Excessive aggression, ADHD- and ASD-like phenotypes in TPH2- and brain ganglioside-deficient mice

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MAASTRICHT UNIVERSITY

**Excessive aggression, ADHD- and
ASD-like phenotypes in TPH2- and brain
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Evgeniy Svirin

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**Excessive aggression, ADHD- and
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and gene-by-environment interaction**

DISSERTATION

to obtain the degree of Doctor at the Maastricht University,

on the authority of the Rector Magnificus,

Prof. dr. Rianne M. Letschert

in accordance with the decision of the Board of Deans,

to be defended in public

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Propositions

1. Partial deficiency of neuronal serotonin following heterozygosity in the *Tph2* gene (*Tph2*^{+/-}) causes abnormal aggression and sociability in stressed male and female mice (*this thesis*).
2. These behavioral changes are accompanied by alterations in dopamine and norepinephrine metabolism, aberrant expression of myelination proteins, markers of stress, and plasticity (*this thesis*).
3. Downregulation of myelination marker PLP1 in *St3gal5*^{-/-} mice deficient for brain gangliosides is associated with dominant behavior, and behaviors reminiscent of autism spectrum disorders (*this thesis*).
4. Inflammatory stress in *St3gal5*^{-/-} mice leads to aberrant expression of pro-inflammatory cytokines in the brain and in the periphery, and aggravated aggressive/dominant behavior (*this thesis*).
5. Abnormalities in myelination and neuroinflammation may constitute common molecular mechanisms leading to behavioral abnormalities in the *Tph2*^{+/-} and *St3gal5*^{-/-} mice (*this thesis*).
6. Modeling of gene-by-environment interaction rather than of genetic or environmental factors alone is useful for animal studies of neurodevelopmental disorders (*impact*).

7. Different types of environmental adversities may lead to similar pathological changes contributing to neurodevelopmental disorders.
8. Multiple risk genes act on early stages of neurodevelopment and the formation of brain circuits, thus conferring broad liability to psychiatric disorders.
9. Validation of experimental animal models of behavioral features related to psychiatric disorders requires the use of multiple tests and a variety of experimental conditions.
10. “It’s not stress that kills us, it is our reaction to it.” — Hans Selye

List of abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HTP	5-hydroxytryptophan
5-HTT/SERT	serotonin transporter
ADHD	attention-deficit/hyperactivity disorder
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
ASD	autism spectrum disorders
B4GALNT1	beta-1,4 N-acetylgalactosaminyltransferase 1
BH4	tetrahydrobiopterin
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
COMT	catechol-O-methyltransferase
COX	cyclooxygenase
CREB	cAMP response element-binding protein
DA	dopamine
DAT	dopamine transporter
DBH	dopamine beta-hydroxylase
DCC	deleted in colorectal carcinoma
DISC1	disrupted in schizophrenia 1
DLB	dark-light box
dIPFC	dorsolateral prefrontal cortex
dmPFC	dorsomedial prefrontal cortex
DOPAC	3,4-Dihydroxyphenylacetic acid
DRD2	dopamine receptor D2
DRN	dorsal raphe nuclei
DTI	diffusion tensor imaging
EAE	experimental autoimmune encephalomyelitis
fMRI	functional magnetic resonance imaging
FST	forced swim test
G×E	gene × environment

GSK-3 β	glycogen synthase kinase 3 beta
HO-1	heme oxygenase 1
HPLC	high-performance liquid chromatography
Hsp70	heat shock protein 70
HVA	homovanillic acid
HPA	hypothalamic-pituitary-adrenal axis
IDO-1	indoleamine-2,3-dioxygenase
IFN- γ	interferon gamma
iNOS	inducible nitric oxide synthase
LC	locus coeruleus
MAG	myelin-associated glycoprotein
MAO	monoaminoxidase
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MHPG	3-Methoxy-4-hydroxyphenylglycol
MRI	magnetic resonance imaging
NE	norepinephrine
NMDA	N-methyl-d-aspartate
OCD	obsessive-compulsive disorder
OFC	orbitofrontal cortex
OL	oligodendrocyte
PLP1	proteolipid protein 1
PNS	peripheral nervous system
PTSD	post-traumatic stress disorder
RIPK1	receptor-interacting protein kinase 1
RIT	resident-intruder test
SIRT	sirtuin
SN	substantia nigra
SNP	single nucleotide polymorphism
SSRI	selective serotonin reuptake inhibitor
SNRI	selective serotonin–norepinephrine reuptake inhibitor
ST3GAL3	beta-galactoside- α 2,3-sialyltransferase-III
ST3GAL5	lactosylceramide alpha-2,3-sialyltransferase

TNFR1	TNF receptor 1
TLR4	toll-like receptor 4
TPH1	tryptophan hydroxylase 1
TPH2	tryptophan hydroxylase 2
vHPC	ventral hippocampus
VMAT2	vesicular monoamine transporter 2
VMH	ventromedial nucleus of the hypothalamus
vmPFC	ventromedial prefrontal cortex
VTA	ventral tegmental area
wt	wildtype

CHAPTER

General introduction

1

Chapter 1. General introduction

1.1 Emotional dysregulation and aggression in neurodevelopmental disorders

Aggression is an adaptive social behavior with multiple possible causes; most of them arise over limited resources where there is a conflict of interests between two or more individuals. The adaptive role of aggression comprises the defense of territory or resources, social position, or group identity (Covington et al., 2019). However, when brought to the extreme, aggression may become maladaptive or pathological. Such forms of aggression may be represented by an overreaction to provoking social stimuli, eventually leading to excessive harm, injury, or death.

Aggression in humans tends to be overrepresented in some individuals affected by mental disorders. Marked aggression often accompanies neurodevelopmental disorders, with the rate of aggression varying across different conditions. According to epidemiological research, the prevalence of abnormal aggression is estimated in the range from 5.7% in attention-deficit/hyperactivity disorder, where children with mostly hyperactive-impulsive presentation show more aggression than children with the inattentive endophenotype (González et al., 2013; Slaughter et al., 2020), to about 68% in autism spectrum disorders (Mazurek et al., 2013). Aggressive behaviors are also overrepresented in bipolar disorder and major depressive disorder (Ballester et al., 2014; Pulay et al., 2008), schizophrenia (Fazel et al., 2014; Rund, 2018; Soyka, 2011), intellectual disability with comorbidities (Tsiouris et al., 2011),

and neurodegenerative disorders such as Alzheimer's disease (Yu et al., 2019) and other forms of dementia (Kunik et al., 2020).

Stress-related disorders are also associated with aggressive behavior, e.g., patients with post-traumatic stress disorder are known to experience sudden uncontrollable bursts of anger and have difficulties with controlling aggressive urges (Van Voorhees et al., 2014). Last but not least, violent behavior is significantly increased among individuals suffering from drug and/or alcohol abuse (Pulay et al., 2008; Rund, 2018) — conditions, which are often comorbid with neurodevelopmental disorders (Gold et al., 2018; Hunt et al., 2018; Weissenberger et al., 2017). Aggression is a complex heterogeneous behavior, which can be qualitatively differentiated into subtypes by several criteria. In light of current views, human aggression is most often categorized as impulsive (reactive) or instrumental (proactive), for which the underlying neurobiological mechanisms differ (Lesch and Merschdorf, 2000).

Besides aggression, other symptoms occur across various neurodevelopmental disorders, including emotional dysregulation (Faraone et al., 2019; Sagar-Ouriaghli et al., 2018), anxiety (Katzman et al., 2017; Rakofsky and Dunlop, 2011; Schatz and Rostain, 2006; Temmingh and Stein, 2015; Vasa et al., 2016), and increased impulsivity (Hoptman, 2015; Najt et al., 2007; Rund, 2018; Saddichha and Schuetz, 2014). Correlations between these symptoms and aggression were studied in both patients and healthy individuals. Suicide risk and risk of violent behavior were shown to be positively correlated with impulsivity, anxiety, and measures of anger in acute psychiatric inpatients (Apter et

al., 1990). A link was also shown between impulsivity and violence in schizophrenia (Rund, 2018).

Correlations between aggression and other psychometrical measures were also shown for non-clinical groups. Measures of aggression were correlated with depressive symptoms in adults (Bjork et al., 1997). In children attention scores negatively correlated with both anxiety and aggression, while aggression was positively correlated with anxiety (Meesters et al., 2007). Some of the authors speculate that such a high degree of comorbidity between these disorders is due to shared neurobiological mechanisms and neuroanatomic substrates, while the brain serotonin (5-HT) system receives much attention as the main candidate for intervention.

1.2 Aggression in females

Female aggression and violent behavior are particularly understudied, as there is a common misconception that aggression lies outside of female behavioral repertoire in many mammalian species, and it has been regarded as self-evident that men are more aggressive than women (Lindberg et al., 2009). However, this view is challenged by research suggesting that while boys are more physically aggressive than girls, boys and girls are virtually equal in scores of verbal and indirect aggression (Björkqvist, 2018). Regarding psychiatric pathology and aggression in women, a meta-analysis showed that about 42% of female prisoners had some personality disorder, and about one in five — antisocial personality disorder (Fazel and Danesh, 2002).

In animals, several types of female aggression were proposed. Maternal aggression is a defensive behavior against a potentially dangerous intruder, intended to protect the offspring. In mice and rats, such aggression is triggered by stimuli from pups, i.e., suckling stimulus, and the presence of an intruder (Bosch, 2013; Bosch et al., 2005). However, rodent maternal aggression is an example of female aggression less applicable to modeling human psychiatric pathology. Social aggression, as intermale territorial aggression, is much less common in female mice (Angoa-Pérez et al., 2012a). The prairie vole (*Microtus ochrogaster*) has recently emerged as a new animal model for investigating the neurobiology of escalated aggression and violence, since ethologically their mating is accompanied by aggressive behavior directed toward both male and female conspecifics (Gobrogge, 2013). Another highly recognized model of female territorial aggression is a Syrian hamster (*Mesocricetus auratus*), as in this species both males and females are highly territorial and females tend to be aggressive and dominant over male intruders (Been et al., 2019; Gutzler et al., 2010). Still, while there are rodent models of female aggression, they represent ethologically adequate behaviors, and little research is directed towards female aggression in pathology, including neurodevelopmental disorders.

1.3 Neuroanatomical basis of aggression and associated impairments in humans and rodents

Data from both human and animal studies highlighted several brain regions implicated in aggression. Multiple studies point out an

essential role of the amygdala in emotional regulation and, in particular, in aggression (Cupaioli et al., 2021; Haller, 2017). The amygdala consists of several regions: basolateral nuclear complex, centromedial nuclear complex, and cortical nucleus. The basolateral complex is a structure that receives most of the amygdalar inputs from multiple brain regions, including the prefrontal cortex (PFC) and thalamus. It also receives dopamine (DA) projections from the ventral tegmental area (VTA) and substantia nigra (SN), norepinephrine (NE) projections from locus coeruleus (LC), and 5-HT projections from dorsal raphe nuclei (DRN) (Deal et al., 2021; Sharp, 2017). The centromedial complex, in turn, is the main output of the amygdala, including projections into the thalamus, hypothalamus (Keifer et al., 2015), and brainstem regions of 5-HT, DA, and NE systems of the brain (Fadok et al., 2018). Moreover, the basolateral complex also sends efferent projections to prefrontal and striatal regions (Rosell and Siever, 2015).

The amygdala was shown to play a major role in the integration of sensory and motivationally salient stimuli. There is a remarkable body of evidence from animal and human studies implicating the amygdala in aggression. Enhanced amygdalar activity upon presentation of threatening facial stimuli was shown in aggressive individuals with intermittent explosive disorder compared to healthy controls (Coccaro et al., 2007). Several studies in humans have shown that total amygdalar volume negatively correlates with psychometric scores of aggression (Matthies et al., 2012; Pardini et al., 2014). The latter study also showed volumetric changes, i.e., a decrease in the volume of the gray matter, of

only the dorsal part of the left amygdala in more aggressive individuals (Gopal et al., 2013). In another study, the volume of the dorsal part of the left amygdala correlated negatively with self-reported measures of aggression. Lower activation of the left dorsal amygdala was also shown in aggressive compared to non-aggressive individuals. In the same study, it was also shown that the amygdala of more aggressive individuals is more reactive to neutral stimuli (Bobes et al., 2013). Involvement of the dorsal but not ventral amygdala suggests that observed changes may arise from the altered amygdalar output.

One of the major amygdalar outputs projects to the medial (m) PFC, which was also implicated in aggression in humans. The mPFC is reciprocally connected with the amygdala and is a pivotal region for the integration of information about the motivational value and affective valence of stimuli, as well as for decision-making and determination of adequate responses and their outcomes (Rudebeck and Murray, 2014; Walton et al., 2007). In more aggressive individuals, volumetric studies showed lower volumes of the gray matter in several regions of the ventromedial (vm) PFC (Ducharme et al., 2011; Gansler et al., 2009). Reciprocal activation was found between the vmPFC and amygdala. Coccaro et al. (2007) showed that in control non-aggressive individuals, who were presented with threatening faces, activity of the amygdala negatively correlated with the activity of the vmPFC, suggesting functional coupling of these structures. Such a relationship was not evident in the aggressive group with intermittent explosive disorder (Coccaro et al., 2007).

Impulsive aggression is thought to develop when the amygdala is released from inhibition by the PFC (Haller, 2017). However, this point of view is questioned, as there is evidence that lesions in the vmPFC not only lead to a higher risk of impulsive aggression (Grafman et al., 1996), but also have a protective effect on the risk of post-traumatic stress disorder (PTSD) development (Koenigs and Grafman, 2009). Moreover, in a study using rats, lesions of the vmPFC led to reduced amygdala activation during decision-making tasks (Saddoris et al., 2005). Blair (2016) hypothesizes that vmPFC does not inhibit the activity of the amygdala, but rather provides information for optimal response choice. Thus, increased aggression accompanying the vmPFC-amygdalar dysconnectivity may arise not from disinhibition, but from the lack of adequate estimation of possible consequences of engagement in impulsive aggression (Blair, 2016).

Notably, neuroimaging studies showed that anxiety, which is often co-morbid with defensive aggression, may predict dysfunctional dorsomedial (dm) PFC- and vmPFC-amygdala connectivity in psychiatrically healthy humans both in goal-directed tasks and during resting state (Kim et al., 2011). Vice versa, in healthy individuals, trait anxiety was predicted by the structural integrity of the amygdala-prefrontal pathway (Kim and Whalen, 2009). The dorsolateral (dl) PFC and orbitofrontal cortex (OFC) receive inputs from the amygdala and other areas that may integrate sensory information and affective signals (Schoenbaum et al., 2006) as part of a network processing emotionally salient stimuli and mediating goal-directed behaviors. Dysfunction in

these structures also may result in impaired emotional regulation and contribute to behavioral disinhibition, leading to aggression and impulsivity (Schoenbaum et al., 2006; Winstanley, 2007). Again, OFC is implicated in a number of anxiety-related disorders. Hypoactive medial OFC is implicated in phobias and panic disorder, as medial OFC fails to properly inhibit inappropriate fear and anxiety. Hyperactive lateral OFC is found in obsessive-compulsive disorder (OCD), which is also characterized by maladaptive behavioral stereotypes guided by anxiety (Milad and Rauch, 2007).

The striatum, especially the Nucleus accumbens, is one of the structures essential for the motivational effects of emotionally salient stimuli. It receives projections from the amygdala and the PFC and is seen as a "limbic-motor" interface, which plays a role in amygdala-mediated behaviors as well (Ambroggi et al., 2008; Cardinal et al., 2003). Lesions in the Nucleus accumbens provoked excessive aggression and muricide in male Wistar rats (Lee and Ueki, 1986; Pucilowski and Valzelli, 1986). In patients, deep brain stimulation in the Nucleus accumbens region led to a reduction of symptoms in anxiety disorder and OCD (Sturm et al., 2003).

The hippocampus also plays a role in abnormal aggression. Disruption of the prefrontal-hippocampal circuitry may result in emotional dysregulation and impaired contextual fear conditioning (Raine et al., 2004). In mouse studies, stress-induced attack behavior was shown to be associated with the activation of ventral hippocampus neurons (Chang and Gean, 2019). Additionally, activation of pyramidal

neurons in the CA2 region of the hippocampus, which are important for social memory, promote social aggression in mice (Leroy et al., 2018).

1.4 Brain monoamine system in neurodevelopmental disorders

Three main monoaminergic systems of the brain, characterized by 5-HT, DA, and NE transmission, mediate the functions of structures involved in the pathogenesis of aggression, impulsivity, and anxiety in neurodevelopmental disorders. The 5-HT system of the brain received particular attention in relation to these behavioral aspects in the past decades.

The main source of brain 5-HT are raphe nuclei located in the brainstem. Diffuse fibers arising from the raphe project to many cortical and subcortical areas. 5-HT receptors are abundant in the brain, and they are present in structures associated with emotional control and aggression, such as the PFC, amygdala, and striatum (Rosell and Siever, 2015). DRN projections to the PFC modulate its activity and thus may contribute to top-down control of impulsive aggression (Rosell and Siever, 2015). 5-HT is widely acknowledged as one of the most important regulators of aggression. In humans, dysregulation of the 5-HT system was associated with self-directed aggression and suicide (Mann, 2001). Human and animal studies linked aberrations in 5-HT signaling and metabolism with aggression and interpersonal violence (Glick, 2015; Virkkunen et al., 1995, 1987). Excessive aggression, muricide, and hyperemotionality were also shown upon pharmacological destruction or

electrical lesioning of DRN in rats (Kostowski et al., 1975; Yamamoto and Ueki, 1977).

Genetic modification of components in the 5-HT system is widely known as a factor underlying excessive aggression in both animals and humans. Both male and female mice with *Tph2* gene inactivation exhibit excessive aggression (Angoa-Pérez et al., 2012b; Gutknecht et al., 2015; Mosienko et al., 2020). Moreover, partial *Tph2* inactivation in mice was shown to result in increased aggression after subjection to stressful conditions (Gorlova et al., 2020). In humans, variants in the *TPH2* gene were also associated with aggressiveness, suicidal behavior, and risk of development of borderline personality disorder (Pavlov et al., 2012). Strikingly, allelic variants of the *TPH1* gene were also associated with anger-related traits in suicide attempters and suicidal behavior itself, while *TPH1* is not present in the brain and is responsible for peripheral 5-HT synthesis only (Pavlov et al., 2012).

Another gene of the 5-HT system implicated in aggression is the 5-HT transporter (*5-HTT/SERT, SLC6A4*). Mice deficient for *5-Htt* showed lowered aggression (Holmes et al., 2002). In humans, homozygosity for the short allele of *5-HTT* was shown to be associated with increased hostility in women (Sysoeva et al., 2009). Deficiency of monoamine oxidase A (MAOA), which is an enzyme catabolizing monoamines, including 5-HT, leads to excessive aggression in animals and humans (Bortolato et al., 2013; Godar et al., 2016). A rare MAOA mutation in humans causes Brunner syndrome, whereby symptoms include elevated impulsive behavior including pyromania and violence (Hunter, 2010).

The DA system of the brain is implicated in the processing of both pleasurable and aversive stimuli (Budygin et al., 2012), as well as in social stress (Deal et al., 2018), and aggression. DA pathways are an integral part of the brain reward system, and lowered sensitivity of the reward system may cause pathological aggression. Alterations in genes of DA pathways were hypothesized to be involved in its etiology (Chen et al., 2005). Polymorphisms in DA receptor D2 gene (*DRD2*) were shown to be associated with aggression in men (Butovskaya et al., 2013). In an epistatic model, interactions associated with aggression were also found between *DRD2* and dopamine transporter (*DAT*), as well as between *DAT* and catechol-O-methyl transferase (*COMT*) (Butovskaya et al., 2013). Lower availability of D2/D3 receptors in the VTA, which leads to enhanced DA release in striatum was shown to be associated with higher impulsivity in humans (Buckholtz et al., 2010).

In mice, agonists of D2/D3 receptors were shown to potentiate defensive behavior and reduce aggressive behavior (Gendreau et al., 2000). D1 receptor inhibition in the Nucleus accumbens was shown to decrease aggression-seeking behavior in mice (Golden et al., 2019). *Dat* knockout mice showed excessive aggression (Rodriguez et al., 2004), and repeated aggression in male mice led to elevated expression of the *Dat* gene in VTA (Bondar et al., 2009). *COMT* variants with lowered enzymatic activity in humans were associated with psychiatric abnormalities and predisposition to aggression (Brennan et al., 2011; Kulikova et al., 2008). *Comt* heterozygous knockout mice show increased aggressive behavior as well (Gogos et al., 1998). Alterations in DA

signaling may induce impulsivity and aggression through weakening inhibitory pathways (Pavlov et al., 2012). As COMT and MAOA also participate in NE metabolism, there might also be a link between the NE system and aggression. Furthermore, in mice, knockout of dopamine beta-hydroxylase (DBH), which is involved in NE synthesis from DA, led to almost complete absence of aggression (Marino et al., 2005). Allelic variants in *DBH* gene were also shown to interact with aggressiveness traits in humans (Hess et al., 2009).

1.5 Neuroinflammation and myelination in the pathogenesis of neurodevelopmental disorders and excessive aggression

While inflammation, in general, is a protective mechanism, intended to remove pathogens and affected cells and stimulate regenerative functions (Kulkarni et al., 2016), negative effects of prolonged inflammation are evident, including those of neuroinflammation in particular. Pro-inflammatory signals and subsequent neuroinflammation may affect CNS function in several ways. First of all, neuroinflammation may lead to neuronal death. Through activation of the death receptor TNF receptor 1 (TNFR1), TNF may induce apoptosis and necroptosis, which are implicated in many human CNS diseases (Yuan et al., 2019). Downstream to TNFR1 activation, receptor-interacting protein kinase 1 (RIPK1) mediates not only necroptosis but also inflammation itself (Ofengeim and Yuan, 2013). In mice, administration of LPS led to elevated apoptotic marker expression and decreased cell survival in the cortex and hippocampus (Khan et al., 2018).

Neuronal cell death in brain structures implemented in aggression may lead to behavioral alterations, e.g., increased aggression in Alzheimer's disease may partly be attributed to monoaminergic neuron loss (Palmer and DeKosky, 1993; Weinshenker, 2008).

There are also more subtle pathways by which neuroinflammation may contribute to behavioral changes. CNS inflammation may affect monoaminergic transmission by altering monoamine synthesis and reuptake. Depressive-like behavior in mice caused by LPS administration was accompanied by activation of indoleamine 2,3-dioxygenase (IDO), an enzyme catabolizing tryptophan into kynurenine, thus possibly lowering tryptophan availability for 5-HT synthesis (Couch et al., 2013; O'Connor et al., 2009). Deactivation of IDO prevented depressive-like behavior (O'Connor et al., 2009). It was shown that IDO may be activated by interferon- γ (IFN- γ) and TNF, while cyclooxygenase (COX) inhibitors prevent IDO induction (Wirleitner et al., 2005). IDO effect, however, may be independent of 5-HT metabolism, as the kynurenine pathway also generates substances that act as either agonists or antagonists of the NMDA (N-methyl-d-aspartate) receptor, which also may contribute to behavioral changes (Dantzer et al., 2008).

Inflammation may lead to non-enzymatic oxidation of tetrahydrobiopterin (BH₄), which is a co-factor of tryptophan- and tyrosine hydroxylases, thus lowering the activity of 5-HT and DA/NE synthesis rate-limiting enzymes (Haroon et al., 2012). Inflammation also affects DA metabolism by altering levels of vesicular monoamine transporter 2 (VMAT2) and DAT (Kazumori et al., 2004; Tien et al., 2013).

DAT availability is also regulated by mitogen-activated protein kinases (MAPKs), which participate in the cellular response to pro-inflammatory cytokines (Morón et al., 2003). Exposure to pro-inflammatory cytokines may also reduce the expression of glutamate transporters in glial cells (Matute et al., 2006) and stimulate glutamate exocytosis from astrocytes (Ida et al., 2008) leading to elevated extracellular glutamate levels and, consequently, glutamate excitotoxicity. This excitotoxicity may lead to alterations in myelination by triggering excitotoxic oligodendrocyte (OL) death (Takahashi et al., 2003).

Myelination is one of the major postnatal CNS developmental milestones that provide efficient neuronal circuit connectivity. Myelin sheaths are electrically insulating structures consisting of a lipid-rich substance wrapped around axons in both the CNS and PNS (**Figure 1.1**).

Figure 1.1. Myelination in the CNS. **A.** Myelin sheaths enwrap axons in the CNS and the PNS. **B.** Saltatory conduction in myelinated axons speeds up the propagation of the action potential. **C.** Two phases of myelination: intrinsic myelination after birth and activity-dependent myelin remodeling (adapted from Stassart et al., 2018; Encyclopædia Britannica, 2012; Bechler et al., 2018; *also see the text*).

In the CNS, it is produced by OLs, while in the PNS Schwann cells are responsible for myelin sheath formation (Salzer, 2015). Myelin sheaths enwrap segments of an axon, forming internodal segments (or internodes), and individual internodes are separated by gaps, so-called nodes of Ranvier, where axonal membrane ion channels are concentrated (Arancibia-Cárcamo et al., 2017). Such structure of myelinated axon provides so-called saltatory propagation of action potential, ensuring faster signal conduction (Huxley and Stämpeli, 1949). For a long time, it was thought that the main functions of myelination are increasing maximum conduction velocity and decreasing axonal energy consumption (Stadelmann et al., 2019). However, growing body of evidence suggests that myelinating OLs are involved in other processes such as supporting axonal energy metabolism via myelin sheaths (Saab et al., 2013). There is also evidence that in the absence of one of the major myelin proteins, proteolipid protein 1 (PLP1), axonal degeneration is observed independently from demyelination and neuroinflammation (Garbern et al., 2002).

Until recently, the prevailing view assumed that myelination processes occurring around birth and in postnatal development result in topographically fixed myelination that remains static in adulthood. Recent discoveries, however, shifted this view. Now CNS myelination is thought to occur in two phases: genetically predefined intrinsic stage, which meets the idea of developmental myelination in early life stages, and adaptive phase. The adaptive phase implies constant *de novo* formation of myelin in the nervous system (Ye et al., 1995) and

remodeling of the myelin sheaths, which was shown to be dependent on neural activity and now is thought to participate in learning and long-term neuroplasticity (Fields and Bukalo, 2020). It was directly shown that inhibition of OL proliferation and new myelin formation while preserving existing myelin sheaths, impairs memory consolidation and retrieval (Pan et al., 2020). Myelin plays multiple roles in the CNS function, and to no surprise, dysregulated myelination may lead to pathology.

Deficits in myelination are observed in numerous neurological and neuropsychiatric disorders. In addition to well-known demyelinating diseases such as multiple sclerosis and leukodystrophies, neurovisualization studies found myelination deficits in late-onset neurodegenerative diseases such as Alzheimer's, Parkinson's diseases and amyotrophic lateral sclerosis (Bartzokis, 2011; Dean et al., 2016; Han et al., 2019; Kolind et al., 2013; Nasrabady et al., 2018), neurodevelopmental disorders such as autism spectrum disorders and ADHD (Gozzi et al., 2012; Lesch, 2019; Onnink et al., 2015), and schizophrenia (Takahashi et al., 2011). In addition, factors such as prenatal SSRI exposure or social isolation, which are detrimental for the CNS development and function, lead to myelination abnormalities, as well as inflammatory disease, e.g., necrotizing enterocolitis in children (Shin et al., 2016).

It was found that social isolation impaired remyelination in the brain of male C57BL/6J mice (Makinodan et al., 2016). Social isolation in juvenile mice led to hypomyelination in the mPFC and lowered mPFC activation when exposed to a stranger mouse, while re-socialization

reverted hypomyelination (Makinodan et al., 2017). Psychological stress, e.g., chronic social defeat stress, was also shown to affect myelination in a strain-specific and region-specific manner. In essence, stress-susceptible B6 mice showed thinner myelin sheaths in vHPC and downregulation of myelination genes in the mPFC, while stress-resilient mice had thicker myelination in the mPFC (Laine et al., 2018). Also, in mouse model of necrotizing enterocolitis, inflammatory disease of the digestive tract, impairments in myelination and changes in myelin protein expression were found (Nino et al., 2018).

Diffusion tensor imaging (DTI) (Chang et al., 2017) studies in children with perinatal exposure to SSRIs had shown that such exposure might lead to abnormalities in the brain white matter (Jha et al., 2016) and may contribute to affect-related behavioral alterations observed in some of these children (Misri et al., 2006; Oberlander, 2012). Such abnormalities in myelination were also evident in rodents perinatally exposed to the SSRI treatment. These rodents displayed behavioral abnormalities, such as increased emotional reactivity and altered social behavior (Jha et al., 2016). It was shown that exposure to SSRI in rodents alters the expression of myelination-related genes. Notably, the effect of SSRI on the expression of these genes depends on the age of the animal. While exposure to SSRI of adult rats led to the upregulation of myelination-related gene expression in the hippocampus, perinatal exposure led to the downregulation of such genes in adult rats (Kroeze et al., 2015).

Such an effect of SSRIs on myelination in the CNS may link 5-HT to myelination processes. Another line of evidence of 5-HT implication in myelination comes from recent research, in which polymorphisms in the *TPH2* gene were associated with alterations in white matter integrity in depressed patients (Ping et al., 2019) and the severity of multiple sclerosis (Natarajan et al., 2012). Ultimately, it was shown *in vitro* that 5-HT directly affects OL development and myelination (Fan et al., 2015). But the list of factors affecting myelination is not limited to 5-HT (**Figure 1.2**). Other neurotransmitters, such as glutamate and DA, also promote differentiation of OLs and myelination via receptors expressed by oligodendrocyte precursor cells OPCs and OLs. Gangliosides GD1a and GT1b were identified as functional receptors for one of the crucial myelination proteins MAG, and they regulate axon-myelin interaction and axonal stability (Schnaar and Lopez, 2009). In turn, pro-inflammatory cytokines released by reactive microglia can impair Glu uptake and trigger excitotoxic OL death (Takahashi et al., 2003). Finally, glycogen synthase kinase 3 beta (GSK-3 β) suppresses myelination, and its inhibition promotes myelination by suppressing inflammation and Notch signaling (Dohare et al., 2018).

