135]. In general, Poly I:C has proven to be a valuable tool to replicate the immunological challenge due to a viral infection, due to the absence of stringent precautions about safety, the good replicability of the cytokinic response and the fact that the compound is easy to purchase. In fact, different types of Poly I:C are commercially available and have been studied with the aim to identify their differences and optimize reproducibility of experiments [134,136]. High molecular weight (HMW) Poly I:C has proven to lead to significant cytokine responses after 2-3 hours from the injection, while the low molecular weight (LMW) leads to a less-significant response [134]. The latter is therefore preferable when the aim is to minimize the abortion rates in the litters while still providing a robust increase in the level of the cytokine cascade [136]. Moreover, recent studies have extensively investigated other confounders that can alter the response to MIA,

taking into account also the housing condition and other mediators [179,180], providing useful guidelines to facilitate the reproducibility and the solidity of findings with MIA models using Poly I:C [181].

Offspring from Poly I:C treated mothers display changes in microglial activity [182,183], synaptic structure and neuronal connectivity [184,185], resulting in behavioral abnormalities including increased anxiety [184], impaired sensorimotor gating, and disruptions in cognitive and social behaviors [186,187], such as reduced social interest and repetitive behaviors. This indicates that these models successfully recapitulate phenotypes associated with common and debilitating disorders, such as ASD, schizophrenia, and depression [188–190]. Moreover, experimental findings suggest that prenatal MIA not only affects early neurodevelopment but also increases vulnerability to secondary environmental stressors later in life, consistent with the "double hit" hypothesis [191]. Furthermore, offspring exposed to MIA during prenatal development exhibit greater vulnerability to stressors during adolescence, resulting in behavioral abnormalities and alterations in brain and immune system function [191].

Taken together, these information indicate that MIA could represent a valid tool to evaluate the effect of a double genetic and immunological challenge in the context of the 16p11.2 deletion. While no studies in the context of 16p11.2 deletion have ever been performed with this kind of double vulnerability, some evidence comes from clinical studies and other ASD models. It has been reported that in carriers of CNV, including the 16p11.2 deletion, the presence of prenatal infections was linked with worse ASD symptoms in deletions (but not in duplications) [192]. In rodent studies, other genetic mutations (like SHANK-3, DISC-1, CX3CR-1) displayed a heightened vulnerability for MIA [193–195]. To thoroughly understand how the interaction between genetic vulnerability and MIA might contribute to the mechanisms underlying socio-cognitive deficits typical of neurodevelopmental disorders, I replicated the impact of a viral infection during mid-pregnancy using a reproducible MIA model based on the intraperitoneal administration of LMW Poly I:C. In this Chapter, I will provide an assessment of social behavior with the social battery that was validated in adolescence in the previous Chapter, to assess if the presence of a prenatal immunological challenge can further determine alterations in 16p112.

Methods

Mice

For this specific experiments, C57BL/6J female mice aged 8 to 14 weeks, with no previous pregnancies were used as breeders, while 16p11.2 males (age range between 8 and 28 weeks) were used as breeders in order to transfer the mutation only through the paternal lineage. A 1:3 (male:female) breeding ratio was chosen to maximize the number of pregnant females simultaneously.

The timeline of the experimental protocol is reported in Figure 25. Females were housed in groups of three animals and, following a one-week acclimation period and a one-week handling protocol (every other day) aimed at reducing stress during subsequent manipulations, they were exposed to bedding from a 16p11.2 deletion male carrier intended for mating. After two days of exposure, the male was introduced into the females' cage, and vaginal plugs were checked twice daily, in the morning and evening, to promptly detect successful mating. Females with vaginal plugs were immediately removed from the mating cage, weighed to establish baseline weight, and transferred individually to cages equipped with nesting materials. The day a vaginal plug was detected was designated as gestational day 0 (GD0).

On GD11, the females were weighed again to confirm pregnancy (defined as a weight gain of at least 3 grams compared to baseline [196]). Pregnant females were then randomly assigned to two treatment groups and received intraperitoneal injections on GD12. On GD12, an intraperitoneal injection (10 ml/kg) of either Poly I:C or sterile, pyrogen-free saline solution (0.9% NaCl) was administered in the morning between 9 and 11 a.m. Two hours after the injection, all females were assessed for signs of sickness behavior linked to the induction of an inflammatory state. This assessment was repeated 24 hours later, along with a second weight measurement.

Females were then left undisturbed until delivery. After birth, the pups were identified by ear-tagging for genotype assessment on postnatal day 21 and they were weaned on day 28, after an assessment of body weight. For each litter, both 16p11.2 deletion carriers and their wild-type siblings were used for experiments, ensuring that no more than one male and one female per genotype underwent behavioral testing to avoid litter effects. On average, we tested three animals

per litter. Pups destined for behavioral testing underwent handling every other day for one week to accustom them to the experimenter before undergoing tests designed to assess alterations in social behavior. These tests were conducted during adolescence to explore early-emerging behavioral alterations.

Maternal immune activation protocol

A dose of 10 mg/kg LMW Poly I:C (Invivogen), with an injection volume of 10 ml/kg was chosen to induce maternal immune activation (MIA). LMW Poly I:C is recommended in all the cases in which a new MIA protocol needs to be established [136] due also to the lower risk of inducing abortion compared with HMW Poly I:C [134]. Moreover, this type of compound has been validated across studies [140,179,197], and its effect on the cytokine cascade in the pregnant dam, in the placenta and in the fetus have been replicated [136]. The dose was chosen based on a previous study indicating that 20 mg/kg IP LMW Poly I:C in pregnant C57BL/6J females does not affect health or litter size while inducing a significant cytokine response [134]. However, as the 16p11.2 deletion model already exhibits increased in utero mortality [119], and because Poly I:C had not been previously tested in combination with this genetic condition, we decided to use the dose of 10 mg/kg to further lower any risk of abortion of transgenic mice. The injection technique for the IP administration of Poly I:C in pregnancy was based on the one described in [196].

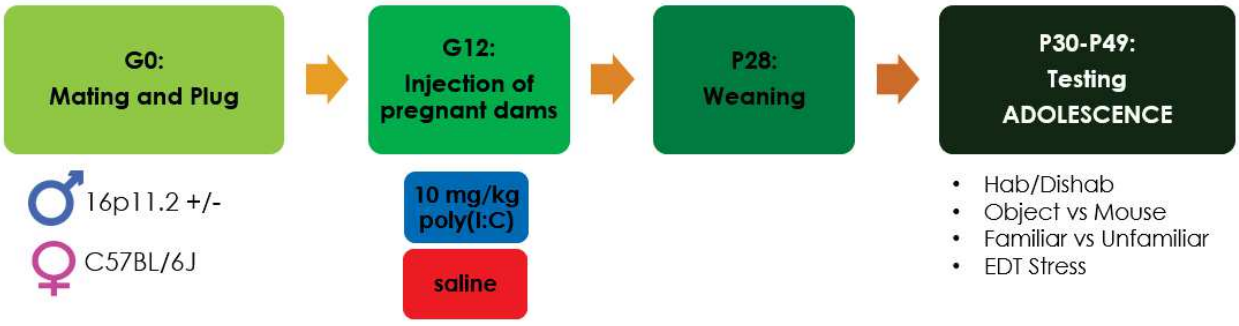


Figure 25: Timeline of the MIA protocol.

Behavioral assessment and statistical analysis

The behavioral assessment was conducted on MIA offspring in adolescence, by following the methods described in CHAPTER 4, for the behavioral testing of adolescent mice. The timing of the experiment was the same as described in the previous chapter, with the exception of the relief discrimination test which was not reported given that, similarly with what was described in CHAPTER 4, both wild-type animals and 16p +/- animals were not able to discriminate even in the control group. The statistical analysis were conducted by considering Genotype and MIA as factors and by using the same statistical approach described in CHAPTER 3.

Results

Maternal immune activation with 10 mg/kg LMW Poly I:C is not affecting litter size and composition, but impacts body weight of the offspring in 16p +/-.

To first confirm the relative safety of the 10 mg/kg dose in inducing selective abortion rates of 16p +/- pups compared with wild-type, I counted all the pups born from the C57BL/6J mothers who were treated either with Poly I:C or saline at GD12. There were no differences in the number of pups born in each litter, in the number of litters lost during the first days of post-natal life, and in the percentage of male and female 16p +/- mice born in each litter. Around 33% of the pups born from both the saline and in the MIA-treated dams were 16p +/- (referred to as "het" – heterozygous - in the graph (Figure 26 A and B)). The number of wild-type males was not different between groups, while the number of wild-type females was significantly higher in the MIA-offspring group compared with the saline-offspring (Figure 26 C).

The body weight at weaning was also assessed, given that 16p +/- tend to be smaller than their siblings at weaning (Figure 26 D). A significant reduction in body weight was observed between the 16p +/- MIA- offspring (16p+/- MIA) and the wild-type MIA-offspring (wt MIA), and between the 16p +/- MIA and the wild-type saline-offspring (wt SALINE). The difference between the 16p +/- MIA and 16p +/- saline-offspring (16p+/- SALINE) was not significant.

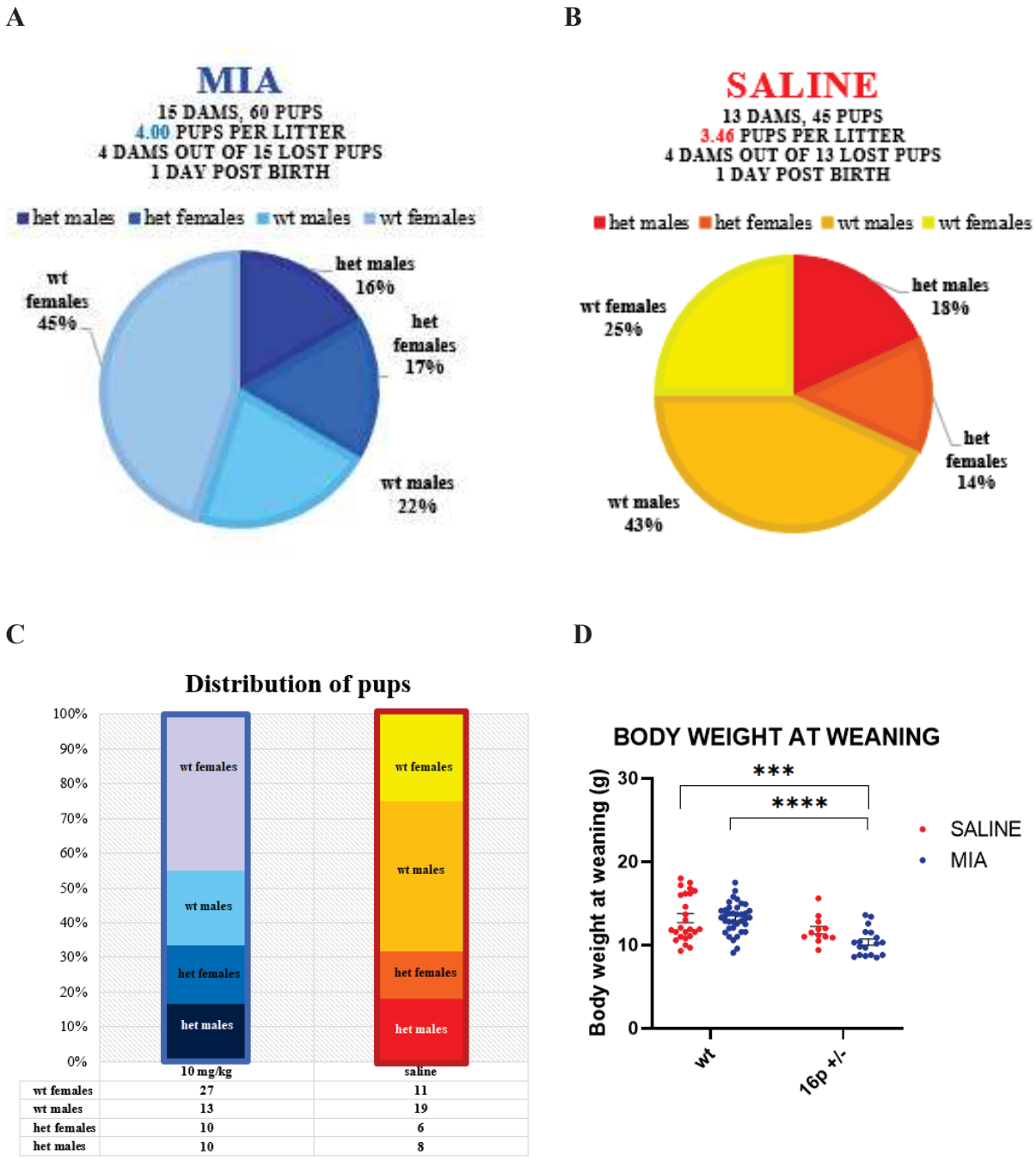


Figure 26: Litter composition and body weight of pups at weaning.

Het = 16p +/-. Wt = wild-type.

A) Distribution of sex and genotypes in the MIA offspring.

In the subtitle, the number of pregnant dams injected with saline (15) and the number of pups at weaning (60) is reported, with an average of 4 pups per litter. 4 dams out of 15 lost all their litter within 24 hours after birth, probably due to cannibalism.

B) Distribution of sex and genotypes in the SALINE offspring.

In the subtitle, the number of pregnant dams injected with saline (13) and the number of pups at weaning (45) is reported, with an average of 3.46 pups per litter. 4 dams out of 13 lost all their litter within 24 hours after birth, probably due to cannibalism. The litter size between MIA and saline was not statistically significant (two-sample independent t-test, p = 0.64).

C) Frequencies of males and females, and 16p +/- and wild-type for MIA and SALINE litters.

The contingency analysis using Fisher's exact test for the MIA group versus the SALINE group did not reveal any statistically significant difference in the distribution of genotypes ($p > 0.9999$, two-sided).

Within the MIA group, 58.33% were 16p+/- and 59.52% were wild type. In the SALINE group, 41.67% were 16p+/- and 40.48% were wild type.

16p +/- represented the 35.90% of the MIA group and 37.04% of the SALINE group while wild-type represented the 64.10% of the MIA group and 62.96% of the SALINE group.

The chi-square analysis of the sex distribution between the MIA and the SALINE in the wild-type groups revealed a statistically significant difference ($\chi^2(1) = 6.567$, $p = 0.0104$, $z = 2.563$). Considering the total wild-type sample, the wild-type MIA males represented the 18.57%, while 38.57% were females, while the wild-type SALINE males were the 27.14% and females the 15.71% were females.

In the 16p +/- group, no statistically significant difference in sex distribution was observed between MIA and SALINE ($p = 0.7385$, two-sided). Within the 16p+/- sample, 16p+/- MIA males represented the 29.41% and females constituted 29.41%, while 16p +/- SALINE males were the 23.53% and females the 17.65%.

D) Body weight in MIA and SALINE offspring.

The analysis of body weight using a 2-way ANOVA indicated a main effect of Genotype ($F_{(1, 88)} = 21.60$, $p < 0.0001$), but no main effect of MIA ($F_{(1, 88)} = 2.191$, $p = 0.1423$) and no significant interaction between Genotype and MIA ($F_{(1, 88)} = 2.377$, $p = 0.1267$). Post-hoc analysis using Sidak's multiple comparisons test indicated a significant mean difference between wild-type MIA and 16p+/- MIA (mean diff = 2.896, 95% CI: 1.301 to 4.491, $p < 0.0001$) and between wild-type SALINE and 16p+/- MIA was significant (mean diff = 2.867, 95% CI: 1.151 to 4.582, $p = 0.0001$). No significant difference was observed between wild-type MIA and wild-type SALINE (mean diff = 0.02876, 95% CI: -1.408 to 1.466, $p > 0.9999$), between wild-type MIA and 16p+/- SALINE (mean diff = 1.482, 95% CI: -0.3619 to 3.325, $p = 0.1836$), between wild-type SALINE and 16p+/- SALINE (mean diff = 1.453, 95% CI: -0.4960 to 3.402, $p = 0.2550$) and between 16p+/- MIA and 16p+/- SALINE (mean diff = -1.414, 95% CI: -3.482 to 0.6544, $p = 0.3495$).

MIA selectively impairs sociability in 16p+/- adolescent mice.

To further investigate the role of the double genetic and immunological challenge in 16p+/- mice, the social behavioral battery was applied in 16p +/- and in wild-type mice whose mother was exposed to MIA or to an injection of saline during pregnancy. The first assessment of sociability, social memory and social novelty was performed through the habituation-dishabituation task in a context of free social interaction.

There was a main effect of Treatment ad a significant interaction between Treatment and Genotype, indicating that MIA can impair social interaction in adolescent mice and that this effect is also influenced by Genotype (Figure 27 A, B, C). In fact, 16p +/- MIA mice had a reduced sociability compared with the other groups throughout all the Trials of this test. The effect was due both to a general reduction in sniffing times during each trial (Figure 27 A), and to a significant reduction in the total sociability (Figure 27 D) compared with all the other groups (although, significance was only at a trend level when compared with the wt MIA group, indicating that MIA alone might produce an intermediate phenotype). However, despite the effect on Sociability, there was no effect of MIA or of the combination between MIA and the 16p +/- condition in the recognition of social novelty, which was preserved in all the groups as indicated by the significant difference between exploration at Trial 4 and 5, and by the Novelty Score (Figure 27 E).

Sociability was also assessed with the Object vs Mouse test. During this test, discrimination occurred mostly during the first 2-min of the test in all the groups, and all the groups significantly preferred the Mouse compared with the Object (Figure 28 A, B). There was an interaction between MIA with Trial and the Variable (Mouse or Object): further post hoc analysis indicated that the MIA group was spending more time sniffing the Object during Trial 1 (min 0-2 of the test) compared with the SALINE group. Moreover, there was a significant effect of Genotype that was led mostly by an increased interest in the Object in 16p +/-. Given that there was also a general higher total exploration of the stimuli in the 16p +/- group compared with wild-type (Figure 28 C), the percentage of preference for the Object or for the Mouse (Figure 28 D) was calculated in order to control for the total exploration time. Further analysis confirmed main effects of Genotype, MIA and Trial in the preference for the Object, but no interactions. In particular, the 16p +/- had a higher

preference for the Object compared with the wild-type, and the MIA group had a tendency towards a higher preference for Object compared with the SALINE group (Figure 28 D, E, F).