Complex evidence suggests the contribution of altered myelination to a broad spectrum of CNS disorders, which may lead to behavioral and cognitive alterations. As it will be shown in the next sections, 5-HT and gangliosides may affect inflammation and myelination in different ways. Thus, some of the deficits of mouse models used in our work may result from inflammation and

demyelination involved in the pathogenetic mechanisms of neurodevelopmental disorders.

Figure 1.2. Factors affecting myelination. Cells of the OL lineage respond specifically to factors that regulate proliferation, migration, differentiation, and survival. A- and b- series gangliosides interact with MAG and are crucial for myelin stability. Activation of AMPA receptors promotes Pre-OL differentiation into mature OLs, and activation of OL NMDA receptors enhances myelination. DA exerts a protective effect on OLs and may participate in differentiation via activation of D3Rs. High levels of extracellular 5-HT acting through 5-HT_{1A} and 5-HT_{2A} may adversely affect OL development and/or myelination. GSK-3β negatively affects OL differentiation and myelination, and its ablation was shown to improve oligodendrocyte precursor cell differentiation and subsequent myelination. Pro-inflammatory cytokines like TNF and IL-1 can impair glutamate uptake and trigger excitotoxic OL death (adapted from Marinelli et al., 2016; *also see the text*).

1.6 Role of serotonin in mechanisms of neurodevelopmental disorders

5-HT action is not limited to neurotransmission in the CNS. 5-HT is widely present in the immune system and also in the intestine. 5-HTP, the precursor of mucosal 5-HT, is synthesized by TPH1 present in mast cells and mucosal epithelium cells. In turn, TPH2 is present in neurons of the enteric nervous system and is involved in neuronal 5-HT synthesis (Israelyan et al., 2019). There are several links between 5-HT and inflammation (**Figure 1.3**). Studies have shown that lack of mucosal 5-HT following *Tph1* knockout leads to an anti-inflammatory effect in a model of experimental colitis. In contrast, *Tph2* knockout leads to an elevated inflammatory response in the intestine, and colonic levels of pro-inflammatory cytokines interleukin 1 beta (IL-1 β), interleukin 6 (IL-6), and tumor necrosis factor (TNF) were found elevated in *Tph2*^{-/-} mice compared to wt littermates (Gershon, 2012). Peripheral circulation of pro-inflammatory cytokines may affect the brain by several pathways. Circulating cytokines may gain access to the brain through cytokine transporters at the blood-brain barrier (Banks, 2009) and activate IL-1 receptor type 1 located on macrophages and endothelial cells of brain venules, inducing prostaglandin E2 synthesis (Konsman et al., 2004). These two pathways lead to the induction of pro-inflammatory cytokines by microglia (Dantzer et al., 2008). Thus, systemic inflammation caused by inactivation of TPH2 may contribute to central inflammation.

Another mechanism that may trigger neuroinflammation in TPH2 deficiency arises from the shift of tryptophan metabolism pathways. Both in the brain and the periphery, tryptophan undergoes utilization by the

Figure 1.3. Complex interactions between the Tph2 deficiency and neuroinflammation. Possible ways by which TPH2 deficiency affects neuroinflammation include both pro- and anti-inflammatory pathways. TPH2 deficiency may lead to a shift of the balance between methoxyindoles pathway and kynurenine pathway towards the latter, producing tryptophane catabolites, which alter neuro-immune regulation. TPH2 deficiency in the enteric neurons leads to colonic inflammation and subsequent systemic inflammation, associated with an elevation of circulating pro-inflammatory cytokines. Cytokines enhance neuroinflammation by being transported through the blood-brain barrier and activating prostaglandin E secretion from the circulating macrophages. On the other hand, deficiency of the neuronal 5-HT may ameliorate its stimulating function on exocytosis from the microglia, thus lowering the levels of pro-inflammatory factors (*see the text*).

kynurenine pathway. Indoleamine-2,3-dioxygenase (IDO-1) is the rate-limiting enzyme of the kynurenine pathway in the brain (Lovelace et al., 2017). Metabolites of this pathway include both neuroprotective and

neurotoxic compounds playing a role in neuroimmune regulation. The imbalance of tryptophan metabolites in the kynurenine pathway may lead to neuroinflammation and induce 5-HT deficiency (Kim and Jeon, 2018). In turn, deficiency of TPH2, the rate-limiting enzyme of the concurring methoxyindoles pathway, may shift the balance towards the kynurenine pathway. The associated decrease in 5-HT levels and the inflammatory reactions associated with another TPH2-associated pathway described above may shift the kynurenine pathway towards toxic tryptophan catabolites.

Brain 5-HT itself appears to modulate the neuroinflammatory process by affecting microglial function. Microglia were shown to express several classes of 5-HT receptors, including 5-HT_{2A}, 5-HT_{2B}, 5-HT₄ (Glebov et al., 2015), 5-HT₅, and 5-HT₇ (Krabbe et al., 2012). The data on whether 5-HT affects pro-inflammatory cytokine exocytosis from microglial cells is inconclusive. Krabbe and coworkers (2012) have shown that in microglial culture pretreated with LPS application of different concentrations of 5-HT does not affect the release of IL-6 or TNF, while complex modulation of LPS-induced cytokine exocytosis by 5-HT was found in blood monocytes (Herr et al., 2017). In cultured human microglial MC-3 cells, activation of 5-HT₇ receptors was shown to elevate the *Il-6* mRNA levels, and this effect was blocked by a selective antagonist of this receptor (Mahé et al., 2005).

Later it was shown that 5-HT stimulates microglial secretion of exosomes via activation of 5-HT_{2A}, _B, 5-HT₄ receptors, but in this particular study exocytosis of pro-inflammatory cytokines, which is

associated with the release of exosomes, was not investigated (Glebov et al., 2015). The link between microglia-derived extracellular vesicles and neuroinflammation, however, is present. In microglial activation caused by LPS-induced inflammatory response in mouse microglial BV2 cell culture, the level of pro-inflammatory IL-6 and TNF in extracellular vesicles was significantly elevated (Yang et al., 2018). Thus, while 5-HT itself was found not to increase exocytosis of pro-inflammatory cytokines, it promotes the release of extracellular vesicles by microglial cells, which, together with neuroinflammation, may increase levels of extracellular pro-inflammatory cytokines.

5-HT was also shown to demonstrate neuroprotective properties. Indirect evidence of such action comes from the well-established anti-neuroinflammatory effect of treatment with selective 5-HT reuptake inhibitors (SSRIs) in several conditions associated with neuroinflammation. There is evidence of a neuroprotective effect of SSRIs against multiple sclerosis in patients (Grech et al., 2019). It is of note that SNPs in the *TPH2* gene in humans were associated with the severity of disability in multiple sclerosis (Natarajan et al., 2012). In a mouse model of diabetic neuropathy, selective 5-HT/NE reuptake inhibitor (SNRI) duloxetine treatment was associated with a lowered number of astrocytes and microglia in the spinal cord. In the sciatic nerve of these mice, treatment with SNRI duloxetine ameliorated axonal degeneration (Tawfik et al., 2018). Administration of SSRI fluoxetine decreased cell death and microglial activation and reduced pro-inflammatory cytokines *Il-1 β* , *Il-6*, and *TNF* mRNA levels in the brain of a

rat model of early brain injury after subarachnoid hemorrhage. Also, in this model, fluoxetine administration decreased protein levels of toll-like receptor 4 (TLR4) (Liu et al., 2018). In a mouse model of Parkinson's disease, administration of norfluoxetine inhibited microglial activation and oxidative stress, thus reducing the degeneration of nigrostriatal DA neurons (Kim et al., 2018).

Further evidence for the implication of the brain 5-HT system in neuroinflammation comes from a mouse model of experimental autoimmune encephalomyelitis (EAE) mimicking human multiple sclerosis. Knockout of *Sert* in mice leads to enhanced 5-HT transmission, markedly reducing susceptibility to EAE (Hofstetter et al., 2005). This finding is in line with reduced inflammatory responses in EAE upon treatment with SSRIs and MAO inhibitors (Grech et al., 2019).

The findings described above show an interaction between neuronal 5-HT and peripheral inflammation, namely enteric inflammation in *Tph2^{+/-}* mice, which may ultimately lead to central inflammation. Strong evidence of complex interaction between microglial cells and 5-HT further underpins the 5-HT system's role in regulating neuroinflammation in the CNS. Thus, we hypothesized that alterations in 5-HT transmission caused by partial or full genetic lack of TPH2 might have a complex effect on neuroinflammation, affecting behavior and contributing to neuropsychiatric disorders.

Striking evidence of direct cellular protective effects of 5-HT was found in the work of Hornedo-Ortega and associates (2018). Treatment of PC12 pheochromocytoma line cells with 5-HT-containing medium

greatly improved cell viability under A β cytotoxicity. The authors also found that treatment with 5-HT enhanced expression of Sirtuin 1 and 2 (SIRT1,2), Heat shock protein 70 (Hsp70), and Heme oxygenase 1 (HO-1) (Hornedo-Ortega et al., 2018). These proteins are known for their neuroprotective properties.

Activation of SIRT1-proliferator-activated receptor-gamma coactivator (PGC)-1 α axis via 5-HT_{2A} receptors was found in rodent cortical cultures and *in vivo* (Fanibunda et al., 2019). Protective effect of SIRT1 was observed in a plethora of neurodegenerative and acute neurological disorders (Zhang et al., 2011). Anti-inflammatory effects of pharmacological SIRT1 activation were described *in vitro* and *in vivo*, in particular through inhibition of matrix metalloproteinases and reduction of cytokine synthesis (Zhang et al., 2020). In experiments with a rat model of hypoxia, pharmacologically enhanced expression of SIRT1 improved the learning and spatial memory of the animals and promoted neuronal survival (Barhwal et al., 2015).

Opposite to SIRT1, inhibition or lack of SIRT2 protein was found to exert a neuroprotective effect in models of Huntington's and Parkinson's diseases (Luthi-Carter et al., 2010; Outeiro et al., 2007), as well as in cellular and mouse models of ischemic stroke (She et al., 2018). One of the major functions of SIRT2 is carried out in OLs. SIRT2 overexpression facilitates OL differentiation and process outgrowth and elevates MBP expression (Ji et al., 2011). It also plays a role in axon-myelin interaction (Li et al., 2007). Hsp70 was also shown to protect neurons and glia in ischemia (Kelly and Yenari, 2002), neurodegenerative

disorders with aberrant protein aggregation, i.e., Parkinson's, Alzheimer's, Huntington's diseases, and some forms of amyotrophic lateral sclerosis (Witt, 2010). Anti-inflammatory properties of this protein after brain injury were also found (Kim et al., 2012). Inducible HO-1 in the brain is usually expressed in glial cells in response to stress. It may protect neurons from oxidative stress; however, chronic overexpression of HO-1 may be detrimental and promote cytotoxicity in neurodegenerative diseases (*reviewed in* Chen 2014).

Thus, by activating the expression of SIRT1,2, Hsp70, and HO-1, brain 5-HT may activate a plethora of neuroprotective mechanisms, including those involved in myelination-related processes. Loss of 5-HT signaling with genetic deficiency of TPH2 may lead to impairments in these pathways, promoting neuroinflammation and impaired myelination.

1.7 Gangliosides in the CNS pathology and neuroinflammation

Impairments of brain ganglioside metabolism are known as a cause of rare severe neurodevelopmental disorders (Trinchera et al., 2018). However, recent studies show the role of genes involved in brain ganglioside synthesis in less pervasive disorders, i.e., ADHD and schizophrenic psychoses (Hall et al., 2020; Klein et al., 2020; Zhao et al., 2018a). Gangliosides or sialoglycolipids are molecules consisting of glycosphingolipid and one or more sialic acid residues. They are ubiquitous in cell membranes in all vertebrates, performing many functions, including protein organization, signaling, and cell adhesion

(Schnaar, 2016). Gangliosides are predominantly found in the CNS, where they play critical roles in development and function (Bowser et al., 2019; Galleguillos et al., 2020; Schnaar et al., 2014). The majority of brain gangliosides consist of derivatives of ganglioside GM3 (Ishii et al., 1999).

Impairments in ganglioside metabolism are associated with several human disorders, most of which are ganglioside catabolic disorders, while only several families with disorders of ganglioside biosynthesis are known (Schnaar et al., 2014). Catabolic disorders include Tay-Sachs disease and Sandhoff disease, arising from dysfunctional hexosaminidases. Both are child-onset disorders, usually rapidly progressing and leading to child death before the age of four (Cachon-Gonzalez et al., 2018; Fernandes Filho and Shapiro, 2004; Sandhoff, 1969). Much more rare disruptions of ganglioside biosynthesis lead to catastrophic neurological deficits, including severe cognitive impairments, seizures, and motor and sensory dysfunctions (Indellicato et al., 2019; Trinchera et al., 2018). These ganglioside biosynthesis disorders arise from mutations in genes encoding GM-3 synthase (*ST3GAL5*) (Bowser et al., 2019; Lee et al., 2016) or GM2/GD2 synthase (*B4GALNT1*) (Boukhris et al., 2013; Harlalka et al., 2013). Loss-of-function mutations in both proteins lead to the complete absence of major brain gangliosides (Trinchera et al. 2018; Schnaar 2010, **Figure 1.4**).

Besides severe neurological phenotypes following the abovementioned disruptions in ganglioside metabolism, changes in ganglioside profile in the brain were also associated with Alzheimer's

Figure 1.4. Brain ganglioside structure and biosynthesis pathways. Knockout of the *St3gal5* (in red) gene leads to a complete absence of major brain gangliosides (adapted from Schnaar 2009; also see the text).

disease and amyloidosis (Ariga, 2017; Bucciantini et al., 2020), Parkinson's disease (Ledeen and Wu, 2018), Huntington's disease (Maglione et al., 2010), and amyotrophic lateral sclerosis (ALS) (Dodge et al., 2015), neurodegenerative conditions which have a well-established link to neuroinflammation.

Indeed, gangliosides in the CNS have an anti-inflammatory effect. Knockout of *B4galnt1* or GD3-synthase (*St8sia1*) in mice led to elevated infiltrating microglia, and double knockout of these genes led to upregulation of inflammation-related genes in the brain, including *Il-1β* and *TNF* (Ohmi et al., 2011). Neurostatin (O-acetyl GD1b) (Abad-Rodríguez et al., 2002) and several other O-acetylated gangliosides were shown to reduce inducible nitric oxide synthase (iNOS) and *Il-6* expression in LPS-activated microglial culture and promote neuronal survival in co-cultures (Yanguas-casás et al., 2019). In rats, GM1 administration relieved negative consequences of developmental lead

exposure, reverting cognitive deficit and cell death in the hippocampus via antioxidant effect, as well as activation of SIRT1 / cAMP response element-binding protein (CREB) / brain-derived neurotrophic factor (BDNF) and anti-apoptotic pathways (Chen et al., 2019; Meng et al., 2016). On the other hand, major brain gangliosides were shown to play a role in platelet activation in case of traumatic brain injury or neurodegeneration, leading to enhanced inflammation upon disruption of the blood-brain barrier (Dukhinova et al., 2018; Kopeikina et al., 2020).

Finally, participation of gangliosides in myelination processes was shown in many cases. One of the major myelination proteins, myelin-associated glycoprotein (MAG), is located on the surface of myelinating glial cells and interacts with neuronal membrane gangliosides to inhibit neurite outgrowth (Vinson et al., 2001). Gangliosides GD1a and GT1b, which are ligands of MAG, are absent in *B4GALNT1* deficiency, and *B4galnt1* knockout mice display axonal degeneration in the CNS and PNS, as well as motor deficits similar to those in *Mag* knockout mice (Pan et al., 2005). We may speculate that aberrant myelination may also play a role in human pathology associated with the loss of brain gangliosides. Notably, despite displaying motor deficits, *B4galnt1*- and *Mag*-knockout mice show motor hyperactivity (Pan et al., 2005). Finally, interaction of ganglioside GM1 and myelin basic protein (MBP) stimulates Schwann cell proliferation in the PNS (Tzeng et al., 1999).

The prominent role of gangliosides in neuroinflammation and myelination suggests that disruption of ganglioside metabolism may be a contributing factor in neuropsychiatric diseases. Moreover, gangliosides

also regulate the function of G-protein coupled receptors, e.g., pharmacological depletion of gangliosides led to lowered 5-HT binding to human 5-HT₇ receptors (Sjögren and Svenningsson, 2007). Interaction between GM1 and 5-HT_{1A} receptors was modeled in molecular dynamics simulation, and authors speculate that such interaction *in vivo* may facilitate the entry of 5-HT (Prasanna et al., 2016). Thus, brain gangliosides may also contribute to the regulation of the 5-HT system.

1.8 Gene × environment interaction and stress in neurodevelopmental disorders

Alongside contribution of genetic predisposition to the risk of neurodevelopmental disorders and excessive aggression, environmental factors contribute to the pathogenesis of these conditions (Lesch et al., 1996; Lesch and Merschdorf, 2000; Lesch and Mössner, 2006). There is a remarkable body of evidence indicating that there is a combined contribution of genetic background and aversive life experiences during childhood, adolescence, and adulthood to the development of antisocial behavior and schizophrenia (Thapar et al., 2007), ADHD (Lesch et al., 2012), autism (Modabbernia et al., 2017), depression (Landrø, 2014), and substance abuse (Lesch, 2005). Family studies of aggressive behavior suggest that in both males and females 50% of the variance in aggressive behavior parameters is explained by environmental factors (Tuvblad and Baker, 2011).

Such aversive experiences include emotional stress, a form of stress evoked by processing negative mental experiences and primarily

triggered by the perception and cognitive evaluation of adverse events rather than a disturbance of physical nature (Fontes et al., 2014). Emotional stress is of particular interest in the context of social interaction. It may contribute to different psychiatric disorders, e.g., emotional neglect, loss of a parent, or child abuse are risk factors for the development of violence and abnormal aggression, depression, and anxiety disorders (Barnow et al., 2001; Heim and Nemeroff, 2001).

In animals, emotional stress was also shown to lead to behavioral abnormalities and aggression (Costa-Nunes et al., 2020, 2014; Gorlova et al., 2019; Pavlov et al., 2019). Maternal separation at a young age and social isolation led to increased aggressive, anxiety- and depressive-like behaviors in mice (Ieraci et al., 2016; Matsumoto et al., 2005; Takeda et al., 2012; Veenema, 2009; Veenema et al., 2007). Recently, increased aggressiveness and depressive-like behavior were reported in BALB/c mice subjected to chronic ultrasound stress (Gorlova et al., 2019).

Brain structures crucial for aggression, impulsivity, and anxiety also play a pivotal role in stress response (Tottenham and Galván, 2016). Chronic restraint stress in C57BL/6 mice was shown to enhance amygdala–PFC transmission, and optogenetic activation of this pathway led to elevated anxiety-like behavior and hyperactivity in unstressed C57BL/6 mice (Lowery-Gionta et al., 2018). Rats subjected to a single-prolonged stress paradigm, which is considered as a model for posttraumatic stress disorder, showed hypoactivation of the PFC associated with elevated activation of the amygdala and striatum (Piggott et al., 2019). Such stress-related changes in the activity of these

structures may be due to modulation by monoaminergic pathways.

The role of the brain monoamine systems in stress response was studied extensively during the past decades. Acute and chronic stress were shown to affect monoamine metabolism in various brain areas in a manner dependent on the stress parameters. Alterations in metabolism of 5-HT, DA, and NE during and after exposure to physical or emotional stress were shown in rodent amygdala, frontal cortex, hippocampus, striatum, as well as midbrain and brainstem structures (Britton et al., 1992; Heinsbroek et al., 1990; Tanaka et al., 2000). Indeed, stress leads to changes in monoamine levels in the PFC (Arnsten, 2015; Feenstra, 2000; Van Bockstaele et al., 1998), which is, as discussed above, crucial for top-down control of aggression, emotional regulation, impulsivity, and anxiety. When the activity of the PFC is altered by monoamine influx, disinhibition of these behaviors may occur. Altered monoaminergic modulation in the striatum (Dunn and File, 1983; Gorlova et al., 2020; Heinsbroek et al., 1990), in turn, may lead to inadequate processing of social stimuli, which also may contribute to excessive aggression. Another mechanism that may contribute to neurodevelopmental abnormalities provoked by stress is impaired myelination, as was discussed above (Lane et al., 2011; Makinodan et al., 2017, 2016).

Another environmental factor widely known for its detrimental effect on neurodevelopment and mental health is systemic inflammation. Prenatal immune challenge, maternal immune activation, is known to be a contributing factor for the risk of autism spectrum disorders in children, as well as for high risk of schizophrenia (Boulanger-Bertolus et

al., 2018; Minakova and Warner, 2018). In a mouse model of maternal immune activation, its interaction with expression of mutant human disrupted in schizophrenia 1 gene (*mhDISC1*) resulted in anxiety, depression-like responses, and altered social behavior (Abazyan et al., 2010).

Notably, infectious diseases in childhood were also shown to be associated with increased risk of these disorders (Hadjkacem et al., 2016; Karlsson and Dalman, 2019), and also OCD and other mental disorders (Orlovska et al., 2017). Pre- and postnatal immune challenges, such as LPS treatment, viral mimic polyriboinosinic-polyribocytidylic acid (poly I:C), or streptococcal antigen exposure are used as models of neurodevelopmental abnormalities arising from immune mechanisms (Cardoso et al., 2015; Haddad et al., 2020; Mora et al., 2020; Piontkewitz et al., 2012). Exposure to prenatal maternal stress was also proven to be detrimental for child neurodevelopment, and implicated mechanisms include inflammation (Manzari et al., 2019; Polanska et al., 2017). Convergent mechanisms may underlie the contribution of pre- and postnatal stress and systemic inflammation to the risk of neurodevelopmental disorders besides genetic factors.

1.9 Mouse models of pathological aggression

Pathological aggression is a common feature of neurodevelopmental disorders; however, effective treatment is challenging. Mouse models can be a powerful tool for the investigation of the pathophysiology of excessive aggression and finding new therapies

(Bortolato et al., 2013). Currently, several approaches are used for modeling excessive aggression in rodents (**Figure 1.5**).

As it was discussed above, stress may contribute to aggression and other behavioral abnormalities both in humans and in rodents. Different stressful conditions are usually used to provoke elevation in aggressive behavior in conventional mouse lines. One of the most used manipulations to provoke excessive aggressive behavior in male mice is social isolation. Socially isolated mice demonstrated alterations in the function of the hypothalamic-pituitary-adrenal (HPA) axis, which makes evident the stressful nature of such isolation (Ieraci et al., 2016), and tend to be more aggressive in the resident-intruder test (Brain et al., 1971; Pinna, 2010). Another stress paradigm potentiating aggression is chronic mild stress, which was shown to provoke increased aggression in male BALB/C mice, as shown in the resident-intruder test (Malki et al., 2016; Mineur et al., 2003; Mutlu et al., 2012; Strekalova et al., 2011). Rat exposure, which is an ethologically valid stressor, as rats are natural predators for mice, caused elevation in aggressive behavior in male C57Bl mice (Costa-Nunes et al., 2014; Vignisse et al., 2017). A novel model of ultrasound emotional stress was also shown to potentiate aggression in male BALB/C mice (Gorlova et al., 2019).

Stress may cause not only an immediate and short-term but also a persistent effect on aggression. In rats, maternal separation during the first two weeks of life significantly increased intermale aggression at 14-16 weeks of age and lowered maternal aggression (Veenema et al., 2006). Interestingly, in mice, such maternal separation stress was shown to

Figure 1.5. Animal models of excessive aggression. Models of animal aggression include genetic models, e.g., knockout of *Tph2*, *Htr1b*, *Dat*, *Maoa*, *Comt*; and mice selectively bred for excessive aggression. Induction of aggression is also reached by environmental stress factors. Gene × environment interaction models combine these factors, i.e., *Tph2*^{+/-} animals subjected to predation stress or *St3gal5*^{-/-} mice with induced systemic inflammation (see the text).

cause opposing changes in intermale and maternal aggression: maternally separated females tended to be more aggressive towards male intruders than wt control females, while in males, maternal separation decreased intermale aggression (Veenema et al., 2007). Post-weaning social isolation or subjugation also caused elevated intermale aggression in mice and rats, as well as hamsters and guinea pigs (Veenema, 2009).

Other methods of modeling excessive aggression in rodents involve selective breeding and genetic manipulations. There are several mouse lines selectively bred for aggressive behavior, such as the North

Carolina 900 and 100 mice, Turku aggressive mice, or short attack latency (SAL) mice (Natarajan et al., 2009). Genetic manipulations leading to abnormal aggression include those affecting DA or 5-HT systems of the brain. Excessive aggression is observed in mice with a genetic lack of 5-HT_{1B} receptor (Saudou et al., 1994), DAT (Rodríguez et al., 2004), MAOA (Cases et al., 1995), TPH2 (Angoa-Pérez et al., 2012b), and COMT (Gogos et al., 1998).

Genetic manipulations may also have the opposite effect. Lowered aggression was found in mice with a lack of DBH (Marino et al., 2005) and the long form of DRD2 (Vukhac et al., 2001). Recently, *Tph2*^{+/-} mice were found to be a promising model of gene × environment (G×E) interaction in excessive aggression, as they do not show behavioral abnormalities under normal conditions, but exhibit excessive aggression after stress (Gorlova et al., 2020).

Hypothesis and aims

In the current study, we employed two mouse models of neurodevelopmental abnormalities: heterozygous *Tph2*-deficient mice and mice with a knockout of *St3gal5* gene. Since *Tph2* heterozygous mice display increased aggressiveness after being subjected to stress, we used rat exposure stress to investigate the possible role of alterations in brain catecholaminergic systems, neuroinflammation, and myelination abnormalities as mechanisms of neurodevelopmental pathology underlying excessive aggression and accompanying behavioral changes in these mice. We also aimed to study these putative non-specific pathogenetic mechanisms in another model of neurodevelopmental abnormalities, *St3gal5* knockout mice, sharing common behavioral features with *Tph2* heterozygous mice. Given possible converging pathways of environmental challenges contributing to neurodevelopmental diseases, we used LPS to induce systemic inflammation (inflammatory stress) to investigate whether *St3gal5* knockout mouse behavior and inflammatory response will be altered compared to those of wild-type mice.

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CHAPTER

2

Altered behaviour, dopamine and norepinephrine regulation in stressed mice heterozygous in *Tph2* gene

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Chapter 2. Altered behaviour, dopamine and norepinephrine regulation in stressed mice heterozygous in *Tph2* gene

Abstract

Gene-environment interaction (G×E) determines the vulnerability of an individual to a spectrum of stress-related neuropsychiatric disorders. Increased impulsivity, excessive aggression, and other behavioural characteristics are associated with variants within the tryptophan hydroxylase-2 (*Tph2*) gene, a key enzyme in brain serotonin synthesis. This phenotype is recapitulated in naïve mice with complete, but not with partial *Tph2* inactivation. *Tph2* haploinsufficiency in animals reflects allelic variation of *Tph2* facilitating the elucidation of respective G×E mechanisms. Recently, we showed excessive aggression and altered serotonin brain metabolism in heterozygous *Tph2*-deficient male mice (*Tph2*^{+/-}) after predator stress exposure. Here, we sought to extend these studies by investigating aggressive and anxiety-like behaviours, sociability, and brain metabolism of dopamine and noradrenaline. Separately, *Tph2*^{+/-} mice were examined for exploration activity in a novel environment and for the potentiation of helplessness in the modified swim test (ModFST). Predation stress procedure increased measures of aggression, dominance, and suppressed sociability in *Tph2*^{+/-} mice, which was the opposite of that observed in control mice. Anxiety-like behaviour was unaltered in the mutants and elevated in controls. *Tph2*^{+/-} mice exposed to environmental novelty or to the ModFST exhibited increased novelty exploration and no increase in

floating behaviour compared to controls, which is suggestive of resilience to stress and despair. High-performance liquid chromatography (HPLC) revealed significant genotype-dependent differences in the metabolism of dopamine and norepinephrine within the brain tissue. In conclusion, environmentally challenged *Tph2*^{-/-} mice exhibit behaviours that resemble the behaviour of non-stressed null mutants, which reveals how GxE interaction studies can unmask latent genetically determined predispositions.

Keywords: Tryptophan hydroxylase-2 (TPH2), Aggression, Dopamine, Noradrenaline, Stress, Mouse

1. Introduction

Pathological aggression is a common feature of many neuropsychiatric diseases (Van Voorhees et al., 2014; Yu et al., 2019; Kunik et al., 2020; Slaughter et al., 2020). Aggression is also associated with a higher incidence of suicide attempts (Dumais et al., 2005; Conejero et al., 2019; Reich et al., 2019). Moreover, as a consequence of the ongoing COVID-19 pandemic, the incidence of mental disorders is expected to rise, as levels of stress, anxiety, aggression and depression increase, both through direct cases of viral illness and through negative social and economic consequences (Chaturvedi, 2020; Wind et al., 2020). Yet, excessive aggression is currently treated in conjunction with other psychiatric disorders using non-specific drugs with serious side effects, such general sedation, dizziness and rebound effect (Lane et al., 2011; Zaman et al., 2018).

The unmet medical need for therapy for aggression requires the development of improved animal models of excessive aggression. Recent genome-wide association studies suggest that there is greater variability in the genetic mechanisms thought to underpin neuropsychiatric disorders in comparison to neurological diseases (Lee et al., 2019), which further support the need to better understand the role of gene x environment interaction in psychiatric pathologies (Lesch, 2005; Lesch and Mössner, 2006). The majority of the translational studies performed to date have employed either conventional laboratory animals that have been exposed to a stressor, e.g., predation (Costa-Nunes et al., 2014; Strekalova et al., 2015), social defeat (Patki et al., 2013), maternal separation (Auth et al., 2018; Kiser et al., 2018; Veenema et al., 2007; Weidner et al., 2019), ultrasound (Gorlova et al., 2019; Pavlov et al., 2019) and others (Mineur et al., 2003; Malki et al., 2016), or they have used naïve genetically manipulated mice and rats (Nelson and Chiavegatto, 2001; Manchia et al., 2017).

Recently, we proposed a new aggression paradigm in which the contribution of gene x environment interaction in the mechanisms of pathological aggression is modelled in heterozygous (*Tph2^{+/-}*) mice by a five-day rat exposure (Gorlova et al., 2020). The interaction of the *Tph2^{+/-}* genotype and stress had also been explored in earlier studies of Gutknecht et al. (2012) and Weidner et al. (2019). Behavioural trends seemed to be present, but they were not significant, which was felt likely to be a consequence of the mild chronic stress paradigm and maternal separation that were employed. Conceptually, the stress studies with the

Tph2^{+/-} mutants were based on the reported relationships between single nucleotide polymorphisms (SNPs) in the *TPH2* gene e.g., rs1843809, rs4570625, and rs6582071 and psychiatric symptoms of excessive aggression (Oades et al., 2008; Perez-Rodriguez et al., 2010; Plemenita's et al., 2015; Laas et al., 2017), personality disorder (Perez- Rodriguez et al., 2010), negative emotionality (Lesch et al., 2012), depression (Wigner et al., 2018), mood disorder and suicidality (Ottenhof et al., 2018). Importantly, the incidence of these associations can be increased by a history of life stressors (Forssman et al., 2014; Xu et al., 2016).

While naive mice with a complete inactivation of *Tph2* gene (*Tph2*^{-/-}) recapitulate many symptoms related to SNPs of the *Tph2* gene, showing increased aggression, decreased sociability (Angoa-Perez et al., 2012; Weidner et al., 2019; Lieb et al., 2019), hyperactivity, impulsive and compulsive behaviours (Jia et al., 2014; Angoa-Perez et al., 2012; Waider et al., 2017) and enhanced fear learning (Lesch et al., 2012; Gutknecht et al., 2015; Waider et al., 2019), but also signs of stress resilience, altered depressive-like behaviour, decreased anxiety and increased neurogenesis (Savelieva et al., 2008; Jia et al., 2014; Gutknecht et al., 2012, 2015; Zhang et al., 2018; Weidner et al., 2019); these changes were not found in unchallenged *Tph2*^{+/-} mice. However, in *Tph2*^{+/-} mice, predation stress elicits excessive aggressiveness with pathological behavioural patterns of impulsive aggression, as well as changes of the brain concentrations of serotonin, its precursor hydroxytryptophan, and its metabolite 5-hydroxyindoleacetic acid, which were studied in different regions of the brain (Gorlova et al., 2020). Remarkably, behavioural changes in mutants

were found to be the opposite to those found in *Tph2^{+/-}* mice exposed to the predation, i.e., males and females on a C57BL/6 J background display a decrease in aggressive and dominant behaviours in response to predation (Gorlova et al., 2020). As such, the use of predation stress model in *Tph2^{+/-}* mice offers a promising model to explore the neurobiology of pathological aggression. In this paradigm, the stressed mutants have been shown to have altered measures of plasticity compared to the controls (Gorlova et al., 2020). These results are in keeping with other molecular abnormalities found in the *Tph2^{+/-}* mice subjected to a maternal separation, where the stressed mutants exhibited altered brain expression of the sodium-dependent serotonin transporter (Lieb et al., 2019) and changes in the DNA methylation of cholecystokinin receptor (Weidner et al., 2019).

Here, we sought to extend our studies with the *Tph2^{+/-}* mice exposed to stress in order to examine how other endpoints are altered following environmental challenges. In particular, we were keen to understand how the gene x environment interactions affect the levels of the monoamines dopamine (DA) and norepinephrine (NE), whose role in aggression is well established in a clinical context (Comai et al., 2012) and in animal studies (Hutchins and Pearson, 1975; Lewis et al., 1994; Adamczyk et al., 2012). We also sought to discover what other behaviours would be affected by the *Tph2^{+/-}* genotype in response to predation stress or to other environmental challenges including exposure to a novel environment and to a modified forced swim test (modFST), which is known to induce helplessness in mice (Strekalova et al., 2016).

Here, we found increased aggression and dominance, and suppressed sociability in the *Tph2*^{+/-} mice that were exposed to a predation stress procedure. In response to stress, anxiety-like behaviours were only observed in the WT mice, but not in the *Tph2*^{+/-}. These behaviours were associated with significant genotype-dependent differences in the concentrations of DA and NE and of their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-methoxy-4-hydroxyphenylglycol (MHPG), respectively, as well as DA and NE turnover rates. *Tph2*^{+/-} mice challenged with a novel environment exhibited increased vertical exploratory activity, and, in the ModFST, they exhibited no floating behaviour potentiation, suggesting the presence of a behavioural resilience to stress. Thus, stressed *Tph2*^{+/-} mutants display a behavioural profile that resembles that of unstressed *Tph2*^{-/-} animals, and the combination of predation stress and the *Tph2*^{+/-} genotype presents itself as a promising model of excessive human aggression induced by changing environmental conditions.

2. Materials and methods

2.1. Animals

We used 10-12-week-old male *Tph2*^{+/-} mice, and their wild-type littermates (*Tph2*^{+/+}) as controls, which were bred and genotyped in the facilities of the University of Würzburg (Gutknecht et al., 2015). 12-week-old male CD1 mice were used as intruders for a resident-intruder test and 2-to-5-month-old male Wistar rats (Charles River, Janvier, France) were used for the predator stress. *Tph2*^{+/-} and *Tph2*^{+/+} littermates

were housed individually, while CD1 mice and rats were housed in groups of five. Animals were kept under controlled laboratory conditions (22 ± 1 °C, 55% humidity, food and water ad libitum, lights on: 21:00 h). Studies were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals (permission issued by Ethical Committee of C. Bernard University of Lyon).

2.2. Study outline

Two cohorts of animals (Fig. 1A&B) were exposed predation stress in which *Tph2^{+/-}* mutants, and littermate controls, were subjected to a daily rat-exposure stress for 5 days (Costa-Nunes et al., 2014, Vignisse et al., 2017; see below). The groups of mice were balanced according to their body weight, and for latency to attack in a baseline resident-intruder test (Couch et al., 2016; Strekalova et al., 2018). Beginning 24 h after the end of the predation stress procedure, the first set of animals were tested in the elevated O-maze (day 6), dark/light box (day 6), and, on days 6–10, in a resident-intruder test, with 3 h inter-test intervals (see below, Fig. 1A). In the second cohort, 24 h after the end of the last predator stress session, the mice were killed and their brains were isolated and dissected (see below) for subsequent HPLC tissue assay (see below, Fig. 1B). An additional cohort of *Tph2^{+/-}* mutants and their littermate *Tph2^{+/+}* control mice were subjected to a novel cage test and to the modified swim test (Fig. 1C). In each part of the experiment, an average of seven to ten mice per group were used (group sizes are indicated in Figure legends).

Fig. 1. Experiment design. *Tph2^{+/+}* and *Tph2^{+/-}* mice were subjected to rat exposure stress for 5 consecutive nights, and 24 h after last stress session both groups were (A) studied in a battery of behavioural tests or (B) killed and brains were dissected for HPLC study. (C) Naïve *Tph2^{+/+}* and *Tph2^{+/-}* mice were studied for 5 consecutive days in novel cage test and modified swim test.

2.3. Predation stress

Mice were introduced into a spacious transparent glass cylinder (15 cm high x 8 cm diameter) and placed into the rat cage as described elsewhere (Costa-Nunes et al., 2014; Vignisse et al., 2017; Gorlova et al., 2020). A 15-h long exposure was performed between 18:00 and 9:00 for five consecutive nights. Mice had free access to food and water in their home cages between the stress sessions. The timing of the rat exposure

model was designed to minimize the impact of food and water deprivation, as the predation period overlaps with the light (inactive) phase of activity of the mice when food and water consumption is minimal. While there is no doubt that food and water deprivation during this period might add to the stress, our earlier studies, with another version of this stress model, revealed that if food and water is made available during the predation stress the C57/Bl6 mice do not consume it (Strekalova et al., 2004; Strekalova and Steinbusch, 2009, 2010). Thus, while the environment undoubtedly results in a mixture of stresses, the principal stress is based on the fear of predation.

2.4. O-maze test The O-maze apparatus (Technoplast, Rome, Italy) consisted of a circular path (runway width 5.5 cm, diameter 46 cm) placed 50 cm above the floor. Two opposing arms were protected by walls (height 10 cm), and the illumination strength was 25 Lux. Anxiety-like behaviour was assessed using previously validated parameters as described elsewhere (Couch et al., 2016; Costa-Nunes et al., 2020). Mice were placed in one of the closed arm compartments of the maze. Total duration of time spent in the open arms of the maze and the number of visits to the open arms were scored as established measures of anxiety-like behaviour during the first 5 min period.

2.5. Dark/light box test

At the start, mice were placed into the dark compartment (15 cm x 20 cm x 25 cm) of the box from where they could visit the lit box (30 cm x 20 cm x 25 cm, Technoplast, Rome, Italy). The time spent by the mice in the lit part of the box, which was set to 25 Lux on the floor of the

apparatus, was recorded over a 5-min period, as described elsewhere (Costa-Nunes et al., 2014; Strekalova et al., 2018).

2.6. Resident-intruder test

The resident-intruder test procedure was carried out on five consecutive days (Strekalova et al., 2004, 2018; Costa-Nunes et al., 2014; Gorlova et al., 2020). Mice were placed individually in an observation cage (30 cm × 60 cm × 30 cm) for 30 min, after which a CD1 mouse was introduced. During the next 4 min, mice were separated by a transparent wall with holes it is, which was removed for the following 4-min-long period. Latency to attack and the duration of attacks were recorded. In addition, measures of neutral social exploration were evaluated; the latency to contact and the number of nose/anal and nose/nose contacts were recorded as described elsewhere (Couch et al., 2016; Veniaminova et al., 2017).

2.7. Novel cage test

A 5-min long novel cage test was carried out to assess exploration in a new environment as described elsewhere (Strekalova et al., 2004; Couch et al., 2016; Veniaminova et al., 2020). Mice were introduced into a standard plastic cage (21 cm × 21 cm × 15 cm) filled with fresh sawdust. The number of exploratory rears was counted in white light for 5 min; the strength of illumination was set to 25 Lux.

2.8. The modified swim test to assess helplessness

Mice were subjected to two swimming sessions with an interval of 24 h. After the first two swim session a third swim session was carried out on day 5 as previously described (Strekalova et al., 2016; Pavlov et al., 2017, 2020). All sessions were 6-min long and were performed by placing a mouse in a transparent cylinder (Ø 17 cm) filled with water (+23°C, water height 13 cm, height of cylinder 20 cm). The duration of floating behaviour that was defined as the absence of any directed movements of the head or body, which was scored by an observer unaware of the identity of the animal with Noldus EthoVision XT 8.5 (Noldus Information Technology, Wageningen, The Netherlands) as described elsewhere (Malatynska et al., 2012). It is of note that the increase in floating behaviour, which is observed on day 5 compared to day 2, is reversible by pre-treatment common antidepressant compounds (Markova et al., 2017). For this reason, the increase in day 5 floating is regarded as a measure of learning in an adverse context and helplessness (Pavlov et al., 2017, 2020).

2.9. Tissue collection

Mice were terminally anaesthetized with an intraperitoneal injection of sodium pentobarbitone. The left ventricle was perfused with 10 ml of ice-cold saline and the brains were removed and dissected (Couch et al., 2013). The prefrontal cortex, striatum, amygdala, hippocampus and dorsal raphe were isolated and stored at -80°C as described elsewhere (Gorlova et al., 2020). Selected brain structures were

microdissected using Paxinos atlas as a guide (Paxinos and Franklin, 2001). As the HPLC method requires a substantial amount of tissue, all brain structures were analysed as a whole.

2.10. High-performance liquid chromatography (HPLC) tissue assay

The concentrations of dopamine, norepinephrine, and their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-methoxy-4-hydroxyphenylglycol (MHPG), respectively, were measured in the parts of the brain mentioned above using HPLC with electrochemical detection using the method of Waider et al. (2017). Dopamine turnover was calculated as a ratio of DOPAC/DA, and norepinephrine turnover was calculated as MHPG/NE. *2.11. Statistical analysis* Data were analysed using GraphPad Prism v.8.3.0 for Windows (San Diego, CA). Three-way, or two-way, or repeated measures ANOVA were employed followed by post-hoc testing were appropriate. For the two-group comparisons in the novel cage test and modFST, unpaired *t*-test were used. The level of confidence was set at 95% ($p < 0.05$). For non-parametric data (O-maze exits) a Kruskal-Wallis rank sum test was performed with post-hoc pairwise comparisons using Wilcoxon rank sum test.

3. Results

3.1. Increased aggressive behaviour in stressed $Tph2^{+/-}$ mice

A three-way ANOVA revealed that there was no interaction between predator stress, genotype, and the day of testing for the latency to attack in the resident-intruder test ($F_{1,21} = 0.095$; $p = 0.76$). There was

also no significant interaction between stress and the day was present independent of genotype ($F_{1,46} < 0.01$, $p = 0.89$, two-way ANOVA). However, a significant interaction, independent of day, was found between predator stress and genotype ($F_{1, 46} = 17.44$, $p < 0.01$, two-way ANOVA). Post hoc analysis revealed that there was a significantly shorter latency to attack in the stressed $Tph2^{+/-}$ group than in either the non-stressed $Tph2^{+/-}$ or the stressed $Tph2^{+/+}$ mice (both $p < 0.01$, Sidak's test). A significant day $\times$ genotype interaction, independent of predator stress, was also present ($F_{1, 23} = 6.215$, $p = 0.02$, two-way ANOVA). Sidak's post-hoc testing revealed that significant decrease in the latency to attack on the day 5 in $Tph2^{+/-}$ group compared to day 1 ($p < 0.01$; Fig. 2A).

Total duration of attacks was found to be dependent on an interaction between predator stress, genotype, and the day of testing as revealed by a three-way ANOVA ($F_{1,21} = 5.046$, $p = 0.04$). Post hoc analysis showed that stress increased the duration of attacks in the $Tph2^{+/-}$ animals on days 1 and 5 compared to non-stressed $Tph^{+/-}$ animals or the stressed $Tph2^{+/+}$ animals (all $p < 0.01$, Bonferroni test). In the mutant mice the duration of attacks was higher on day 5 than on day 1 ($p = 0.01$; Fig. 2B). Thus, predator stress appears to have the opposite effect on the duration of attacks in the $Tph2^{+/+}$ animals subjected to.

3.2. Stressed $Tph2^{+/-}$ mice display a decrease of neutral social exploration

Both latency and number of nose/anal contacts were dependent on a stress, genotype and the day of testing ($F_{1,21} = 7.06$, $p = 0.015$ and $F_{1,20} = 12.13$, $p < 0.01$, respectively, three-way ANOVA). Post hoc testing revealed a significant increase in the latency to nose/anal contact on day

5 in stressed *Tph2^{+/-}* animals in comparison to stressed *Tph2^{+/+}* animals and to the non-stressed *Tph2^{+/-}* animals on the same day ($p = 0.03$ and $p = 0.04$, respectively, Bonferroni test) and in comparison to stressed *Tph2^{+/-}* on day 1 ($p = 0.02$; Fig. 2C). On day 5, stress significantly reduced the number of nose/anal contacts in the stressed *Tph2^{+/-}* compared to the stressed *Tph2^{+/+}* animals ($p < 0.01$), while in *Tph2^{+/+}* mice stress increased the number of contacts ($p = 0.04$).

Three-way ANOVA also showed the presence of an interaction between stress, genotype, and the day for both latency to nose/nose contact and the total number of nose/nose contacts ($F_{1,20} = 6.23$, $p = 0.02$ and $F_{1,21} = 13.46$, $p < 0.01$, respectively). On day 5, the latency to a nose/nose contact was significantly longer in the predator stressed *Tph2^{+/-}* mice as compared to the stressed *Tph2^{+/+}* animals ($p = 0.04$). Number of nose/nose contacts was significantly elevated in the stressed *Tph2^{+/+}* animals from day 1 to day 5 ($p < 0.01$), which was not observed in the *Tph2^{+/-}* animals.

Resident-intruder test

Neutral social exploration

Fig. 2. Opposing effects of predator stress on aggressive behaviour on *Tph2*^{+/+} and *Tph2*^{+/-} mice. (A) In the resident-intruder test, *Tph2*^{+/-} showed significant decrease in latency to attack on day 5 compared to day 1. (B) On both day 1 and day 5 stressed *Tph2*^{+/-} mice displayed a significant increase in duration of attacks in comparison to non-stressed *Tph2*^{+/-} and stressed *Tph2*^{+/+} groups on corresponding days. Total duration of attacks was longer on day 5 than on day 1 in *Tph2*^{+/-} animals. (C) Latency to nose/anal contact was significantly elevated in stressed *Tph2*^{+/-} on day 5 in comparison to non-stressed *Tph2*^{+/-} and stressed *Tph2*^{+/+} animals on the same day, and to

stressed *Tph2^{+/-}* on day 1. (D) Number of nose/anal contacts was significantly increased on day 5 in stressed *Tph2^{+/+}* mice compare to stressed *Tph2^{+/-}* mice on the same day and to stressed *Tph2^{+/+}* mice on day 1. (E) Latency to nose/ nose contact was significantly higher in stressed *Tph2^{+/-}* mice on day 5 than in stressed *Tph2^{+/+}* group on the same day. (F) In stressed *Tph2^{+/+}* number of nose/nose contacts was significantly higher on day 5 than on day 1. * $p < 0.05$, repeated measures three-way ANOVA and post hoc Bonferroni's test or Sidak's tests (see the text). 5–7 animals per group were used. Bars represent Mean $\pm$ SEM.

*3.3. Anxiety-like behaviour after stress exposure does not alter in *Tph2^{+/-}* mice*

In the elevated O-maze, using Kruskal-Wallis rank sum test it was found that there was an interaction between the genotype and stress (chi-squared = 11.932, df = 3, p -value = 0.0076). Subsequent pairwise comparisons using Wilcoxon rank sum test revealed that there were significant differences between the stressed *Tph2^{+/+}* group and both the *Tph2^{+/-}* stressed ($p = 0.030$) and non-stressed animals ($p = 0.026$). Stressed *Tph2^{+/-}* mice displayed shorter latency to exit to an open arm than non-stressed *Tph2^{+/-}* ($p = 0.03$, Tukey's test, *data not shown*). In stressed *Tph2^{+/+}* animals the number of exits was significantly lower in comparison to non-stressed controls ($p = 0.03$; Fig. 3A). The main effect of genotype, but not main stress effect or stress x genotype interaction was significant for the time spent the open arms of the O-maze ($F_{1,20} = 6.12$, $p = 0.02$; $F_{1,20} = 1.55$, $p = 0.23$ and $F_{1,20} = 4.087$, $p = 0.06$, respectively; Fig. 3B). Thus, in essence, *Tph2^{+/-}* mice do not exhibit the anxiety-like behaviours that are observed in *Tph2^{+/+}* group in the elevated O-maze.

Fig. 3. Stress-induced increase in anxiety-like behaviour in $Tph2^{+/+}$, but not in $Tph2^{+/-}$ mice. (A) In comparison to non-stressed $Tph2^{+/-}$, stressed $Tph2^{+/-}$ mice demonstrated significantly increased latency to exit to open arms of the O-maze, while (B) $Tph2^{+/-}$ and $Tph2^{+/+}$ mice differed significantly in time spent therein. No significant differences were found in (C) number of entries into the lit box in the dark-light box test and (D) time spent in the lit box. * $p < 0.05$, Kruskal- Wallis rank sum test and two-way ANOVA and post hoc Tukey's test (see the text). 7–9 animals per group were used. Bars represent Mean $\pm$ SEM.

In the dark-light box test, no significant stress $\times$ genotype interaction was found for latency to entry into the lit box, number of entries and time spent in the lit box ($F_{1,20} = 0.027$, $p = 0.87$; $F_{1,20} =$

1.903, $p = 0.18$ and $F_{1,20} = 0.95$, $p = 0.34$, respectively; Fig. 3C,D), as well as no significant stress effects ($F_{1,20} = 1.56$, $p = 0.23$; $F_{1,20} = 0.95$, $p = 0.34$ and $F_{1,20} = 1.9$, $p = 0.18$, respectively; Fig. 3C,D) and no genotype effects ($F_{1,20} = 0.48$, $p = 0.49$; $F_{1,20} = 2.13$, $p = 0.16$ and $F_{1,20} < 0.01$, $p > 0.99$, respectively; Fig. 3C,D).

3.4. Stress-induced changes in dopamine metabolism in the brain of Tph2^{+/-} mice

There was a significant interaction between stress and genotype for the concentration of dopamine in the amygdala and prefrontal cortex, but not in hippocampus, dorsal raphe or striatum ($F_{1,27} = 5.75$, $p = 0.02$; $F_{1,28} = 8.41$, $p < 0.01$; $F_{1,28} < 0.01$, $p = 0.98$; $F_{1,27} = 0.04$, $p = 0.84$ and $F_{1,26} = 0.05$, $p = 0.82$, respectively, two-way ANOVA). In the hippocampus, dorsal raphe, and striatum the concentration of dopamine was significantly affected by stress ($F_{1,28} = 28.58$, $p < 0.01$; $F_{1,27} = 5.08$, $p = 0.03$ and $F_{1,26} = 47.73$, $p < 0.01$, respectively). A post-hoc analysis revealed significantly lower concentrations of dopamine in the amygdala of the stressed Tph2^{+/-} mice in comparison to both the non-stressed Tph2^{+/-} and the stressed Tph2^{+/+} animals ($p < 0.01$ and $p = 0.02$, respectively Tukey's test. Fig. 4A). The concentration of dopamine in the prefrontal cortex was decreased in stressed Tph2^{+/-} mice compared to the non-stressed mutants ($p < 0.01$; Fig. 4D). Finally, the concentration of dopamine was lower in the stressed controls and mutants in comparison with non-stressed groups in the hippocampus (both $p < 0.01$, Tukey's test; Fig. 4G) and striatum (both $p < 0.01$; Fig. 4M).

Fig. 4. Stress-induced alterations in concentrations of dopamine, its metabolite DOPAC, and dopamine turnover rate in *Tph2*^{+/+} and *Tph2*^{+/-} mice. (A) Dopamine concentration in the amygdala was significantly lower in

stressed *Tph2^{+/-}* mice in comparison to both stressed *Tph2^{+/+}* and non-stressed *Tph2^{+/-}* mice. (B) DOPAC concentration in the amygdala showed a significant increase in stressed *Tph2^{+/+}* mice compared to non-stressed *Tph2^{+/+}* group and stressed *Tph2^{+/-}* mice. (C) The dopamine turnover rate in the amygdala was significantly increased in stressed groups compared to non-stressed animals of the same genotype. (D) In the prefrontal cortex, dopamine concentration was significantly lower in stressed *Tph2^{+/-}* in comparison to stressed *Tph2^{+/+}* group. (E) No differences in the DOPAC concentration between groups were observed. (F) The dopamine turnover was significantly higher in stressed *Tph2^{+/-}* mice compared to both non-stressed *Tph2^{+/-}* and stressed *Tph2^{+/+}* animals. (G) In the hippocampus in both *Tph2^{+/+}* and *Tph2^{+/-}* significant decrease in dopamine concentration under stress condition was observed in comparison to non-stressed animals of the same genotype. (H) No significant differences between the groups were found in the DOPAC concentrations in the hippocampus. (I) Dopamine turnover rate in the hippocampus was higher in both stressed groups in comparison to non-stressed group of the same genotype. (J) Dopamine concentration in the dorsal raphe was not changed significantly between the groups. (K) There was a significant increase in the DOPAC concentration in stressed compared to non-stressed *Tph2^{+/+}* mice in the dorsal raphe, while (L) no alterations were found in the dopamine turnover ratio. (M) In the striatum, dopamine concentration showed a significant decrease in stressed mice compared to non-stressed groups. (N) DOPAC concentration was significantly elevated in the striatum of non-stressed *Tph2^{+/-}* compared to stressed *Tph2^{+/-}* group. (O) No significant group differences were found in the dopamine turnover ratio in the striatum. * $p < 0.05$, two-way ANOVA and post hoc Tukey's test. 7–9 animals per group were used. Bars represent Mean $\pm$ SEM. DA = dopamine, DOPAC = 3,4-Dihydroxyphenylacetic acid.

In contrast to the levels of dopamine, there was found to be an interaction between stress and genotype for DOPAC in the amygdala and in the dorsal raphe, but not in prefrontal cortex, hippocampus, or striatum ($F_{1,28} = 12.86$, $p < 0.01$; $F_{1,27} = 4.25$, $p < 0.05$; $F_{1,28} = 1.09$, $p = 0.31$; $F_{1,28} = 0.76$, $p = 0.39$ and $F_{1,26} = 3.87$, $p = 0.06$, respectively). Stress alone significantly affected DOPAC concentrations in the prefrontal cortex and striatum ($F_{1,28} = 4.42$, $p = 0.04$ and $F_{1,26} = 21.49$, $p < 0.01$,

respectively) and post hoc analysis revealed that in the amygdala of stressed *Tph2^{+/+}* mice the DOPAC concentration was elevated compared to non-stressed *Tph2^{+/+}* animals or predator stressed *Tph2^{+/-}* mice (both $p < 0.01$, Tukey's test; Fig. 4B). The concentration of DOPAC was increased in stressed *Tph2^{+/+}* mice in comparison with non-stressed controls in dorsal raphe ($p = 0.06$; Fig. 4K). It was also elevated in striatum of stressed *Tph2^{+/+}* compared to the non-stressed mutants ($p < 0.01$; Fig. 4N).

A significant interaction between stress and genotype on the dopamine turnover rate was found in the prefrontal cortex ($F_{1,28} = 11.30$, $p < 0.01$, two-way ANOVA), but not in amygdala, hippocampus, dorsal raphe, or striatum ($F_{1,27} = 1.13$, $p = 0.30$; $F_{1,28} < 0.05$, $p = 0.86$; $F_{1,27} = 1.77$, $p = 0.19$ and $F_{1,26} = 0.46$, $p = 0.5$, respectively). However, stress had significant main effect on dopamine turnover in amygdala, hippocampus, and dorsal raphe ($F_{1,27} = 30.07$, $p < 0.01$; $F_{1,28} = 51.13$, $p < 0.01$ and $F_{1,27} = 5.03$, $p = 0.03$, respectively). Post hoc analysis revealed an increase in turnover rate in the stressed controls and mutants in comparison with non-stressed groups in the amygdala ($p = 0.02$ and $p < 0.01$, Tukey's test, respectively; Fig. 4C) and hippocampus (both $p < 0.01$; Fig. 4I), while in the prefrontal cortex of stressed *Tph2^{+/-}* mice it was increased compared to both non-stressed *Tph2^{+/-}* or *Tph2^{+/+}* animals (both $p < 0.01$; Fig. 4F).

3.5. Altered brain metabolism of norepinephrine in stressed *Tph2^{+/-}* mice

There was no significant interaction between stress and genotype for norepinephrine concentrations in amygdala, prefrontal cortex, hippocampus, dorsal raphe and striatum ($F_{1,28} < 0.01$, $p = 0.89$; $F_{1,28} = 0.11$, $p = 0.75$; $F_{1,28} = 1.77$, $p = 0.19$; $F_{1,27} = 1.21$, $p = 0.28$ and $F_{1,28} = 0.78$, $p = 0.39$, respectively, two-way ANOVA). Stress significantly affected concentrations of norepinephrine in prefrontal cortex, hippocampus, and striatum ($F_{1,28} = 7.96$, $p < 0.01$; $F_{1,28} = 6.00$, $p = 0.02$ and $F_{1,28} = 50.82$, $p < 0.01$, respectively). Post hoc analysis revealed that there was a significant increase in norepinephrine levels in the stressed *Tph2^{+/-}* and *Tph2^{+/+}* mice compared to non-stressed animals of respective genotype (both $p < 0.01$, Tukey's test; Fig. 5M).

MHPG concentrations exhibited a stress x genotype interaction in the amygdala, but not in the prefrontal cortex, hippocampus, dorsal raphe, or striatum ($F_{1,28} = 13.45$, $p < 0.01$; $F_{1,28} = 0.86$, $p = 0.36$; $F_{1,28} = 1.7$, $p = 0.2$; $F_{1,27} = 0.42$, $p = 0.52$ and $F_{1,28} < 0.01$, $p = 0.78$, respectively). Main effects for stress and genotype were observed in the hippocampus ($F_{1,28} = 28.75$, $p < 0.01$ and $F_{1,28} = 6.34$, $p = 0.02$, respectively; Fig. 5H). In the stressed *Tph2^{+/-}* group, the MHPG level in the amygdala was significantly lower than in both the non-stressed *Tph2^{+/-}* mutants and stressed *Tph2^{+/-}* mice (both $p = 0.01$, Tukey's test; Fig. 5B).

Fig. 5. Stress-induced alterations in concentrations of norepinephrine, its metabolite MHPG, and norepinephrine turnover rate in *Tph2*^{+/+} and *Tph2*^{+/-} mice. (A) In the amygdala, no significant group

differences were observed in the norepinephrine concentration. (B) MHPG concentration was significantly lower in the stressed *Tph2^{+/-}* compared to both stressed *Tph2^{+/+}* and non-stressed *Tph2^{+/-}* mice. (C) Norepinephrine turnover rate was significantly lower in stressed *Tph2^{+/-}* in comparison to non-stressed *Tph2^{+/-}* mice. No significant changes between the groups were found in (D) norepinephrine concentration and (E) MHPG concentration in the prefrontal cortex. (F) However, norepinephrine turnover rate in the prefrontal cortex was significantly lower in stressed *Tph2^{+/+}* compared to non-stressed *Tph2^{+/+}* mice. (G) No significant group changes in the norepinephrine concentration were observed in the hippocampus. (H) In the hippocampus, MHPG concentration was elevated significantly in both genotypes in stressed groups compared to non-stressed animals, but (I) no significant changes between the groups were found in the norepinephrine turnover rate. (J - L) No significant group changes in the norepinephrine metabolism were found in the dorsal raphe. (M) In the striatum, in both genotypes, there were significant increases in the norepinephrine concentrations in stressed groups compared to non-stressed animals of the same genotype. (N) MHPG concentration in the striatum did not show any significant changes between the groups. (O) Norepinephrine turnover ratio was significantly lowered in both genotypes in stressed groups in comparison to non-stressed animals. * $p < 0.05$, two-way ANOVA and post hoc Tukey's test. 7–9 animals per group were used. Bars represent Mean $\pm$ SEM. NE = norepinephrine, MHPG = 3-Methoxy-4-hydroxyphenylglycol.