Social Novelty was also assessed with the Familiar vs Unfamiliar test (Figure 29 A and B). The effects observed at the habituation dishabituation task were replicated, with both 16p +/- and wild-type being able to discriminate social novelty independently from the MIA or SALINE condition. However, a significant interaction between the Variable and the Genotype was observed during the test, further indicating that the 16p+/- were spending more time sniffing the Familiar mouse compared with the wild-types, while the time spent with the Unfamiliar mouse was not different between groups. The total exploration time was not differently affected by Genotype and by the MIA factors, indicating similar amounts of total exploration in all the groups (Figure 29 C).

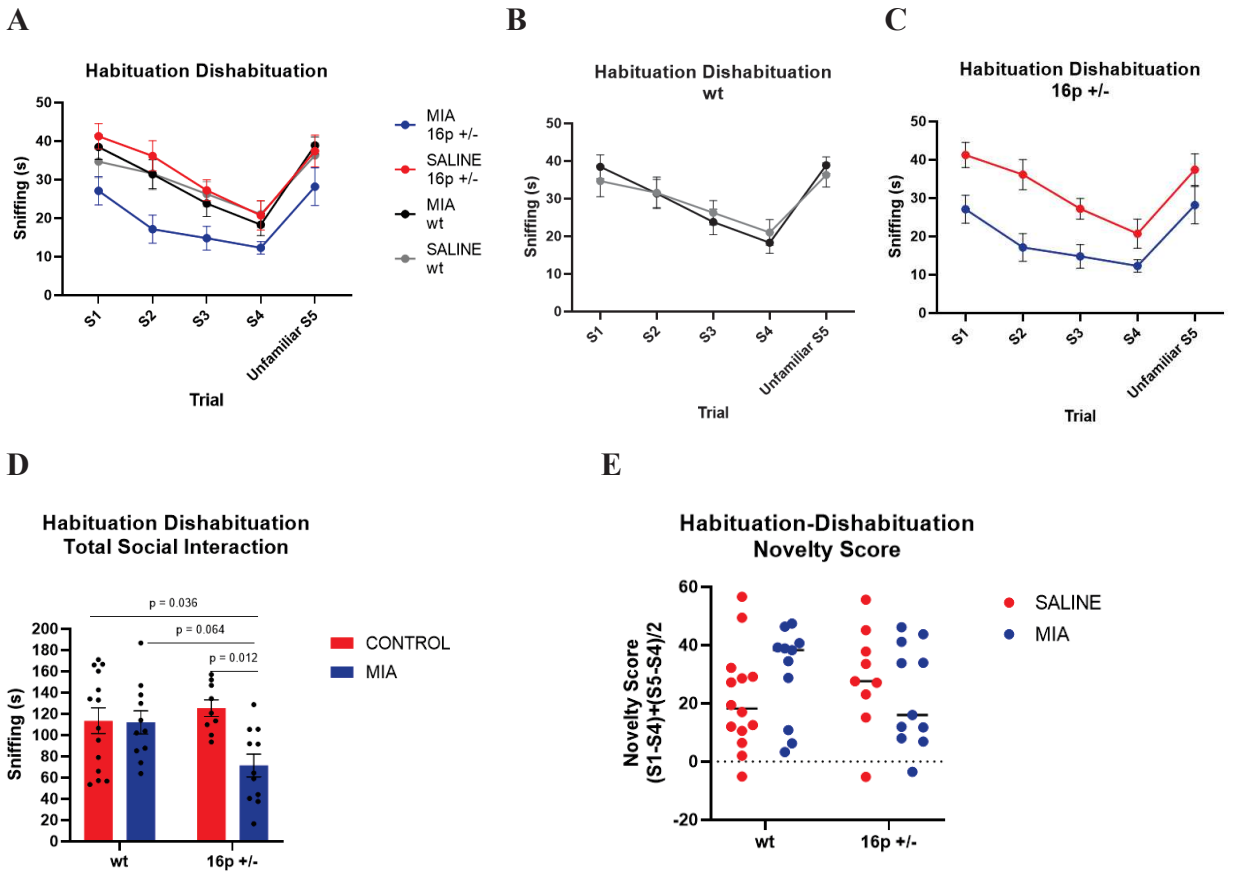


Figure 27: Habituation Dishabituation task in adolescent 16p +/- and wild-type with and without MIA.

A 3-way ANOVA (Genotype, MIA as factors, Trial as repeated measure) revealed a significant effect of **Trial** ($F_{(3.503, 143.6)} = 31.04$, $p < 0.0001$), and of **MIA** ($F_{(1, 41)} = 5.508$, $p = 0.0238$), while the main effect of Genotype was not significant ($F_{(1, 41)} = 2.098$, $p = 0.1551$). There was a significant interaction between **Genotype and MIA** ($F_{(1, 41)} = 5.908$, $p = 0.0195$). Other interactions were not significant, including Trial x Genotype ($F_{(4, 164)} = 0.1522$, $p = 0.9618$), and Trial x MIA ($F_{(4, 164)} = 0.7615$, $p = 0.5517$) and the three-way interaction Trial x Genotype x MIA ($F_{(4, 164)} = 1.028$, $p = 0.3945$). To further understand the effect of Genotype and MIA, data were collapsed by obtaining the average of

all the Trials, for each subject. A 2-way ANOVA (Genotype, MIA as factors) indicated a significant effect of **MIA** ($F_{(1, 41)}$ = 5.508, p = 0.0238), with higher mean values in the SALINE group compared with MIA (31.26 vs 25.05), and of the interaction **MIA x Genotype** ($F_{(1, 41)}$ = 5.908, p = 0.0195). The main effect of Genotype was not significant ($F_{(1, 41)}$ = 2.098, p = 0.1551).
The Tukey's post-hoc qith multiple comparisons correction showed significant differences for **wt SALINE vs. 16p+/- MIA** (q = 4.020, p = 0.0337), **wt MIA vs. 16p+/- MIA** (q = 3.883, p = 0.0426), and **16p+/- SALINE vs. 16p+/- MIA** (q = 4.537, p = 0.0133). No significant differences were found for wt SALINE vs. wt MIA (q = 0.08881, p > 0.9999), wt MIA vs. 16p+/- SALINE (q = 0.8532, p = 0.9304), or wt SALINE vs. 16p+/- SALINE (q = 0.9814, p = 0.8988).
To assess differences in social novelty recognition, the sniffing at Trial 4 and 5 were compared across group with a 3-way ANOVA (Genotype, MIA as factors and Novelty (Trial 4 vs Trial 5) as repeated measures). There was a significant effect of **Novelty** ($F_{(1, 41)}$ = 77.98, p < 0.0001) but no main effect of MIA ($F_{(1, 41)}$ = 1.969, p = 0.1681), Genotype ($F_{(1, 41)}$ = 2.462, p = 0.1243) or their interactions: Novelty x MIA ($F_{(1, 41)}$ = 0.1800, p = 0.6736); Novelty x Genotype ($F_{(1, 41)}$ = 0.3281, p = 0.5699); MIA x Genotype ($F_{(1, 41)}$ = 2.410, p = 0.1283); Novelty x MIA x Genotype ($F_{(1, 41)}$ = 0.6242, p = 0.4340).
Sidak's multiple comparisons revealed that social novelty was significantly recognized in all the groups. 16p +/- MIA (t = 4.098, p = 0.0008); 16p +/- SALINE (t = 3.904, p = 0.0014); wt MIA (t = 5.314, p < 0.0001); wt SALINE (t = 4.456, p = 0.0003).

A) Habituation Dishabituation Task. Sniffing for each Trial and for each experimental group.

The graph represents the social interaction of all the experimental groups across the 5 trials of the Habituation Dishabituation test. During the Habituation phase (Trial 1 - 4) there was a consistent decline of interaction for all the groups, indicating normal social memory. The final increase in social interaction during Trial 5 (Dishabituation) indicates normal recognition of social novelty. The blue line represents the average sniffing of the 16p +/- MIA group, indicating a general reduction of sociability during the whole test.

B) Habituation Dishabituation Task. Sniffing for each Trial in the wild-types.

Performance of the wild-type groups (SALINE and MIA) at the habituation/dishabituation task. A decrease from Trial 1 to 4 is observed, indicating social memory during the Habituation phase. During Trial 5 (Dishabituation) sniffing was increasing in response to social novelty.

C) Habituation Dishabituation Task. Sniffing for each Trial in the 16p +/-.

Performance of the 16p +/- groups (SALINE and MIA) at the habituation/dishabituation task. A significant decrease is observed in the 16p +/- MIA group (blue line). A decrease from Trial 1 to 4 is observed, indicating social memory. During Trial 5 (Dishabituation) sniffing was increasing in response to social novelty. 16 p +/- with MIA display a significant reduction of sniffing all over the Trials.
A 2-way ANOVA was conducted to further explore the effect of MIA in the 16p +/- Genotype. There was a significant main effect of **Trial** ($F_{(3.177, 57.19)}$ = 13.17, p < 0.0001), and of **MIA** ($F_{(1, 18)}$ = 13.28, p = 0.0019). The interaction between Trial and MIA was not significant ($F_{(4, 72)}$ = 1.042, p = 0.3918). The Sidak's multiple comparisons test for the 16p +/- group comparing MIA and SALINE shows a significant difference in Trial 2 (mean difference = -19.02, 95% CI = -34.44 to -3.591, adjusted p = 0.0119) and Trial 3 (mean difference = -12.41, 95% CI = -24.14 to -0.6737, adjusted p = 0.0352) with higher social interaction in the SALINE group. In Trial 1, significance was only at a trend level (mean difference = -14.15, 95% CI = -28.31 to 0.01992, adjusted p = 0.0504). No significant differences are observed in Trial 4 (mean difference = -8.419, 95% CI = -21.17 to 4.332, adjusted p = 0.2874) or Trial 5 (mean difference = -9.261, 95% CI = -27.57 to 9.052, adjusted p = 0.5915).

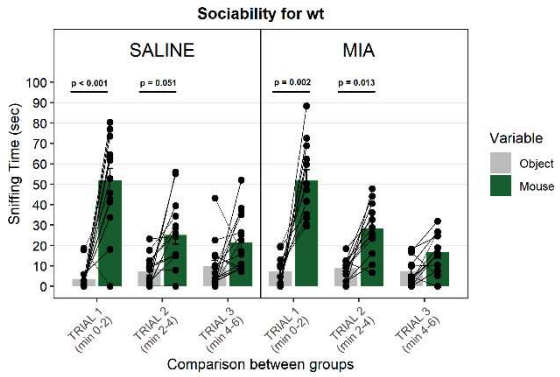
D) Habituation Dishabituation Task: Total Sociability.

Total sociability was calculated as the sum of all the interactions from Trial 1 to Trial 4 (Habituation phase). A 2-way ANOVA revealed a significant interaction between **Genotype and MIA** ($F_{(1, 41)}$ = 5.504, p = 0.0239), and a main effect of **MIA** ($F_{(1, 41)}$ = 6.144, p = 0.0174). The main effect of Genotype was not significant (F(1, 41) = 1.651, p = 0.2060). The Tukey's multiple comparisons indicates significant differences between **wt SALINE and 16p+/- MIA** (q = 3.987, p = 0.0357) and between **16p+/- MIA and 16p+/- SALINE** (q = 4.582, p = 0.0122). A trend was found in the comparison between **wt MIA and 16p+/- MIA** (q = 3.635, p = 0.0640). The remaining comparisons were not significant: wt MIA and wt SALINE (q = 0.1405, p = 0.9996); wt MIA and 16p+/- SALINE (q = 1.134, p = 0.8533); wt SALINE and 16p+/- SALINE (q = 1.060, p = 0.8763).

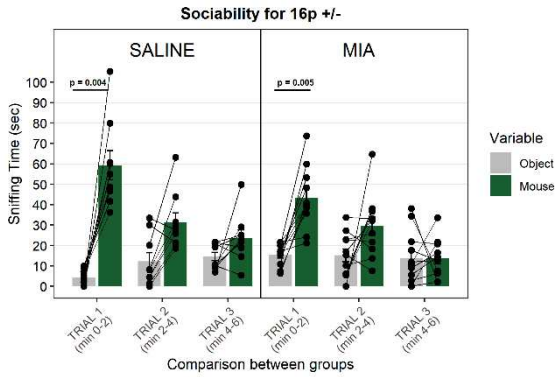
E) Habituation Dishabituation Task: Novelty Score.

The Novelty Score (calculated as the average difference between Trial 1 and 4, and Trial 5 and 4) indicates normal recognition of social novelty in all the groups, without significant differences: [2-way ANOVA, no significant effects. Genotype x MIA ($F_{(1, 41)}$ = 2.190, p = 0.1465); Genotype ($F_{(1, 41)}$ = 0.0001085, p = 0.9917); MIA ($F_{(1, 41)}$ = 0.08262, p = 0.7752)].

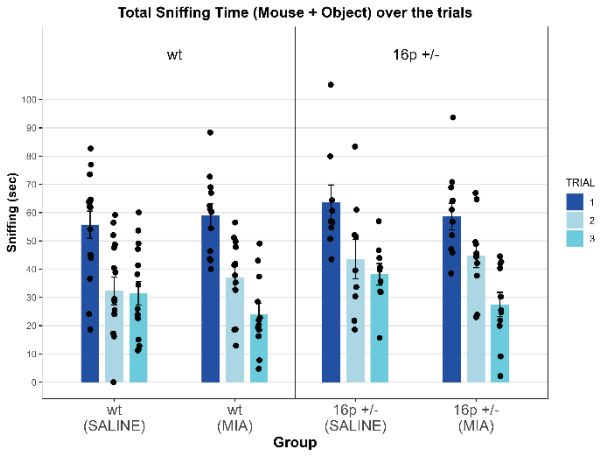
A


Sociability for wt
SALINE
MIA
p < 0.001
p = 0.051
p = 0.002
p = 0.013
Sniffing Time (sec)
Variable
Object
Mouse
TRIAL 1 (min 0-2)
TRIAL 2 (min 2-4)
TRIAL 3 (min 4-6)
Comparison between groups

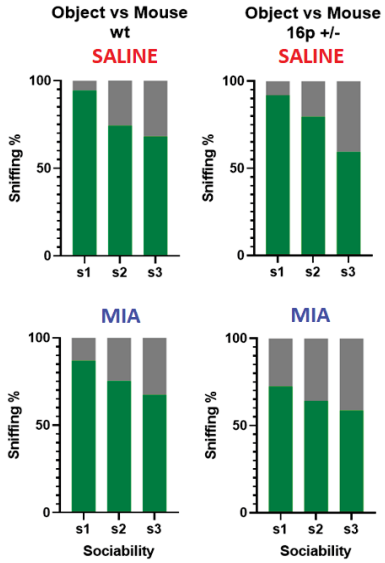
B


Sociability for 16p +/-
SALINE
MIA
p = 0.004
p = 0.005
Sniffing Time (sec)
Variable
Object
Mouse
TRIAL 1 (min 0-2)
TRIAL 2 (min 2-4)
TRIAL 3 (min 4-6)
Comparison between groups

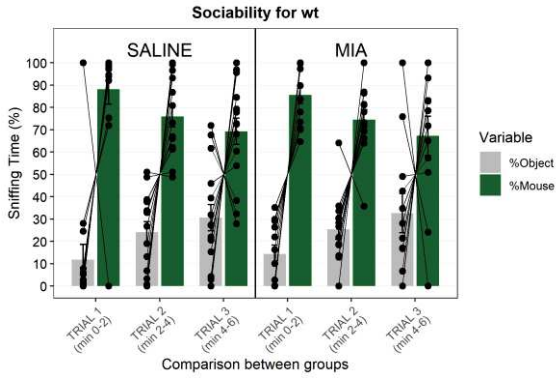
C


Total Sniffing Time (Mouse + Object) over the trials
wt
16p +/-
Sniffing (sec)
TRIAL
wt (SALINE)
wt (MIA)
16p +/- (SALINE)
16p +/- (MIA)
Group

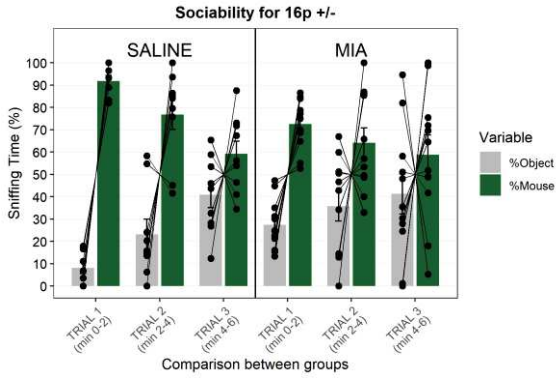
D


Object vs Mouse wt
SALINE
Object vs Mouse 16p +/-
SALINE
MIA
MIA
Sniffing %
s1
s2
s3
Sociability

E


Sociability for wt
SALINE
MIA
Sniffing Time (%)
Variable
%Object
%Mouse
TRIAL 1 (min 0-2)
TRIAL 2 (min 2-4)
TRIAL 3 (min 4-6)
Comparison between groups

F


Sociability for 16p +/-
SALINE
MIA
Sniffing Time (%)
Variable
%Object
%Mouse
TRIAL 1 (min 0-2)
TRIAL 2 (min 2-4)
TRIAL 3 (min 4-6)
Comparison between groups

G

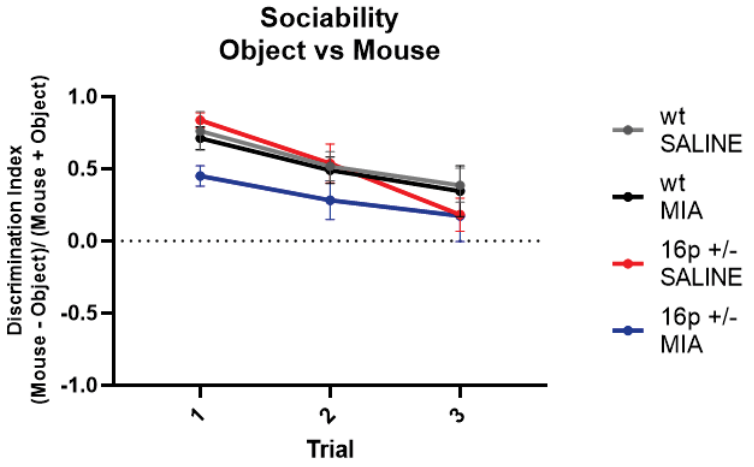


Figure 28: Sociability (Object vs Mouse) in adolescent 16p +/- and wild-type with and without MIA.

A non-parametric test (f2.ld.f1) was performed after assessing the non-normality of the data distribution. Genotype, MIA were used as factors, while Trial * Variable (Object vs Mouse) was used as a repeated measure. **Genotype** had a significant main effect ($F = 9.5538$, $df = 1$, $p = 0.0020$) while MIA did not ($F = 0.4433$, $df = 1$, $p = 0.5055$) nor did the interaction between Genotype and MIA ($F = 0.3220$, $df = 1$, $p = 0.5704$). The effect of **Trial x Variable**, was significant ($F = 64.4208$, $df = 4.4795$, $p < 0.0001$) as well as the interaction between **MIA and Trial × Variable** ($F = 4.0753$, $df = 4.4795$, $p = 0.0017$). No significance was found in any of the remaining interactions: Genotype and Trial × Variable ($F = 1.0397$, $df = 4.4795$, $p = 0.3887$), Genotype, MIA, and Trial × Variable ($F = 1.2016$, $df = 4.4795$, $p = 0.3069$).

To further clarify the effect of Genotype, a non-parametric test (f1.ld.f1) was performed by including Genotype and Variable only as factors. There was a significant effect of Genotype ($p = 0.0063$, $F = 7.47$, $df = 1$), of the Variable ($p < 0.0001$, $F = 165.47$, $df = 1$) and of the Genotype x Variable interaction ($p = 0.0139$, $F = 6.05$, $df = 1$). The Dunn test for Mouse shows no significant difference between the groups (wild-type = 97.70, 16p +/- = 98.91, and $p = 0.7736$). However, there is a significant difference with increased sniffing time for the **Object** in 16p +/- (wild-type = 22.08, 16p +/- = 38.45, and $p = 0.0004$)

Given the **MIA x Trial x Variable** interaction, further tests were conducted in order to evaluate the effect of MIA, comparing the MIA vs SALINE groups. For the variable **Object**, a significant difference was found in **Trial 1** ($Z = 3.182$, $p = 0.0012$), but not in Trial 2 ($Z = 1.462$, $p = 0.1445$) or Trial 3 ($Z = 0.531$, $p = 0.5946$). For the variable Mouse, no significant differences were observed between groups across all trials (Trial 1: $p = 0.0994$; Trial 2: $p = 0.5728$; Trial 3: $p = 0.0622$).

A) Sociability Test (Object vs Mouse) in the wild-type group.

Object vs Mouse test in wild-type animals, comparing MIA and SALINE. A paired-t-test was performed for every group and every trial to further understand the specific behavior of each group in each Trial. For the wild-type : SALINE, a significant difference was observed in Trial 1 ($p < 0.0001$), but no significant differences were found in Trial 2 ($p = 0.0021$) or Trial 3 ($p = 0.0339$). For the wt MIA, significant differences were observed in Trial 1 ($p = 0.0001$) and Trial 2 ($p = 0.0005$), while no significant differences were found in Trial 3 ($p = 0.0099$).

B) Sociability Test (Object vs Mouse) in the 16p+/- group.

Object vs Mouse test in 16p+/- animals, comparing MIA and SALINE. **Discrimination occurs during the first 2 minutes of the test.** A significant difference was observed in Trial 1 ($p = 0.0002$), while no significant differences were observed in Trial 2 ($p = 0.0098$) or Trial 3 ($p = 0.1141$). For the 16p +/- MIA, significant differences were observed in Trial 1 ($p = 0.0002$), but no significant differences were found in Trial 2 ($p = 0.0597$) or Trial 3 ($p = 0.9735$).

C) Total stimuli exploration in the Sociability Test.

Total exploratory behavior was calculated for all the trials and for each experimental condition. A RM-ANOVA was performed (Genotype, MIA as factors and Trial as RM) with a significant main effect of **Genotype** ($F = 54.8886$, $df = 2$, $p < 0.0001$). No significant main effect was observed for MIA ($F = 0.7046$, $df = 2$, $p = 0.4971$) and of Trial ($F =$

0.1180, df = 2, p = 0.8889). The interaction between Genotype and MIA was not significant (F = 2.3179, df = 2, p = 0.1046).
This further suggests that the main effect of Genotype observed in the test is probably driven by an increased exploratory behavior in 16p+/-.

D) Comparison of the preference for Object and Mouse between wild-type and 16p +/- for each MIA or SALINE group.

To control for the different exploration times observed in 16p+/- the **Percentage of preference** was calculated for all the test (i.e %Object = Object/(Object + Mouse)*100). The graph represents the percentage of preference for the Mouse (Green) and the Object (Grey) for each experimental group, allowing comparisons within the same type of MIA condition. Analysis were performed (f2.ld.f1) using Genotype, MIA as factors, while Trial was used as repeated measure, while %Object was chosen as variable of interest. Significant main effects were found for **Genotype** (F=1, df=1, p=0.0489), **MIA** (F=1, df=1, p=0.0463), and **Trial** (F=1.7965, df=1, p<0.0001). The interactions were not significant including: Genotype x MIA (F=1, df=1, p=0.2004), Genotype x Trial (F=1.7965, df=1, p=0.5941), MIA x Trial (F=1.7965, df=1, p=0.0716), and Genotype x MIA x Trial (F=1.7965, df=1, p=0.4416).
The Kruskall-Wallis test for %Object comparing MIA and SALINE revealed a significant difference (H = 3.45, p = 0.0631), followed by the Dunn test that was not significant (p = 0.0908) but indicated a **trend toward higher preference for the Object in the MIA group.**

E) Sociability Test (Object vs Mouse) in the wild-type group, expressed as % of preference.

(%Object = Object/(Object + Mouse)*100; %Mouse = Mouse/(Object + Mouse)*100)
The effect of **Genotype** was tested with the Kruskal-Wallis test for %**Object**, showing a significant effect with a p-value of 0.0472 (H = 3.94, df = 1). Following this, the Dunn test revealed a significant difference between 16p +/- and wild-type (Z = 89.99, p = 0.0313), with **16p +/- showing a higher preference for the Object compared with wild-type.**

F) Sociability Test (Object vs Mouse) in the 16p +/- group, expressed as % of preference.

The graph represents the % of preference for Object and for Mouse in wt and in 16p+/- animals.

G) Discrimination Index.

To facilitate the comparison between groups, the DI (Mouse – Object)/(Mouse + Object) is here represented. No differences between the groups were observed in the DI, with a 3-way RM-ANOVA indicating that the Genotype (p = 0.0881) and the MIA groups were both only approaching significance (p = 0.0827)

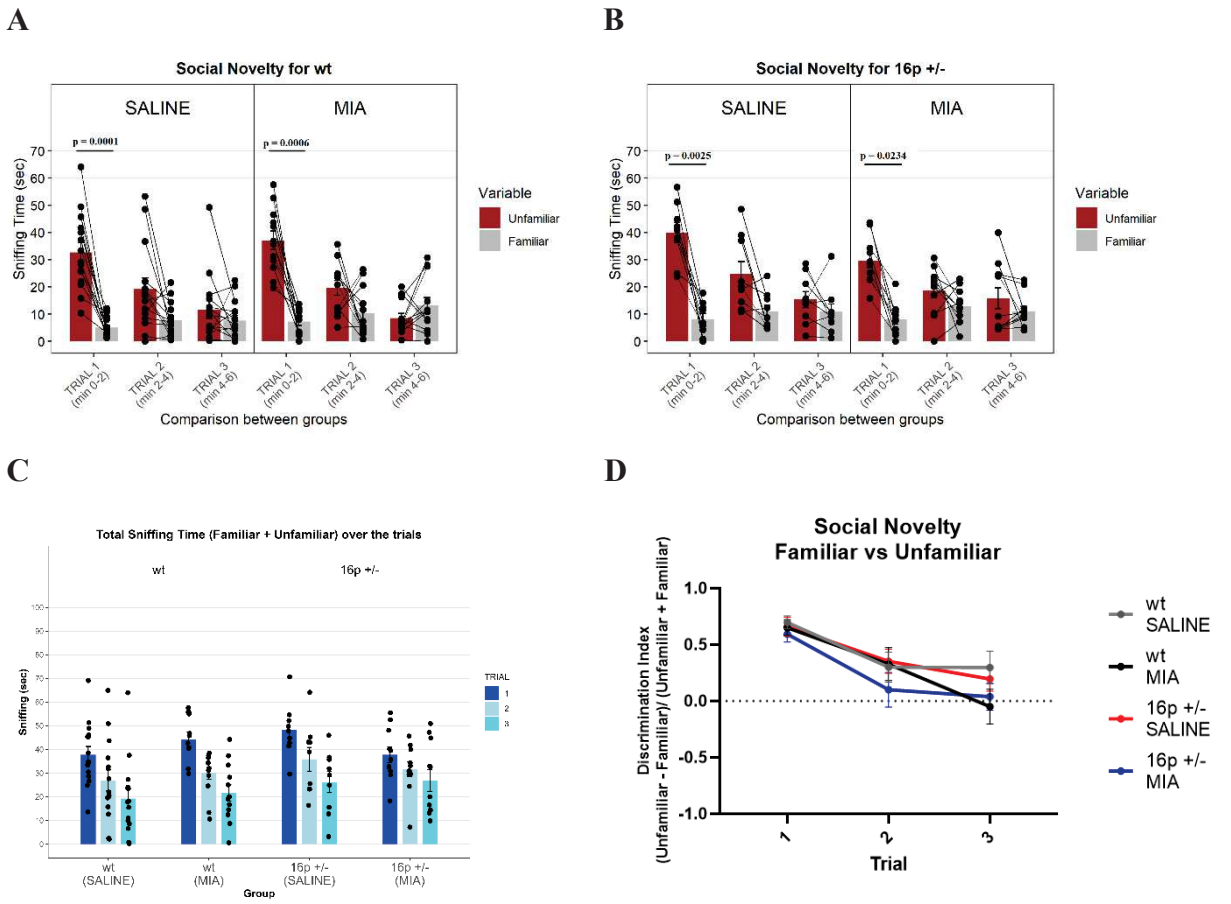


Figure 29: Social Novelty (Familiar vs Unfamiliar) in adolescent 16p +/- and wild-type mice with or without MIA.

A non-parametric test (f2.ld.f1) was performed after assessing that data distribution was not normal. Genotype, MIA were used as factors, while Trial x Variable (Object vs Mouse) was used as a repeated measures. There was a significant effect of **Genotype** ($p = 0.0219$, $F = 5.25$) and of the **Trial x Variable** interaction ($p < 0.0001$, $F = 46.29$). No significance was observed in the remaining tests, including MIA ($p = 0.5082$, $F = 0.44$), Genotype x MIA ($p = 0.1865$, $F = 1.74$), Genotype x Trial x ($p = 0.5905$, $F = 0.71$), MIA x Trial x Variable ($p = 0.5080$, $F = 0.84$), and Genotype x MIA x Trial x Variable ($p = 0.8281$, $F = 0.39$). The following test assessed the effect of Genotype without considering the Trials, by using a f1.ld.f1 model (Genotype and Variable). Genotype did not have a significant effect ($p = 0.065$, $F = 3.40$, $df = 1$) but the interaction **Genotype x Variable** (Familiar vs Unfamiliar) was significant ($p < 0.0001$, $F = 134.91$, $df = 1$).

The Dunn test comparing the sniffing time for **Familiar** shows only a trend level (wild-type = 24.97, 16p +/- = 31.17, $p = 0.0794$) while for the Unfamiliar it is not significant (wild-type = 64.23, 16p +/- = 71.35, $p = 0.2435$). These results indicate no significant differences between the groups.

In the wild-type group SALINE, the time spent sniffing the Familiar stimuli was (20.35 ± 2.71), and the Unfamiliar was (63.43 ± 7.79). In the wild-type with MIA, the time spent sniffing the Familiar was (30.75 ± 5.10), while the Unfamiliar was (65.22 ± 5.39).

In the 16p+/- group with SALINE, the time spent on Familiar stimuli was (30.12 ± 5.24), while on Unfamiliar stimuli was (80.09 ± 8.18). In the 16p +/- with MIA, the time spent on Familiar stimuli was (32.03 ± 3.17), while on Unfamiliar stimuli was (64.20 ± 6.27).

A) Social Novelty Test (Familiar vs Unfamiliar) in the wild-type group.

In the SALINE subgroup, significant discrimination between Familiar and Unfamiliar is seen at Trial 1 ($p = 0.0001$), but not in Trial 2 ($p = 0.4117$) and Trial ($p > 0.9999$).

In the MIA subgroup, there is a significant preference for the Unfamiliar mouse in Trial 1 ($p = 0.0006$) but not in Trial 2 ($p > 0.9999$) or Trial 3 ($p > 0.9999$).

B) Social Novelty Test (Familiar vs Unfamiliar) in the 16p +/- group.

For the 16p +/- SALINE, there is a significant preference for Unfamiliar during Trial 1 ($p = 0.0025$) but not in Trial 2 ($p = 0.6255$) and Trial 3 ($p > 0.9999$).

For 16p +/- with MIA, preference for Unfamiliar is significant during Trial 1 ($p = 0.0234$), and not in Trial 2 ($p > 0.9999$), and Trial 3 ($p > 0.9999$).

C) Total stimuli exploration in the Social Novelty Test.

No significant effect of Genotype ($F = 1$, $p = 0.1059$) or MIA ($F = 1$, $p = 0.8805$) were observed for the total exploration time (non-parametric test (f2.ld.f1)). There was a significant effect of the Trial ($F = 1.8188$, $p < 0.0001$), but no significant interactions between Genotype and MIA ($F = 1$, $p = 0.1183$), Genotype and Trial ($F = 1.8188$, $p = 0.5437$), or MIA and Trial ($F = 1.8188$, $p = 0.8093$) or at the three-way interaction among Genotype, MIA, and Trial ($F = 1.8188$, $p = 0.2645$).

D) Discrimination Index.

To facilitate the comparison between groups, the DI (Unfamiliar – Familiar)/(Unfamiliar + Familiar) is here represented. A positive DI indicates preference for the Unfamiliar mouse. At the 3-way RM ANOVA, a significant main effect of Trial was found ($F_{(1.894, 81.45)} = 20.45$, $p < 0.0001$). No significant main effect of Genotype, $F_{(1, 43)} = 0.4177$, $p = 0.5215$), of MIA ($F_{(1, 43)} = 3.606$, $p = 0.0643$) was found. There were no significant interactions: Trial and Genotype ($F_{(2, 86)} = 0.1137$, $p = 0.8926$); Trial and MIA ($F_{(2, 86)} = 0.6862$, $p = 0.5062$); Genotype and MIA ($F_{(1, 43)} = 0.05900$, $p = 0.8092$); Trial, Genotype, and MIA ($F_{(2, 86)} = 0.9469$, $p = 0.3919$).

MIA does not impair the ability to discriminate negative affective states of conspecifics in 16p +/- adolescent mice and in their wild-type littermates.

Emotion discrimination task was performed for the negative affective state condition (Stress). Discrimination was significant for all the groups, especially in the first 2 minutes of the test as indicated by the interaction Trial x Variable and by the DI (Figure 30 A, B, D). However, while in the wt SALINE group the discrimination was significant during the first 4 minutes of the test (Figure 30 A), in all the other experimental groups discrimination did not reach significance during the first 2 minutes of the task, but remained at a trend level (Figure 30 A, B) as indicated also by the DI ((Figure 30 D). The absence of main effects of Genotype or of MIA or of their interactions further indicates that such differences were not driven by these factors. Moreover, no significant differences were observed in the total exploration time, indicating similar exploration of stimuli in all the experimental groups (Figure 30 C).

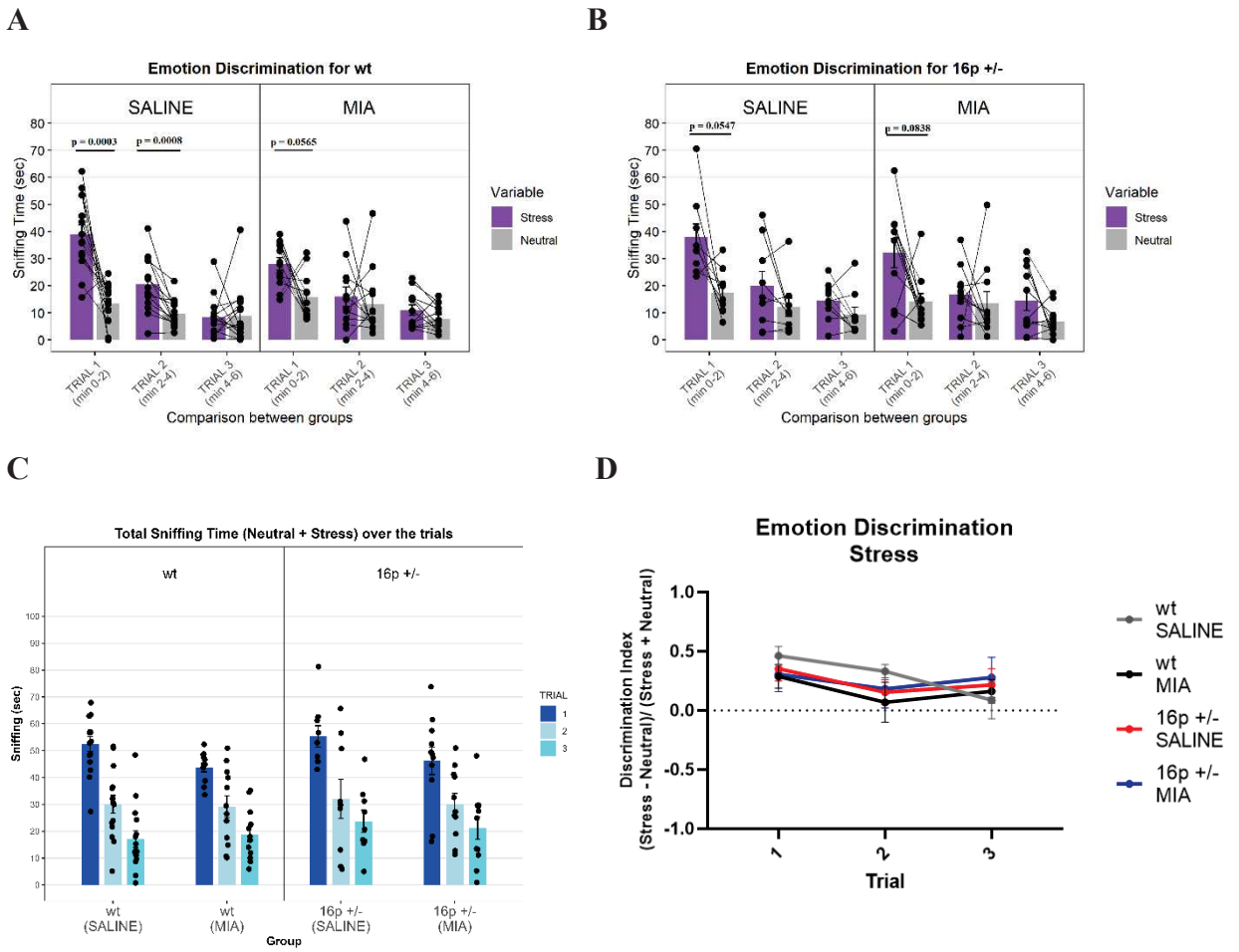


Figure 30: Emotion Discrimination (Stress) in 16p +/- and wild-type adolescents with and without MIA.