There was a significant interaction between genotype and stress for norepinephrine turnover rate in the amygdala, but not in prefrontal cortex, hippocampus, dorsal raphe, or striatum ($F_{1,28} = 11.31$, $p < 0.01$; $F_{1,28} = 0.25$, $p = 0.62$; $F_{1,28} < 0.01$, $p = 0.95$; $F_{1,27} = 0.59$, $p = 0.45$ and $F_{1,28} = 2.35$, $p = 0.14$, respectively). Norepinephrine turnover rate was significantly lower in the stressed *Tph2^{+/-}* compared to the non-stressed *Tph2^{+/-}* in the amygdala ($p < 0.01$, Tukey's test; Fig. 5C). Stress alone significantly changed norepinephrine turnover rate in prefrontal cortex, hippocampus, and striatum ($F_{1,28} = 18.32$, $p < 0.01$; $F_{1,28} = 6.17$, $p = 0.02$ and $F_{1,28} = 27.84$, $p < 0.01$, respectively). Norepinephrine turnover rate

was decreased in the prefrontal cortex of stressed $Tph2^{+/-}$ in comparison to controls ($p = 0.01$; Fig. 5F). It also was decreased in the striatum of stressed $Tph2^{+/-}$ and $Tph2^{+/+}$ mice compared to the non-stressed groups of same genotypes ($p < 0.01$ and $p < 0.05$, respectively; Fig. 5O).

3.6. $Tph2^{+/-}$ mice display increased novelty exploration and reduced helplessness in the modified swim test

In the modFST test, there was an interaction between day and genotype in the duration of floating, but not in the latency to float or the number of floating episodes ($F_{2,22} = 3.73$, $p = 0.04$; $F_{2,22} = 3.06$, $p = 0.07$ and $F_{2,22} = 0.69$, $p = 0.51$, respectively, two-way ANOVA). A main effect of the day was observed for the latency to float and number of floating episodes ($F_{1,134,12.47} = 9.80$, $p < 0.01$ and $F_{1,901,20.91} = 6.44$, $p < 0.01$, respectively). No significant group differences in latency to float were found on day 1 ($p = 0.14$, Bonferroni test; Fig. 6A). Latency to float was shortened in $Tph2^{+/-}$ mice on day 5 compared to day 1 ($p = 0.02$, Bonferroni test; Fig. 6A), the number of floating episodes was increased in $Tph2^{+/-}$ on day 5 compared to day 1 ($p = 0.04$; Fig. 6B). On day 5, the duration of floating was significantly shorter in $Tph2^{+/-}$ than in the $Tph2^{+/+}$ animals ($p < 0.01$; Fig. 6C).

In the novel cage test, $Tph2^{+/-}$ mice showed significantly higher number of exploratory rears than their $Tph2^{+/+}$ littermates ($p = 0.04$, unpaired t -test; Fig. 6D). Together, observations in these two behavioural tests suggest that there is a lower propensity to float and increased exploratory activity in the naïve $Tph2^{+/-}$ mice when they are exposed to

environmental challenges, which may reflect increased stress-resilience in these animals.

Fig. 6. Reduced propensity to float and increased vertical exploratory activity in naïve *Tph2*^{+/-} mice. In the modified swim test, (A) naïve *Tph2*^{+/-} showed significant decrease in the latency to the first immobility episode between days 1 and 5 of the test. (B) Total number of immobility episodes significantly increased from day 1 to day 5 in naïve *Tph2*^{+/-} mice. (C) Total duration of floating was significantly lower in naïve *Tph2*^{+/-} mice compared to naïve *Tph2*^{+/+} mice on day 5 of the test. Both in *Tph2*^{+/+} and *Tph2*^{+/-} mice total immobility time was significantly lower on day 1 in comparison with day 2 and day 5. (D) In the novel cage test, naïve *Tph2*^{+/-} mice showed significantly higher number of rearings in comparison to naïve *Tph2*^{+/+} mice. **p* < 0.05; in A-C repeated measures two-way ANOVA and post hoc Tukey's test; in D unpaired t-test. 5–8 animals per group were used. Bars represent Mean ± SEM.

4. Discussion

Tph2^{+/-} mice subjected to the predation stress paradigm displayed a marked increase of aggression, dominance and a suppression of neutral sociability, whereas the stressed *Tph2*^{+/+} group showed opposing changes in social behaviour. Rat exposure paradigm increased anxiety-like behaviour in *Tph2*^{+/+} control mice but did not alter it in the *Tph2*^{+/-} mutants. The separate cohort of *Tph2*^{+/-} mice exhibited increased exploration in a novel environment and a lack of potentiation of floating behaviour over the course of repeated sessions of the ModFST. The HPLC tissue assays revealed profound changes in the content and metabolism of brain DA and NE in the stressed and in the non-stressed *Tph2*^{+/-} mice, suggesting that this monoamine dysregulation might contribute to the altered behaviours that are observed in the mutants.

In accordance with previous results, we found that *Tph2*^{+/-} mice subjected to a five-day predation stress paradigm displayed excessive aggression, which was the opposite of the changes found in the littermate controls (Gorlova et al., 2020) and reminiscent to aggressive behaviour typical of non-stressed *Tph2*^{-/-} mice (Gutknecht et al., 2012, 2015). Additional analysis of the social behaviour showed that the stressed *Tph2*^{+/-} mice also displayed increased measures of dominance and decreased scores of neutral social exploration, which are known to correlate with the appearance of aggressive traits in small laboratory rodents, including the naive null *Tph2*^{-/-} mutants (Weidner et al., 2019). Again, control mice exposed to predation stress model exhibited the opposite response in these forms of social behaviour. In particular, in the

Tph2^{-/-} group, rat exposure stress did not elicit anxiogenic-like behaviour in the elevated O-maze test, in contrast to the *Tph2*^{+/+} controls. Changes in the dark-light box test did not reach statistical significance, but numerically the data suggest their similar changes in direction to above-mentioned group differences in anxiety. Lowered anxiety levels have been reported in naïve and stressed *Tph2*^{-/-} mice (Mosienko et al., 2012; Gutknecht et al., 2012, 2015; Jia et al., 2014; Weidner et al., 2019), and the present findings further supports the view of phenotypical resemblance between naïve *Tph2*^{-/-} and stressed *Tph2*^{+/-} mice (Gorlova et al., 2020).

Previous work with stress paradigm that was employed here demonstrated altered brain concentrations and metabolism of serotonin, a major regulator of aggression and anxiety (Nelson and Chiavegatto, 2001; Abela et al., 2020; Quah et al., 2020), in the stressed *Tph2*^{+/-} mice (Gorlova et al., 2020). The serotonergic system closely interacts with brain dopaminergic system of the brain, reciprocally modulating dopaminergic activity (Millan et al., 1998; De Deurwaerdere et al., 2005; Seo et al., 2008; Rosell and Siever, 2015). Lower levels of dopamine were previously measured in the brains of naïve *Tph2*^{-/-} mice in the hippocampus and in the frontal cortex (Gutknecht et al., 2012). Similarly, in the present study, stress resulted in diminished DA concentrations in the amygdala and in the prefrontal cortex of *Tph2*^{+/-} mice that was not found in *Tph2*^{+/+} animals. In the stressed *Tph2*^{+/-} mice, DA concentrations were also lower in the hippocampus and striatum in comparison with naïve mutants. Thus, changes in the dopaminergic system of the stressed

Tph2^{+/-} mice are strikingly different from those found in the *Tph2*^{+/+} animals and overly resemble those observed in *Tph2*^{-/-} mice; they can underpin stress-induced excessive aggression and other behavioural changes reported in this study.

Changes to the serotonergic system are often associated with changes to NE neurotransmission, particularly, in amygdala (Pucilowski et al., 1987; Millan et al., 1998). Here, stress exposure resulted in similar genotype-related increases of NE concentration and NE turnover in the striatum, elevated concentrations of NE metabolite MHPG in the hippocampus and decreased NE turnover in the prefrontal cortex. However, genotype differences between stressed groups were shown for MHPG concentrations in the amygdala, whose values in the *Tph2*^{+/-} group were lower than in stressed *Tph2*^{+/+} mice. Stress significantly decreased this measure, as well as NE turnover in the amygdala of *Tph2*^{+/-} mice, but not in the littermate *Tph2*^{+/+} controls. These data further show the contribution of amygdala and suggest the role of NE in described here stress-induced behavioural abnormalities of *Tph2*^{+/-} mice.

Together, the HPLC data presented here provides evidence for genotype-governed differences in the stress-induced changes of DA and NE regulation in the amygdala and prefrontal cortex. These are brain areas in which genotype changes in the TPH2-deficient mice were also shown for serotonin precursor 5-hydroxytryptophan (5-HTP). This led to the suggestion that these inter-related changes in the three monoamines in the amygdala might underlie excessive aggression and altered social behaviour in general of stressed *Tph2*^{+/-} mutants. This

suggestion is keeping with previously reported monoamine changes in the amygdala of stressed *Tph2*^{+/-} mice in relation to GABA metabolism (Waider et al., 2017), as well as the altered methylation profile of CCK in this brain structure (Weidner et al., 2019). These findings are in line with the well-documented primary role of the amygdala in the regulation of aggression (Haller et al., 2017) and stress response (Tottenham and Galvan, 2016). Moreover, they are also in agreement with clinical data showing that the amygdala has a key function in the response to emotional stimuli of valence in carriers of SNPs of the *TPH2* gene G-844T and G-703T (Brown et al., 2005; Canli et al., 2005).

Regarding other brain structures that could be potentially involved in behavioural changes reported here in the *Tph2*^{+/-} mice, it should be noted that prefrontal cortex was shown to regulate amygdala-dependent processing of social cues in fMRI studies in humans with acute tryptophan depletion (Passamonti et al., 2012). Reduced inhibitory control of the amygdala by the prefrontal cortex is considered to result in impulsive aggression (Jackson and Moghaddam, 2001; Haller, 2017). Here, we found diminished DA levels in the prefrontal cortex in stressed *Tph2*^{+/-} group. These changes might lead to a decrease in the top-down control of the amygdala (Rosenkranz and Grace, 2001) resulting in excessive aggression that is in keeping with earlier studies. For example, the hypoactivation of the prefrontal cortex in rats was associated with elevated activation of the amygdala in a single-prolonged stress paradigm (Piggott et al., 2019). Chronic restraint stress in C57BL/6 mice was shown to enhance amygdala-prefrontal cortex interactions, and

optogenetic activation of these circuits elicited anxiety-like and hyperactivity in naïve mice (Lowery-Gionta et al., 2018). The changes of NE regulation observed here after stress in the prefrontal cortex may alter the function of the amygdala that is keeping with previous findings (Van Bockstaele et al., 1998).

Our previous study showed a decrease of 5-HTP and the serotonin metabolite 5-Hydroxyindoleacetic acid (5-HIAA) in the striatum of stressed *Tph2*^{+/-} mice, but not in other experimental groups (Gorlova et al., 2020). Together with the findings of a decreased DOPAC concentration in the striatum in stressed *Tph2*^{+/-} mice, this suggests a role for this brain area in differential effects of stress in these mutants compared to the *Tph2*^{+/+} controls. The striatum, and, particularly, the nucleus accumbens, is essential in the so called ‘limbic-motor’ interface, which is functionally connected to the amygdala (Cardinal et al., 2003; Ambroggi et al., 2008) and to the prefrontal cortex (Del Arco and Mora, 2008; Janak and Tye, 2015). In rats, lesions of the nucleus accumbens provoked excessive aggression and muricide behaviour (Lee and Ueki, 1986; Pucilowski and Valzelli, 1986). Whereas social defeat stimulated dopamine signalling by increasing both DA release and uptake (Anstrom et al., 2009; Deal et al., 2018), isolation stress suppressed baseline DA levels, but increased the DA-mediated response to various stimuli (Yorgason et al., 2016; Karkhanis et al., 2016). While nucleus accumbens is distinct from dorsal region of striatum functionally, many data suggest unidirectional stress-induced changes in DA metabolism in these areas (Abercrombie et al., 1989; Porcelli et al., 2012). A decrease of DA

concentrations in the striatum of stressed mice in our study may resemble previously reported effects of chronic isolation stress on basal DA levels in rats (Yorgason et al., 2016; Karkhanis et al., 2016). Thus, in addition to the changes reported here in the whole striatum, the studies highlighted above argue for the importance of the striatal DA system in the mechanisms of stress associated with social determinants.

The stress model employed here is based largely on social stress. The model was designed to minimize the impact of physical stressors such as food and water deprivation, and the loss of consummatory behaviour in mice during the rat exposure was shown to be secondary to the fear of predation (Strekalova and Steinbusch, 2009, 2010). Yet, the role of these stressors, as well as other potential factors cannot be completely ruled out (Nakamura et al., 1990; Bekkevold et al., 2013), thus, the nature of the applied stress model should be considered in the broader context.

Separate experiments on *Tph2*^{+/-} mice revealed elevated vertical exploratory activity in a situation of novelty and a lack of increase in floating behaviour between days 2 and 5, which is a marker of enhanced acquisition of despair behaviour (Strekalova et al., 2016; Pavlov et al., 2020). Both features can be interpreted in a light of increased resilience to environmental challenges and stressors, as diminished rearing activity in the novel cage and increased floating behaviour were associated with a susceptibility of mice to chronic stress (Strekalova et al., 2004; Cline et al., 2012, 2015). Elevated locomotor activity in home cage conditions was reported in naïve *Tph2*^{-/-} mice (Zhang et al., 2018), which is reminiscent

of the finding reported here. Notably, increased aggression scores were found to inversely correlate with the stress response and the development of depressive behaviour in mice (Strekalova et al., 2004; Comai et al., 2012). Together, these findings suggest the presence of similar behavioural patterns in *Tph2*^{+/-} mice and the null mutants, which were also described to show decreased signs of stress resilience (Savelieva et al., 2008; Jia et al., 2014; Weidner et al., 2019).

5. Conclusions

Thus, the present findings suggest that *Tph2*^{+/-} mutants exposed to the predation stress model display changes in social behaviour that are the opposite of those observed in control mice. In summary, these results, along with data on anxiety-like behaviour and the outcome from studies on mutants challenged with novelty and repeated swimming, suggest that under stress conditions, *Tph2*^{+/-} demonstrate behavioural profile that is reminiscent of naïve *Tph2*^{-/-} animals. This behavioural repertoire is associated with increased aggressive behaviour, decreased stress-induced anxiety and other signs of stress resilience, such as reduced floating and elevated exploratory activity. In addition to previously demonstrated changes in serotonin metabolism in the amygdala, altered dopamine concentration and turnover in this brain structure are likely to mediate excessive aggression and reduced sociability of stressed *Tph2*^{+/-} mice. These neurochemical changes, along with genotype differences in monoamine regulation the prefrontal cortex and striatum and a lack of changes in DOPAC levels in the dorsal raphe in stressed mutants are likely to contribute to the behavioural phenotype of

these mice. In conclusion, present paradigm can be considered as a promising model of gene x environment interactions underlying excessive human aggression.

Author statement

All authors confirm that they have contributed to the manuscript “Altered behavior, dopamine and norepinephrine regulation in stressed mice heterozygous in TPH2 gene”, have read and approved its final form and Response to reviewers.

Ethical statement

All experiments described in the manuscript “Altered behavior, dopamine and norepinephrine regulation in stressed mice heterozygous in TPH2 gene” by Tatyana Strekalova, Evgeniy Svirin, Jonas Waider, Anna Gorlova, Raymond Cespuglio, Allan Kalueff, Igor Pomytkin, Angelika G. Schmitt-Boehrer, Klaus-Peter Lesch, and Daniel Anthony were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals upon approval by the local governmental body of animal care and welfare.

Declaration of Competing Interest

All authors express a lack of any conflict of interests in connection with the manuscript “Altered behavior, dopamine and norepinephrine regulation in stressed mice heterozygous in TPH2 gene”.

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Predation stress causes excessive aggression in female mice with partial genetic inactivation of tryptophan hydroxylase-2: evidence for altered myelination-related processes

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Chapter 3. Predation stress causes excessive aggression in female mice with partial genetic inactivation of tryptophan hydroxylase-2: evidence for altered myelination-related processes

Abstract

The interaction between brain serotonin (5-HT) deficiency and environmental adversity may predispose females to aggravated aggression. Specifically, complete inactivation of the gene encoding tryptophan hydroxylase-2 (TPH2) results in the absence of neuronal 5-HT synthesis and excessive aggressiveness both in male and female null mutant (*Tph2*^{-/-}) mice. Heterozygous male mice (*Tph2*^{+/-}) have a moderate reduction in 5-HT, and when exposed to stress also display increased aggression. Here, we subjected female *Tph2*^{+/-} mice to a five-day predation stress comprising rat exposure and assessed their emotionality and social interaction/aggression-like behaviors. *Tph2*^{+/-} females exhibited excessive aggression and increased dominant behavior. Stressed mutants displayed altered gene expression of 5-HT receptors *Htr1a* and *Htr2a*, glycogen synthase kinase-3 β (GSK-3 β), and c-fos as well as several myelination-related markers in the prefrontal cortex: myelin basic protein (*Mbp*), proteolipid protein 1 (*Plp1*), myelin-associated glycoprotein (*Mag*) and myelin oligodendrocyte glycoprotein (*Mog*). The expression of the plasticity markers synaptophysin (*Syn*) and

AMPA receptor subunit *GluA2* were not affected by genotype. Moreover, in a separate experiment, naïve female *Tph2*^{+/-} mice showed signs of enhanced stress resilience in the modified swim test with repeated swimming sessions. Taken together, an interaction of a moderate reduction in brain 5-HT with environmental challenges results in behavioral changes in female mice that resemble aggression-related behavior and resilience of stressed male mutants that altogether is comparable to a phenotype of null mutants lacking neuronal 5-HT. Alteration in myelination-associated processes may point to a molecular mechanism in the manifestation of aggressive behavior.

1. Introduction

Aggression is a behavior that is frequently accompanied by violence, and as such, results in numerous social and health adverse effects. World Health Organization categorizes violent behavior, the incidence of which continues to increase, among the top 20 causes of disability worldwide [1]. Although women are less aggressive than men, and female aggression is often expressed in more indirect forms, it is important to note of women are just as likely to aggress against a partner as men, but men cause more serious physical and psychological harm [2]. Recently, an increased incidence of female aggressive behavior in individuals with neuropsychiatric disorders [3] and more frequent crime statistics involving women have been reported [4]. This rise demands a better understanding of the molecular mechanisms that underpin female aggression, but the neurobiology of female aggression is largely unstudied. The use of experimental animal models to investigate the

neurobiology of female aggression is limited, as this behavior is outside of the normal repertoire of mouse and rat behavioral assessments that commonly focus on male aggression [5,6].

Female aggression can result from decreased synthesis of neuronal serotonin (5-HT); studies employing complete inactivation of the gene encoding tryptophan hydroxylase-2 (TPH2), a key enzyme of 5-HT synthesis in the brain, have revealed a higher scores of aggression in female *Tph2*^{-/-} mice [7–10]. In humans, the *Tph2* gene polymorphism G-703T was found to contribute to anger-related traits and the expression of anger in women [11]. Variations in the *TPH2* gene were also associated with higher incidences of anxiety disorder in women and peripartum major depression [12,13].

Accumulating evidence highlights the importance of gene × environment interaction in neuropsychiatric conditions [2,14–17]. This implicates both genetic factors and stressful experience interacting at the molecular level in the neurobiology of aggression. Mechanistic studies addressing this interaction in the context of female aggression are scarce. Nevertheless, female aggression was demonstrated to be influenced by environmental adversities including stress both in animal experiments [2,6,18] and in clinical studies, where verbal and physical aggression was associated with traumatic stress experience [19].

he relevance of gene × environment interaction in the manifestation of pathological aggression is further supported by studies in male mice heterozygous for *Tph2* gene inactivation, which exhibit a moderate brain 5-HT reduction of 15-20% [7,8]. *Tph2*^{+/-} mice showed

unaltered social behavior at baseline, but after subchronic rat exposure stress demonstrated markedly increased levels of aggression and dominancy, and reduced sociability compared to wild type controls [20,21]. These changes were accompanied by profound alterations in the brain metabolism of 5-HT, dopamine, and norepinephrine. Altogether, this suggested a phenotype of stressed *Tph2*^{+/-} male mice reminiscent of naïve *Tph2* null mutants.

The effects of environmental challenges and stress on aggression are gender-specific [6]. In rodents, a decrease in aggressive and dominant behaviors was reported in females subjected to a maternal separation paradigm in C57BL6 mice [22], and following social isolation stress in Wistar rats [23], while males showed increased aggression in these studies. Here, we aimed to clarify how gene × environment interaction affects aggressive behavior in female *Tph2*^{+/-} mice and whether aggression in stressed female *Tph2*^{+/-} mice displays similarities to male mutants. Owing to sex differences in the neurobiology of aggression under stressful conditions, we hypothesized that female *Tph2*^{+/-} mice would not demonstrate the abnormally elevated aggressive behavior found in male mutants. We adopted a previously validated five-day rat exposure paradigm with includes an element of restraint, by virtue of limiting the extent free movement available to the *Tph2*^{+/-} female mice. This approach has been shown to induce changes in monoamine transmitters, neurogenesis, oxidative stress [24,25], as well as aggressive behavior in male *Tph2*^{+/-} mice [20,21]. The rat exposure

procedure applied here has been shown to result in increases in blood levels of CORT in C57BL/6 mice at 6 and 24 h post-stress [25].

In the current study, social interaction/aggression-like behaviors of stressed female mice were scored using tests of home cage social interaction and food competition [26–28]. Potential molecular changes were investigated in the prefrontal cortex, a brain area implicated in the mechanisms of both aggression and responses to stress. Based on previous findings in *Tph2*^{-/-} males [7,29,30], we studied gene expression of 5-HT receptors *Htr1a* and *Htr2a*. We also examined gene expression of a pro-inflammatory cytokine interleukin-1 β (IL-1 β) and glycogen synthase kinase-3 β (GSK-3 β), a marker of distress and degeneration, whose changes can accompany aberrant serotonergic processes [31] and regulate aggression and stress responses [32]. Expression of plasticity markers AMPA receptor subunit GluA2, synaptophysin (Syp), and a marker of neuronal activation c-fos were also measured [24,33,34]. Gene expression relating to brain myelination was also explored based on our previous findings in stressed male *Tph2*^{+/-} mice [35] and established relationships between myelination and the 5-HT system [36] and stress [37]. Gene expression of myelin basic protein (Mbp), proteolipid protein 1 (Plp1), myelin-associated glycoprotein (Mag) and myelin oligodendrocyte glycoprotein (Mog), was measured.

Finally, we sought to determine whether female *Tph2*^{+/-} mice resemble features of *Tph2*^{+/-} males in the broader context of emotional resilience to environmental challenges found in the modified swim test (modFST) and

in tests for anxiety-like behavior [20,38] in naïve and stressed female *Tph2*^{+/-} mutants.

2. Results

2.1 Predation stress induces aggressive and dominant behavior in Tph2^{+/-} females

In the novel cage test, the number of exploratory rears did not differ significantly between *Tph2*^{+/+} and *Tph2*^{+/-} mice ($t=0.6140$, $df=22$, $p=0.55$, unpaired t-test. Figure 1A). Time spent in the lit box of the dark-light box test also was not significantly different between these groups ($t=1.378$, $df=18$, $p=0.19$, unpaired t-test. Figure 1B).

The latency to crawl-over, number of crawl-overs, and total duration of this behavior, as a measure of home cage dominance, were significantly different between the groups as studied in the home cage ($H=15.14$, $p<0.01$, $H=17.73$, $p<0.01$ and $H=17.39$, $p<0.01$, respectively; Kruskal-Wallis test. Figure 1C-E). The latency to crawl-over in the stressed *Tph2*^{+/-} group was significantly shorter in comparison to both non-stressed *Tph2*^{+/-} and stressed *Tph2*^{+/+} (wild type) animals (both $p<0.01$, Dunn's test). The number of episodes and the duration of crawl-over behavior were significantly higher in the stressed mutant mice in comparison to non-stressed *Tph2*^{+/-} animals and stressed controls (all $p<0.01$). While there was no significant group difference in number of animals displaying following behavior (all $p=0.07$, Fisher's exact test. Figure 1F), in comparison to both non-stressed *Tph2*^{+/-} and stressed *Tph2*^{+/+} mice, the number of animals displaying agonistic (attacking)

behavior was significantly higher in the stressed *Tph2*^{+/-} group (both $p=0.02$, Figure 1G). None of the non-stressed mice exhibited following or attacking behaviors, regardless the genotype (Figure 1F,G).

Figure 1. Behavioral features of naïve and stressed *Tph2*^{+/-} female mice. (A) No alterations in the exploratory behavior of naïve *Tph2*^{+/-} mice were found

in the novel cage test and in **(B)** the time spent in the lit box (controls: n=14, mutants: n=10). **(C)** Significantly lowered latency to crawl-over, and significantly elevated number of crawl-overs **(D)** and duration of crawl-over behavior **(E)** in the social interaction in the home cage were found in stressed *Tph2^{+/-}* group. **(F)** There was no significant group difference in the percentage of the animals exhibiting following behavior in the social interaction in the home cage. **(G)** In the social interaction in the home cage, agonistic behavior was displayed by a significantly higher percentage of animals in the stressed *Tph2^{+/-}* group, in comparison with non-stressed *Tph2^{+/-}* mice or stressed wild type animals. **(H)** In the food competition test, a significantly higher number and **(I)** duration of attacks in stressed *Tph2^{+/-}* group was observed. **(J)** No significant group differences in the time spent in the open arms were found in the O-maze (C-J: no stress: n=9; stress, n=7.). WT – *Tph2^{+/-}*, * – p<0.05 vs same-genotype non-stressed group, # – p<0.05 vs stress-matched WT group.

In the food competition test, significant differences were found between the groups in both the number and the duration of attacks (H=14.57, p<0.01, and H=14.57, p<0.01, respectively. Figure 1H, I). Post-hoc analysis revealed that, in comparison to both non-stressed *Tph2^{+/-}* group and stressed *Tph2^{+/-}* mice, the number and duration of attacks were significantly elevated in the stressed *Tph2^{+/-}* group (both p=0.01, Dunn's test). In a similar manner to the home cage assay, none of the non-stressed mice exhibited following or attacking behaviors in the food competition test, regardless the genotype (Figure 1H, I). In the O-maze, Kruskal-Wallis testing showed significant group difference in the time spent in the open arms (H=14.19, p<0.01. Figure 1J). The only significant difference was found between non-stressed wild type mice and stressed mutants (p<0.01); post-hoc analysis did not show significant differences between genotype-matched or stress-matched groups. Kruskal-Wallis

test did not demonstrate any significant group differences in the food intake ($H=0.17$, $p=0.99$, Kruskal-Wallis test. Figure A1).

2.2 Altered gene expression of selected molecular markers in the prefrontal cortex of stressed *Tph2*^{+/-} mice

Two-way ANOVA revealed a significant main effect of genotype ($F_{1,21}=21.40$, $p<0.01$) and no significant stress $\times$ genotype interaction ($F_{1,21}=0.93$, $p=0.35$) in *Htr1a* expression. Irrespective of the stress factor, a significant decrease in the expression of the *Htr1a* was found in *Tph2*^{+/-} animals (Figure 2A). No significant stress $\times$ genotype interaction was shown in the expression of *Htr2a* ($F_{1,20}=1.240$, $p=0.28$, two-way ANOVA. Figure 2B), though both main effects of stress and genotype significant altered expression ($F_{1,20}=26.58$, $p<0.01$, and $F_{1,20}=10.59$, $p<0.01$, respectively, two-way ANOVA). *Htr2a* expression was significantly higher in the stressed animals independently of the genotype, and significantly lower in the mutant groups irrespective of the stress factor. These data suggest differential regulation of expression of 5-HT receptor subtypes by stress and partial *Tph2* inactivation.

For *Gsk-3 β* expression, a significant stress $\times$ genotype interaction was observed ($F_{1,16}=16.47$, $p<0.01$, two-way ANOVA. Figure 2 C). In comparison to non-stressed *Tph2*^{+/-} animals, post-hoc analysis revealed significantly higher *Gsk-3 β* expression in both the stressed *Tph2*^{+/-} group, and the non-stressed *Tph2*^{+/+} mice (both $p<0.01$, Tukey's test). *GluA2* expression was not significantly affected by stress $\times$ genotype interaction ($F_{1,22}=0.248$, $p=0.62$. Figure 2D), and only a significant main effect of stress was observed ($F_{1,22}=4.331$, $p=0.05$). Specifically, stress elevated

GluA2 expression compared to non-stressed animals irrespectively of their genotype.

No stress $\times$ genotype interaction was found for both *c-fos* and *Syp* expression ($F_{1,22}=0.437$, $p=0.52$, and $F_{1,22}=1.149$, $p=0.30$, respectively, two-way ANOVA), though main effects of genotype or stress on gene expression were observed. The expression of *c-fos* was significantly higher in *Tph2*^{+/-} mice in comparison with control animals, irrespectively of stress ($F_{1,22}=6.63$, $p=0.02$, two-way ANOVA. Figure 2E). Irrespectively of the genotype, the expression of *Syp* was significantly higher in stressed animals than in controls ($F_{1,22}=5.24$, $p=0.03$. Figure 2F).

Stress $\times$ genotype interaction significantly affected *Plp1* expression ($F_{1,19}=4.949$, $p=0.04$, two-way ANOVA). Post-hoc analysis revealed significantly lower expression of *Plp1* in stressed *Tph2*^{+/-} mice in comparison to non-stressed *Tph2*^{+/-} mice ($p=0.02$, Tukey's test, Figure 3A). No significant differences were observed between *Tph2*^{+/+} stressed and naïve mice ($p=0.07$). For *Mbp* and *Mag* expression, ANOVA revealed significant stress $\times$ genotype interaction ($F_{1,16}=16.68$, $p<0.01$ and $F_{1,18}=7.610$, $p=0.01$ respectively, Figure 3B, C). Compared to the non-stressed *Tph2*^{+/-} group, the expression of *Mbp* and *Mag* was significantly lower in both stressed *Tph2*^{+/-} ($p=0.01$ and $p=0.02$, respectively, Tukey's test) and non-stressed *Tph2*^{+/+} mice ($p<0.01$ and $p=0.03$, respectively). ANOVA revealed no significant interaction for *Mog* expression ($F_{1,19}=4.098$, $p=0.06$, two-way ANOVA. Figure 3D), though a significant main effect of stress was observed ($F_{1,19}=10.08$, $p<0.01$). In comparison

to non-stressed mice, stressed animals had a significantly lower expression level of *Mog* irrespective of their genotype.