The analysis of Stress vs Neutral using a non-parametric test (f2.ld.f1) reveals no significant main effects of Genotype ($F_{(1)} = 0.28$, $p = 0.5986$) or MIA ($F_{(1)} = 0.60$, $p = 0.4400$). However, there is a significant interaction between Trial × Variable ($F_{(3.47)} = 33.31$, $p < 0.0001$) indicating that the dynamics of discrimination vary across Trials. No significant interactions are found between Genotype and MIA ($F_{(1)} = 0.46$, $p = 0.4961$), Genotype and Trial × Variable ($F_{(3.47)} = 0.89$, $p = 0.4546$), or MIA and Trial × Variable ($F_{(3.47)} = 0.67$, $p = 0.5921$). Similarly, the three-way interaction among Genotype, MIA, and Trial × Variable is not significant ($F_{(3.47)} = 0.68$, $p = 0.5861$).

A) Emotion Discrimination (Stress vs Neutral) in the wild-type group.

In the wild-type group with SALINE, significant differences are observed between the time spent sniffing the Stress and Neutral stimuli in Trial 1 ($p = 0.0003$) and Trial 2 ($p = 0.0008$). However, no significant effect was found in Trial 3 ($p > 0.9999$).

In the wild-type group with MIA, no significant differences were detected in any trial. In Trial 1 ($p = 0.0565$) is detected only at a trend level, while no significance was observed in Trial 2 ($p > 0.9999$), and Trial 3 ($p = 0.3787$).

B) Emotion Discrimination (Stress vs Neutral) in the 16p +/- group.

In the 16p +/- group with SALINE, no significant effects were found across trials. Trial 1 ($p = 0.0547$) was showing a trend, while Trial 2 ($p = 0.9023$), and Trial 3 ($p = 0.4184$) did not show significant differences between sniffing of Stress and Neutral stimuli.

In the 16p+/- group with MIA, no significant effects were observed in any trial. Trial 1 ($p = 0.0838$), Trial 2 ($p > 0.9999$), and Trial 3 ($p = 0.2150$).

C) Emotion Discrimination (Stress vs Neutral): Total exploration of stimuli.

The total exploration time was analyzed with a 2-way RM ANOVA (Genotype, MIA as factors, Trial as RM). The analysis showed no significant main effect of Genotype ($F_{(1, 43)} = 0.706$, $p = 0.405$) or Challenge ($F_{(1, 43)} = 1.317$, $p = 0.257$). The interaction between Genotype and Challenge was also not significant ($F_{(1, 43)} = 0.093$, $p = 0.762$). There was a significant main effect of Trial ($F_{(2, 86)} = 103.393$, $p < 0.0001$) but the interaction between Genotype and Trial

was not significant ($F_{(2, 86)} = 0.324$, $p = 0.7241$), as well as interaction between Challenge and Trial ($F_{(2, 86)} = 2.564$, $p = 0.0829$) and the three-way interaction among Genotype, Challenge, and Trial ($F_{(2, 86)} = 0.128$, $p = 0.8798$).

D) Discrimination Index.

To facilitate the comparison between groups, the DI (Stress - Neutral)/(Stress + Neutral) is here represented. There was a significant effect of Trial ($F_{(1.677, 72.12)} = 3.509$, $p = 0.0429$), but no effect of Genotype ($F_{(1, 43)} = 0.02268$, $p = 0.8810$), MIA ($F_{(1, 43)} = 0.2879$, $p = 0.5944$). There was no significant effect of the interactions Genotype × Trial ($F_{(2, 86)} = 0.8278$, $p = 0.4405$), MIA × Trial ($F_{(2, 86)} = 1.048$, $p = 0.3552$), Genotype × MIA ($F_{(1, 43)} = 0.4674$, $p = 0.4979$), and Genotype × MIA × Trial ($F_{(2, 86)} = 0.5362$, $p = 0.5869$).

Discussion

The analysis of social behavior in the context of the combined genetic (16p +/-) and prenatal environmental (MIA) challenges revealed that the interaction between these factors significantly affects the social behavior of mice during adolescence, consistently with the double-hit hypothesis. These alterations were observed in sociability, which is one of the social domains that constitutes a major feature of ASD [1]. The importance of this finding is confirmed by the fact that the effect on sociability was significant across distinct paradigms: the habituation/dishabituation test (a free social interaction context) and the Object vs. Mouse test (a choice-related context). In both paradigms, sociability was reduced only under the condition where both the genetic and environmental challenges (16p11.2 deletion and MIA) coexisted, indicating that the combination of these two factors is essential for producing such effects.

Importantly, significant sociability deficits were not observed in the wild-type group with MIA, suggesting that the prenatal inflammatory insult on its own, with the chosen administration route (IP), dose, timing (GD12) and type of Poly I:C (LMW), was insufficient to induce significant changes in behavior. A very recent study induced MIA with similar timing, dose, and administration route of LMW Poly I:C (although in C57BL/6N mice), and tested the behavioral outcomes of the offspring in adulthood [180]. Interestingly, they did not find significant effects of MIA in sociability with the 10 mg/kg dose and with a 20 mg/kg dose as well [180] (although the results were varying between different laboratories). Similarly, another study using HMW Poly I:C, which is expected to induce a higher cytokine response [134], did not find sociability deficits in adolescent MIA offspring [198]. These findings support the idea that this model of MIA specifically affects behavior in the context of a pre-existing vulnerability, such as in 16p +/- mice, further reinforcing the notion that MIA amplifies a genetic vulnerability (16p +/-) into an altered phenotype.

It must be noted that in the Object vs. Mouse test, where animals could express a preference between a social (Mouse) and non-social (Object) stimulus, 16p +/- MIA animals were still preferring the social stimulus compared with the non- social, despite showing an increased interest for the Object. This might mean that the drive for social interaction is maybe blunted in those mice, but not abolished. This effect could be dependent on the dose that was used in this experiment: in

fact, the absence of effects on litter size and in the number of 16p +/- pups born are further indicating that overall, 10mg/kg LMW Poly I:C is not affecting strongly the survival and in general the phenotype of these animals. Given the recent evidence [180] that also 20 mg/kg LMW Poly I:C is not impacting behavior of wild-type mice, replicating this experiment with that same dose in 16p +/- will probably provide more robust evidence of the link between social impairments and MIA in the context of this deletion. This would provide critical insights into the significance of the prenatal inflammatory insult in shaping behavioral outcomes.

In the habituation/dishabituation test, social novelty recognition was not affected by the combination of 16p +/- and MIA. However, in the Familiar vs Unfamiliar test, also assessing social novelty, an increased exploration of the familiar mouse was observed in the double-challenged group. As in ASD novelty itself often presents as a challenging stimulus for the affected individual, these findings could indicate that despite the absence of overt novelty avoidance, the 16p +/- MIA mice were displaying an increased interest in the less-stimulating familiar choice compared with wild-type littermates. A similar effect is observable also in the Object vs Mouse test, where the Objects represents the less-stimulating choice. However, for the moment this is just a speculation; if confirmed by further evidence, this could potentially represent another subtle indication of an ASD-like phenotype in 16p +/- only emerging after MIA.

The reduced exploration of stimuli observed in the habituation/dishabituation test appears to be in contrast with the normal exploration of stimuli seen in all other tests (Object vs Mouse, Familiar vs Unfamiliar, Stress vs Neutral). This discrepancy can be explained by the differences in the design and purpose of the tests. In the habituation/dishabituation test, the mouse has the option to explore or not, as the environment contains only a single social stimulus (another mouse) and an open space with bedding, allowing relatively free movement and the opportunity to avoid or even escape from interaction if desired. In contrast, the three-chamber test places the mouse in a more restricted environment, where it is required to make a choice between two types of stimuli: one social and one non-social (or two social, one familiar and one unfamiliar). Still, the mouse could spend time in the area without stimuli, but the setup is more confined and the mouse cannot fully avoid exploration and generally must engage with at least one of the two stimuli. This difference in design means that the three-chamber test inherently forces the animal to interact with its

surroundings. On the other hand, the habituation/dishabituation test allows for a clearer measure of whether the animal displays a preference for social interaction or chooses to disengage.

Interestingly, no significant effects were observed in the EDT Stress, which measures the ability to detect negative affective states. This result could indicate that this ability is not significantly affected in the MIA-16p +/- model or that emotion discrimination is a domain relying on circuits whose sensitivities to environmental challenges occurs later in life or during a different phase of pregnancy. Interestingly, a previous study investigated, among others, the levels of mRNA markers for PV and SOM in the mPFC of a mouse model of 16p11.2 deletion, reporting a reduced PV expression in the prelimbic (PL), infralimbic (IL) and medial orbital (MO) cortex [122]. Previous evidence indicates that in the PL cortex PV interneurons are involved in sociability [102,103], while SOM are involved in the circuits relevant for emotion recognition [103]. The presence of alterations only for PV expression and not for SOM expression in PL cortex seems to mirror the deficits in social domains that we have demonstrated in the 16p+/- MIA. Further studies on the activity of these neuronal populations in 16p +/- during the behavioral tasks, i.e with in vivo fiberphotometry, could be a high priority for future research in this field, in order to address the specific neuronal contributions to sociability deficits.

The results from these experiments underscore the vulnerability of 16p +/- mice to inflammation when it occurs in the intrauterine life. Further investigations into microglial morphology and activation status are essential to confirm the involvement of inflammation in the CNS during early prenatal development and their contribution to behavioral outcomes in adolescence. This would help identify the mediators driving these behavioral changes and provide a clearer understanding of the underlying mechanisms. Notably, previous studies have reported that MIA itself induces a vulnerability to post-natal challenges [199], and adolescent challenges [191], such as social stressors. The MIA-16p +/- framework is particularly promising for evaluating whether these mice exhibit a heightened sensitivity to such social challenges, which are commonly experienced by individuals with NDD [12,160]. In parallel, the effect of other types of challenges, such as a second viral-like challenge in adolescence or social isolation, could further elucidate the mechanisms underlying the vulnerability to adolescent stressors, thereby contributing to a more comprehensive understanding of a "third-hit" hypothesis.

In conclusion, these results indicate that adolescence is a critical phase for studying the effects of MIA vulnerability in mice, and that specific behavioral alterations relevant for ASD can be evident during adolescence. A better understanding of the cell populations and circuits involved in determining social behavior in adolescence could shed new light on the mechanisms underlying such deficits. Given that MIA was supposedly inducing an inflammatory state in the 16p +/- brain (which in future will be further assessed with assessments of the microglial morphology and expression of inflammatory marker), in the next Chapter I will provide a comprehensive assessment of the role of microglia in shaping social behavior in adolescent mice.

CHAPTER 6

Depletion of microglia in adolescent mice impairs discrimination of others' emotions and alters the activity of somatostatin-positive neurons in the prelimbic cortex.

Abstract

Adolescence represents a critical window for brain development, marked by significant synaptic pruning, particularly in the prefrontal cortex. This process refines cortical circuits by balancing excitatory and inhibitory activity, which is essential for socio-cognitive functions such as decision-making, emotional regulation, and social behavior. The medial PFC (mPFC) is especially relevant to social cognition, hosting neuronal subpopulations such as SOM+ and PV+ interneurons, which are critical for emotion recognition and sociability, respectively. Adolescence is characterized by heightened sensitivity of these circuits to environmental and intrinsic factors. The interplay between microglia and these neuronal populations is believed to shape social behaviors by regulating the excitatory-inhibitory balance, yet the mechanisms remain poorly understood. By abolishing microglia in two different stages of adolescence, we provide evidence that microglia mediates the development of social abilities in adolescence, and that the effect is phase-specific.

In early adolescence, immediately preceding the peak of synaptic pruning, microglial depletion leads to deficits in emotion recognition without affecting sociability, and the deficits are paired with alterations in synaptic morphology and spine-type. Conversely, in mid adolescence, microglial depletion disrupts both sociability and emotion recognition, but the effects are not paired with changes in the synaptic morphology or spine-type. These findings suggest that sociability relies on mechanisms which are reaching maturation after synaptic pruning, while emotion recognition appears to require continuous microglial modulation throughout adolescence. Additionally, microglial depletion in mid adolescence was shown to disrupt the activity of SOM + interneurons in the PL cortex, whose calcium activity was completely reverted in response to the affective states of conspecifics.

This work underscores adolescence as a critical period for the development of social abilities and highlights the importance of microglial-neuronal interactions in shaping these behaviors, offering insights into potential mechanisms underlying sociability and emotion recognition deficits in neurodevelopmental disorders.

Introduction

Adolescence has long been recognized as a period of increased susceptibility to psychiatric disorders, including schizophrenia and mood disorders [149], due to the extensive refinement of cortical circuits that occurs during this phase, particularly in the PFC [148,151,200]. Experience-mediated synaptic pruning during adolescence is essential for developing an appropriate excitatory-inhibitory balance in cortical circuits, marking the final phase of central nervous system remodeling [201–203]. This process eliminates redundant or less-used synapses, enhancing the efficiency of neural networks and refining neural circuits based on experiential learning.

One of the brain regions significantly impacted by pruning is the PFC [13,153,200], which is critical for various cognitive functions, including decision-making, attention, and emotional regulation. The PFC is composed of multiple sub-regions collectively engaged in complex cognitive processes and in the "top-down" executive modulatory control over socio-cognitive functions [154–156]. Therefore, it is recognized as a fundamental hub for social cognition.

Anatomically, the PFC can be divided into two main parts: the lateral and the medial regions. While the lateral PFC is more involved in broader cognitive functions that indirectly contribute to social cognition [204], the medial PFC, including the anterior cingulate cortex (ACC), is more directly linked to social cognition. These medial regions play essential roles in emotional self-awareness and the perception of others affective states [205–207]: evidence from human lesion studies has shown that damage to the mPFC is associated with deficits in accurately interpreting emotions in others, often resulting in abnormal social behaviors [206,208].

In rodents, the human PFC is paralleled by the PL, IL, and ACC [13]. Consistent with findings in humans, the rodent mPFC has been implicated in various social functions, including perception of others, social motivation to engage in relationships with others and the ability to discriminate the emotions of conspecifics [13,103]. These social abilities appear to be closely linked to specific neuronal subpopulations of inhibitory interneurons, such as SOM+ and PV+ neurons [102,103,107]. These neurons are particularly noteworthy as they modulate the excitatory-inhibitory balance in circuits associated with emotion processing. Moreover, these subpopulations mediate distinct

social domains: SOM + neurons in the PL cortex are critical for emotion recognition [103], while PV+ neurons are strongly associated with sociability [102,107].

Adolescence is a key phase of sensitivity for circuits involving these neuronal populations, as it is a critical window for the maturation of the excitatory-inhibitory balance for which PV+ and SOM + neurons are key players [153,209]. Consistent with this, during adolescence the levels of PV and SOM expression are increased in the PFC, coinciding with significant developmental changes [210]. Both the excitatory-inhibitory balance and the social behaviors it underpins appear to be influenced by the interplay between microglia and neurons. This dynamic interaction between neuronal and glial populations likely plays a fundamental role in shaping the emotional and cognitive maturation of the PFC during adolescence [153,200]. However, despite advancements in understanding the anatomical and functional changes that occur during adolescence, the intrinsic relationship between neuronal populations and glial cell activity—particularly microglia—remains poorly understood. Recent studies have highlighted distinct patterns of activity in neuronal populations at different stages of rodent adolescence, meaning early and late adolescence, with specific phases of development associated with unique functional dynamics [153]. Notably, the peak of synaptic pruning in the mPFC occurs around PND 30 [200,211], marking the transition from early to mid-adolescence. Microglia, known for their involvement in synaptic remodeling and pruning, may play a pivotal role in the refinement of inhibitory networks during adolescence.

Given that there is some evidence on the role of microglia in social behavior [212,213], but less is known about microglia and social behavior in adolescence (especially recognition of emotions), the aim of this Chapter is to identify the role of microglia in shaping social behavior in different stages of adolescence, and to elucidate their interplay with specific neuronal populations. Uncovering these mechanisms could provide crucial insights into the fine-tuning of adolescent brain development and its link with social behavioral deficits, potentially offering novel therapeutic avenues for NDD.

Methods

Mice

For the analysis of the behavioral effects of the microglia depletion, adolescent male and female C57BL/6J mice were used, while for the fiberphotometry experiment adolescent males and females Somatostatin cre-recombinase mice (SOM-Cre, Jackson: 013044) on a C57BL/6J background were used. C57BL/6J adolescent mice were used also for the spine analysis. SOM-Cre mice were heterozygous, and their genotype was determined by PCR of ear snip tissue. Animals were housed two to four per cage in a climate-controlled (22 ± 2 C) and specific pathogen-free animal facility, with ad libitum access to food and water throughout, a standard environmental enrichment (material for nest and cardboard house), and with a 12-hour light/dark cycle (7pm/7am schedule). Experiments were run during the light phase (between 10am-5pm). All mice were handled on alternate days during the week preceding the first behavioral testing. Experimenters were blind to mouse treatments during testing. All the animals were left in the home-cage with siblings until weaning, occurring at PND21. C57BL/6J adolescent mice paired for sex and age with the experimental mice were used as stimuli and habituated to the experimenter and to the testing box with the same schedule used for the observers, but by carefully avoiding any contamination between the odors between stimuli and observers. All the stimuli were unfamiliar for the observers.

Microglia depletion

PLX5622 was synthetized in our Institution ad added (1200 ppm) to rodent chow (Mucedola) following Spangemberg et al. [214]. Prior to administration, the effectiveness of the diet was tested in wild type mice to confirm microglial depletion (a depletion of at least 80% of microglia was observed after 9 days of diet). The PLX5622 supplemented diet was not different from the CTRL in the general composition, with the exception for the color, which was added to prevent involuntary shifting with the diet type. Diet was administered daily, in pellets, in the home cage, in order to allow ad libitum access.

Behavioral protocol

All the behavioral paradigms used in this chapter (Habituation Dishabituation, Emotion Discrimination Stress) have been fully describer earlier in the previous chapters.

Behavioral assessment in C57BL/6J adolescent mice

For the C57BL/6J mice who underwent only behavioral testing, mice were left with their mother until PND21 and then weaned. For the Early Adolescence experiment (PND 21- 31) mice received the PLX5622 or the CTRL diet since the first day after weaning. Handling was performed immediately after weaning to habituate the animal to the experimenter. Both mice and the amount of food left in the cage were weighted daily in order to verify a normal growth curve and the amount of food eaten. On PND 28 the habituation dishabituation task was performed, and then the animals were habituated to the EDT apparatus for 2 consecutive days. EDT Stress was performed on PND 31 and animals were then sacrificed for the further analysis. For the Mid Adolescence experiment (PND 32-42) animals were weaned and PND21 and handled, but the PLX5622 or CTRL diet was administered only starting from PND 32. Mice and the amount of food left in the cage were weighted daily. On PND 39 behavior was assessed with the Habituation Dishabituation task. After 2 consecutive days of habituation to the EDT apparatus, on PND 42 the EDT Stress was performed. Brains from both the Early Adolescence and the Mid Adolescence experiments were collected for the immunohistochemistry and spine density analysis.

Fiberphotometry in SOM-Cre adolescent mice

All the animals were left in the home cage with their mother until weaning at PND21. At PND 15, they underwent surgery for the viral vector injection, followed by the implant of the optic fiber around PND 30. The PLX5622 or CONTROL diet was administered starting from PND 32 (Mid Adolescence). All the normal rodent food was removed from the cage and substituted with the CONTROL chows or the one supplemented with PLX5622. Mice and the amount of food left in the cage were weighted daily. After signal checking, mice were habituated for 3 consecutive days to the EDT apparatus and to the cable. They underwent the EDT Stress test between PND 40 and 42, while recording for the calcium activity. At the end of the experiments (PND 42) brains were collected for further analysis.

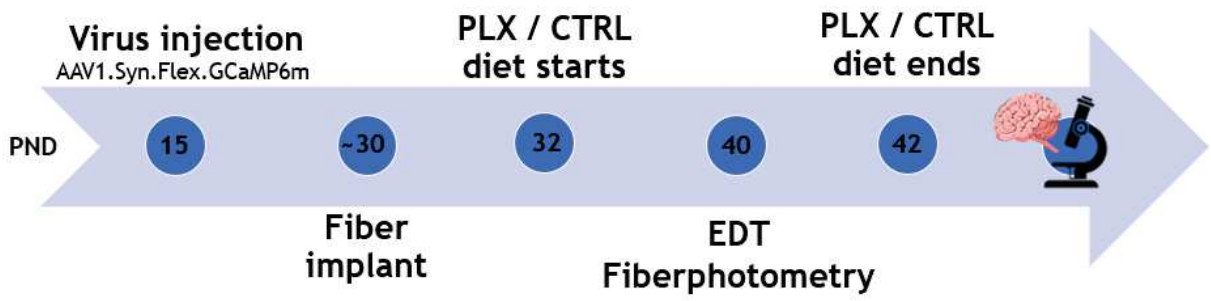


Figure 31: Timeline of the fiberphotometry experiment.

PND = post natal day. Following viral injection and fiber implant, animals were treated with the PLX or the CTRL diet and after habituation to the testing apparatus, EDT Stress was performed. Brains were then collected for further IHC and placement analysis.

Viral Injections and Fiber Implantation

Naïve mice were prepared for stereotaxic surgeries at postnatal day 14-16. All of them were anaesthetized with a mix of isoflurane (3%) in Oxygen (O2, 1%) by inhalation. Analgesic (Ketorolac/saline 1:10, 0.01ml per gram bodyweight) and antibiotics (Baytril 0.03 ml) was administered subcutaneously before the animal was mounted onto a stereotaxic frame (Kopf) linked to a digital reader. Throughout surgery isoflurane was maintained at 1.5%. Brain coordinates of viral injection in the mPFC were chosen to target PL cortex in accordance with the mouse brain atlas but adjusted to the smaller brain size. The pipette was inserted at a 10 degree angle to avoid puncturing the sagittal artery, and the following coordinates were used anterior-posterior (AP) +1.3 mm; medial-lateral (ML) +/- 0.5 mm; dorsal-ventral (DV) -1.3 mm. The animals were allowed to recover from anesthesia under a heating lamp until they were able to move, then immediately returned to the home cage with the mother. We infused and adeno-associated virus (AAV) through a borosilicate micropipette connected to a 10µl Hamilton syringe filled with mineral oil. AAV1.hSyn.Flex.GCaMP6m.WPRE.SV40 (100838-AAV1) was used as viral vector, 0.3 ul volume of virus diluted 1:5 in saline, titer 2.1 x 10(13), injected at 0.1ul/min in one hemisphere. The pipette was maintained in position for 4 min to allow proper diffusion and then slowly withdrawn. The injected volumes and targets were adjusted based on pilot injections and immunostaining to verify virus expression. At PND30, mice underwent a second stereotaxic

surgery for optic fiber implant. Anesthesia, analgesic and antibiotics procedures were the same as described above. Brain coordinates of optic fiber implant in the mPFC were AP +1.8, ML +/- 0.5, DV -2.1, based on Arruda-Carvalho et al [215].

In-vivo fiberphotometry recordings and analysis

To analyze calcium fluctuations, the fluorescence signal emitted by GCaMP6m-expressing SOM+ neurons was recorded using fiber photometry system. A signal processor (RZP5, Tucker Davis Technologies) was used to control two light sources (465-nm LED, CLED_465; 405-nm LED, CLED_405, Doric Lenses), which were modulated at 211 and 539 Hz, respectively. The two wavelengths were combined by a fluorescence minicube (Doric Lenses) and transmitted through an optical patch cable (Doric Lenses) to the mouse head implant. Emitted fluorescence was collected by the same patch cable, delivered back to the same minicube through a 525-nm filter and sent to a photoreceiver (Femtowatt Silicon Photoreceiver, DC-750 Hz; Newport). Real-time signals were acquired, lowpass filtered (3 Hz) and demodulated with Synapse Essentials software (Tucker Davis Technologies). Anymaze tracking system program (Stoelting, Ireland) was used. Data were extracted from TDT files and analyzed using custom MATLAB scripts.

Before starting the analysis, bleaching was corrected by fitting a double exponential decay to the fluorescent signal, capturing the exponential decay of the isosbestic. Subsequently, the GCaMP raw signal was divided by the fitted decay, and Z-score normalization was performed to standardize the signal by expressing it in terms of units of standard deviation. For the PSTH analysis, the signal was aligned to behavioral outcomes (entrance in the zone), considering a baseline preceding the events (T=5 seconds) and a post-baseline following the events (T=10 seconds). The PSTH quantities were then obtained by subtracting the mean of the baseline activity from the restricted signal and then dividing by the standard deviation of the same. Average plots of PSTH were represented as mean and standard error of the mean (S.E.M.), combining all the behavioral outcomes across all the mice. Areas under the curve (AUC) of the peristimulus curves have been computed over an interval of 10 seconds after the entrance in the zone (at time T=0), and subdivided into altered and neutral cases, if they corresponded to the entrance in the altered or neutral zone. Identification of calcium signal events was performed via custom MATLAB scripts, using the findpeaks function to find the local maxima of calcium signals. Such maxima were considered calcium events if they overcame thresholds on the minimum event height (MinHeight,

set as the average of the signal), minimum event prominence (MinProm, set as the standard deviation of the signal), minimum distance between consecutive events (MinDist = 1 second). The event frequency in a given time window was computed by dividing the number of events identified in such window by its length. Peak intensity was calculated as the ΔF/F value of the event.

Histology

At the end of the behavioral procedures, we checked viral expression and the position of the optic fibers, as well as microglial depletion with an IBA-1 staining. Mice were deeply anesthetized (urethane 20%) and transcardially perfused with 4% paraformaldehyde in PBS at pH 7.4. Brains were dissected, fixed in 4% PFA overnight at 4°C, and cryoprotected in 30% sucrose in PBS at 4°C.

For the IBA-1 staining, on the first day, coronal sections of the brain were cut with a thickness of 40 µm using a HM450 microtome (Thermo Fisher Scientific). The sections were washed three times in PBS for 10 minutes each. Blocking was performed to reduce non-specific binding by incubating the sections in a solution containing 10% normal goat serum (NGS) and 0.3% Triton X-100 in PBS, for 4 hours at room temperature (RT). Sections were then incubated overnight at 4°C in a primary antibody solution with PBS with 1% NGS and 0.5% Triton X-100, containing the anti-Iba1 antibody (Rabbit, FUJIFILM Wako Pure Chemical Corporation, cat n. 019-19741) at a dilution of 1:500. On the second day, the sections were washed three times for 10 minutes each in PBS containing 0.5% Triton X-100 and then incubated for two hours at RT in a secondary antibody solution (PBS with 1% NGS and 0.5% Triton X-100, anti-Rabbit antibody conjugated to Alexa Fluor 488 (1:1000, Molecular Probes, A-11008). Sections were then washed three times for 5 minutes each in PBS containing 0.5% Triton X-100, followed by a 5-minute wash in PBS, and a 15-minute wash in PBS with DAPI (1:1000). Finally, the sections are rinsed three additional times in PBS for 10 minutes, mounted on slides and covered with cover slips.

For the viral expression and optic fiber, free-floating sections of selected areas were washed in PBS three times for 10 min, blocked by incubation in PBS plus 10% normal goat serum (NGS) and 0.1% Triton X-100 for 30 min (all at room temperature, 20–23 °C), and subsequently incubated in PBS plus 1% NGS and 0.3% Triton X-100 overnight at RT with the following primary antibodies: rabbit GFP polyclonal antibody (1:1000, Abcam, A11122). Incubated slices were washed three times in PBS for 10 min at room temperature, incubated for 2 h at room temperature

with the following secondary antibody: Alexa Flour 488 goat anti-rabbit IgG (1:1000, Molecular Probes, A-11011) in PBS plus 1% NGS and 0.3% Triton X-100. Subsequently, slices were washed three times in PBS for 10 min at room temperature. Fluorescence images were obtained with a confocal microscope (Leica, CTR6500) using a 504 nm filter.

Spine analysis

Cardiac perfusion was first performed with 0.1M phosphate buffer (PB) at room temperature, followed by ice-cold 1.5% paraformaldehyde (PFA) in 0.1M PB. The brain was then post-fixed in 1.5% PFA in 0.1M PB for 40 minutes at 4°C. After post-fixation, the brain was washed twice with 0.1M PB to prepare it for sectioning. The brain was cut coronally around the region of interest to create a slice of 2–3 mm in thickness. This slice was made to ensure clear visibility of both the beginning and the end of the region (PL cortex). DiI crystals were then applied using a thin needle by delicately touching the region of interest on both sides of the 2–3 mm coronal slice. The slice was left in 0.1M PB for 12–24 hours in the dark at room temperature to allow the DiI to diffuse. Following the diffusion step, the slice was fixed in 4% PFA in 0.1M PB for 45 minutes to 1 hour at 4°C. Subsequently, 100–150 μm coronal slices were prepared using a vibratome in 0.1M PB on ice. The first slice was discarded. Finally, the slices were mounted on glass slides with Fluoromount mounting medium (Sigma-Aldrich) and sealed with nail polish to prepare them for confocal imaging.

For the DiOlistic labeling tubing was treated with polyvinyl pyrrolidone (PVP), using a stock solution of 20 mg/ml PVP in 100% ethanol. This stock solution was diluted freshly for each use by mixing 80 μl of the stock with 4920 μl of ethanol 100%. The dilute PVP solution was then drawn into the tubing using a syringe, ensuring it extended across the BioRad coating station. After allowing the solution to sit for 8 minutes, it was purged from the tubing, which was left to dry while the remaining dye preparation steps were completed. For coating the particles, 3 mg of dye (DiI, DiIC18(3)) was first placed into a locking snap-cap Eppendorf tube and suspended in 150 μl of methylene chloride by vortexing. Concurrently, 150 mg of tungsten particles (M-20) were spread onto a clean glass slide. The suspended dye solution was then added to the tungsten particles on the glass slide, rapidly spreading the mixture to form a thin film. After drying for several minutes, the particles were then carefully scraped from the slide with a razor blade onto wax filter

paper, producing a fine powder. The coated particles were transferred into a 15 ml conical tube and suspended in 3750 µl of MilliQ water. After brief vortexing and sonication for 1–2 minutes, the slurry was drawn into the precoated PVP tubing using a syringe with a snugly fitting piece of silicone tubing. The particles were allowed to settle for 2–3 minutes, and excess water was carefully removed with the syringe. The tubing was then dried gently using nitrogen gas at a low flow rate (0.2–0.4 lpm). Once dried, the tubing was cut into pieces (referred to as "bullets") using a tubing cutter. Finally, the bullets were stored in a covered or tinted scintillation vial with a desiccant pellet at 4°C, where they remained viable for several months. Shooting was conducted using the BioRad Helios Gene Gun, with parameters adjusted based on tissue type and condition. For initial optimization, helium pressure was set between 100–200 psi. A clean Isopore membrane filter with 3.0 µm pores was placed between the gun nozzle and the tissue to evenly disperse the particles. Membranes were replaced after three to four uses or sooner if clumping was observed, which often indicated membrane damage. The gun was positioned perpendicular to the tissue at a distance of 1–3 cm. After shooting, tissues were proceeded. Imaging was performed using a confocal microscope equipped with a 63x objective lens and a 543 nm laser for excitation. A z-stack was acquired, with 0.5 µm step intervals between slices to capture the three-dimensional structure of the sample. The zoom setting was maximized at 2, and the resolution was set to a minimum of 1024 pixels. Scanning was conducted at a mean of 2–4 scan lines per frame, with a scan speed set to level 3 to balance speed and image quality. Z-stack images were analyzed with ImageJ, focusing on slices where the spines were clearly in focus. The following measurements were taken for each spine: Spine Length: measured using the straight-line function, capturing the total distance from the base to the tip of the spine; Spine Head Width: measured at the widest point of the spine head; Neck Width: measured at the narrowest point of the spine neck. Once all the spines in an image were measured, the total dendrite length within the field of view was calculated. This was done using the free-hand line function to trace the length of all visible dendrites.

Results

Microglia depletion in early adolescence (PND 21 – 31)

First, we assessed body weight and the amount of food eaten in order to assess the absence of any impact of PLX5622 diet on the normal growth of mice. No differences were observed between the two groups. Moreover, a significant effect of Time indicates that the body weight is increasing over the days, indicating normal growth (Figure 34).

Effects on sociability

Microglia depletion during early adolescence did not produce effects in sociability as measured with the habituation dishabituation task, where a normal reduction of the total time spent sniffing stimuli was observed during the Habituation from Trial 1 to Trial 4, as well as a normal increase at Trial 5 (Dishabituation) which indicates normal social novelty recognition (Figure 36 A). The total interaction time was measured as the sum of sniffing from Trial 1 to Trial 4 but no differences were observed between the two groups, indicating that PLX5622 diet in early adolescence is not affecting sociability or social novelty recognition (Figure 36 B).

Effects on discrimination of negative affective states

The performance at the EDT task (Stress vs Neutral) indicated that PLX-treated mice were not able to discriminate between a stressed and a neutral conspecific (Figure 36 D), ability that was preserved in the CTRL-treated (Figure 36 C). The PLX-treated mice displayed a general increase in the exploration of the stimuli (Figure 36 F), but were not significantly preferring the stressed one, especially during the first trial where discrimination usually occurs. However, the differences between groups were not confirmed by the 3-way RM ANOVA that did not find any significant main effect of the Diet. The Discrimination Index further indicates a difference between the CTRL and the PLX-treated, however the difference was only approaching a trend level (Figure 36 E).

Effects on dendritic spines

The number, length, morphology and type of the dendritic spines in the PL cortex were evaluated to understand if the alterations in social behavior could be explained by changes in the synapses.

The analysis was performed on all the neurons. At PND 31 a significant increase in the length of spines and a reduction in the percentage of stubby spines was observed in the PLX-treated group compared with controls. All the other parameters were not significantly different between the two groups (Figure 33 A).

Microglia depletion in mid adolescence (PND 32-42).

The analysis of food consumption and of body weight during PLX and CTRL diet administration indicated no difference between the two groups, confirming that also in mid adolescence the diet is not having a significant impact on growth overall (Figure 35).