Figure 2. Expression of 5-HT receptors, *Gsk-3β*, *GluA2*, *c-fos* and *Syp* in the brain of stressed *Tph2*^{+/-} mice. (A) Compared to control groups, *Htr1a* expression was significantly lowered in *Tph2*^{+/-} animals. (B) In comparison to non-stressed animals, in stressed groups *Htr2a* expression was significantly higher. Irrespectively of stress, *Htr2a* expression was higher in wild type groups. (C) Significantly higher *Gsk-3β* expression in both stressed *Tph2*^{+/-} group, and non-stressed *Tph2*^{+/+} mice was observed in comparison to non-stressed *Tph2*^{+/-} animals. (D) A significant main effect of stress was observed for the *GluA2* subunit, whereby expression was elevated irrespectively of the genotype in stressed groups. (E) Expression of the *c-fos* was higher in *Tph2*^{+/-} mice than in wild-type mice irrespectively of stress. (F) In stressed animals, expression of *Syp* was higher than in non-stressed animals, irrespectively of the genotype. WT – wild type, * – $p < 0.05$ vs same-genotype non-stressed group, # – $p < 0.05$ vs stress-matched WT group. 6-9 mice per group were used.

Two-way ANOVA did not reveal any significant interaction between stress and genotype for the expression of the IL-1 β ($F_{1,22}=0.226$, $p=0.64$), and no post-hoc group differences were observed (Figure A2).

*2.3 Naïve female *Tph2*^{+/-} mice show signs of decreased learning of adverse memories and helplessness as a manifestation of stress resilience*

In the modFST, in comparison to wild type mice, *Tph2*^{+/-} mice demonstrated a significantly smaller increase in floating duration in first two minutes of the test session between days 2 and 5 ($U=15$, $p < 0.01$, Mann-Whitney test; Figure A3 A). In the latency to float and the duration of floating, there was no significant interaction between day and genotype, though a main effect of the test day was found ($F_{1,14} = 91.79$ and $F_{1,12} = 89.22$, respectively, both $p < 0.01$, repeated measures two-way ANOVA; Figure A3 B,C). No significant group differences in the latency and duration of floating were found on either day of the test.

Figure 3. Elevated expression of myelination-related genes in the prefrontal cortex of non-stressed *Tph2*^{+/-} mice. (A) Significantly lower expression of *Plp1* was observed in stressed *Tph2*^{+/-} mice in comparison to the non-stressed *Tph2*^{+/-} group (B, C) Compared to non-stressed *Tph2*^{+/-} group, expression of *Mbp* and *Mag* was significantly lower in both stressed *Tph2*^{+/-} and non-stressed *Tph2*^{+/-} mice. (D) In comparison to non-stressed mice, stressed animals had a significantly lower expression level of *Mog* irrespective of the genotype. WT – wild type, * – p<0.05 vs same-genotype non-stressed group, # – p<0.05 vs stress-matched WT group. 6-9 mice per group were used.

3. Discussion

Our study revealed aggressive and dominant behaviors in female *Tph2*^{+/-} mice subjected to predation stress, resembling a behavioral profile reported for stressed male *Tph2*^{+/-} mutants and mice with a complete inactivation of *Tph2*. Wild type stressed controls did not show any of these changes. We also found a decrease in gene expression of *Plp1*, *Mbp*, and *Mag* in the prefrontal cortex of stressed mutants, which may reflect aberrant myelination processes that might underlie their stress-induced aggression and dominance behavior. Baseline expression of *Gsk-3β* was lower in the non-stressed *Tph2*^{+/-} mice than in wild type animals. Unlike wild type mice, mutants showed relatively increased *Gsk-3β* expression under stress conditions. The lowered basal expression of *Gsk-3β* in female *Tph2*^{+/-} mutants may also explain a diminished increase in behavioral despair during repeated swimming in the modFST, a sign of stress resilience.

The increased aggression and dominance in stressed mutants were accompanied by genotype effects on the prefrontal cortex expression of *Htr1a* and *Htr2a*. Both receptors are known to modulate aggressive behavior [39–41]. The expression of *Htr1a* and *Htr2a* were decreased in *Tph2*^{+/-} females regardless of stress, which is also a feature of *Tph2*^{-/-} mutants; it was explained by potentially higher sensitivity of this receptor on a protein level resulting from the central 5-HT deficiency [42]. However, the *Tph2*^{+/-} males subjected to predation stress did not affect *Htr1a* and *Htr2a* expression. Thus, for *Htr1a*, the sex-dependent behavioral effects that has been reported after the pharmacological

targeting of *Htr1a* in rodents [43] suggests that there is likely to be a differential role for this receptor in abnormal aggression in males and females. Moreover, lowered gene expression of *Htr6* reported in stressed male *Tph2^{+/-}* mice [21] was also found in stressed female mutants in the present study.

The predation stress used in this work was previously shown to increase 5-HT turnover in the prefrontal cortex of stressed male *Tph2^{+/-}* mice [21] and significantly correlate with measures of aggressiveness (Bazhenova and Lesch, unpublished results). Surprisingly, stressed *Tph2^{+/-}* males exhibited unaltered 5-HT levels in the prefrontal cortex, while wildtype mice showed significant increases in 5-HT levels under these conditions. These abnormalities might arise from compromised 5-HT metabolism in the prefrontal cortex of stressed mutants that result in disrupted cortical top-down control of limbic structures regulating aggression, including the amygdala, and is thus could underpin the social abnormalities of the stressed female *Tph2^{+/-}* mice.

As compromised serotonin metabolism in the *Tph2^{+/-}* mutants can independently result in altered regulation of appetite, satiety, and metabolic processes, in which changes in monoamine levels and changes in the expression of their receptors can play a major role [44], excessive aggression in stressed mutants in our study might be food deprivation state-dependent. Preliminary studies on *Tph2^{+/-}* stressed mice housed under normal conditions did not reveal any changes in social behavior in the food competition test (Strekalova and Costa-Nunes, unpublished results). In the present study, we used a food deprivation challenge, a

well-established inducer of aggression in male mice [45,46] and hierarchical dominance behaviors in female mice [47]. Further studies are warranted to address the issue as to how the changes in serotonin receptor expression, the effects of food deprivation and aggression in stressed *Tph2*^{+/-} mice are related.

Genetic deficits in 5-HT function are well-established to result in developmental abnormalities of brain connectivity [36,48–50]. Compromised frontostriatal white matter integrity and connectivity is believed to underlie increased impulsivity and aggression [51–53]. Here, for the first time we report increased expression of genes encoding myelination-related proteins in the prefrontal cortex of naïve *Tph2*^{+/-} female mice, and its significant decrease following predation stress. Previously work showed a decreased expression of *Mbp* and *Mag* in naïve *Tph2*^{+/-} males [35]. Thus, the present findings in naïve *Tph2*^{+/-} females may mirror compensatory effects of the overexpression of myelination factors that is neutralized by stress, leading to impaired connectivity and maladaptive aggression in these animals. The stress-induced decrease of myelination-related marker expression was previously reported in numerous rodent models of stress, such as chronic unpredictable stress, social defeat and social isolation, immobilization stress and early-life stress [54,55].

Moreover, others have previously demonstrated a relationship between the myelination in the prefrontal cortex and aggression and emotional dysregulation. Reduced thickness of the myelin sheath in the prefrontal cortex was reported to correlate with increased aggression

caused by juvenile isolation [56]. Group housing was shown to ameliorate both aggressive behaviors and the myelination deficit in another study of social isolation in mice [37,57]. In rats, overexpression of the myelin transcription factor 1 (*MyT1*) promoting differentiation of oligodendrocytes, which is also regulated by PLP1 and MBP [58], ameliorates anxiety-like and compulsive behaviors [59]. Aberrant myelination is believed to underlie impaired brain connectivity and be associated with impulsive and aggressive behaviors, contributing to neurodevelopmental disorders, such as attention deficit hyperactivity disorder (ADHD), autism spectrum disorders (ASD), and schizophrenia [60,61]. We speculate that the observed changes in the expression of myelination proteins in stressed *Tph2*^{+/-} mice may mirror deficient development of white matter and brain connectivity and underpin the excessive aggression observed here.

Other molecular processes can potentially contribute to abnormal social behavior of stressed *Tph2*^{+/-} mice. Genotype differences in the expression of brain *c-fos* argues for a role for this factor in the aggressive behavior of stressed female *Tph2*^{+/-} mice. In males, by comparison, *c-fos* expression was increased in the amygdala and prefrontal cortex of stressed mice of both genotypes [21]. Overexpression of *c-fos* in the hippocampus of *Tph2*^{+/-} mice is accompanied by increased freezing in the fear conditioning paradigm; a trend both towards molecular and behavioral changes was reported in the *Tph2*^{+/-} mutants [8,62]. It can be speculated that the over-expression of this immediately early gene as found in the stressed *Tph2*^{+/-} groups twenty-four hours after the last

manipulation might be owing to increased conditioning after the handling procedure. While chronic stress has been reported to suppress expression of *Syp*, a marker of neuronal plasticity [63,64], here *Syp* expression was elevated in *Tph2*^{+/-} mice regardless of stress exposure. Elevated expression of *Syp* in *Tph2*^{+/-} females may indicate compensatory plasticity processes related to the upregulation of myelination in naïve mutants and may further contribute to their stress resilience shown in the modFST. Upregulated myelination markers may also relate to the decreased baseline expression of *Gsk-3β*, a key indicator of helplessness behavior, in naïve mutants [38]. Previous studies point to a reciprocal relationship between GSK-3β and myelination-related factors, e.g., *Mbp* [65,66] that is in keeping with our findings of increased gene expression of the latter molecules found in naïve mutants.

In the present study, stress-induced overexpression of *Gsk-3β* and *GluA2* was not affected by the mutation. Similar results were found in the brain of stressed *Tph2*^{+/-} in males for *Gsk-3β*, but *GluA2* was upregulated selectively in the male mutants [21]. This challenges the view that these transcripts play a pivotal role in the aggression elicited in stressed *Tph2*^{+/-} females [25,33] and further suggests that differences sex difference result in the differential regulation of aggression in the *Tph2*^{+/-} mice. For GSK-3β, given that the level of the phosphorylated form of this kinase is the principal determinant of its activity, activity has been shown to correlate with *Gsk-3β* gene expression changes [67]. However, the further assessment of the level of GSK-3β phosphorylation might be useful to

confirm this association and its role in the behavioral abnormalities of *Tph2*^{+/-} females reported here.

While molecular changes reported here are based on gene expression data, they are likely to also result in the changes in the level of protein expression. Similar studies reporting stress-induced increases of aggressive behavior in mice killed on the next day after the last manipulation showed congruent changes on gene and protein levels for the markers investigated here [32], and for myelin proteins [68].

4. Materials and Methods

4.1 The animals and housing conditions

We used 12-week-old *Tph2*^{+/-} female mice, and their wild type littermates as controls, which were bred and genotyped in the facilities at the Institute of Molecular Medicine, New University of Lisbon, Portugal as previously described [8]. Mice of the same genotype were housed in standard cages in groups of 5 under controlled laboratory conditions (22±1°C, 55% humidity) and maintained on a reversed 12-hour light/dark cycle (lights on at 19:00), with food and water provided ad libitum. All mice were tested during the dark phase of light/dark cycle. Laboratory housing conditions and experimental procedures were set up and maintained in accordance with Directive 2010/63/EU of 22 September 2010 and had been approved by the Ethics Committee of the New University of Lisbon (No. 0421/000/000/2013). All efforts were undertaken to minimize the potential discomfort of experimental animals. Experimental protocols conformed to directive 2010/63/EU and

were compliant with ARRIVE guidelines (<http://www.nc3rs.org.uk/arrive-guidelines>).

4.2 Study design

Female *Tph2*^{+/-} mice and their wild type littermates (*Tph2*^{+/+} controls) were studied for baseline behavior in novel cage and dark-light box paradigms (Figure 4, Experiment 1). Mice from four cages per genotype were studied: two cages per genotype per stress condition. Thereafter, they were subjected to a five-day rat exposure stress and investigated for social behaviors in their home cage, and in food competition tests and the elevated O-maze. The sequence of the behavioral tests was designed in a manner to minimize any potential effects of the testing procedure on the experimental animals and on the outcome of the subsequent tests [69,70]. In total, mice from four cages per genotype were studied: two cages per genotype per stress condition. Mice were sacrificed 24 h after the last behavioral test, and their brains were dissected for qRT-PCR assay. During this study, daily food intake was monitored (see below). A separate cohort of mice was studied in the modFST, in which the animals were exposed to three 6-min swim sessions on days 1, 2, and 5. The learning of adverse context is defined by an increase in floating behavior from day 2 to day 5 (Figure 4, Experiment 2) [38]. On average, 7-10 animals per group were used in molecular and behavioral assays, group sizes are indicated in figure legends.

Experiment 1	Novel cage test +4 h Dark-light box Day 1	15h rat exposure stress Days 1-5	+4 h O-maze Day 6	16 h food deprivation Social interaction in home cage +1 h Food competition test Day 7	+24 h Brain dissection for RT-PCR Day 8
Experiment 2	6 min swimming Day 1	+24 h 6 min swimming Day 2	+72 h 6 min swimming Day 5		

Figure 4. Experiment design. (Experiment 1). Female *Tph2^{+/-}* mice and their wild type littermates were studied for baseline behavior. Thereafter, they were subjected to a 5-day rat exposure predation stress. Mice were studied in a battery of behavioral tests for aggression and anxiety-like behavior, then their brains were dissected for qRT-PCR assay. **(Experiment 2)** Separate cohort of mice was used for modFST. qRT-PCR - quantitative reverse transcription polymerase chain reaction assay.

4.3 Novel cage

Vertical exploratory activity of mice was studied in the novel cage test under red light as described elsewhere [34,70,71]. Briefly, mice were placed into a plastic cage and the number of exploratory rears was counted during a five-minute period under red light.

4.4 Dark-light box

The dark-light box consisted of two plexiglass compartments, dark box (15×20×25 cm) and light box (30×20×25 cm), connected by a tunnel. Mice were placed into the dark compartment, from where they could visit the light compartment, illuminated by bright light (300 lx intensity). Total duration of time spent in the light compartment was scored over 5 min [72].

4.5 *Rat exposure stress*

Mice were introduced into a spacious transparent glass cylinder (15 cm high x 8 cm diameter) and placed into the rat cage between 18:00 and 9:00 for five consecutive nights as described elsewhere [20,25]. Mice had free access to food and water in their home cages between the stress sessions. The timing of the rat exposure model was designed to minimize the impact of food and water deprivation, as the predation period overlaps with the light (inactive) phase of activity of the mice when food and water consumption is minimal [73,74]. As the analysis of aggressive behavior in *Tph2^{+/-}* male mice that were exposed to a five-day predation stress only exhibited a significant increase of aggressiveness on day 5 [75], we used the same five-day stress procedure as minimally sufficient for the induction of aggression in the current study.

4.6 *Home cage interaction*

In all experimental groups, dominant, aggressive, and other social behaviors in a home cage were assessed during a ten-minute period under low lighting (5 lux) after a 16 h food deprivation. In this study, daily food intake was measured three days prior and one day after the behavioral test. The top of a home cage was replaced by a transparent cover, and mice were scored for the latency, total duration and number of episodes of crawl-over, following and agonistic (attacking) behaviors, and the number of mice expressing these behaviors [26,27]. The social interaction behavioral parameters employed here were validated in previous studies on female mice [27].

The crawl-over behavior, considered as a manifestation of hierarchical dominance [76–78], was defined as the movement of a mouse over the body of the partner; predominantly head first crossing transversely from one side to the other [77,79]. Following behavior, another sign of hierarchical dominance in female mice [47] was defined as the aggressive and rapid chasing of a fleeing counter-partner where the maximum distance between the animals was one body length (adapted from [78]). Agonistic (attacking) behavior was defined by the occurrence of physical attack of one mouse against another, which involved kicking, wrestling, biting, and rolling over the body of the counter-partner (adapted from [80,81]).

4.7 *Food competition test*

The food competition test was carried out immediately after the recording of the home cage behavior (see 2.6). Pairs of 16 h food-deprived mice from different cages and the same experimental group were placed in a plastic observation cage (21×27×14 cm) and allowed to compete for a piece of beef meat (2 g) for 10 min under low lighting (5 lux). The number and duration of attacks were scored [26,27]. Same definitions of social behavior as in a home cage interaction situation were used; these parameters were validated in previous studies on female mice [26].

4.8 *Elevated O-maze*

The apparatus consisted of a circular path (runway width 5.5 cm, diameter 46 cm) that was placed 45 cm above the floor. Two opposing

arms were protected by walls (closed area, height 10 cm). The apparatus was placed on a dark surface to maintain control over lighting conditions during testing, which were kept constant at 25 lux. Mice were placed in one of the closed-arm areas of the apparatus. Behavior was assessed using previously validated parameters during a 5-min observation period. The latency of the first exit to the open arms of the maze, the number of exits to the open arms and time spent in open arms were recorded [82].

4.9 *Modified forced swim test*

The modified forced swim test (modFST) was used here as a model that seeks to mimic the neurobiological changes that involve the enhanced learning of adversities and result in a helplessness in a particular context [38]. Mice were subjected to two swimming sessions with an interval of 24 h. After the first two swim sessions, a third swim session was carried out on day 5 as previously described [38,83,84]. All sessions were 6-min long and were performed by placing a mouse in a transparent cylinder ($\varnothing$ 17 cm) filled with water (23°C, water height 13 cm, height of cylinder 20 cm). The floating behavior was defined as the absence of any directed movements of the head or body, and was scored by an observer unaware of the identity of the animal with Noldus EthoVision XT 8.5 (Noldus Information Technology, Wageningen, The Netherlands) as described elsewhere [85]. The duration of floating behavior was assessed in 2-minute intervals; the latency to float was measured. It is of note that in this model, the increase in floating behaviour, which is observed on day 5 compared to day 2, is reversible by pre-treatment with antidepressant compounds [86–88]. For this reason,

the increase in day 5 floating is regarded as a measure of excessive conditional learning and helplessness in an adverse context [83,84]. The increase in floating behavior during the first observation interval from day 2 to day 5 was expressed in percent and interpreted as a measure of learning in an adverse context, and helplessness [83,84,86].

4.10 Brain dissection and tissue collection

Mice were terminally anaesthetized with an intraperitoneal injection of sodium pentobarbitone, the left ventricle was perfused with 10 ml of ice-cold saline [71]. The brains were removed and the prefrontal cortex was isolated and stored at -80°C as described elsewhere [75,89].

4.11 Quantitative real-time PCR (qRT-PCR)

RNA extraction and cDNA synthesis were performed as described elsewhere [88]. Total mRNA was isolated from each sample with TRI Reagent (Invitrogen, Carlsbad, CA, USA). During first-strand cDNA synthesis 1 μg total RNA was converted into cDNA using random primers and Superscript III transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR was performed using the SYBR Green master mix (Bio-Rad Laboratories, Philadelphia, PA, USA) qRT-PCR was performed using the SYBR Green master mix (Bio-Rad Laboratories, Philadelphia, PA, USA). qRT-PCR was performed in a 10 μl reaction volume containing a SYBR Green master mix (5 μl), RNase-free water (3 μl), specific forward and reverse primers used at the concentration 20 pmol/ μl (1 μl) and cDNA (1 μl). The initial denaturation step for qRT-PCR was at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s and annealing at

60°C for 30 s. The sequences of primers used are listed in Supplementary Table A1; all primers were purchased from Life Technologies (Carlsbad, CA, USA). All samples were run in triplicate. Relative gene expression was calculated using the $\Delta\Delta C_t$ method and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene and to the expression of the control sample as described elsewhere [34,90].

4.12 *Statistical analysis*

Data analysis was performed using GraphPad Prism software version 8.3 (San Diego CA, USA). Normally distributed data were analyzed using an unpaired t-test or a two-way ANOVA test followed by the Tukey's correction for the pairwise comparisons of the group means of behavioral and molecular data. Specifically, the Tukey's test was used for the post-hoc analysis of gene expression results, as each RT PCR assay in this study, was carried out separately for each transcript and because the confidence intervals obtained for the values of the mRNA concentrations and the fold changes of all investigated transcripts do not include zero values. Further corrections for multiple comparisons, or the use of a three-way ANOVA, for distinct transcripts was not considered appropriate owing to altered variances, the method of normalization, and because corrections across multiple independent experiments would be expected to give rise to an increase in false negatives. Nonparametric data were analyzed by Kruskal-Wallis test and Dunn's post-hoc test. Fisher's exact test was performed for analysis of contingency tables. Statistical significance was set at $p < 0.05$. Data are shown as mean $\pm$ SEM.

5. Conclusions

Taken together, our findings show that an interaction between partial genetic inactivation of neuronal Tph2 expression and environmental adversity results in aggressive and dominant behaviors in female *Tph2*^{+/-} mice. Naïve female *Tph2*^{+/-} mice show decreased learning of adverse memories and helplessness, a sign of stress resilience. These behaviors are reminiscent to changes in *Tph2*^{+/-} males and null mutants of both sexes lacking Tph2. For the first time, we report altered expression of myelination markers in naïve and stressed female *Tph2*^{+/-} mice. These data encourage speculation regarding impaired brain connectivity in these mice, which likely contributes to the increased aggression and dominance observed in the stressed *Tph2*^{+/-} mice. Further studies are required to shed light on the detailed mechanisms of the relationships between serotonin deficiency, stress, and myelination in the context of gene × environment interaction and female aggression.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Directive 2010/63/EU of 22 September 2010 and had been approved by the Ethics Committee of the New University of Lisbon (No. 0421/000/000/2013). Experimental protocols conformed to directive 2010/63/EU and were compliant with ARRIVE guidelines (<http://www.nc3rs.org.uk/arrive-guidelines>).

Data Availability Statement: In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section “MDPI Research Data Policies” at <https://www.mdpi.com/ethics>. You might choose to exclude this statement if the study did not report any data.

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Conflicts of Interest: Declare conflicts of interest or state “The authors declare no conflict of interest.” Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. Any role of the funders in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results must be declared in this section. If there is no role, please state “The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results”.

Appendix A

Supplementary methods

Table A1. Primer sequences for mRNA expression analysis

Gene	Primer	Sequence
Htr1a	Forward	5'-GACAGGCGGCAACGATACT-3'
	Reverse	5'-CCAAGGAGCCGATGAGATAGTT-3'
Htr2a	Forward	5'-TAATGCAATTAGGTGACGACTCG-3'
	Reverse	5'-GCAGGAGAGGTTGGTTCTGTTT-3'
GSK-3β	Forward	5'-GCACTCTTCAACTTTACCACTCA-3'
	Reverse	5'-CGAGCATGTGGAGGGATAAG-3'
GluA2	Forward	5'-GCGTGGAAATAGAAAGGGCC-3'
	Reverse	5'-ACTCCAGTACCCAATCTTCCG-3'
c-fos	Forward	5'-CGGGTTTCAACGCCGACTA-3'
	Reverse	5'-TTGGCACTAGAGACGGACAGA-3'
Syp	Forward	5'- TGTGTTTGCCTTCCTCTACTC-3'
	Reverse	5'- TCAGTGGCCATCTTCACATC-3'
Plp1	Forward	5'-CCAGAATGTATGGTGTTCCTCCC-3'
	Reverse	5'-GGCCCATGAGTTTAAGGACG-3'
Mbp	Forward	5'- TCACAGCGATCCAAGTACCTG-3'
	Reverse	5'-CCCCTGTCACCGCTAAAGAA-3'
Mag	Forward	5'-GGTACATGGCGTCTGGTATTTC-3'
	Reverse	5'-ACTTGTGTGCGGGACTTGAAG-3'
Mog	Forward	5'-TCATGCAGCTATGCAGGACAA-3'
	Reverse	5'-TTTCGGTAGAGGTGAACCACT-3'
IL-1β	Forward	5'- CTTCCAGGATGAGGACATGAGCAC -3'
	Reverse	5'-TCATCATCCCATGAGTCACAGAGG -3'
GAPDH	Forward	5'-ATGACCACAGTCCATGCCATC -3'
	Reverse	5'-GAGCTTCCCGTTCAGCTCTG -3'

Supplementary results

Daily food intake of *Tph2*^{+/-} mice

The Kruskal-Wallis test did not reveal significant differences in the average daily food intake measured during the observation period ($H=0.17$, $p=0.99$, Kruskal-Wallis test. Figure A1).

Figure A1. Daily food intake of *Tph2*^{+/-} mice. No significant group difference in the average daily food intake during the observation period was found. WT – wild type.

Expression of *Il-1β* in the prefrontal cortex of *Tph2*^{+/-} mice

No significant differences were found by two-way ANOVA in the expression of *Il-1β* in the prefrontal cortex (interaction: $F_{1,22}=0.226$, $p=0.64$; stress: $F_{1,22}=0.062$, $p=0.81$; genotype: $F_{1,22}=2.635$, $p=0.12$. Figure A2).

Figure A2. Expression of *Il-18* in the prefrontal cortex of *Tph2*^{+/-} mice. No significant group differences were found in the expression of *Il-18*. WT – wild type, 6-9 mice per group were used.

Tph2^{+/-} mice display reduced potentiation of floating in the modFST paradigm

The change in floating duration in the first two minutes of the test session between days 2 and 5 in *Tph2*^{+/-} animals was significantly smaller than in wild type mice (see *ms text*, Figure A3 A). In the latency to float and the duration of floating, only the main effect of the test day was found (see *ms text*, Figure A3 B,C). Post-hoc analysis revealed significant decrease in latency to float and significant increase in the duration of floating on days 2 and 5 compared to day 1 irrespective of the genotype (both $p < 0.01$, Šídák's multiple comparisons test).

Figure A3. Floating behavior in the modified swim test. (A) Significantly lower increase of floating duration from day 2 to day 5 in *Tph2*^{+/-} mice. **(B)** Significant decrease in latency to float on days 2 and 5 compared to day 1 was observed irrespectively of the genotype. **(C)** There was significant increase in the duration of floating on days 2 and 5 compared to day 1 irrespectively of the genotype. WT – wild type, **p*<0.01 vs. wild type, #*p*<0.01 vs same genotype on day 1. 11-13 animals per group were used.

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CHAPTER

4

ASD-like behaviours, a dysregulated inflammatory response and decreased expression of PLP1 characterize mice deficient for sialyltransferase ST3GAL5

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Chapter 4. ASD-like behaviours, a dysregulated inflammatory response and decreased expression of PLP1 characterise mice deficient for sialyltransferase ST3GAL5

Abstract

Gangliosides are glycosphingolipids, which are abundant in brain, are known to modulate ion channels and cell-to-cell communication. Deficiencies can result in aberrant myelination and altered immune responses, which can give rise to neurodevelopmental psychiatric disorders. However, to date, little mechanistic data is available on how ganglioside deficiencies contribute to the behavioural disorders. In humans, the loss of lactosylceramide- α -2,3-sialyltransferase (ST3GAL5) leads to a severe neuropathology, but in *St3gal5* knock-out (*St3gal5*^{-/-}) mice the absence of GM3 and associated a-, b- and c-series gangliosides is partially compensated by 0-series gangliosides and there is no overt behavioural phenotype. Here, we sought to examine the behavioural and molecular consequences of GM3 loss more closely. Mutants of both sexes exhibited impaired conditioned taste aversion in an inhibitory learning task and anxiety-like behaviours in the open field, moderate motor deficits, abnormal social interactions, excessive grooming and rearing behaviours. Taken together, the aberrant behaviours are suggestive of an autism spectrum disorder (ASD)-like syndrome. Molecular analysis showed decreased gene and protein expression of proteolipid protein-1 (Plp1) and over expression of proinflammatory cytokines, which has been associated with ASD-like

syndromes. The inflammatory and behavioural responses to lipopolysaccharide (LPS) were also altered in the *St3gal5*^{-/-} mice compared to wild-type, which is indicative of the importance of GM3 gangliosides in regulating immune responses. Together, the *St3gal5*^{-/-} mice display ASD-like behavioural features, altered response to systemic inflammation, signs of hypomyelination and neuroinflammation, which suggests that deficiency in a- and b-series gangliosides could contribute to the development of an ASD-like pathology in humans.

Keywords: lactosylceramide alpha-2,3-sialyltransferase (ST3GAL5), major brain gangliosides, autism spectrum disorder (ASD), aggression, proteolipid protein 1 (PLP1), neuroinflammation, mice.

1. Introduction

Gangliosides are glycosphingolipids that facilitate neuronal membrane protein organization, signalling and adhesion (Lopez and Baez, 2018; Schnaar et al., 2014). Brain gangliosides also regulate microglia and cytokine-mediated immune responses, including microglial activation, myelination, CNS development, and platelet activation (Bowser et al., 2019; Dukhinova et al., 2018; Kim et al., 2002). The ganglioside GM3, is generated by alpha-2,3-sialyltransferase 5 (ST3GAL5) or GM3-synthase (Ishii et al., 1999), which is the precursor of the principal brain gangliosides including GM1, GD1a, GD1b, GD3, GT1b and GQ1b. Abnormalities in the *ST3GAL5* gene disrupt GM3 synthesis, leading to intellectual disability, microcephaly, epilepsy, blindness and

deafness, somatic growth failure and metabolic syndrome (Gordon-Lipkin et al., 2018; Trinchera et al., 2018).

Emerging evidence suggests that ganglioside deficiency may have other effects in addition to severe neuropathologies observed in individuals with *ST3GAL5* deficiency. Recent GWAS studies have reported an association between SNPs encoding alpha-2,3-sialyltransferase 3 and factors regulating GM function, and the incidence of schizophrenia, attention-deficit/hyperactivity disorder (ADHD) or autism spectrum disorders (ASD). In particular, in a large-scale integrative analysis of genome-wide association studies (GWAS), comprising of 20,183 ADHD cases and 35,191 controls, using DEPICT analysis of gene prioritization, pathway and tissue/cell type enrichment analysis, *ST3GAL3* was the top gene associated with ADHD ($P=1.19\times 10^{-2}$) (Zhao et al., 2018). Furthermore, the homozygous loss-of-function mutation of *SLC39A8*, a well-established schizophrenia biomarker, was shown to result in serum manganese (Mn) abnormalities, a causal factor of glycosyltransferases dysfunction. This mechanism was suggested to underlie the pathophysiology of schizophrenia (Mealer et al., 2020). A more recent GWAS also revealed a relationship between increased expression of a *ST3GAL3* transcript in the human fetal brain and a risk for ADHD and schizophrenia (Hall et al., 2020). Moreover, children with ASD often displayed increased anti-ganglioside antibody levels (Mostafa and Al-Ayadhi, 2011; Yang et al., 2018) and changes in the ganglioside expression has also been proposed as a biomarker for schizophrenia (Sarbu et al., 2018).