Effects on sociability

Microglia depletion during mid adolescence induced an increased sociability at the habituation dishabituation task, in PLX compared with the CTRL group (Figure 38 A). The total time spent sniffing the familiar stimulus in the Habituation phase (Trial 1 - Trial 4) was significantly higher in the PLX group compared with CTRL (Figure 38 B) indicating an increased interest for social interaction. The amount of social interaction in the PLX group was similar with the one of early adolescent mice (Figure 37). However, both PLX and CTRL mice were able to discriminate social novelty (Figure 38 A), as suggested by the increased sniffing during the Dishabituation phase (Trial 5) compared with the last Habituation trial (Trial 4), indicating normal recognition of social novelty with no group differences.

Effects on discrimination of negative affective states

The discrimination between stress and neutral stimuli was impaired in adolescent mice treated with PLX5622 compared with controls (Figure 38 C and D). While in the CTRL group could significantly discriminate stress, with a significant preference especially during the first 2 minutes of the test (Figure 38 C), no discrimination was seen in the PLX group, in none of the Trials, with mice spending the same amount of time exploring both the stimuli (Figure 38 D). The significant difference in the discrimination index indicate that while the CTRL displayed a significant preference for the stressed demonstrator, this was not true for the PLX-treated group (Figure 38 E). Moreover, the total exploration for both the stimuli was not different between the two groups,

further confirming that the deficits was not linked with a reduction in the exploratory behavior (Figure 38 F).

Effects on dendritic spines

The analysis of spine morphology and type at PND42 revealed no significant differences between the PLX and CTRL groups. Both groups exhibited similar protrusion density, spine width and length, and percentage of distribution of the spine types, indicating that the treatment with PLX did not affect these parameters and therefore was not interfering with synaptic pruning at this time point (Figure 33 B).

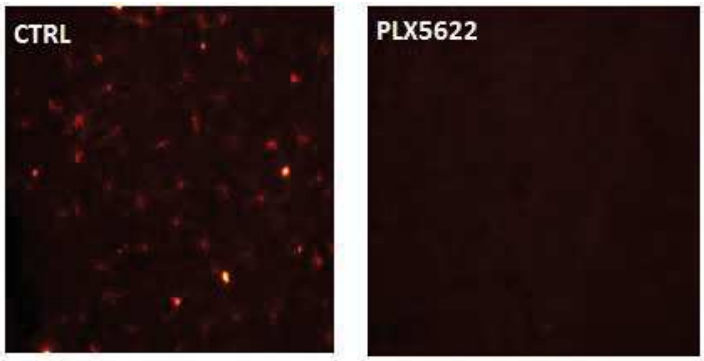


Figure 32: Representative image of the PLX effect after 10 days of PLX.

The image represents the microglia in the PL cortex of adolescent mice after IHC staining for IBA-1. In the CTRL treated group microglia is still present, while in the PLX group it's abolished.

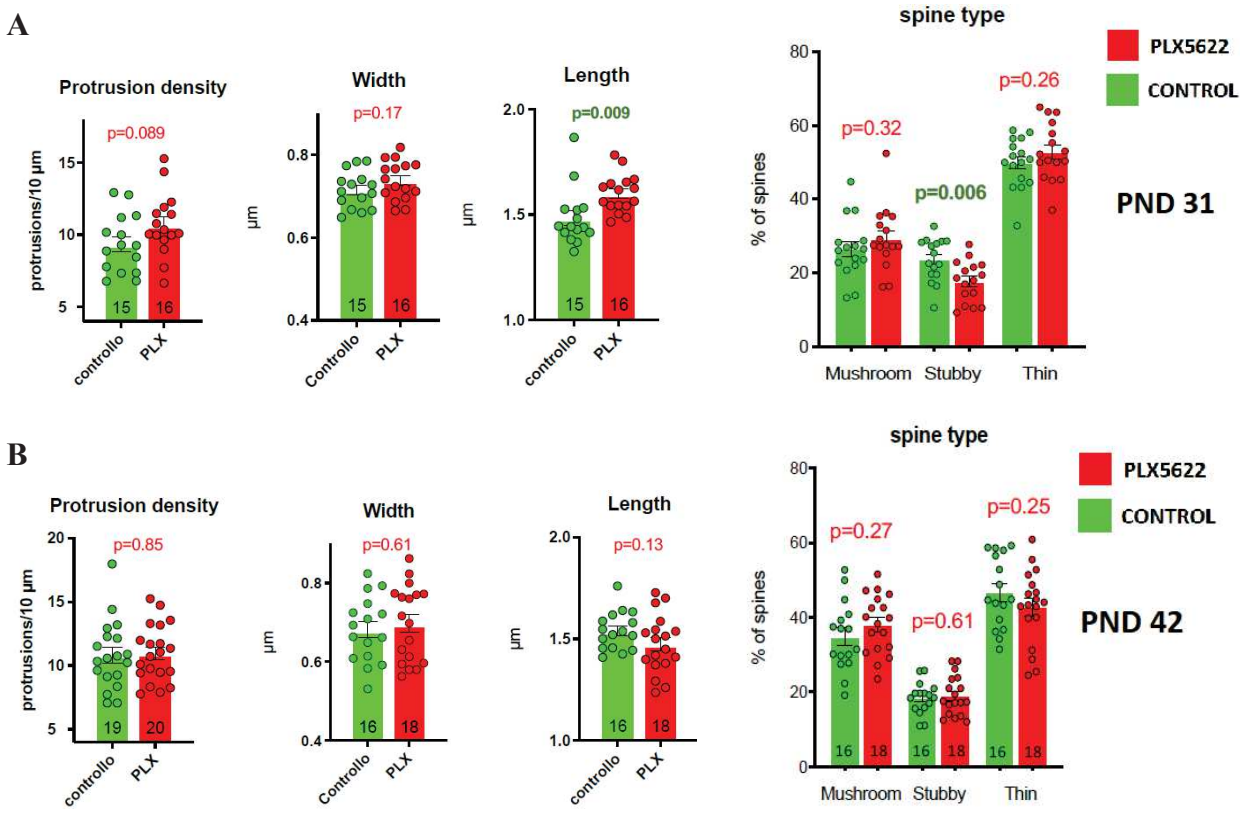


Figure 33: Analysis of dendritic spines (all neurons) in PLX or CTRL mice in Early and Mid-Adolescence.

Width: measured at the narrowest point of the spine neck. Length: measured using the straight-line function, capturing the total distance from the base to the tip of the spine.

A t-test for independent samples was performed to compare all the conditions and p-value are reported on the figures.

A) Early Adolescence

Analysis for early adolescence were performed on 4 male mice, on all the neurons. Animals were sacrificed on PND 31 after PLX or CTRL diet.

B) Mid Adolescence

Analysis in Mid adolescence were performed on 8 male mice, on all the neurons. Animals were sacrificed on PND 42 after PLX or CTRL diet.

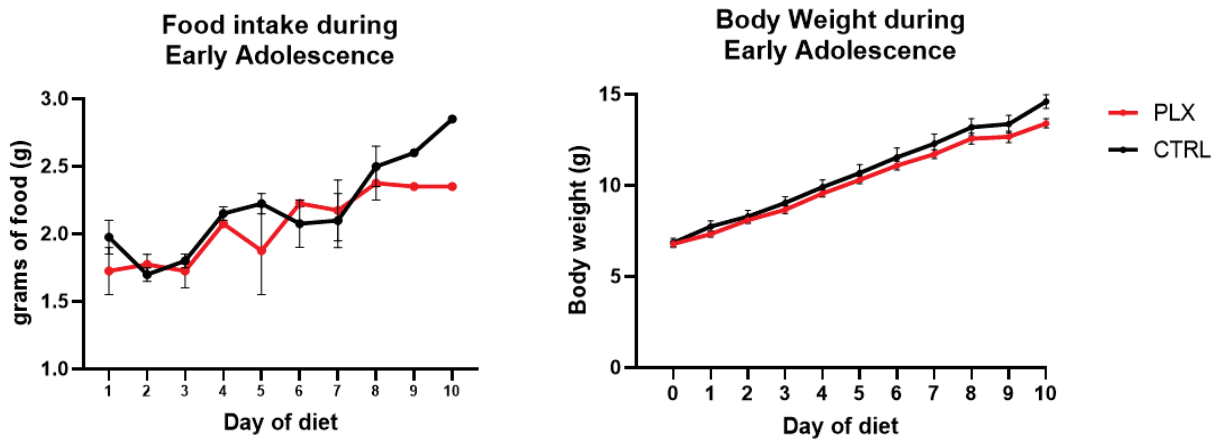


Figure 34: Early Adolescence (PND21-31) food consumption and body weight

N(CTRL) = 9 (5 males, 4 females) N(PLX) = 10 (5 males, 5 females)

The graph on the left represent the daily measurements of body weight for CTRL and PLX treated mice. The graph on the right represents the body weight assessed daily for the PLX and the CTRL treated mice. No significant differences were observed between the two groups. The mixed-effects model analysis of food intake reveals no significant effects of Time ($F(1.847, 2.873) = 6.708$, $p = 0.0830$), Diet ($F(1, 2) = 2.552$, $p = 0.2513$), or their interaction ($F(9, 14) = 0.7914$, $p = 0.6296$). The mixed-effects model analysis of body weight during shows a significant effect of Time ($F(1.241, 10.18) = 636.8$, $p < 0.0001$) indicating a normal growth-curve and a significant interaction between Time and Diet ($F(10, 82) = 2.020$, $p = 0.0414$). Diet alone does not have a significant effect ($F(1, 9) = 1.151$, $p = 0.3113$). However, the Sidak's multiple comparisons post hoc test reveals no significant differences in mean body weight between the PLX and CTRL groups at any specific time point. The largest difference occurs at time point 10, where the mean difference is -1.213 (95% CI: -3.514 to 1.089, adjusted $p = 0.4217$), but not statistically significant.

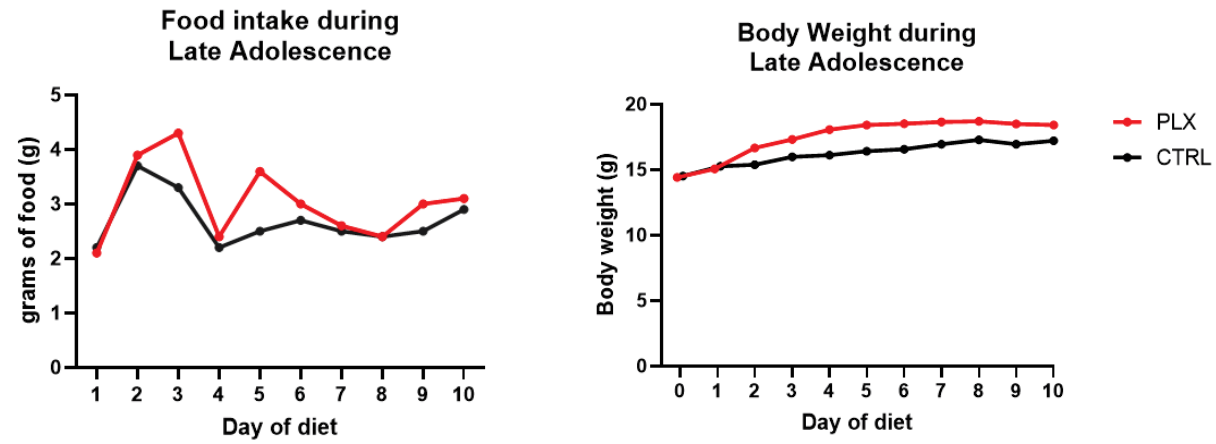


Figure 35: Mid Adolescence (PND 32-42) food consumption and body weight

N(CTRL) = 22 (15 males, 7 females) N(PLX) = 23 (16 males, 7 females)

On the left side, the amount of food eaten daily. On the right, the body weight of mice assessed daily.

The two-way repeated measures ANOVA analyzing food intake shows a significant interaction between Time and Diet ($F_{(10, 20)} = 3.162$, $p = 0.0136$) but not main effect of Time ($F_{(1.421, 2.842)} = 5.460$, $p = 0.1067$), or Diet ($F_{(1, 2)} = 2.666$, $p = 0.2441$). The Sidak's multiple comparisons test for food intake between PLX and CTRL groups reveals no significant differences across all time points. The largest mean difference is observed at time point 3 (-2.400, 95% CI: -22.92 to 18.12, adjusted $p = 0.7438$), but it is not statistically significant. The two-way repeated measures ANOVA analyzing body weight shows no significant main effects or interactions. Time and Diet ($F_{(10, 20)} = 0.9603$, $p = 0.5044$), Time ($F_{(1.052, 2.104)} = 8.766$, $p = 0.0923$), Diet ($F_{(1, 2)} = 0.2272$, $p = 0.6806$).

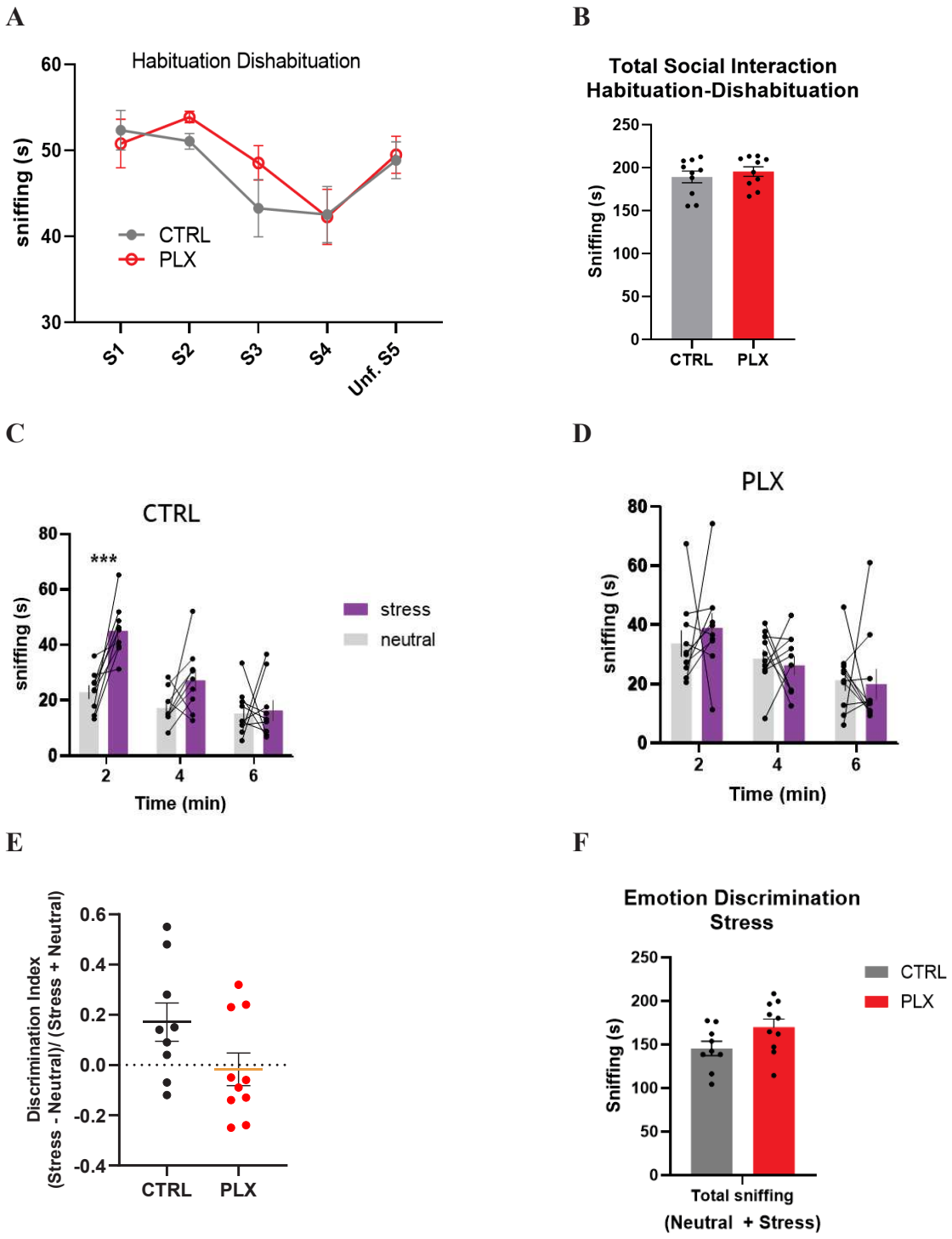


Figure 36: Early Adolescence: Habituation Dishabituation and Emotion Discrimination (Stress) in PLX-5622 and CTRL.

N(CTRL) = 9 (5 males, 4 females) N(PLX) = 10 (5 males, 5 females)

A) Habituation Dishabituation task. Sniffing during the trials.

No significant differences were observed between the CTRL and the PLX treated mice at the Habituation Dishabituation task.

The two-way repeated measures ANOVA shows a significant main effect of Trial ($F_{(3.12, 56.1)} = 8.20$, $p = 0.0001$). There is no significant main effect of Diet ($F_{(1, 18)} = 0.373$, $p = 0.5492$), and no significant interaction between Trial and Diet ($F_{(4, 72)} = 0.856$, $p = 0.4947$).

B) Habituation Dishabituation task: total sociability.

Total sociability was calculated as the sum of sniffing of all the Habituation trials (from Trial 1 to Trial 4). The unpaired t-test comparing total sociability in CTRL and PLX groups reveals no significant difference ($t_{(18)} = 0.712$, $p = 0.4857$).

C) Emotion Discrimination: Stress. CTRL group.

A significant main effect of Trial is observed ($F_{(2, 34)} = 71.17$, $p < 0.0001$). The main effects of Diet ($F_{(1, 17)} = 3.730$, $p = 0.0703$) and Emotion ($F_{(1, 17)} = 4.316$, $p = 0.0532$) approach significance. None of the interactions are significant, including Trial × Diet ($F_{(2, 34)} = 0.5686$, $p = 0.5716$), Trial × Emotion ($F_{(2, 34)} = 2.648$, $p = 0.0854$), Diet × Emotion ($F_{(1, 17)} = 3.564$, $p = 0.0762$), and Trial × Diet × Emotion ($F_{(2, 34)} = 0.6922$, $p = 0.5074$). In the CTRL group, the two-way repeated measures ANOVA reveals significant main effects of Trial ($F_{(1.92, 30.7)} = 32.2$, $p < 0.0001$) and Emotion ($F_{(1, 16)} = 9.93$, $p = 0.0062$). Additionally, the interaction between Trial and Emotion is significant ($F_{(2, 32)} = 10.5$, $p = 0.0003$). The Sidak's multiple comparisons test for the CTRL group reveals a significant difference between Neutral and Stress in Trial 1, with a mean difference of -22.0 (95% CI: -32.8 to -11.2, adjusted $p = 0.0002$). For Trial 2, the difference of -9.90 (95% CI: -22.3 to 2.51, adjusted $p = 0.1356$) is not significant, and in Trial 3, the difference of -1.02 (95% CI: -13.4 to 11.3, adjusted $p = 0.9948$) is also not significant.

D) Emotion Discrimination: Stress. PLX group.

The two-way repeated measures ANOVA in the PLX group shows a significant main effect of Trial ($F_{(1.80, 32.4)} = 7.50$, $p = 0.0028$). There is no significant main effect of Emotion ($F_{(1, 18)} = 0.0231$, $p = 0.8809$), and no significant interaction ($F_{(2, 36)} = 0.517$, $p = 0.6004$).

E) Discrimination Index of the Emotion Discrimination: Stress

The DI was calculated as [(Stress- Neutral)/(Stress+Neutral)]. A positive DI indicates a preference for the stressed stimulus. The unpaired t-test comparing CTRL and PLX groups shows no significant difference but only a tendency towards a reduction in PLX-treated animals ($t_{(17)} = 1.888$, $p = 0.0763$).

F) Total exploration time during the Emotion Recognition: Stress.

The paired t-test comparing CTRL and PLX groups for the total sniffing of the stimuli during the Emotion Discrimination Stress shows no significant difference ($t_{(8)} = 1.458$, $p = 0.1830$), despite a small increase in the total exploration time in the PLX group (difference between groups: 20.10 ± 13.79).

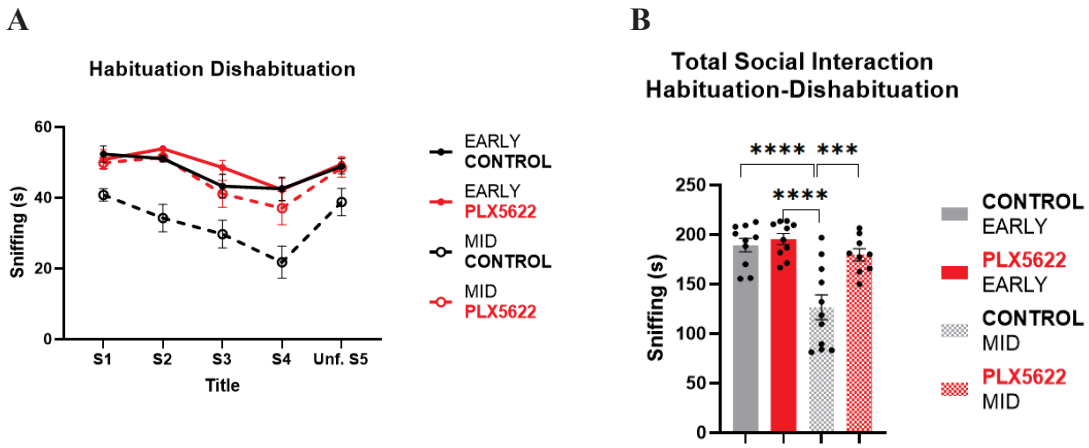


Figure 37: Habituation Dishabituation Task. Comparison between Early and Mid-Adolescence.

A) Habituation Dishabituation

While PLX was not producing significant alterations in early adolescence, the treatment was increasing the sociability in mid adolescence. This figure indicates that the levels of sociability in mid adolescence, in animals treated with PLX, is similar with the one observed in early adolescence in both PLX and in the CTRL.

B) Total sociability

The 2-way ANOVA on total social interaction shows that interaction between Diet and Phase was statistically significant ($F_{(1, 36)} = 7.179$, $p = 0.011$) as well as the main effect of phase ($F_{(1, 36)} = 20.42$, $p < 0.0001$) and of Diet ($F_{(1, 36)} = 11.56$, $p = 0.0017$). Sociability in late adolescence in the control group was significantly reduced compared with all the other conditions: compared with late - PLX ($p = 0.0008$); with early PLX ($p < 0.0001$); with early CTRL ($p < 0.0001$).

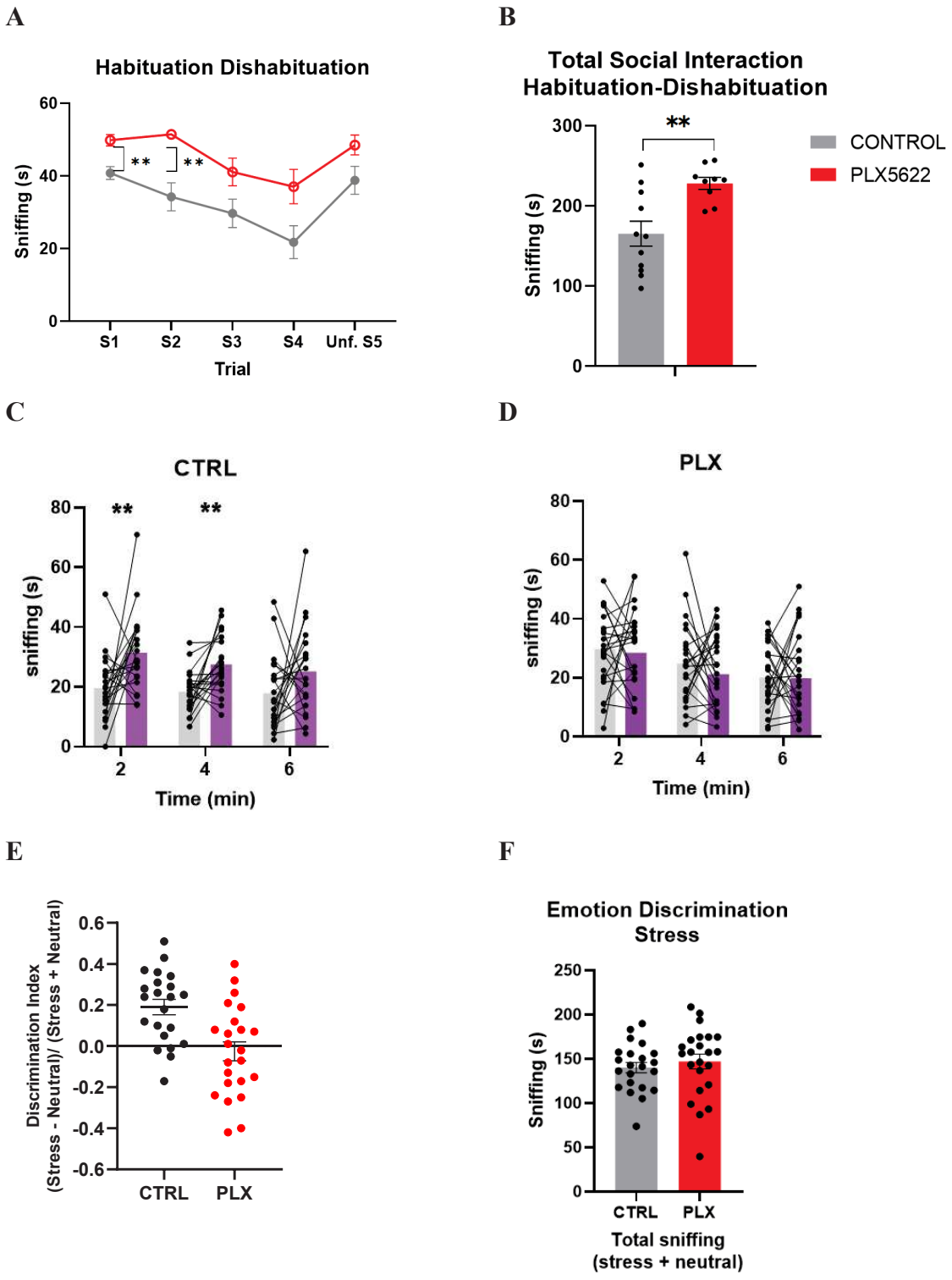


Figure 38: Mid Adolescence: Habituation Dishabituation and Emotion Discrimination (Stress) in mice treated with PLX5622 or with CTRL diet.

A) Habituation Dishabituation task.

The two-way repeated measures ANOVA for Habituation Dishabituation test reveals significant main effects of Trial ($F_{(3.263, 58.73)} = 13.57$, $p < 0.0001$) and Diet ($F_{(1, 18)} = 11.34$, $p = 0.0034$). The interaction between Trial and Diet is not

significant ($F_{(4, 72)} = 0.9546$, $p = 0.4378$). The Sidak's multiple comparisons test shows significant differences between PLX and CTRL in Trial 1 (mean difference = 9.03, 95% CI: 2.30 to 15.76, adjusted $p = 0.0059$) and Trial 2 (mean difference = 17.19, 95% CI: 4.97 to 29.41, adjusted $p = 0.0058$). No significant differences are observed in Trial 3 (mean difference = 11.40, 95% CI: -4.24 to 27.05, adjusted $p = 0.2296$), Trial 4 (mean difference = 15.30, 95% CI: -3.46 to 34.06, adjusted $p = 0.1452$), or Trial 5 (mean difference = 9.72, 95% CI: -3.91 to 23.34, adjusted $p = 0.2467$). The two-way repeated measures ANOVA analyzing recognition of Social Novelty (sniffing during Trial 4 vs Trial 5) reveals significant main effects of Novelty ($F(1, 14) = 17.50$, $p = 0.0009$) and Diet ($F(1, 14) = 18.32$, $p = 0.0008$), but the interaction between Novelty and Diet is not significant ($F(1, 14) = 0.6411$, $p = 0.4367$). The Sidak's multiple comparisons test shows significant differences between Trial 4 and 5 within both the PLX ($p = 0.0451$) and CTRL ($p = 0.0100$) groups.

B) Habituation Dishabituation task: total sociability.

Total sociability was calculated as the sum of sniffing of all the Habituation trials (from Trial 1 to Trial 4). The unpaired t-test shows a significant difference, with higher mean in the PLX (228.1) compared to CTRL group (165.4) ($t_{(18)} = 3.367$, $p = 0.0034$).

C) Emotion Discrimination: Stress. CTRL group.

N(CTRL) = 22 (15 males, 7 females).

The three-way ANOVA shows a significant main effect of Trial ($F_{(2, 114)} = 8.480$, $p = 0.0004$). There is no significant main effect of Diet ($F_{(1, 114)} = 0.745$, $p = 0.3900$) or Emotion ($F_{(1, 114)} = 3.496$, $p = 0.0641$). The interaction between Diet and Emotion is significant ($F_{(1, 114)} = 8.398$, $p = 0.0045$). None of the other interactions are significant: Trial × Diet ($F_{(2, 114)} = 0.6192$, $p = 0.5402$), Trial × Emotion ($F_{(2, 114)} = 0.3105$, $p = 0.7337$), or Trial × Diet × Emotion ($F_{(2, 114)} = 0.4138$, $p = 0.6621$).

To assess if the CTRL group could discriminate the affective states of stimuli, a two-way repeated measures ANOVA in the CTRL group was performed. There was significant main effect of Emotion ($F_{(1, 36)} = 15.3$, $p = 0.0004$), but no significant effect of Trial ($F_{(1.75, 63.0)} = 2.10$, $p = 0.1364$), and of the interaction between Trial and Emotion ($F_{(2, 72)} = 0.631$, $p = 0.5348$). The mean sniffing time of the Neutral is 18.9, and for the Stress it is 26.7, resulting in a significant mean difference of -7.79 (95% CI: -11.8 to -3.75). The Sidak's multiple comparisons test reveals significant differences between the sniffing for Neutral and Stress in the first two Trials. In Trial 1, the mean difference is -10.9 (95% CI: -21.2 to -0.662, adjusted $p = 0.0340$), and in Trial 2, the mean difference is -7.25 (95% CI: -13.7 to -0.824, adjusted $p = 0.0229$). However, in Trial 3, the mean difference of -5.17 (95% CI: -15.1 to 4.73, adjusted $p = 0.4867$) is not significant.

D) Emotion Discrimination: Stress. PLX group.

N(PLX) = 23 (16 males, 7 females)

The two-way repeated measures ANOVA reveals a significant main effect of Trial ($F_{(2.00, 79.8)} = 7.15$, $p = 0.0014$). There is no significant effect of Emotion ($F_{(1, 40)} = 0.391$, $p = 0.5352$), and of the interaction between Trial and Emotion ($F_{(2, 80)} = 0.253$, $p = 0.7772$). The mean sniffing time of the Neutral is 24.9, while for the Stress it is 23.2, resulting in a mean difference of 1.68. The Sidak's multiple comparisons test for the PLX group reveals no significant differences between the Neutral and Stress across all Trials. In Trial 1, the mean difference is 1.27 (95% CI: -8.45 to 11.0, adjusted $p = 0.9835$). In Trial 2, the mean difference is 3.59 (95% CI: -6.46 to 13.6, adjusted $p = 0.7597$). In Trial 3, the mean difference is 0.171 (95% CI: -9.26 to 9.60, adjusted $p > 0.9999$). This indicates that mice treated with PLX cannot discriminate negative affective states in conspecifics.

E) Discrimination Index of the Emotion Discrimination: Stress

The unpaired t-test comparing DI between CTRL and PLX groups shows a significant difference ($t_{(43)} = 3.620$, $p = 0.0008$). The mean for CTRL is 0.1900, while for PLX it is -0.02522, with a mean difference of -0.2152 (SEM = 0.05946, 95% CI: -0.3351 to -0.09531).

F) Total exploration time during the Emotion Recognition: Stress.

The unpaired t-test comparing total exploration of the stimuli during EDT reveals no significant difference between CTRL and PLX ($t_{(43)} = 0.6666$, $p = 0.5086$). The mean sociability for CTRL is 140.1, while for PLX it is 146.9, with a mean difference of 6.830 (SEM = 10.25, 95% CI: -13.83 to 27.49).

Microglia depletion in Mid Adolescence alters the activity of SOM+ neurons in the PL cortex of mice.

SOM-Cre mice were tested with the emotion discrimination stress paradigm while simultaneous fiber photometry recordings were performed to measure the activity of SOM+ interneurons in the PL cortex (Figure 39 C). The behavioral findings replicated those from previous experiments, confirming a deficit in emotion discrimination in the PLX-treated group (Figure 39 A) as indicated by the reduced DI in the PLX-treated group (Figure 39 B).

Fiber photometry revealed distinct patterns of SOM+ interneuron activity during the test (Figure 39 C). In the CTRL group, SOM+ activity was higher during interaction with the stressed stimulus and lower during the interaction with the neutral stimulus (Figure 39 C). Conversely, in the PLX-treated group, this pattern was inverted, and activity was higher during interaction with the neutral stimulus compared to the stressed stimulus (Figure 39 C). Analysis of the area under the curve (AUC) confirmed this difference in SOM+ activity between groups, with significant differences between the PLX and the CTRL in the activity of SOM+ neurons in response to sniffing of the Neutral stimulus (Figure 39 E). The changes in SOM+ activity were task-specific, aligning with the episodes of interaction involving the stressed or neutral demonstrators (Figure 39 C).

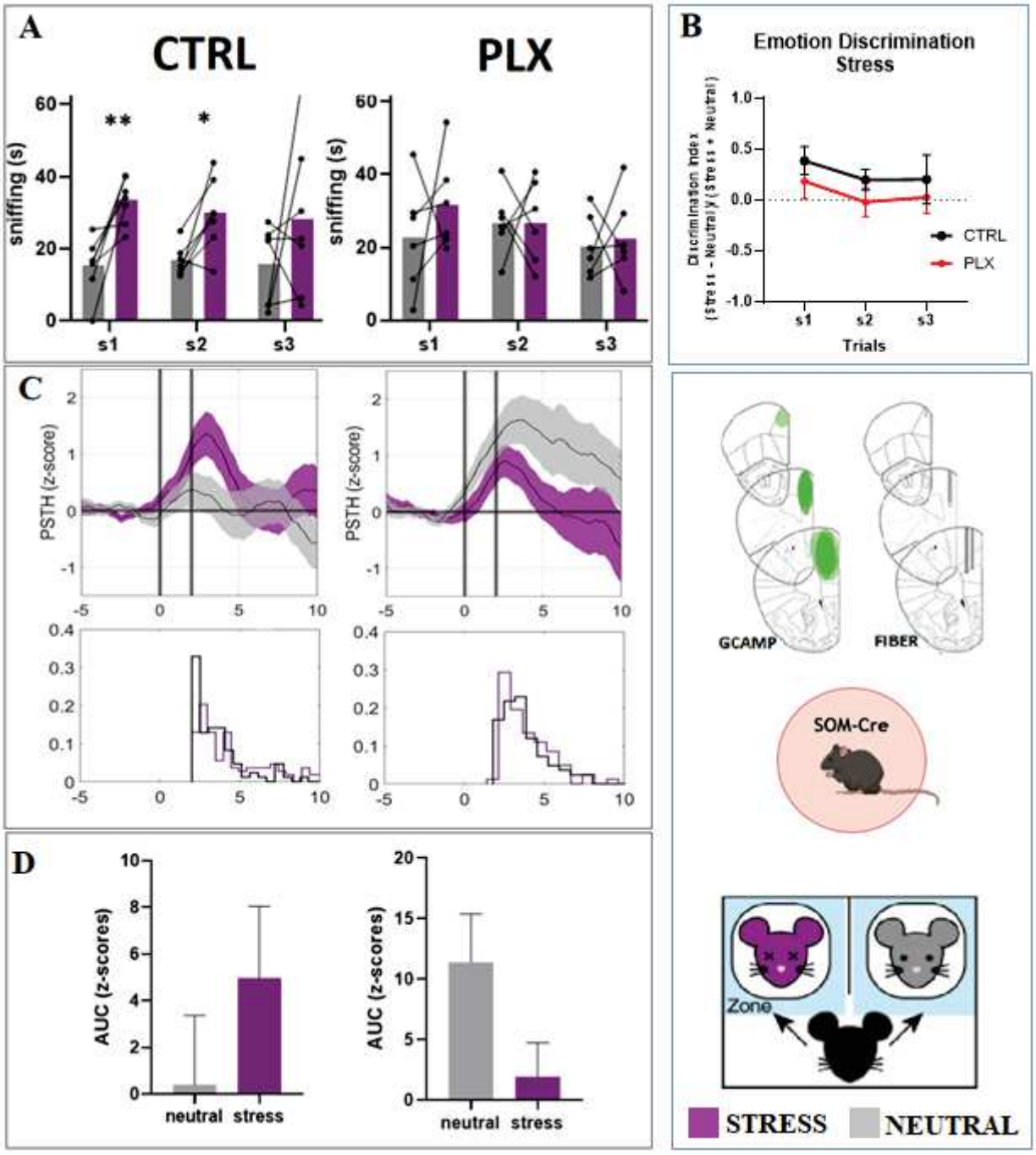


Figure 39: Fiberphotometry experiment in SOM-Cre adolescent mice treated with PLX or with the CTRL diet.