The mechanisms underlying a link between the path physiology of neurodevelopmental disorders and dysregulated brain gangliosides implicate neuroinflammation as a risk factor of these pathologies (Dunn et al., 2019; Matta et al., 2019; Meyer, 2013), as gangliosides are known to regulate immune responses in which they have an anti-inflammatory role (Galleguillos et al., 2020; Sipione et al., 2020). Specifically, gangliosides have been shown to regulate the effects of tumour necrosis factor (TNF) (Tagami et al., 2002), microglial and platelet activation (Dukhinova et al., 2018; Sotnikov et al., 2013), nitric oxide synthase (NOS2), and ICAM-1 and MCP-1-mediated signalling (Kim et al., 2002; Ryu et al., 2002). For microglia treated with lipopolysaccharide (LPS), interleukin-1 β (IL-1 β) or ATP, gangliosides have been shown to have anti-inflammatory effects (Galleguillos et al., 2020). Moreover, neuroinflammation and neuro-immune system dysregulation are particularly well established as clinical features of ASD (Gevezova et al., 2020; Siniscalco et al., 2018). However, mechanistic studies of the role of the principal CNS gangliosides in neurodevelopmental psychiatric syndromes are still lacking.

ST3GAL5 dysfunction in humans has been modelled in *St3gal5*^{-/-} mice (Yamashita et al., 2003), but these mutants only partly re-capitulate clinical abnormalities, which may be attributable to the compensatory synthesis of 0-series gangliosides GD1 α and GM1b. However, *St3gal5*^{-/-} mice do lack the major CNS gangliosides GM3, GM1, GD1a, GD3, GT1b, GQ1b and display alterations in activity, insulin receptor sensitivity, platelet activation and neuronal damage following brain trauma

(Dukhinova et al., 2018; Kopeikina et al., 2020; Niimi et al., 2011; Trinchera et al., 2018). While *St3gal5*^{-/-} mice appear to only to display selective neurodegeneration in the Organ of Corti, they do afford the opportunity to explore the consequences of the brain ganglioside deficiency rather than complete absence (Niimi et al., 2011).

Here, we hypothesized that *St3gal5*^{-/-} mice would display features of neuropsychiatric developmental pathologies and that these changes, given the anti-inflammatory actions of brain gangliosides, would be exacerbated by an inflammatory challenge with LPS. Therefore, we studied male and female *St3gal5*^{-/-} mice for social, restricted-repetitive behaviours to determine whether the animals would display ASD-like features (Haratizadeh et al., 2021), as well as evaluation of inhibitory learning and the expression of myelination and inflammation markers in brain cortex and spleen.

2. Methods

2.1. Animals

Eight twelve-week-old *St3gal5*^{-/-} and C57BL/6 mice were bred as described elsewhere (Dukhinova et al., 2018); six-week-old C57BL/6 mice were used in social interaction test. Mice were housed under standard conditions; all protocols complied 2010/63/EU and ARRIVE guidelines (see Supplementary file, SF).

2.2. Study design

Mice were examined for social interaction, rearing and conditioned taste aversion (Fig. S1A); in the separate groups, open field

grooming, motor performance, the mRNA levels of myelination factors and protein concentrations of Plp1 in the brain cortex were studied. A cohort of mice was investigated for social behavior or mRNA expression of proinflammatory cytokines in the cortex and spleen 6h following the i.p. administration of LPS (0.1 mg/kg) or PBS (Fig. S1B). For protocols used and group sizes, see SF and Figure legends; on average, 9 mice per group were used for the behavioural studies and 5 mice per group were used in molecular assays.

2.3. Behavioural tests

Experiments were performed during the dark period of light cycle, followed by offline analysis (Anymaze, Stoelting, Dublin, Ireland); customized equipment was used. Briefly, in the social interaction test, unfamiliar naïve juvenile mouse of the same sex was exposed to adult mice after its habituation to the apparatus, for 10 min (Couch et al., 2016; Veniaminova et al., 2017). The number of rears, latency, total duration, and number of episodes of attacking behavior and dominant-like behaviours: following and mounting, and neutral social exploration: nose-nose and nose-anal contacts were recorded. The number of mice displaying following and mounting were scored in the study with LPS. Inhibitory learning was studied in the conditioned taste aversion model, where mice were injected with LiCl, causing nausea, after their exposure to a sucrose solution. A loss of sucrose preference thereafter was taken as a memory measure. Grooming scores in the open field center and time spent therein were investigated. To assess motor functions, latencies to fall or to descend and the incidence of falling or sliding were studied in

the Pole and Wire tests, respectively (de Munter et al., 2020; Veniaminova et al., 2020).

2.4. Induction of systemic inflammation

Mice were injected with LPS dissolved in PBS (Sigma, St.Louis, MO, USA) at the dose 0.1 mg/kg, i.p., or PBS (Couch et al, 2013, 2016). Previous studies reported a marked pro-inflammatory response both in the brain and peripheral tissue 6 h after the administration of LPS (Couch et al., 2013). At this time point, significant changes in floating behavior, novelty exploration and social interaction were observed in the mice (Couch et al., 2013; Strekalova and Anthony, unpublished results).

2.5. Real-time polymerase chain reaction (RT-PCR) and Western Blot

Mice were terminally anaesthetized with isoflurane inhalation and perfused intracardially with 20 ml of PBS, brain cortex and spleen were dissected, homogenized, and stored until use under -80°C (Dukhinova et al., 2018; for details on brain cortex dissection, see *SF*, Fig. S2). RT-PCR of samples of brain cortex and spleen were examined for the expression of *Il-1 β* , *Il-6* and *TNF* and Western blot analysis of Plp1 expression in the brain cortex were performed (Ponomarev et al., 2011; Veremeyko et al., 2018). The pro-inflammatory cytokines were selected for gene expression analysis based on their pivotal roles in local and systemic immune processes (Kany et al., 2019) and for their established association with neuropsychiatric disorders (Dunn et al., 2019; Matta et al., 2019). Furthermore, previous studies in our laboratories have revealed the presence of altered gene expression of *Il-1 β* , *Il-6* and *TNF* in mice with exhibiting aberrant social, emotional and cognitive features

following exposure to stress, LPS challenge, or other adverse conditions (Couch et al, 2013, 2016; Gorlova et al., 2019; Pavlov et al, 2017, 2019). Relative gene expression was calculated using the $\Delta\Delta CT$ method and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene and to the expression of the control sample, see Table S1. The choice of the reference gene was based on our previous experiments, in which, in comparison with other housekeeping genes, GAPDH demonstrated relatively stable expression in the tissue obtained in mouse models of systemic inflammation and stress (Couch et al, 2013, 2016; Pavlov et al., 2017). Relative expression of Plp1 protein was calculated as fold change relative to β -actin.

2.6. Statistics

Data were analyzed using GraphPad Prism v.8.01 (San Diego, CA, USA). Two- or three-way ANOVA was employed for multiple group comparisons, followed by Tukey's or Sidak's tests, respectively. Data were checked for normality using Shapiro-Wilk test. Data from LPS-treated groups were additionally normalized to PBS-treated groups of the respective genotypes. The level of significance was $p < 0.05$.

3. Results

3.1. *St3gal5^{-/-} mice display anxiety-like behavior, stereotypy and abnormalities in learning and social interaction*

A two-way ANOVA revealed a significant sex $\times$ genotype interaction for the number of following events ($p < 0.05$), but no group differences ($p > 0.05$, Tukey's test; Fig. 1A). There was a significant sex $\times$

genotype interaction in the duration of following behavior ($p < 0.05$), which was longer in male *St3gal5*^{-/-} mice than in controls ($p < 0.05$) and unaltered in females ($p < 0.05$; Fig. 1B). There was a main effect of genotype on the latency to follow ($p < 0.05$; Fig. 3A); mounting and attacking behaviors did not exhibit a sex $\times$ genotype interaction (Figs. S2B–G).

The number of nose-anal contacts exhibited a sex $\times$ genotype interaction ($p < 0.05$, two-way ANOVA), this measure was higher in *St3gal5*^{-/-} male mice than in wild type group ($p < 0.05$, Tukey's test; Fig. 1C) that was not shown for females ($p < 0.05$). Congruent changes were found in the duration of this behavior as suggested by a significant effect of genotype (Fig. S4A). There were no significant effects of sex, genotype, or their interaction on the latency to nose-anal contact (all $p > 0.05$). Genotype significantly affected the duration of this behavior ($p < 0.05$), which was increased in *St3gal5*^{-/-} animals in comparison with controls irrespective of sex (both $p < 0.05$, unpaired t-test, Fig. S4B). (All statistical values of behavioural analysis are presented in Table S2).

Only main effects of sex and genotype were significant for the number of nose-nose contacts ($p < 0.05$, respectively, two-way ANOVA), where this measure was lower in mutants than in controls ($p < 0.05$) and lower in females ($p < 0.05$, Fig. 1D). Changes in the latency to nose-nose contacts, as well as other measures of social interaction were unchanged, except for the main effect of genotype on the duration of nose-nose contacts ($p < 0.05$; Figs. 4C and D).

Two-way ANOVA showed significant effect of genotype, but not sex $\times$ genotype interaction for the time mice spent in the center of the open field ($p < 0.05$ and $p > 0.05$, respectively). This behavior was shorter in *St3gal5*^{-/-} groups than in controls ($p < 0.05$; Fig. 1E). center was significantly affected by genotype where it was increased in the mutants ($p < 0.05$; Fig. 1F), but here there was no sex $\times$ genotype interaction ($p < 0.05$ and $p > 0.05$, respectively). While there was no sex $\times$ genotype interaction in the number of rears ($p > 0.05$), there was a significant main effect of genotype and sex on this measure (both $p < 0.05$), which was higher in female mutants than in controls and male mutants (both $p < 0.05$; Fig. 1G).

During recall session of the conditioned taste aversion test, all PBS-treated groups and LiCl-treated mutants exhibited no significant difference in sucrose preference from the 50%-chance level (all $p > 0.05$, one-sample t-test; Fig. 1H), while the LiCl-treated wild-type groups exhibited a decrease ($p < 0.05$ for both males and females).

3.2. Motor deficits and lowered brain expression of myelin marker Plp1 in St3gal5^{-/-} mice

Genotype significantly affected the latency to fall in the Wire test ($p < 0.05$), which was shorter in *St3gal5*^{-/-} mice than in controls ($p < 0.05$; Fig. 1I). No group differences in the incidence of falling were observed (both $p > 0.05$, Fisher's test; Fig. 1J). In the Pole test, we found increased incidence of sliding in male mutants ($p < 0.05$) and no changes in the latency to descend ($p > 0.05$; Fig. 1 K, L). (Statistical values for the behavioral analysis and gene expression data are presented in Tables S2–

S3).

Figure 1. *St3gal5*^{-/-} mice display behavioral and molecular abnormalities. (A-D) Social behavior (n=14 in each group). (E-G) Anxiety-like and stereotypic behavior (n=10 per group for males, n=8 per group for females). (H)

Associative inhibitory learning (n=6 in each group). **(I-L)** Motor functions (n=6 in each group). **(M-O)** Expression of mRNA of myelination transcripts and Plp1 protein (n=6 in each group). *p<0.05 vs. wild-type group, #p<0.05 vs. male group, \$p<0.05 vs. 50%-chance level. 'Genotype' – significant effect of genotype. Two-way ANOVA and Tukey's test; one-sample t-test; Chi-square and Fisher's exact test; data present as Mean $\pm$ SEM except J and L (*see the text*).

Two-way ANOVA revealed a significant genotype effect on the concentration of *Plp1* mRNA ($p < 0.05$), but for the other myelin transcripts (all $p > 0.05$; Fig. 1M,N, Fig. 5). In *St3gal5*^{-/-} mice, the level of *Plp1* mRNA and relative fold protein expression of Plp1 were lower than in controls ($p < 0.05$; Fig. 1O,P); the latter measure was significantly affected by genotype only ($p < 0.05$).

3.3. Altered behavioral and molecular response of *St3gal5*^{-/-} mice to LPS

A Chi-square test revealed a significantly higher incidence of following in males *St3gal5*^{-/-}-LPS-treated animals compared to the *St3gal5*^{-/-}-PBS-treated and control-LPS-treated groups (both $p < 0.01$, Fisher's test; Fig. 2A); no such differences were found in females ($p > 0.05$). The duration of following was significantly affected by a sex $\times$ genotype interaction ($p < 0.05$, two-way ANOVA). In comparison with the LPS-treated wild type groups, normalized to PBS-groups, the duration of following was unchanged in male LPS-treated mutants and decreased in the LPS-treated female mutants ($p < 0.05$, Tukey's test; Fig.2B, Fig. S6A,B). However, no difference in the number of following episodes or the latency to follow was observed (Fig. S6A,B). (For statistical values of behavioural analysis, see Table S4).

Figure. 2. Altered behavioural and molecular response of *St3gal5*^{-/-} mice to LPS challenge. (A–F) Dominant and aggressive behavior (n = 6 in each group). (G–I) Expression of pro-inflammatory cytokines in the brain and (J–L) in the spleen, (n = 4–6 in each group). *p < 0.05 vs. wild-type group, #p < 0.05 vs. PBS group. &p < 0.05 vs male group. Int. – significant interaction. M - males, F - females. Two-way and three-way ANOVA, Tukey's and Sidak's post -hoc tests; Chi-square and Fisher's exact test; data present as Mean $\pm$ SEM except A and C (see the text).

There was a significant difference in the incidence of mounting in males and females (both $p < 0.01$, Chi-square test). This behavior was not displayed by the PBS-treated male mutants. Among LPS-treated *St3gal5*^{-/-} groups, the incidence was higher in males and lower in females compared to controls (both $p < 0.05$; Fig. 2C). Normalized to the PBS-groups, the duration of mounting was unaltered in the LPS-treated mice (all $p > 0.05$, two-way ANOVA; Fig. 2D). There was significant interaction, but no significant group differences in the number of mounting episodes or latency to mounting (Fig. S6 C,D).

There was a significant difference in the incidence of attacks in the males ($p < 0.01$, Chi-square test), which was s higher in the *St3gal5*^{-/-}-LPS-treated group than in the *St3gal5*^{-/-}-PBS treated group or the wild type LPS-treated mice (both $p < 0.05$, Fisher's test; Fig. 2E). LPS-treated mutants exhibited an increased duration, normalized to PBS- t-test; Fig. 2F). No differences in the number of attacks or latency to groups, of attacks in comparison to *St3gal5*^{-/-}-PBS group ($p < 0.05$, attack were observed (Fig. S6E,F).

For brain expression of mRNA for cytokines IL-1 β , IL-6 and TNF, significant sex $\times$ genotype $\times$ treatment interactions were observed following LPS administration (all $p < 0.01$, three-way ANOVA). *Il-1 β* mRNA and *TNF* mRNA concentrations were higher in LPS-treated animals than in PBS-treated groups (all $p < 0.05$, Sidak's test; Fig. 2G). In the LPS-treated groups, *Il-1 β* mRNA and *TNF* mRNA was lower in the *St3gal5*^{-/-} males than in wild-type mice and higher in *St3gal5*^{-/-} females than in controls and male mutants (all $p < 0.01$). Wild-type mice, but not

St3gal5^{-/-} groups showed significant increases in *Il-6* mRNA after LPS administration in comparison with PBS-treated groups ($p < 0.01$ and $p > 0.05$, for both males and females, respectively). LPS-treated mutants of both sexes had lower concentrations of *Il-6* mRNA and *TNF* mRNA than LPS-treated wild-type mice (both $p < 0.01$; Fig. 2 H and I). In males, *TNF* mRNA levels were higher than in females among PBS-treated mutants and LPS-treated wild type and mutant mice (all $p < 0.01$). (Statistical values for the gene expression data are presented in Supplementary Tables S5–7). Spleen levels of *Il-1 β* mRNA and *TNF* mRNA, but not of *Il-6* mRNA were significantly affected by sex $\times$ genotype $\times$ treatment interaction ($p < 0.01$, three-way ANOVA). LPS-treated groups, except in wild-type females, showed higher *Il-1 β* mRNA concentrations (all $p < 0.01$, Sidak's test; Fig. 2 J). In the LPS-treated groups of mutants, *Il-1 β* mRNA concentrations were lower in males and higher in females than in the wild type groups (both $p < 0.01$). There was a significant interaction between treatment and genotype for spleen *Il-6* mRNA concentrations ($p < 0.01$). This measure was increased in all LPS-treated groups in comparison to the PBS-treated animals and was higher in *St3gal5*^{-/-} groups than in the wild-type mice (all $p < 0.01$, Tukey's test; Fig. 2K). Only mutants, both males and females, displayed a significant LPS-induced increase of *TNF* mRNA concentrations in comparison with PBS-treated groups; this increase was significantly higher in females (all $p < 0.01$, Fig. 2 L). (Statistical values for the gene expression data are presented in Supplementary Tables S5–7).

4. Discussion

We observed substantial abnormalities in dominant behavior and neutral sociability in the *St3gal5*^{-/-} mice. The *St3gal5*^{-/-} mutants also ate motor deficits, systemic inflammation, and signs of hypomyelination. In addition, *St3gal5*^{-/-} mutants exhibited aberrant behavioural and molecular responses to LPS. Naïve mutants showed decreased gene and protein expression of the major myelin component Plp1, and overexpression of pro-inflammatory cytokines in the brain and spleen. LPS administration to *St3gal5*^{-/-} mice elicited increased dominant/attacking behaviours in males and decreased dominance in females. Relative to the changes in wild-type mice, the expression of cytokines in the LPS-challenged *St3gal5*^{-/-} mice was reduced in the brain and exacerbated in the spleen.

These abnormalities are reminiscent of the established phenotypes of ASD in clinic (Thapar et al., 2017) and in animal models (Haratizadeh et al., 2021). The Diagnostic and Treatment Manual for Mental Disorders, Fifth Edition (DSM-5) defines ASD by the expression of deficient social communication and interaction, restricted-repetitive behaviours and developmental impairments (DSM-5R, 2013). The core ASD-like characteristics in rodents can be recapitulated by the expression of aberrant reciprocal social behavior, stereotypy and cognitive deficits (Haratizadeh et al., 2021; Veniaminova et al., 2017, 2020).

For example, in mice, abnormal interactions with counter partners, excessive following, dominance, increased grooming and

rearing, impaired inhibitory learning, are all signs of ASD-like behavior (Das et al., 2019; Lewis et al., 2007; Onore et al., 2013; Tatsukawa et al., 2019). We observed increased following behavior in male mutants, decreased social exploration in female mice, and aberrant nose-anal interaction in both sexes which are suggestive of social dysfunction. Social behaviours were also abnormally altered by the administration of LPS which is in keeping with findings that LPS exacerbates ASD-like behaviours in other mouse models including BTBR mice, a genetic model of ASD (Das et al., 2019; McFarlane et al., 2008; Onore et al., 2013). The impaired conditioned taste conditioning suggests cognitive rigidity in *St3gal5*^{-/-} animals, an important element of ASD-like phenotype (Haratizadeh et al., 2021). The presence of motor deficits in the *St3gal5*^{-/-} mice also agrees with other studies in which an association between ASD syndrome and motor dyscoordination has been described (Das et al., 2019; Lewis et al., 2007; Thapar et al., 2017).

Clinical studies suggest a pivotal role of altered immunity and response to systemic inflammation in the behavioural/cognitive signs of ASD (Ashwood et al., 2011; Jyonouchi et al., 2012; Napolioni et al., 2013). In comparison to controls displaying developmental disabilities other than autism, plasma levels of cytokines IL-1 β , IL-6, IL-8 and IL-12p40 were increased in children with ASD, which correlated with the severity of the disease (Ashwood et al., 2011). The over-production of pro-inflammatory cytokines was also demonstrated by utilizing cultured and stimulated peripheral blood monocytes from children with ASD (Enstrom et al., 2010; Jyonouchi et al., 2012). Over-expression of IL-1 β , IL-6, IL-17

and TNF has also been reported in the adult autistic brain (Napolioni et al., 2013; Theoharides et al., 2016; Wei et al., 2013).

Our data showing central and peripheral over-production of IL-1 β , IL-6, and TNF are in keeping with these findings, as well as with previous demonstrations of systemic inflammation and up-regulated brain expression of IL-1 β , IL-6, IL-17, IL-18, IL-33, TNF and astrocyte/microglia activation in animal models of ASD (Das et al., 2019; Prata et al., 2017; Wei et al, 2012, 2016). We also found the upregulation of IL-1 β in the double transgenic *St3gal5*-deficient 5xFAD female mice (Dukhinova et al., 2019). The synergistic interaction of abnormal baseline production of IL-1 β , IL-6 and TNF in mutants and LPS-induced systemic inflammation is reminiscent of a ‘double hit’ phenomenon (Carlezon et al., 2019; Couch et al, 2013, 2016; Onore et al., 2013; Yasumatsu et al., 2020) and could explain the exacerbation of the changes in social behavior observed in the *St3gal5*^{-/-} mice. At the same time, no differences were found in counts for mononuclear and polymorphonuclear white blood cells between controls and mutants (Ponomarev, unpublished results). The current study also revealed the presence of muted responses in cytokine expression following LPS in the mutants. This is in keeping with observations that cytokine production is reduced in the monocytes of children with ASD after TLR-targeted stimulation (Iyonouchi et al., 2012) and with decreased brain expression of IL-6 and TNF in *St3gal5*^{-/-} mice after brain injury (Dukhinova et al., 2018).

Sex-specific expression profiles of TNF, TGF- β , IFN- γ , IL-17 and IL-6 were recently reported in ASD patients (Eftekharian et al., 2018).

St3gal5^{-/-} mice display a sex bias in cytokine expression and this bias may underlie the sex-dependent behavioural changes reported here and elsewhere (Niimi et al., 2011). Similarly, LPS-induced social and repetitive behaviours in C57BL6 mice were sex-dependent (Carlezon et al., 2019).

Pro-inflammatory changes in mutants are likely to be owing to the lack of gangliosides of the a-, b- and c-series, which exert anti-inflammatory functions (Schnaar, 2016). This imbalance is, seemingly, not compensated by expression in the *St3gal5*^{-/-} mice of GD1α and GM1b, whose role in inflammation has been not shown (Trinchera et al., 2018). Congruently, signs of deficit in myelination, which is regulated by a- and b-series gangliosides, is unlikely to be compensated by the 0-gangliosides in the *St3gal5*^{-/-} mutants. The decreased Plp1 expression in the *St3gal5*^{-/-} mice is in keeping with the myelin changes observed in carriers of *St3gal5* gene variants that are associated with ganglioside deficiency (Bowser et al., 2019). Decreased Plp1 expression may also result from a bi-directional relationship with neuroinflammatory processes (Groh et al., 2018), that can compromise synaptic plasticity, brain connectivity, and motor function (Thapar et al., 2017), as well as give rise to aberrant social behavior. For example, studies have linked hypomyelination in the medial prefrontal cortex with behavioural abnormalities in a mouse ASD model (Makinodan et al., 2017).

Thus, genetic deficiency in a-, b- and c-series of ganglioside in the brain, due to the genetic lack of ST3GAL5 enzyme, leads to the abnormalities that are reminiscent to the ASD-like syndrome. We would

not argue that gangliosides deficiency is likely to be a causal element of ASD pathology, but it could be a contributing factor in some cases. Our work warrants future investigation into the potential link between ASD and deficits in brain glycoprotein sialylation and a- and b-series ganglioside synthesis. In particular, further studies are needed to examine the correlation between the molecular and behavioural changes, whether the ASD-like phenotype can be recovered with a therapeutic intervention, and to better understand the impact of the myelin associated changes. Yet, given ASD is a disorder of highly variable genetics with as-yet-unknown pathophysiology, the *St3gal5^{-/-}* mice provide a useful animal model to explore ASD-like features associated with ganglioside deficiency.

Declaration of competing interest

On behalf of all authors, I would like to state that none of the authors involved in the work have any competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbih.2021.100306>.

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Supplementary File

Methods

Animals

Eight to twelve-week-old male and female *St3gal5*^{-/-} mice and C57BL/6 mice used as wild-type controls and six-week-old counter partners in the social interaction test were bred as described elsewhere (Dukhinova et al., 2019, 2018; Sotnikov et al., 2013). Male mice were housed individually in standard plastic cages (27x22x15 cm), female mice were housed in groups of three-five per cage. Mice were maintained on reversed 12-h light/dark cycle, under controllable laboratory conditions (22±1°C, 55% humidity, room temperature 22°C, lights were on at 19:00), food and water were available ad libitum. All efforts were undertaken to minimize the potential discomfort of experimental animals. Experimental protocols conformed to directive 2010/63/EU and were compliant with ARRIVE guidelines (<http://www.nc3rs.org.uk/arrive-guidelines>) and were approved by local veterinarian committee of School of Biomedical Sciences, Faculty of Medicine, the Chinese University of Hong Kong, Shatin, Hong Kong.

Study design

The social behaviour of the *St3gal5*^{-/-} mice was studied in two sets of experiments (Fig S1A&B). In cohort A, mice were first employed to examine social interaction and rearing behaviours (n=14 in each group and then they were used to detect the ability to induce conditioned taste aversion (n=6 in each group; Fig.S1A). In cohort B, the mice were used to investigate social behaviour or cytokine expression in following the i.p. administration of LPS (0.1mg/kg) or PBS (n=6 per group). Additional groups of mice were used to study behaviour in the open field and grooming (n=10 per group for males, n=8 per group for females). The motor function of *St3gal5*^{-/-} mice and wild-type animals was evaluated in the Pole test and Wire test (n=6 in each group). The mRNA levels for myelin proteins and protein concentrations of PLP1 in the brain cortex were studied in separate cohorts (n=3-6). For all protocols used, *see below*.

Figure S1. Experimental design of studies with social interaction test. (A) *St3gal5*^{-/-} and wild-type mice of both sexes were tested in the social test on day 1, and in the conditioned taste aversion on days 2-3. **(B)** *St3gal5*^{-/-} and wild-type mice of both sexes with LPS or PBS. After 6 hours post administration, half of the animals was studied in the social interaction test, and other half was killed, their brain and spleen were dissected and were collected for consequent RT PCR assay.

Behavioral tests

Behavioral tests were carried out during an active period of the animals' light cycle (09:00–21:00) and analyzed offline by the experimenter who was unaware of the genotype of each animal studied. One test was run per day. Mice of both genotypes were tested simultaneously. Behavioural equipment was thoroughly cleaned with water between each test.

Social interaction test

The social interaction test was adapted from a previously described method (Couch et al., 2016; Gorlova et al., 2020, 2019; Veniaminova et al., 2017). Each mouse was allowed to habituate to a new plastic cage (43 x 27 x 19 cm) in which an unfamiliar group-housed naïve juvenile mouse of the same sex, that was five-week-old, was placed for 10 min. the number of rears was scored. The latency, total duration, and number of events of aggressive (attacking) behaviour and dominant-like behaviours: following and mounting, and two forms of neutral social exploration: nose-nose and nose-anal contact were scored. In the study with LPS, the number of mice with the incidence of indicated types of dominant-like

behaviour and neutral social exploration was scored during two-minute intervals.

Conditioned taste aversion

During the training session, mice were deprived from water between 17.00 and 14.00 (for 21 h) and then were allowed to drink 2.5% sucrose solution for 30 min in one-bottle paradigm (Strekalova, 2008, 2009). Thereafter, mice received an i.p. injection with solution of lithium chloride (LiCl, 0.24 M) at the dose 2% of body weight or PBS. After injection, animals were allowed to have access to sucrose solution for 1.5 h and thereafter they were subjected to a water deprivation for 12 h. On the next day, a test for recall was performed. Mice were given a choice between tap water or 1% sucrose solution in a two-bottles paradigm for 8h. The amount of consumed liquid was determined by weighing the bottles before and after a drinking session, the preference to sucrose solution was calculated as percentage of consumed sucrose solution from total amount of liquid drunk by following formula:

$$\text{Sucrose preference} = 100\% \times \frac{\text{Amount of sucrose solution consumed, g}}{\text{Total amount of liquid consumed, g}}$$

A decrease of sucrose preference during recall session in comparison with a chance level is considered as a sign of taste aversion, i.e., inhibitory associative learning.

Wire test

The Wire test was used to assess motor abilities of mice as described previously (de Munter et al., 2020; Veniaminova et al., 2020). Mice were allowed to grip onto a horizontal hanging wire (0.3 cm in diameter at 60 cm above the surface) using their four limbs, the latency to fall was scored in two consecutive sessions and the mean was calculated. The incidence of falling events was recorded as well.

Pole test

Mice were placed on top of a vertically standing bar (diameter 1.1 cm, height 60 cm) and allowed to climb down to a horizontal surface as described elsewhere (de Munter et al., 2020; Veniaminova et al., 2020). The latency to descend, measured as time spent by an animal to reach

the ground with all four paws and events of sliding were recorded as measures of motor functions, in two consecutive trials.

Open field test

Mice were placed in the central arena of a customized square open field (40 x 40 cm), which was illuminated with a red light. Their behaviour was video recorded for 10 min as described elsewhere (Lim et al., 2016). The time spent in the central zone (30 x 30 cm), and duration of grooming were analysed offline using Any-maze software (Anymaze, Dublin, Ireland).

Real-time polymerase chain reaction (RT-PCR)

qRT-PCR was performed using the SYBR Green master mix (Bio-Rad Laboratories, Philadelphia, PA, USA). qRT-PCR was performed in a 10µl reaction volume containing a SYBR Green master mix (5 µl), RNase-free water (3 µl), specific forward and reverse primers used at the concentration 20 pmol/ul (1 µl) and cDNA (1 µl). The initial denaturation step for qRT-PCR was at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 seconds and annealing at 60°C for 30 seconds. The sequences of primers used are listed in *Table S1* (see below); all primers were purchased from Life Technologies (Carlsbad, CA, USA). All samples were run in triplicate.

Western blot

Samples of brain cortex (0.1 g of wet tissue) were lysed in 1 ml of lysis buffer (50 mM of Tris-HCl (pH=7.4), 150 mM of NaCl, 2%SDS, 1% Sodium Deoxycholate, 5 mM EDTA, 1% NP40 (Tergitol Solution, Sigma, St. Louis, MO, USA), and 1:100 protease inhibitor cocktail (cat#1860932, Thermo Fisher Scientific Hong Kong, Hong Kong), and lysates were passed through 18G needle 10 times, vortexed for 5 min and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was collected and 5 µl of supernatant was mixed with 195 µl of Laemmli buffer. Then 10 µl of samples were resolved on SDS electrophoresis using Bolt™ 8% Bis-Tris Plus Mini Gels (Ref. NW00082BOX, Invitrogen, Thermo Fisher Scientific Hong Kong, Hong Kong) according to manufacturer's recommendations using Bolt™ MES SDS Running buffer, voltage of 200V for 25 minutes.

Table S1. Primer sequence for mRNA expression analysis

Gene	Primer	Sequence
Plp1	Forward	5'-CCAGAATGTATGGTGTCTCTCCC-3'
	Reverse	5'-GGCCCATGAGTTTAAGGACG-3'
Mbp	Forward	5'- TCACAGCGATCCAAGTACCTG-3'
	Reverse	5'-CCCCTGTCACCGCTAAAGAA-3'
Mag	Forward	5'-GGTACATGGCGTCTGGTATTTTC-3'
	Reverse	5'-ACTTGTGTGCGGGACTTGAAG-3'
Mog	Forward	5'-TCATGCAGCTATGCAGGACAA-3'
	Reverse	5'-TTTCGGTAGAGGTGAACCACT-3'
Il-1β	Forward	5'- CTTCCAGGATGAGGACATGAGCAC -3'
	Reverse	5'-TCATCATCCCATGAGTCACAGAGG -3'
Il-6	Forward	5'-CCTTCTTGGGACTGATGCTGGTG-3'
	Reverse	5'- AGGTCTGTTGGGAGTGGTATCCTC-3'
Tnf	Forward	5'-AGCCGATGGGTTGTACCTTG- 3'
	Reverse	5'- GTGGGTGAGGAGCACGTAGTC -3'
Gapdh	Forward	5'-ATGACCACAGTCCATGCCATC -3'
	Reverse	5'-GAGCTTCCCGTTTCAGCTCTG -3'

Immunoblotting was performed using Imobilon-PSQ polyvinylidene difluoride (PVDF) membranes (cat# ISEQ00010, Millipore, Bedford, MA, USA). PVDF membranes were incubated in a 99%-methanol solution for 1

min (Sigma, St. Louis, MO, USA), then washed with mQ H₂O for 5 min, and then incubated in Tris/Glycine transfer buffer (cat#161-0734, Bio-Rad Pacific Limited, Hong Kong) for 15 min. Transfer was performed on ice using ice-cold transfer buffer with constant voltage 80V for 2h. After transfer, the membrane was treated with a 5% BSA the TBST, containing

50 mM Tris-HCl (pH=8.2), 150 mM NaCl, 0.05% Tween-20 (Sigma, St. Louis, MO, USA) for 1 h at the room temperature and subsequently incubated with antibodies to PLP1 (1:1000, cat#ab28486, Abcam, Cambridge, U.K.) and β -Actin (1: 1000, cat#4967; Cell Signalling, Beverly, MA, USA) at 4°C overnight that was followed by the incubation with secondary horseradish peroxidase-conjugated secondary (HRP) antibodies (1:1000, cat#7074S, Cell Signaling, Beverly, MA, USA), for 1h at room temperature on a rotary shaker. Bands were visualized using Peroxide Detection Reagent from ECL Substrate kit (cat#ab133406, Abcam, Cambridge, U.K.) according to manufacturer's recommendations.

β -Actin was used as a loading control. Quantitative analysis of relative expression levels of PLP1 was normalized to β -Actin as we reported earlier (Veremeyko et al., 2018).

Results

Dominant and aggressive behavior

Statistical values of behavioral analysis are also presented in **Table S2**. No significant interaction between sex and genotype was found for the latency to following ($p=0.34$, two-way ANOVA, Fig. S2). A significant main effect of genotype was observed ($p<0.05$, two-way ANOVA). Latency to following in wild-type mice was significantly higher compared to *St3gal5*^{-/-} groups irrespectively of the genotype ($p=0.02$, unpaired t-test). Two-way ANOVA showed no significant differences in the latency to mounting, number of mounting events, and mounting duration (all $p>0.05$, two-way ANOVA). In males, no significant differences were found in the latency to attack, number or duration of attacks.

Figure S2. Dominant and aggressive behaviors. (A) Latency to following was significantly lower in *St3gal5*^{-/-} animals irrespective of sex. (B-D) Parameters of mounting and (E-F) parameters of attacks in males were not changed significantly. WT – wild type. KO – *St3gal5*^{-/-}. Data are presented as Mean $\pm$ SEM.

Neutral social exploration

Duration of both nose-anal and nose-nose contacts was affected by the genotype only (both $p < 0.05$, two-way ANOVA, Fig.S3). No significant changes in the latencies to nose-anal and nose-nose contacts were revealed by two-way ANOVA.

Figure S3. Neutral social exploration in *St3gal5*^{-/-} mice. (A) Duration of nose-anal contacts. (B) Latency to nose-anal contact. (C) Duration of nose-nose contacts. (D) Latency to nose-nose contact. WT – wild type. KO – *St3gal5*^{-/-}. Data are presented as Mean $\pm$ SEM.

RT-PCR and Western blot

Two-way ANOVA did not reveal any significant differences in the expression of *Mog* (all $p > 0.05$, two-way ANOVA, Fig.S4A). PLP1 relative-fold protein expression was significantly affected by genotype only ($p < 0.01$, two-way ANOVA, Fig. S4B). A significant decrease of PLP1 relative-fold protein expression was found in *St3gal5*^{-/-} mice in comparison to wild-type controls. (Statistical values of Western-blot analysis are also presented in *Table S3*).

Figure S4. (A) Expression of *Mog* in the prefrontal cortex of *St3gal5*^{-/-} mice. (B) Western blot analysis of PLP1 expression in the brain of wild-type (WT) and *St3gal5*-deficient (*ST3*^{-/-}) mice of both sexes. Western blot analysis was performed as described in *Methods* and representative image for staining with anti-PLP1 antibody is shown. Data are presented as Mean ± SEM.

Changes in social behaviors in the LPS-treated mice

Aggressive and dominant behavior

Three-way ANOVA found no significant differences in the latency to the following events (all $p > 0.05$, two-way ANOVA, Fig. S5A). For the latency to the mounting events three-way ANOVA revealed a significant sex $\times$ genotype interaction ($p = 0.01$, Fig. S5B). *St3gal5*^{-/-} female mice exhibited a significantly higher latency to mounting events compared to wild-type female group irrespectively of the LPS administration ($p = 0.04$, unpaired t-test). Significant main effects of both treatment, genotype, and sex were found for the number of following events (all $p < 0.05$, three-way ANOVA, Fig.S5C). Significant sex $\times$ genotype $\times$ treatment interaction was also found for the number of mounting events ($p < 0.05$, three-way ANOVA, Fig. S5D). Female LPS-treated wild-type group exhibited a significantly higher number of mounting events than LPS-treated wild-type male group and *St3gal5*^{-/-} LPS-treated female group (both $p < 0.05$, Sidak's test). Interaction of treatment and sex significantly affected the duration of following ($p < 0.05$, three-way ANOVA, Fig.S5E). Irrespectively of genotype, LPS-treated female groups exhibited higher duration of following compared to LPS-treated males, and same effect was observed in PBS-treated groups (both $p < 0.05$, Tukey's test). Duration of mounting was significantly affected by the interaction of sex, genotype, and treatment ($p < 0.05$, three-way ANOVA, Fig.S5F). Compared to control-LPS-treated male group and *St3gal5*^{-/-}-LPS-treated female group, female LPS-treated wild-type group exhibited a significantly higher duration of mounting (both $p < 0.05$, Sidak's test) In male mice, significant differences were found neither in the number of attacks nor in the latency to attack in the social interaction test (all $p > 0.05$, three-way ANOVA, Fig.S5G,H). (Statistical values of behavioral analysis are also presented in *Tables S5A,B*.)

Figure S5. Aggressive and dominant behavior in LPS-treated groups, normalized to the wild-type PBS groups. (A) Latency to following was not affected significantly by any of the factors. **(B)** Significant sex $\times$ genotype interaction was found for the latency to mounting. **(C)** Number of following events was significantly affected by the main effects of sex, genotype, and treatment, but not by their interactions. **(D)** Number of mounting events was higher in LPS-treated wild-type female mice, than in LPS-treated mutant female mice, and LPS-treated wild-type male mice. **(E)** Duration of following was affected by treatment $\times$ sex interaction. **(F)** Duration of mounting events was elevated in LPS-treated wild-type female mice than in the LPS-treated mutant female mice, and LPS-treated wild-type male mice. **(G)** Number of attacks and **(H)** latency to attack in male were not affected by either genotype, sex, or treatment. WT – wild type. KO – *St3gal5*^{-/-}. Int. – interaction. * $p < 0.05$ vs. wild type group, & $p < 0.05$ vs. group of males. Data are presented as Mean $\pm$ SEM.

Neutral social exploration

Statistical values of behavioral analysis are also presented in *Tables S6A,B*. No significant differences were found by three-way ANOVA in the latency to nose-anal contacts, and in the latency, duration, and number of nose-nose contacts (all $p > 0.05$, three-way ANOVA. Fig.S6A,B,D,F). For the duration of the nose-anal contacts, three-way ANOVA revealed a significant effect of the genotype ($p = 0.04$, Fig.S6C). Irrespective of the sex and LPS treatment, *St3gal5*^{-/-} mice showed enhanced duration of the nose-anal contacts. The number of nose-anal contacts also was found to be significantly affected by the genotype only ($p < 0.05$, Fig.S6E). *St3gal5*^{-/-} mice exhibited significantly higher number of the nose-anal contacts compared to wild-type mice irrespective of sex and treatment.

Figure S6. Social interaction in LPS-treated groups. (A, B) No significant differences were found in the latencies to nose-anal or nose-nose contacts, as well as in the (D) duration and (F) number of nose-nose contacts. (C, E) In the duration and number of nose-anal contacts, only genotype effect was significant. per group. WT – wild type. KO – *St3gal5*^{-/-}. M – male mice, F – female mice. Int. – interaction. *p<0.05. Data are presented as Mean ± SEM.

Expression of the cytokines in the brain and the spleen normalized to sex- and genotype-matched PBS groups

Two-way ANOVA revealed significant sex × genotype interaction for the normalized to the expression in the sex-and genotype-matched PBS-treated control groups expression of *Il-1β* mRNA, *Il-6* mRNA, and *Tnf* mRNA in the brain of LPS-treated mice (all p<0.01, two-way ANOVA. Fig.S7A,B). In both wild-type male and female mice an increase in the concentration of the *Il-1β* mRNA in the brain after LPS administration

was significantly higher than in the corresponding *St3gal5^{-/-}*-LPS groups (both $p < 0.05$, Tukey's test). In male wild-type mice, the increase of this measure was significantly higher than in wild-type group of females ($p < 0.01$, Tukey's test). In *St3gal5^{-/-}*-LPS-treated mice, an increase in the *Il-1 β* mRNA concentration upon LPS administration was more pronounced in female mice, compared to *St3gal5^{-/-}*-LPS-treated males ($p < 0.01$, Tukey's test). As for *Il-1 β* , LPS-induced augmentation of brain concentration of *Il-6* mRNA was greater in the wild-type LPS-treated groups than in *St3gal5^{-/-}* animals (both $p < 0.01$, Tukey's test). In both genotypes such increases were significantly higher in male animals than in female groups (both $p < 0.01$). Similar increases were found for brain concentration of *Tnf* mRNA, whose increases were higher in the wild-type groups than in mutants (both $p < 0.05$, Tukey's test). In male wild-type-LPS-treated animals, the elevation in concentration of *Tnf* mRNA in the brain was higher than in wild-type-LPS-treated female mice ($p < 0.01$, Tukey's test), and opposite changes were found in the *St3gal5^{-/-}* groups ($p < 0.01$, Tukey's test). (Statistical values of behavioral analysis are also presented in Table S7).

In the spleen, normalized concentrations of *Il-1 β* mRNA and *Il-6* mRNA were significantly affected by the interaction of sex and genotype (both $p < 0.05$, two-way ANOVA. Fig.S7C,D). Elevation of *Il-1 β* mRNA concentration upon LPS administration was significantly higher in wild-type males compared to *St3gal5^{-/-}* males and wild-type females (both $p < 0.01$, Tukey's-test). In wild-type-LPS-treated female group, increase in the *Il-6* mRNA concentration was significantly higher in comparison to both male wild-type-LPS group and female *St3gal5^{-/-}*-LPS-treated animals (both $p < 0.01$, Tukey's-test). For the *Tnf* mRNA concentration in the spleen, only the main factor of genotype was significant ($p < 0.01$, two-way ANOVA). Elevation in the concentration of the *Tnf* mRNA in the spleen in the *St3gal5^{-/-}*-LPS-treated groups was significantly higher than in the wild-type-LPS-treated groups irrespectively of the sex.

Figure S7. Expression of pro-inflammatory cytokines normalized to sex-and genotype-matched groups. (A, B) All the LPS-treated groups of both sexes showed a significant elevation of the mRNA concentrations of all the analyzed cytokines in the brain. In mutant males, elevation in the concentrations of *Il-1 β* mRNA and *Tnf* mRNA were lower compared to wild-type males. The same was found for the *Il-6* mRNA and *Tnf* mRNA in the female groups. *Il-1 β* mRNA concentration in females showed opposite changes to those in males. **(C,D)** In the spleen of LPS-treated mice, mRNA concentrations of all the cytokines were elevated significantly in comparison to PBS-treated groups, except *Tnf* in wild-type females. In male mice, *Il-1 β* and *Tnf* mRNA were elevated significantly higher in wild-type-LPS mice in comparison to the *St3gal5*^{-/-}-LPS animals. *Il-6* in males and all three cytokines in females were significantly elevated in LPS-treated mutants vs. LPS-treated wild-type animals in the spleen. WT – wild type. KO – *St3gal5*^{-/-}. **p*<0.05 vs. PBS group, **p*<0.05 vs. wild-type group. Data are presented as Mean $\pm$ SEM.

Table S2. Summary of comparisons between wild type and *St3gal5*^{-/-} male and female groups in the measures of behavior (two-way ANOVA and post-hoc Tukey's test; Chi-square and Fisher's exact test). Fig 1. A-L, Fig. S2-S3

Two-way ANOVA						
Behavior	Parameter	Interaction, F	Interaction, p	Genotype, F	Genotype, p	Sex, F Sex, p
Dominant and aggressive behavior						
Following	Latency	0.96	0.34	4.35	<0.05	1.22 0.28
	Duration	5.48	0.02	4.18	<0.05	1.02 0.32
	Number	5.47	0.02	0.19	0.66	1.27 0.26
Mounting	Latency	1.49	0.23	0.21	0.65	0.34 0.56
	Duration	0.35	0.56	0.43	0.52	1.78 0.19
	Number	0.12	0.79	0.06	0.81	3.81 0.06
Neutral social exploration						
N-A contact	Latency	0.60	0.45	0.04	0.85	3.24 0.09
	Duration	0.47	0.50	5.65	0.02	0.76 0.39
	Number	22.63	<0.01	11.65	<0.01	15.12 <0.01
N-N contact	Latency	0.61	0.44	1.20	0.28	0.53 0.47
	Duration	0.70	0.41	12.49	<0.01	0.46 0.50
	Number	1.32	0.26	4.99	0.03	5.53 0.02
Open field and repetitive behavior						
Open field	Center time	0.15	0.70	13.2	<0.01	0.02 0.87
Grooming	Frequency	0.7	0.41	21.82	<0.01	1.71 0.20
Rears	Number	3.70	0.06	4.83	0.03	7.44 <0.01
Aversive memory formation						
Sucrose preference in the CTA		0.02	0.89	1.23	0.28	4.85 0.04
Motor functions						
Wire test	Latency to fall	0.14	0.71	20.83	<0.01	1.41 0.24
Pole test	Latency to descend	0.16	0.70	2.35	0.14	2.71 0.12

Table S2 (continued)

Tukey's tests						
<i>Behavior</i>	<i>Parameter</i>	<i>WT ♂ vs ♀, p</i>	<i>KO ♂ vs ♀, p</i>	<i>♂ KO vs WT, p</i>	<i>♀ KO vs WT, p</i>	
<i>Dominant and aggressive behavior</i>						
Following	Latency	>0.99	0.46	0.07		0.90
	Duration	0.79	0.09	0.03		>0.99
	Number	0.09	0.82	0.31		0.41
Mounting	Latency	0.97	0.58	0.92		0.72
	Duration	0.54	0.95	0.73		>0.99
	Number	0.68	0.37	>0.99		0.98
<i>Neutral social exploration</i>						
N-A contact	Latency	0.89	0.29	0.90		0.98
	Duration	>0.99	0.68	0.07		0.72
	Number	<0.01	0.93	<0.01		0.72
N-N contact	Latency	>0.99	0.71	0.42		>0.99
	Duration	0.60	>0.99	0.25		0.02
	Number	0.84	0.07	0.90		<0.05
<i>Open field and repetitive behavior</i>						
Open field	Center time	0.97		>0.99	0.11	0.04
	Frequency	0.35		0.99	0.06	<0.01
	Number	>0.99		0.03	0.94	0.01
<i>Inhibitory learning</i>						
Sucrose preference in the CTA		0.37		0.48	0.90	0.81
<i>Motor functions</i>						
Wire test	Latency to fall	0.70		0.94	0.02	<0.01
	Latency to descend	0.49		0.81	0.85	0.53

Table S2 (continued)

Fisher's exact test		
Parameter	Males, <i>p</i>	Females, <i>p</i>
Incidence of falling	>0.99	0.45
Incidence of sliding	0.01	0.06
Unpaired t-test (attacks, males)		
Parameter	<i>t</i> , <i>df</i>	<i>p</i>
Duration of attacks	<i>t</i> =0.9785, <i>df</i> =19	0.34
Number of attacks	<i>t</i> =0.6429, <i>df</i> =19	0.53
Latency to attack	<i>t</i> =0.4683, <i>df</i> =19	0.64

Table S3. Summary of comparisons in expression of myelination markers in the brain of wild type and *St3gal5*^{-/-} male and female mice (two-way ANOVA and post-hoc Tukey's test). Fig. 1 M-P, Fig. S4.

<i>Expression of myelination proteins</i>								
Two-way ANOVA								
	Gene	Interaction, F	Interaction, p	Genotype, F	Genotype, p	Sex, F	Sex, p	
mRNA	Mbp	0.50	0.49	0.05	0.83	0.5	0.49	
	Plp1	0.86	0.37	26.91	<0.01	0.86	0.37	
	Mag	0.06	0.81	<0.01	>0.99	0.06	0.81	
	Mog	<0.01	>0.99	0.37	0.55	<0.01	>0.99	
Western blot	Plp1	2.49	0.15	331.2	<0.01	3.22	0.11	
Tukey's tests								
	Gene	WT ♂ vs ♀, p	KO ♂ vs ♀, p	♂ KO vs WT, p	♀ KO vs WT, p			
mRNA	Mbp	>0.99	0.75	0.91	0.99			
	Plp1	>0.99	0.57	<0.01	<0.05			
	Mag	>0.99	0.98	>0.99	>0.99			
	Mog	>0.99	>0.99	0.97	0.97			
Western blot	Plp1	>0.99	0.16	<0.01	<0.01			

Table S4. Summary of comparisons in the measures of dominant and aggressive behavior after LPS-treatment (two-way ANOVA, post-hoc Tukey's test. Chi-square and Fisher's exact test, Fig. 2 A-F.

Two-way ANOVA (LPS groups, normalized to sex- and genotype-matched PBS groups)						
<i>Behavior</i>	<i>Parameter</i>	<i>Interaction, F</i>	<i>Interaction, p</i>	<i>Genotype, F</i>	<i>Genotype, p</i>	<i>Sex, F</i> <i>Sex, p</i>
<i>Dominant and aggressive behavior</i>						
Following	Latency	1.01	0.33	1.86	0.19	0.92
	Duration	7.47	0.01	2.10	0.16	2.23
	Number	4.78	0.04	0.05	0.83	0.34
Mounting	Latency	1.26	0.30	1.22	0.32	0.42
	Duration	6.39	0.02	0.76	0.39	0.76
	Number	4.77	0.04	0.66	0.43	1.41
<i>Neutral social exploration</i>						
N-A contact	Latency	0.13	0.72	0.50	0.49	0.05
	Duration	0.45	0.51	0.19	0.67	<0.01
	Number	0.07	0.79	2.38	0.14	0.14
N-N contact	Latency	<0.01	0.93	1.44	0.24	0.93
	Duration	1.31	0.27	0.02	0.88	2.20
	Number	0.30	0.59	<0.01	0.95	3.41
Tukey's tests						
<i>Behavior</i>	<i>Parameter</i>	<i>WT ♂ vs ♀, p</i>	<i>KO ♂ vs ♀, p</i>	<i>♂ KO vs WT, p</i>	<i>♀ KO vs WT, p</i>	
<i>Dominant and aggressive behavior</i>						
Following	Latency	0.52	>0.99	0.36		>0.99
	Duration	0.03	0.82	0.80		0.04
	Number	0.24	0.67	0.35		0.52
Mounting	Latency	>0.99	>0.99	>0.99		>0.99
	Duration	0.11	0.65	0.65		0.11
	Number	0.11	0.89	0.77		0.18

Table S4 (continued)

Neutral social exploration									
N-A contact	Latency		0.98		>0.99		0.87		>0.99
	Duration		0.97		0.96		0.86		>0.99
	Number		0.97		>0.99		0.59		0.80
N-N contact	Latency		0.92		0.88		0.80		0.86
	Duration		0.28		>0.99		0.89		0.80
	Number		0.35		0.79		0.99		0.97
Chi square test									
Parameter		Males				Females			
Incidence of following		X ² (3, N=24)=13.33, p<0.01				X ² (3, N=24)=4.444, p=0.22			
Incidence of mounting		X ² (3, N=24)=22.3, p<0.01				X ² (3, N=24)=27.86, p<0.01			
Incidence of attacks		X ² (3, N=24)=15.42, p<0.01				Not measured			
Fisher's exact test									
Parameter		Males, p				Females, p			
	WT (PBS vs. LPS)	KO (PBS vs LPS)	PBS (WT vs. KO)	LPS (WT vs. KO)	WT (PBS vs. LPS)	KO (PBS vs LPS)	PBS (WT vs. KO)	LPS (WT vs. KO)	
Incidence of following	>0.99	0.01	>0.99	0.01	0.15	0.47	>0.99	>0.99	
Incidence of mounting	>0.99	0.01	>0.99	0.01	<0.01	>0.99	>0.99	<0.01	
Incidence of attacks	0.61	0.03	>0.99	<0.01	Not measured				
Unpaired t-test (Male LPS groups, normalized to genotype-matched PBS groups)									
Duration of attacks		t=2.230, df=10				p<0.05			
Two-way ANOVA (Male LPS groups, normalized to WT PBS group)									
Parameter	Interaction, F	Interaction, p	Genotype, F	Genotype, p	Sex, F	Sex, p			
Number of attacks	4.19	>0.05	2.75	0.11	1.62	0.22			
Latency to attack	2.29	0.15	1.94	0.18	1.61	0.22			

Table S5A. Summary of comparisons in the measures of cytokine expression in the brain and the spleen upon LPS administration normalized to sex-matched wild-type PBS controls (three-way ANOVA, Sidak's test). Fig. 2 G-L.

Three-way ANOVA												
Cytokine	LPS (L)		Sex (S)		Genotype (G)		L × S interaction		L × G interaction		L × S × G interaction	
	F	p	F	p	F	p	F	p	F	p	F	p
Brain												
Il-1β	180.3	<0.01	11.87	<0.01	<0.01	0.93	11.33	<0.01	10.50	<0.01	55.98	<0.01
Il-6	212.8	<0.01	21.97	<0.01	109.5	<0.01	30.25	<0.01	132.0	<0.01	13.14	<0.01
Tnf	815.2	<0.01	500.5	<0.01	280.4	<0.01	331.0	<0.01	427.9	<0.01	166.1	<0.01
Spleen												
Il-1β	80.45	<0.01	1.57	0.22	5.28	0.03	4.84	0.04	0.25	0.62	27.40	<0.01
Il-6†	263.8	<0.01	1.54	0.23	57.41	<0.01	1.97	0.17	51.43	<0.01	0.14	0.71
Tnf	84.08	<0.01	18.41	<0.01	34.39	<0.01	8.67	<0.01	59.13	<0.01	31.93	<0.01
Sidak's tests												
Cytokine	Males vs. females				Wild type vs. KO				PBS vs. LPS			
	WT PBS (♂ vs ♀)	KO PBS (♂ vs ♀)	WT LPS (♂ vs ♀)	KO LPS (♂ vs ♀)	♂ PBS (WT vs KO)	♀ PBS (WT vs KO)	♂ LPS (WT vs KO)	♀ LPS (WT vs KO)	♂ WT (PBS vs. LPS)	♀ WT (PBS vs. LPS)	♂ KO (PBS vs. LPS)	♀ KO (PBS vs. LPS)
Brain												
Il-1β	>0.99	>0.99	<0.01	<0.01	0.79	0.75	<0.01	<0.01	<0.01	0.07	0.03	<0.01
Il-6	>0.99	>0.99	<0.01	0.57	>0.99	>0.99	<0.01	<0.01	<0.01	0.08	<0.01	>0.99
Tnf	>0.99	<0.01	<0.01	<0.01	<0.01	>0.99	<0.01	<0.01	<0.01	<0.01	<0.01	0.01
Spleen												
Il-1β	>0.99	>0.99	<0.01	>0.05	>0.99	0.90	<0.01	<0.01	<0.01	<0.01	0.98	<0.01
Il-6	>0.99	>0.99	>0.99	0.59	>0.99	>0.99	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Tnf	>0.99	0.96	0.87	<0.01	0.80	>0.99	0.49	<0.01	0.80	<0.01	>0.99	<0.01

† see Table S5B for two-way ANOVA.

Table S5B. Summary of comparisons of consolidated data on expression of Il-6 in the spleen upon LPS administration normalized to sex-matched wild-type PBS controls (two-way ANOVA, Tukey's test). Fig. 2 G-L.

<i>Il-6 expression in the spleen</i>			
Two-way ANOVA (treatment × genotype interaction)			
<i>Interaction, F</i>		<i>Interaction, p</i>	
55.73		<0.01	
Tukey's test			
PBS WT vs. KO	LPS WT vs. KO	WT PBS vs. LPS	KO PBS vs. LPS
>0.99	<0.01	<0.01	<0.01

Table S6A. Summary of comparison in the measures of dominant and aggressive behavior after LPS treatment normalized to a sex-matched wild-type PBS control group (three-way ANOVA, Sidak's test). Fig. S5-S6.

Three-way ANOVA															
Behavior	Parameter	LPS (L)		Sex (S)		Genotype (G)		L × S interaction		L × G interaction		S × G interaction		L × S × G interaction	
		F	p	F	p	F	p	F	p	F	p	F	p	F	p
Dominant and aggressive behavior															
Following	Latency	2.79	0.09	1.10	0.30	1.75	0.19	0.23	0.63	0.59	0.45	0.09	0.76	0.76	0.39
	Duration†	8.49	<0.01	11.65	<0.01	0.72	0.40	4.39	0.04	0.15	0.70	0.12	0.73	0.95	0.34
	Number	5.57	0.02	5.57	0.02	5.50	0.02	1.20	0.28	1.04	0.32	0.81	0.37	0.14	0.71
Mounting	Latency†	3.56	0.07	0.57	0.45	1.97	0.17	0.49	0.49	<0.01	0.96	6.70	0.01	1.78	0.26
	Duration	3.33	0.08	0.60	0.44	0.30	0.35	0.90	0.35	0.60	0.44	6.72	0.01	5.83	0.02
	Number	3.35	0.07	0.90	0.35	1.15	0.29	1.44	0.24	0.67	0.42	6.05	0.02	4.87	0.03
Neutral social exploration															
N-A contact	Latency	<0.01	0.93	1.27	0.27	0.07	0.79	<0.01	0.98	0.17	0.67	2.20	0.15	0.14	0.71
	Duration	2.71	0.11	1.09	0.30	4.52	0.04	0.03	0.87	0.56	0.46	0.46	0.50	0.04	0.84
	Number	3.19	0.08	0.48	0.49	4.09	<0.05	0.17	0.68	2.13	0.15	0.03	0.86	0.01	0.92

Table S6A (continued)

Sidak's tests																
		Males vs. females					Wild type vs. KO					PBS vs. LPS				
Behavior	Parameter	WT PBS (♂ vs ♀)	KO PBS (♂ vs ♀)	WT LPS (♂ vs ♀)	KO LPS (♂ vs ♀)	♂ PBS (WT vs KO)	♂ LPS (WT vs KO)	♀ PBS (WT vs. KO)	♀ LPS (WT vs. KO)	♂ WT (PBS vs. LPS)	♂ KO (PBS vs. LPS)	♀ WT (PBS vs. LPS)	♀ KO (PBS vs. LPS)			
Dominant and aggressive behavior																
Following	Latency	>0.99	>0.99	0.91	>0.99	>0.99	0.76	>0.99	>0.99	>0.99	0.87	0.97	0.99			
	Duration	>0.99	0.93	<0.05	0.21	>0.99	>0.99	0.94	>0.99	>0.99	>0.99	0.03	0.61			
	Number	>0.99	0.94	0.86	0.48	>0.99	0.88	0.93	0.52	>0.99	0.90	0.62	0.40			
Mounting	Latency	>0.99	0.86	0.60	0.56	>0.99	0.96	0.86	0.17	>0.99	0.96	0.60	>0.99			
	Duration	>0.99	>0.99	0.02	0.75	>0.99	0.75	>0.99	0.02	>0.99	0.95	0.06	>0.99			
	Number	>0.99	>0.99	0.02	0.94	>0.99	0.90	>0.99	0.03	>0.99	>0.99	>0.05	>0.99			
Neutral social exploration																
N-A contacts	Latency	>0.99	0.98	>0.99	0.84	>0.99	0.94	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99			
	Duration	>0.99	>0.99	>0.99	>0.99	>0.99	0.96	0.98	0.73	>0.99	0.96	>0.99	0.97			
	Number	>0.99	>0.99	>0.99	>0.99	>0.99	0.7	>0.99	0.65	>0.99	0.86	>0.99	0.65			
N-N contacts	Latency	>0.99	0.9	>0.99	>0.99	0.86	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99			
	Duration	>0.99	>0.99	0.42	>0.99	>0.99	>0.99	>0.99	0.97	>0.99	>0.99	0.93	>0.99			
	Number	>0.99	>0.99	0.56	>0.99	>0.99	>0.99	0.99	0.81	0.96	>0.99	>0.99	>0.99			

† see Table S6B for two-way ANOVA.