In the box on the bottom-right of the Figure, a summary of the experiment is reported. After viral injection and optic fiber implant, Som-Cre mice were tested in the EDT – Stress with simultaneous recording of the activity of SOM+ interneurons.

A) Behavior of the SOM-Cre adolescent mice during the recording of Ca+ activity with fiberphotometry.

The rest replicates the results observed previously in Mid Adolescence, with PLX-treated mice showing an impairment in the ability to discriminate between the stressed and the neutral stimulus.

B) Discrimination Index

A reduced discrimination index, approaching zero, is observed in the PLX group, further indicating the absence of discrimination in SOM-Cre PLX-treated mice compared with CTRL during fiberphotometry acquisition.

C) Activity of SOM+ neurons in the CTRL (left) and in the PLX (right) treated animals.

The PTSH (peri-stimulus) represents the 5 seconds before and after a sniffing event, in which the observer was sniffing a neutral (gray) or a stressed (purple) stimulus. The second row represents the likelihood to have a sniffing event for the neutral (black) or for the stress (purple) for all the PTSH, to show the alignment of the activity peak of SOM+ with the sniffing event. In the CTRL group, the activity of SOM+ was higher during the sniffing of the stressed stimulus, for which this experimental group was showing a preference, and lower for the neutral. In the PLX group the activity of SOM+ neurons was higher during the sniffing of the neutral than of the stress, in line with the behavioral data indicating no preference between the two stimuli.

D) Area under the curve for the SOM+ activity.

The area under the curve (AUC) was calculated to measure the different activity of SOM+ neurons in CTRL and in PLX. The AUC was higher when CTRL animals were sniffing the stressed stimulus compared with the sniffing of the neutral. The opposite pattern was observed in the PLX treated animals, where the AUC was higher for the neutral than for the stressed stimulus. Further analysis revealed a significant difference in the neuronal response in the interaction with the Neutral stimulus between CTRL and PLX.

The mixed-effects model analysis on the AUC reveals a significant interaction between Emotion and Diet ($F_{(1, 165)} = 4.703$, $p = 0.0315$), but no main effects of Emotion ($F_{(1, 165)} = 0.5766$, $p = 0.4487$) or Diet ($F_{(1, 189)} = 1.441$, $p = 0.2315$). The Sidak's multiple comparisons test reveals a significant difference between CTRL and PLX for the SOM+ activity in response to the Neutral, with a predicted mean difference of -10.92 (95% CI: -21.53 to -0.3096, adjusted $p = 0.0423$). The activity in response to the Stress was not significant with predicted mean difference of 2.977 (95% CI: -7.095 to 13.05, adjusted $p = 0.7572$).

Discussion

In this Chapter, I presented evidence that microglia depletion induces phase-specific changes in social behavior during adolescence, which could be driven by distinct mechanisms. In early adolescence, the phase immediately preceding the peak of synaptic pruning in mice, microglia depletion leads to deficits in emotion discrimination without affecting sociability. In contrast, during mid adolescence, microglia depletion results in deficits in both sociability and emotion discrimination. These findings suggest that sociability relies on mechanisms that reach maturation only after PND 31, whereas emotion recognition appears to be influenced by microglial modulation throughout adolescence.

Interestingly, the sociability of 16p +/- mice treated with PLX during mid adolescence mirrors the levels of social interaction observed in PLX- and control-treated mice in early adolescence. This resemblance suggests that PLX treatment delays behavioral maturation, maintaining the same level of sociability that were observable in the earlier phase, instead than following a normal reduction in the post-puberal phase. Given that synaptic pruning peaks around PND 31 [200], and that the habituation tests in early and mid-adolescence were performed around PND 28 and PND 39 respectively, it is plausible that sociability-specific maturation occurs between these time points and that it is not mediated by synaptic pruning. It was demonstrated that gamma-band (30–80 Hz) prefrontal activity exhibits a marked increase during early adolescence (P28–P35), followed by a decrease during mid adolescence (P36–P43), and a rebound to adult-like activity [153]. These dynamics could indicate that the mechanism underlying the development (and therefore reduction) of sociability in mid-adolescence could be linked with gamma-band activity which is expected to decline in this phase. The above mentioned study shows that PLX treatment in early adolescence increases basal spine densities on dendrites of layer 2/3 pyramidal neurons (L2/3 PYRs), which are putatively responsible for the gamma oscillations together with the PV+ interneurons [153]. However, the study doesn't provide the same information about PLX depletion in mid adolescence. It is important to note that PV and of SOM are highly expressed in the mPFC during adolescence [210], and that the expression of SOM is decreasing from PND 28 to adulthood, while PV shows a significant increase comparing PND28 to PND42, especially in PL cortex of male mice [210]. It is possible that in mid adolescence the gamma oscillation could depend particularly on PV+

interneurons [104], and therefore that microglia depletion is in turn affecting the PV+ interneuron activity in mid-adolescence. An in-vivo recording of the activity (i.e. with fiberphotometry) of PV+ interneurons during social interaction with microglia depletion in mid adolescence could probably help clarifying these mechanisms.

Another major finding of this Chapter is that in adolescence emotion recognition is linked to the activity of SOM + interneurons in the PL cortex. This replicates previous findings in adult mice [103,216]: in fact, similar with the study on adults, increased calcium signaling in SOM + neurons in the PL cortex was observed during interactions with demonstrators in altered affective states, but not with neutral ones. However, PLX treatment disrupts this response, altering SOM + neuron activity (which is higher in interactions with neutral demonstrators), suggesting that microglia depletion in adolescence impacts SOM-mediated circuits critical for emotion recognition. This mechanism is not likely supported by alterations in synaptic pruning, since the analysis of dendritic spines indicated that their maturation and their length is not affected by PLX, at least in mid adolescence. However, the analysis was performed on all neurons of the PL cortex and not only on SOM +. Previous studies (although performed in the first two weeks of life of the mice) indicate that microglia depletion increases SOM+ synapse numbers [98] and that the modulation of SOM+ cells by microglia is mediated through chemokines such as CX3CL1 (which inhibits axonal growth and complexity), and CXCL12 (which promotes axonal complexity without affecting total axonal length) [217]. The exact mechanisms through which microglia modulates SOM+ activity in adolescence have not been studied yet, however similar processes could be involved and would require further investigation.

In adolescence, the role of the neuro inflammation has often indicated that stress-induced effects from environmental factors, such as social isolation [218], disrupted sleep patterns [159], infections [219] or substance exposure [220], could participate in disturbing the development of neural circuits leading to behavioral alterations and mental disorders. One recent study has paired the double vulnerability to MIA with the effect of stressors in adolescence, indicating a possible combination between the two [191]. The findings in this chapter offer an intriguing framework for exploring the mechanisms underlying reduced sociability in MIA-exposed 16p +/- mice, which were tested in late-adolescence as well. The observed phenotype may represent the opposite of microglia depletion, potentially linked to microglial hyper-activation. This hypothesis warrants further investigation:

one promising approach would be to test the effects of PLX treatment in 16p-MIA mice during adolescence to determine whether it can reverse the observed sociability deficits. If this mechanism is validated, it would indicate that the interplay between microglia and PV+ interneurons during adolescence can be a key area for further exploration in the context of the 16p11.2 deletion.

CHAPTER 7

Summary of the findings, limitations and future directions.

Summary of the findings

In this work, I conducted a comprehensive evaluation of the social behavior of 16p +/- mice, focusing initially on adulthood and assessing behavior across two distinct genetic backgrounds to account for potential genetic modulators outside the deleted locus.

In the C57BL/6J inbred background, no differences were observed between 16p +/- and wild-type littermates in measures of sociability, social novelty, social memory, and emotion discrimination for relief and stress. This suggests that 16p +/- mice behave similarly to their wild-type littermates and do not display the social impairments typically associated with ASD in humans. A plausible explanation is that the genetic mutation alone is insufficient to induce ASD-like phenotypes and may require an additional challenge, either genetic or environmental, to manifest relevant symptoms.

In the CD1 background, I confirmed no differences in sociability, social novelty, and social memory between the two groups. However, emotion discrimination results were inconclusive, as CD1 mice in general, regardless of genotype, did not exhibit clear emotion discrimination. This lack of differentiation could be attributed to intrinsic limitations of the test in this background or potentially to deficits in wild-type CD1 mice raised in the same cage as 16p +/- littermates. To clarify this, a study comparing wild-type littermates of 16p +/- mice in the CD1 background with pure CD1 mice would provide valuable insights (we have preliminary findings in other strains supporting this hypothesis).

Following the "double-hit" hypothesis, I tested whether an immunological challenge mimicking a viral infection could affect the social behavior of 16p +/- mice and their wild-type littermates beyond the acute phase. I demonstrated that injection with Poly I:C induced an activated state in microglia after 24 hours, with full recovery observed after 10 days. Behavioral testing conducted after the resolution of the acute viral-like challenge revealed no differences between 16p +/- and wild-type mice in sociability, social novelty, social memory, or emotion recognition. However, the challenge itself had a genotype-independent effect on emotion recognition, impacting both 16p +/- and wild-type mice. This finding suggests that the post-acute phase following a viral-like challenge may compromise affective state recognition in mice, a domain which is potentially linked to

inflammation. Further tests, including controls for potential olfactory deficits, are planned to confirm this hypothesis, including also a comprehensive assessment of the microglial morphology and reactivity in the 16p +/- and wild-type mice.

To better clarify if the absence of more subtle impairments could be present in earlier stages of the 16p +/- mice, I next extended to adolescence the assessment of social behavior in 16p +/- mice by exploring potential sub-threshold deficits during adolescence, a critical period of brain remodeling. Testing 16p +/- mice during this phase revealed no differences in sociability, social novelty, memory, or affective state discrimination compared to their wild-type littermates. However, emotion recognition for relief was not significant in either genotype, suggesting that the recognition of positive emotions may not yet be fully developed during adolescence. Similar to findings in the CD1 background, it remains unclear whether this lack of recognition is an intrinsic feature of adolescence or a result of housing conditions with both 16p +/- and wild-type mice. Additional controls using pure C57BL/6J animals could help clarify whether the observed effects are age-dependent.

One intriguing finding emerged from the sociability test, where 16p +/- adolescents displayed increased object exploration compared to wild-type littermates. This suggests a potential preference for less complex social stimuli, possibly reflecting an interest in lower-complexity interactions. This mild phenotype led to further exploration of the "double-hit" hypothesis during adolescence, specifically by inducing maternal immune activation prenatally to assess its effects on offspring.

MIA significantly impaired sociability in adolescent 16p +/- mice, affecting both free social interactions during the habituation-dishabituation task and object-versus-mouse discrimination tasks. In particular, social interaction was significantly reduced in 16p +/- compared with wild-type mice during the habituation dishabituation task, and in 16p +/- mice an increased interest for the object (during the Object vs Mouse task) was observed despite their continued to prefer significantly the mouse. Additionally, during the social novelty task, despite 16p +/- mice were preferring the novel social stimulus, their exploration for the familiar mouse was higher compared with wild-type. These findings suggest that the 16p deletion, when combined with MIA, may produce an ASD-like trait characterized by a preference for less-stimulating environmental cues. However, other social domains, including social novelty and emotion recognition, were not

affected by MIA. To explore the underlying mechanisms, microglial morphology and status will be assessed to confirm whether these behavioral alterations are linked to MIA-induced inflammation. Further experiments using different doses of Poly I:C are planned to refine the understanding of these effects.

Given the role of MIA in precipitating the behavioral phenotype of 16p11.2 mice, and given that MIA is strongly linked with inflammation in the brain, in the last Chapter I aimed to dissect the role of microglia in adolescent social behavior, by manipulating microglial activity at two stages of adolescence. Microglial depletion impaired emotion discrimination in both early and mid-adolescence, though effects on sociability were present only in mid-adolescence, with increased social interaction observed in depleted mice. Dendritic spine morphology was affected only in early adolescence, suggesting that synaptic pruning by microglia may play a role in early deficits, while later deficits might involve alternative mechanisms of microglial modulation of neuronal activity. Interestingly, emotion discrimination deficits were accompanied by altered SOM+ interneuron activity. In microglia-depleted mice, during interactions with neutral stimuli, SOM+ neurons displayed increased activity in the PL cortex, with a reversed pattern of activation compared to wild-type controls. This suggests that the microglial modulation of SOM+ neurons is critical for emotion recognition deficits observed in depleted mice. These findings point to a key role for SOM-microglia interactions in regulating affective state recognition.

Given the observed effects on sociability, future experiments will use PV-Cre mice to selectively monitor calcium activity in PV+ neurons in mid-adolescence, aiming to better elucidate the interplay between microglia and PV+ neurons during sociability. This work has the potential to uncover critical mechanisms underlying the mild social deficits observed in adolescent 16p +/- mice, particularly in the context of environmental challenges like MIA.

Future directions

Despite helping unraveling potential mechanisms involved in the 16p11.2 deletion and in social behavior in ASD, this study has several gaps that need to be addressed in future experiments, some of which are already underway.

First, while this study did not focus on sex differences, future experiments will address this factor, as ASD is more prevalent in males. Sex effects will be analyzed using existing data and by increasing the sample size in experiments involving MIA and immunological challenges.

Second, locomotor activity might have impacted the effects observed in many of these experiments. It is also true that the total exploration time was not different between groups in most of the tests that were performed, therefore impairments in locomotion are unlikely to be impacting the behavioral outcomes. However, to further confirm this, an assessment of locomotor activity is currently ongoing to control for this potential confounder.

To further support the hypothesis of inflammatory vulnerability in 16p +/- mice, a detailed assessment of microglial morphology and reactivity is required. This should include evaluations across different background (C57BL/6J and CD1), developmental stages (adolescence and adulthood), and in both baseline and challenged conditions. The latter assessments should be conducted during both the acute phase and 10 days post-challenge in adulthood, as well as in adolescent mice exposed to MIA. Morphological analyses will build on methods already applied in this study and incorporate additional verification of reactive states through the quantification of CD68 and IBA-1 expression in microglia using immunohistochemistry. Moreover, fluorescence-activated cell sorting (FACS) will be used to quantify microglial expression of inflammatory cytokines, such as IL-1b, IL-17, IL-10, IL-6 and TNFalpha, along with surface markers of microglial activity (including CD68, CD45, CD11b, CD11c) providing a comprehensive evaluation of microglia 16p +/- and in wild-type littermates.

Microglial depletion experiments must be extended to include adulthood in both C57BL/6J mice, and in 16p +/- and wild-type littermates, to further confirm that depletion impacts on neuronal activity selectively during neurodevelopment and not in later phases of life, during pruning or during experience mediated modulation of neuronal activity.

Additionally, ongoing experiments aim to assess the long-term effects of MIA during adolescence, determining whether observed deficits are persistent or limited to this developmental stage. Moreover, to further evaluate comprehensively the role of MIA in 16p +/- mice, MIA will be induced with both higher doses (20 mg/kg, IP) and lower doses (2 mg/kg IP) of LMW Poly I:C in order to evaluate the role of the intensity of the inflammation in determining the deficits.

The ultimate goal of this research is to elucidate the mechanisms underlying the disruptions observed in 16p +/- mice following MIA. Building on findings from C57BL/6J and SOM-Cre adolescent mice, the same experimental protocols described in CHAPTER 6 will be applied to MIA-challenged 16p +/- mice to clarify the roles of PV+ and SOM + interneurons. Fiber photometry will be used to assess neuronal activity, and optogenetic manipulation will follow to determine whether inhibition or stimulation of these interneurons can differentially impact behavior during microglial depletion. These experiments aim to confirm the contribution of interneurons to social behavior and the effects of microglial activity. The experiment will be paired with a quantification of the number of the SOM + and the PV+ interneurons in 16p+/- mice and in littermates, following IHC. Moreover, as the treatment with PLX5622 is not site-specific but induces microglial depletion at the whole-brain level, the mPFC specificity of the microglia-SOM+ interplay in determining the behavioral outcomes should be addressed with future experiments. Microglial depletion through the injection of clodronate-containing liposomes [221] in mPFC could be evaluated as a method for region-specific microglial manipulation. However, this technique has an intrinsic limitation due to the risk of inducing of an inflammatory state [222], which could act as a potential confounder in a vulnerable age such as adolescence. Other approaches could take advantage of a recently developed protocol that allows region-specific ablation of microglia via local injection of diphtheria toxin in a double-transgenic mouse (CX3CR1-Cre^{ERT2} x iDTR) [223]. However, this kind of approach would require breeding the double-transgenic mouse line with the SOM-Cre line, thereby increasing the number of mutations in mice and potentially leading to artifacts due to the genetic background rather than on the manipulation. An ideal approach should allow microglia depletion combined with minimal-invasiveness and a good control over the time-window of the manipulation. However, given the absence, to my knowledge, of an approach with all these features, both the above mentioned approaches could be considered to integrate the findings reported in this work and shed more light on the regional specificity of the microglial-SOM+ interneuron interplay.

Comprehensively, this study seeks to uncover mechanisms associated with the 16p11.2 deletion, with the aim of supporting strategies for identifying potential preventive and therapeutic strategies for social deficits in ASD. The ultimate goal is to improve the outcomes for individuals with this condition, and hopefully broadening their clinical relevance also for people affected by idiopathic ASD.

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