Table S6B. Summary of comparisons of consolidated data on behavior and cytokine expression after LPS treatment normalized to a sex-matched wild-type PBS control group (two-way ANOVA, Tukey's test). Fig. S5-S6

<i>Dominant and aggressive behavior</i>			
<i>Behavior</i>	<i>Interaction</i>	<i>Interaction, F</i>	<i>Interaction, p</i>
Two-way ANOVA			
Following duration	Treatment × Sex	4.61	0.04
Latency to mounting	Sex × Genotype	6.50	0.01
Tukey's test			
Following duration	PBS ♂ vs. ♀	LPS ♂ vs. ♀	PBS ♂ vs. ♂
	0.78	<0.01	0.93
Latency to mounting	♂ WT vs. KO	♀ WT vs. KO	WT ♂ vs. ♀
	0.84	0.04	0.58
			0.11

Table S7. Summary of comparisons of the measures of cytokine expression in the brain and the spleen upon LPS administration normalized to sex- and genotype-matched PBS control groups (two-way ANOVA, Tukey's test). Fig S7.

Two-way ANOVA					
<i>Gene</i>	<i>Interaction, F</i>	<i>Interaction, p</i>	<i>Genotype, F</i>	<i>Genotype, p</i>	<i>Sex, F</i>
Brain					
Il-1β	129.8	<0.01	250.1	<0.01	112.0
Il-6	15.09	<0.01	246.6	<0.01	53.70
Tnf	587.6	<0.01	808.6	<0.01	435.7
Spleen					
Il-1β	16.07	<0.01	15.40	<0.01	21.73
Il-6	4.951	0.04	13.39	<0.01	11.86
Tnf†	3.507	0.08	105.3	<0.01	0.37

Table S7. (continued)

Tukey's test				
Gene	WT ♂ vs ♀, <i>p</i>	KO ♂ vs ♀, <i>p</i>	♂ KO vs WT, <i>p</i>	♀ KO vs WT, <i>p</i>
Brain				
Il-1β	<0.01	0.94	<0.01	0.02
Il-6	<0.01	0.11	<0.01	<0.01
Tnf	<0.01	0.09	<0.01	0.03
Spleen				
Il-1β	<0.01	0.97	<0.01	>0.99
Il-6	<0.01	0.82	0.74	<0.01
Tnf	0.79	0.35	<0.01	<0.01

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CHAPTER
General discussion

5

Chapter 5. General discussion

5.1 Genetic and environmental factors in the pathogenesis of neurodevelopmental disorders and excessive aggression

Family studies suggest that genetic and environmental factors each explain about 50% of the variance in aggressive behavior (Tuvblad and Baker, 2011). Excessive aggression is often accompanying neurodevelopmental disorders such as ADHD and ASD (Oades et al., 2008; Søndena et al., 2014).

Known risk factors for neurodevelopmental disorders include early-life stress such as loss of parents, emotional neglect, child abuse, which all are risk factors for the development of anxiety disorders, depression (Heim and Nemeroff, 2001), and pathologic aggression and violence in adulthood (Carr et al., 2013). Such factors constitute emotional stress, defined as a state primarily triggered by the perception and cognitive evaluation of adverse events rather than disturbances of physical nature (Fontes et al., 2014). This type of stress is of particular significance in the context of social environments and is clinically relevant, as its consequences may have a cumulative effect (Barthas et al., 2020), and the social environment may show as both a risk and a protective factor (Pickett et al., 2009).

Another environmental factor that may contribute to the pathogenesis of neurodevelopmental disorders is inflammation, in particular, that caused by infectious diseases (Debost et al., 2019; Søndena et al., 2014). Such diseases suffered during critical periods, e.g.,

during intrauterine and early postnatal development, as well as adolescence, may cause enduring neurodevelopmental abnormalities (Debost et al., 2019; Søndena et al., 2014). While severe negative effects caused by infectious disease or maternal immune activation during prenatal development are widely recognized and include disorders such as ASD and schizophrenic psychoses (Estes and McAllister, 2016; Guma et al., 2019; Knuesel et al., 2014), consequences of inflammatory stress during adolescence are often overlooked. However, they may contribute, in addition to prenatal and perinatal inflammation, to the pathogenesis of neurodevelopmental disorders (Chan and Frankovich, 2019).

In most cases, environmental factors should not be regarded as the sole cause of neurodevelopmental abnormalities. Genetic factors, as mentioned above, were shown to increase the risk of neurodevelopmental disorders, and, in particular, explain about 50% of the variance in aggressive behaviors (Tuvblad and Baker, 2011). Among different genetic factors contributing to the predisposition to neurodevelopmental disorders and associated aggressive behavior, variations in genes related to the brain 5-HT system are considered to be major risk factors (Lesch et al., 2012; Lesch and Merschdorf, 2000). They include genes encoding, e.g., TPH2, SERT, and MAOA (Cupaioli et al., 2021; Homberg and Lesch, 2011; Kolla and Bortolato, 2020). Another gene shown to contribute to the risk of neurodevelopmental disorders, including ADHD and intellectual disability, is *ST3GAL3* (Hall et al., 2020; Klein et al., 2020), encoding β -galactoside- α 2,3-sialyltransferase-III, one

of the enzymes participating in brain ganglioside synthesis (Schnaar, 2016).

Investigation of gene $\times$ environment interaction (GxE) is the approach of choice for studying mechanisms of pathological aggression and neurodevelopmental brain pathology. Thus, valid experimental animal models are needed for such studies. In this work, we used two models of GxE in pathological aggression. In **Chapters 2 and 3**, *Tph2*^{+/-} mice of both sexes were studied for aggression after exposure to predation stress. This type of stress reproduces elements of an emotional stress paradigm, as far as it is delivered only by visual and olfactory contact with the predator, without any physical danger to the mouse (Gorlova et al., 2020). This paradigm has been substantiated and is considered ethologically valid, as rats are natural predators for mice, and the presence of predators or their chemical cues negatively affects the neurophysiological state and behavior of rodents (Adamec et al., 2006).

Unlike *Tph2*^{-/-} mice, which completely lack neuronal 5-HT and exhibit severe behavioral abnormalities including highly increased aggression (Gutknecht et al., 2015; Mosienko et al., 2012), *Tph2*^{+/-} mice have brain 5-HT levels about 30% lower than wt controls and do not exhibit explicit behavioral alterations. We showed, however, that after the rat exposure stress, *Tph2*^{+/-} mice of both sexes demonstrate abnormal aggression and decreased neutral social interaction measures (**Chapters 2, 3**). Of particular interest is the fact that we found increased aggression in *Tph2*^{+/-} female mice (**Chapter 3**), as female aggression is considered to be outside of the normal behavioral repertoire of mice. Of note, in the

modified swim test *Tph2*^{+/-} mice of both sexes exhibited signs of higher stress resilience compared to wt controls, which is known to accompany increased aggression and dominance in other aggression studies and was also found in mice with trait aggression (Strekalova et al., 2011).

In **Chapter 4**, we for the first time investigated the social behavior of male and female mice with a deficiency of major brain gangliosides due to the knockout of the *St3gal5* gene. In these mice, we observed altered social and cognitive behavior alongside compromised motor functions. While naïve female *St3gal5*^{-/-} mice showed decreased social interaction and no aggression, increased dominant behavior was evident in naïve male *St3gal5*^{-/-} mutants (**Chapter 4**). After assessment of the baseline behavior, mice were subjected to a model of infectious stress, i.e., systemic inflammation was induced by injections of LPS (Couch et al., 2016, 2013; Trofimov et al., 2017). It led to a significant increase in the aggressive and dominant behavior in male *St3gal5*^{-/-} mice and a decrease in dominant behavior in *St3gal5*^{-/-} female mutants.

Thus, in both genetic lines, the combination of genetic background and stress of different nature led to abnormal aggression in male mutant mice and altered social behavior in both sexes. These changes resemble features of neurodevelopmental disorders, e.g., ADHD and ASD, and these models could constitute useful paradigms for the investigation of gene × environment interaction mechanisms in neurodevelopmental disorders. Additionally, female aggression in stressed *Tph2*^{+/-} mice may be used as a model for research of sex-specific aspects of the neurobiology of aggression. Apart from behavioral

alterations, we also showed stress-related molecular and neurochemical changes in both models.

*5.2 Alterations of monoamine metabolism and changes in the expression of 5-HT receptors, distress and plasticity markers, as well as myelination-related proteins in stress-related aggression of *Tph2* heterozygous mice*

In male *Tph2*^{+/-} mice, we showed stress-related changes in concentrations and turnover of DA and NE in multiple brain structures related to social and emotional behavior, i.e., amygdala and PFC (**Chapter 2**). Concentrations of 5-HT, its precursor 5-HTP, and its metabolite HIAA were previously assessed in brains of male *Tph2*^{+/-} mice exposed to the same predation stress paradigm we used in **Chapter 2**. The study of Gorlova and coworkers (2020) revealed decreased 5-HTP and HIAA concentrations in brains of stressed male *Tph2*^{+/-} mice with no further stress-related decrease in 5-HT concentration, indicating the existence of certain compensatory mechanisms maintaining normal development and, in particular, brain 5-HT metabolism in naïve *Tph2*^{+/-} mice (Gorlova et al., 2020).

However, while under stress conditions the increase of the 5-HT concentration in the PFC and the striatum was evident in wt controls, no such changes were found in *Tph2*^{+/-} mutants, possibly due to a ceiling effect of a partial deficiency of *Tph2* on 5-HT metabolism. 5-HT system of the brain is in a tight functional connection with DA and NE systems (**Chapter 1**).

We showed changes in the brain DA metabolism of stressed male *Tph2*^{+/-} mice (**Chapter 2**), partly resembling those previously found in the *Tph2*^{-/-} mutants. Decreased concentrations of DA in the amygdala and striatum of stressed *Tph2*^{+/-} mice and enhanced DOPAC/DA turnover in these regions together with strikingly lower DA concentration in the striatum of both stressed and non-stressed *Tph2*^{+/-} mice provide evidence for massive changes in the DA system of the *Tph2*-deficient animals. These observations are in accordance with the data on reciprocal modulation of the DA system by the 5-HT system of the brain (De Deurwaerdère et al., 2005; Millan et al., 1998; Rosell and Siever, 2015; Seo et al., 2008).

Modulation of aggression by the DA system and its interaction with the 5-HT system is known also from human studies of aggressive behavior and suicide (Ryding et al., 2008). These two systems regulate frontostriatal and amygdalar activity, which is crucial for impulsivity and aggression control (**Chapter 1**). Alterations in dopamine system were also found in patients with ASD. Abnormalities in mesocorticolimbic and prefrontal-striatal dopaminergic pathway, which includes DA projections from VTA to striatum and prefrontal cortex, were hypothesized to cause abnormal social behaviors in ASD (Pavăl and Micluția, 2021), which is in accordance with our data. Depressed DA system activity in patients with ADHD was also shown using neuroimaging techniques, suggesting lowered dopaminergic modulation of fronto-striatal networks (Del Campo et al., 2011).

Interaction between DA and NE systems of the brain and a possible role of NE in ADHD were described in the literature (Del Campo et al., 2011), and we also showed stress-related changes in the NE metabolism. In both genotypes, the rat exposure stress caused an increase of NE concentration in the striatum and NE metabolite MHPG concentration in the hippocampus (**Chapter 2**). The only genotype-specific change observed was decreased level of MHPG and, subsequently, NE turnover rate in the amygdala. Given the well-known role amygdala plays in the regulation of social cue processing and aggression (Cupaioli et al., 2021), we may suggest the role of NE in the regulation of pathologic aggression in the *Tph2*^{+/-} mouse line. However, while the role of NE is known in depression, e.g., negative coupling between 5-HIAA and NE concentrations in the CSF known in normal subjects but absent in depressed patients, data on NE participation in the pathogenesis of aggression is scarce and requires further investigation (Geraciotti et al., 1997).

The role of different 5-HT receptors in aggression was extensively studied over the past decades. In **Chapter 3**, we assessed the expression of 5-HT receptor genes *Htr1a* and *Htr2a*, encoding 5-HT receptors 1A and 2A, respectively. Expression of the *Htr1a* mRNA was decreased in the PFC of female *Tph2*^{+/-} mice irrespective of stress. This effect may reflect compensatory changes due to lowered 5-HT baseline concentration observed in *Tph2*^{+/-} animals (Waider et al., 2019). Previously, no alterations were reported in the *Htr1a* mRNA expression in *Tph2*^{+/-} males, irrespective of stress (Gorlova et al., 2020). Such discrepancy may reflect

sex differences in compensatory processes observed in the *Tph2*^{+/-} 5-HT system, as sex differences, i.e., higher 5-HT transmission in different brain regions of females compared to males is known both for mice and rats (Carlsson and Carlsson, 1988; Domínguez et al., 2003; Jones and Lucki, 2005).

The concentration of *Htr2a* mRNA was also decreased in the PFC of female *Tph2*^{+/-} mice, and, unlike *Htr1a*, it was significantly increased in both genotypes after stress (**Chapter 3**). This increase may play a role in abnormal aggression observed in stressed *Tph2*^{+/-} mice, as it was previously shown that upregulation of 5-HT_{2A} receptors in the PFC (Godar et al., 2019) and administration of 5-HT_{2A} agonists (Juárez et al., 2013) reduce aggression in mice. Enhanced sensitivity of 5-HT_{1A} receptor is also known for *Tph2*^{+/-} mice (Araragi et al., 2013). While such effect was not investigated for 5-HT_{1A} yet, the difference in its sensitivity between genotypes might explain putative differential effects of the stress-related increase of its expression. Given the abovementioned findings, we may speculate that while compensatory changes in the 5-HT, DA, and NE systems of *Tph2*^{+/-} mice enable close to normal function of these mice, decompensation under stress conditions leads to changes resembling features of neurodevelopmental disorders, including aggression. These changes were also accompanied by altered expression of several markers of distress and plasticity.

Baseline expression of *Gsk-3β* in the PFC of female *Tph2*^{+/-} mice was lowered in comparison to control mice. After stress exposure, the expression increased to the level of control mice. Control mice did not

show the stress-related change in *Gsk-3 β* expression (**Chapter 3**). This molecule is known as a marker of distress and is implicated in a wide spectrum of cellular functions, including inflammation (Gocho et al., 2013); in particular, it mediates an increase of pro-inflammatory IL-1 β and TNF levels (Green and Nolan, 2012) and inhibits expression of anti-inflammatory IL-10 (Jope et al., 2017). GSK-3 β was found to participate in the development of affective disorders, including depression (Markova et al., 2017; Pavlov et al., 2017; Strekalova et al., 2016).

Previously, in male mice with lowered TPH2 enzymatic activity due to a knock-in of human R439H mutation, suppression of aggressive behavior was shown upon introduction of *Gsk-3 β* heterozygous knockout (Beaulieu et al., 2008). Multiple studies also showed that an inhibitor of GSK-3 β , lithium, which is widely used for the treatment of neurological and psychiatric disorders, has prominent anti-aggressive and anti-inflammatory properties (Beurel and Jope, 2014; Müller-Oerlinghausen and Lewitzka, 2010). Given the well-known association between GSK-3 β activity and aggression, the increase of *Gsk-3 β* expression in stressed female *Tph2^{+/-}* mice may be related to observed aggressive behavior. As in our study expression of *Gsk-3 β* in stressed *Tph2^{+/-}* mice was not significantly different from that of control mice, we speculate that the rise above the baseline level and its interaction with other alterations on a molecular and cellular level rather than the absolute *Gsk-3 β* expression level itself may contribute to the behavioral abnormalities (**Chapter 3**).

Notably, despite observed *Gsk-3 β* expression, we did not find increased expression of the pro-inflammatory cytokine *Il-1 β* in the PFC

of *Tph2*^{+/-} male and female mice irrespective of stress. Moreover, a trend towards lowered expression in *Tph2*^{+/-} males and females was observed, (**Chapter 3**, (Svirin et al., 2021)). As it was discussed in **Chapter 1**, 5-HT deficiency may shift neuroinflammation towards opposite directions through several pathways. The trend to lower expression of *Il-1β* in *Tph2*^{+/-} mice may be explained by decreased microglial activation due to elimination of its stimulation by 5-HT. No signs of neuroinflammation were reported for *Tph2*^{-/-} mice as well.

Predation stress also led to a significant increase in the expression of *Syp* and *GluA2* in both genotypes (**Chapter 3**). Previously, increased *GluA2* expression was found in the PFC of male *Tph2*^{+/-} mice and wt controls subjected to predation stress, with no genotype differences (Gorlova et al., 2020). Increased *Syp* expression was found in the predation stress model in rats, where a significant elevation of *Syp* mRNA expression was observed in the amygdaloid complex, and a trend towards higher expression was observed in the PFC (Campos et al., 2013). In both cases of *Syp* and *GluA2*, the lack of significant expression difference between genotypes in both stress conditions prevents us from concluding their roles in behavioral alterations observed in stressed *Tph2*^{+/-} mice.

For the first time, we demonstrated changes in the expression of myelination-related proteins in the brain of *Tph2*^{+/-} mice (**Chapter 3**, (Svirin et al., 2021)). In the PFC of naïve *Tph2*^{+/-} female mice, we showed a significant increase in *Mbp* and *Mag* expression as well as a trend in *Plp1* expression. These alterations were reverted by stress, while no stress-related changes were found in the PFC of control female mice, and

only *Mog* expression was decreased upon stress in both genotypes, with no genotype difference (**Chapter 3**). Notably, in *Tph2*^{+/-} male mice we did not find significant differences in the expression of *Plp1* and *Mbp* in the PFC, while in the hippocampus mRNA expression of both proteins was decreased in *Tph2*^{+/-} male mutants (Svirin et al., 2021). These differences between male and female mice are in line with sex differences in myelination previously reported in both mice and rats.

Age-related sex differences in the structure of white matter were found in Long-Evans rats (Yang et al., 2008), and MBP expression was higher in the OFC of adult female Long-Evans rats (Darling and Daniel, 2019). Considerable sex differences in *Mbp*, *Plp1*, *Mag*, and *Mog* expression were also shown in a rat model of gestational nicotine administration, where these genes were significantly upregulated in adolescent males and, conversely, downregulated in females receiving gestational nicotine (Cao et al., 2013). Remarkably, in the mouse model of prenatal cigarette exposure, increased aggressive behavior was found in male but not female mice (Yochum et al., 2014). In a rat model of adolescent alcohol drinking, myelinated fiber density in the PFC was significantly lowered in male but not in female animals (Tavares et al., 2019). In mice, early weaning was shown to affect white matter structure in the basolateral amygdala only in males, and authors linked the developmentally premature formation of myelin observed in male mice to anxiety-related behavioral changes (Ono et al., 2008).

Thus, there is a considerable body of evidence suggesting that myelination processes are sex-specific both during normal development

and in pathological conditions, which is in line with our findings in *Tph2*^{+/-} mice (**Chapter 3**, (Svirin et al., 2021)). As it was discussed in **Chapter 1**, myelination deficits are increasingly recognized as a common pathology underlying neuropsychiatric and neurodevelopmental disorders, and it is known that dysregulation of myelogenesis in both directions may lead to pathology. Our data suggest that myelination abnormalities may contribute to the behavioral deficits observed in *Tph2*^{+/-} mice; however, further investigation using complementary methods is needed.

5.3 ST3GAL5-deficient mice as a model of neurodevelopmental disorders: social behavior abnormalities, molecular changes, and effects of inflammatory stress

In **Chapter 4** we investigated *St3gal5* null mutant mice, another model proposed to resemble common features of neurodevelopmental disorders (Niimi et al., 2011). Previously, gene encoding another brain sialyltransferase, *St3gal3*, was found to be associated with the incidence of ADHD, ASD, and schizophrenia (Hall et al., 2020; Mealer et al., 2020; Zhao et al., 2018b), as well as childhood aggression (Ip et al., 2021). While disruption of brain ganglioside synthesis in humans arising from dysfunction of *St3gal5* gene leads to devastating failure of neurodevelopment, *St3gal5*^{-/-} mice do not show a severe phenotype, possibly due to the existence of a compensatory mechanism, i.e., compensatory synthesis of o-series gangliosides. Thus, we speculate that

St3gal5^{-/-} mice are a plausible model for partial brain ganglioside deficiency.

We sought to study baseline behavior of *St3gal5*^{-/-} mice, as no data could be found in the available literature. We showed substantial alterations in the dominant behavior and neutral sociability of *St3gal5*^{-/-} mice, as well as other behavioral alterations, resembling ASD-like features, i.e., repetitive behavior (**Chapter 4**). Naïve *St3gal5*^{-/-} male mice exhibited a substantial increase in dominant behavior, and a similar trend was also found in *St3gal5*^{-/-} female mice. However, no significant differences were shown in attacking behavior in naïve *St3gal5*^{-/-} male mice. Behavioral alterations were accompanied by increased expression of pro-inflammatory cytokines *Il-1β* and *Tnf* both in the PFC and the spleen of *St3gal5*^{-/-} animals. Altered systemic immune regulation and neuroinflammatory changes are well-documented in patients with ASD (Thom et al., 2019) and in BTBR mice considered to represent an ASD model (Careaga et al., 2015; Zhang et al., 2019).

As neuroinflammation is one of the common mechanisms of neuropsychiatric disorders, and anti-inflammatory action of major brain gangliosides was shown in many studies (discussed in **Chapter 1**), we hypothesized that brain ganglioside deficient mice might be susceptible to inflammatory stress. We investigated whether the interaction of genetic background of these mice with environmental adversity such as systemic inflammation aggravates the altered social and aggressive behavior. Additionally, we investigated the expression of pro-inflammatory cytokines *Il-1β*, *Il-6*, and *Tnf* in the brain and in the

periphery in order to test whether LPS administration causes a different inflammatory response in *St3gal5*^{-/-} mice compared to wt controls.

We demonstrated profound sex differences in the behavioral reaction to the LPS administration. While systemic inflammation led to a substantial increase in dominant and aggressive behaviors in *St3gal5*^{-/-} males, in female mice such increase was observed in wt control mice. Sex differences were also found in cytokine expression response to LPS administration. In general, compared to wt controls, *St3gal5*^{-/-} mice of both sexes had a lower degree of increase in the expression of the pro-inflammatory cytokines in the brain and a higher degree in the periphery. However, LPS-induced increase of *Il-1β* expression both in the brain and in the periphery was significantly higher in *St3gal5*^{-/-} females than in wt controls, while in male wt control mice the rise of *Il-1β* expression was higher than in *St3gal5*^{-/-} males. Thus, there are remarkable sex-specific effects of both the *St3gal5* knockout alone and its interaction with inflammatory stress on social and aggressive behavior. Of note, sex-specific differences in the severity of outcomes were shown for many genetic rodent models of neurodevelopmental disorders (Mossa and Manzini, 2021). In most cases, male mice had more severe outcomes, and, in the case of mice with paralogous human truncating variant introduced into the chromodomain-helicase-DNA binding protein 8 (CHD8) gene, male mice exhibited increased anxiety-like behaviors after experiencing stressful events (Jung et al., 2018).

Sex differences in the prevalence and severity of neurodevelopmental disorders are well-known, e.g., ASD and ADHD

incidence is higher in men [60]. Some authors attribute these differences to sex-specific neuro-immune regulation (Hanamsagar and Bilbo, 2016; May et al., 2019; McCarthy and Wright, 2017), and this explanation would be in line with our findings of differential inflammatory response and changes in aggressive and dominant behaviors in *St3gal5^{-/-}* mice. Gender differences in aggression were also found in humans: while men are overall more physically aggressive than women, women tend to indulge in more indirect aggression than men (Björkqvist, 2018), and such difference is known, in particular, for patients with ADHD (Rucklidge, 2010).

As there is a substantial body of evidence suggesting links between brain gangliosides and myelination, we also assessed the mRNA expression of myelination-related proteins MBP, PLP1, MAG, and MOG in the PFC of naïve *St3gal5^{-/-}* mice. In mutants of both sexes, we showed about two-fold decrease of expression of PLP1 both on mRNA and protein levels. No changes in the expression of the other three myelin proteins were found. Interestingly, a mutation in the *Plp1* gene leading to its decreased expression was found in rabbits with paralytic tremor disease, and myelin lipid content in these animals was altered (Sypecka and Domańska-Janik, 2005). It was shown that *Plp1* knockout (*Plp1^{-/-}*) mice, as well as patients with a lack of PLP1, develop length-dependent axonal degeneration progressing with age, with no histological signs of demyelination (Garbern et al., 2002). Local deficits of axonal transport were also found in the internodes myelinated by PLP1-deficient OLs (Gruenenfelder et al., 2011). Given the abovementioned results, we may

suggest that decreased expression of PLP1 in the PFC of *St3gal5*^{-/-} mice may arise from aberrant regulation of OLs in the lack of major brain gangliosides. A decrease in PLP1 may be a factor contributing to white matter abnormalities affecting brain circuits involved in the regulation of social and aggressive behaviors. However, further histological and molecular analysis would be necessary to shed light on the myelination abnormalities suspected in these animals.

5.4 Dysregulated myelination and neuroinflammation as putative common mechanisms of neurodevelopmental disorders and pathologic aggression

In both experimental mouse models of neurodevelopmental disorders used in current research, we showed abnormal aggression and other neurodevelopmental disorder-like behaviors, revealed only by stress, either emotional or infectious. For the first time, such behavioral features were shown for *Tph2*^{+/-} female mice and *St3gal5*^{-/-} mice. We also showed molecular signs of inflammation and cellular stress in both models and accompanying alterations in the expression of myelination-related proteins.

Neuroinflammation is considered one of the substantial mechanisms of neurodevelopmental and neuropsychiatric disorders. Being primarily a normal protective response to infection or tissue injury, abnormal inflammation, which can be caused by stress or infectious agents, may result in cell and tissue damage (Ofengeim and Yuan, 2013; Yuan et al., 2019). In particular, systemic inflammation may cause

damage to the BBB and, thus, cause neuroinflammation in the CNS (Banks and Erickson, 2010; Dantzer et al., 2008).

Inflammation and immune system dysfunction were found in ASD, ADHD, depression, schizophrenia, and other disorders (Anand et al., 2017; Dunn et al., 2019; Prata et al., 2017; Robson et al., 2017). There is also a strong link between inflammation and aggression. In patients with depression an association between aggression levels and immune dysregulation was found. Prolonged social stress was shown to enhance both inflammation and depressive and aggressive traits (Takahashi et al., 2018). In addition, in intermittent explosive disorder and psychosis pathological aggression was found to be associated with increased levels of pro-inflammatory cytokines (Coccaro et al., 2015). Moreover, in Alzheimer's disease, in which a marked increase in both systemic and CNS inflammation is present (Forloni and Balducci, 2018), aggression traits are linked to impairments in frontolimbic circuits (Trzepacz et al., 2013). Myelination deficits are found in neurodevelopmental disorders, such as ASD, ADHD, and schizophrenia (Gozzi et al., 2012; Lesch, 2019; Onnink et al., 2015), in which aggression is a frequent comorbidity. Inflammation and myelination abnormalities are in a complex interplay and may contribute to the pathogenesis of neurodevelopmental disorders.

Impairments in myelination of frontolimbic brain circuits may lead to disrupted top-down control which is crucial for the suppression of aggressive behavior and optimal response choice. In the models we used in our work, dysmyelination may arise due to several mechanisms. In the

Tph2^{+/-} mouse model, brain 5-HT deficit may affect oligodendroglia cell lineage both directly, as it was shown in co-cultures of neurons and OLs (Fan et al., 2015), and by regulation of SIRT2 expression which was shown to affect MBP expression, oligodendrocyte differentiation, and axon-myelin interaction (Ji et al., 2011; Li et al., 2007). An increase in the expression of *Gsk-3β* found in *Tph2*^{+/-} female mice after stress may suppress myelogenesis (Dohare et al., 2018), which is in accordance with the decrease in the expression of myelination-related proteins which we showed in these mice (**Chapter 3**).

Although we did not find increased expression of pro-inflammatory *Il-1β* in *Tph2*^{+/-} mutants, further research will be needed to uncover the neuroinflammatory status of these mice. IL-1β was shown to trigger OL death (Takahashi et al., 2003), and injections of LPS were shown to lead to demyelination in neonatal rats (Singh et al., 2017).

As systemic inflammation and pro-inflammatory cytokines are shown to have a detrimental effect on OLs and myelination, we may speculate that it might be a pathway affecting the expression of PLP1 in *St3gal5*^{-/-} mice, which have increased pro-inflammatory cytokine expression both in the periphery and in the CNS (**Chapter 4**). However, as we showed decreased expression of only the *Plp1* gene and not *Mbp*, *Mag*, and *Mog*, we may suggest that some compensatory mechanism exists. MAG is a protein crucial for the regulation of axon-myelin stability and has highly specific binding to gangliosides GD1a and GT1b which are absent in the brain of *St3gal5*^{-/-} mice. Unlike in the brain of *B4galnt1* knockout mice, however, o-series ganglioside GD1α, which also

has a high affinity to MAG (Sawada et al., 1999), is present in the brain of *St3gal5*^{-/-} mutants. This ganglioside may provide a rescue pathway for maintaining axon-myelin stability, and prevent severe phenotype observed in both *B4galnt1* and *Mag* knockout mice (Schnaar, 2010), but such partial compensation may lead to deficient axon-OL interaction mirrored by PLP1 underexpression. Such underexpression, in turn, may lead to axon degeneration and impaired axonal transport disrupting brain connectivity, which, as discussed above, may lead to impaired cognitive control of aggression.

Several mechanisms may underlie the relationship between myelination abnormalities and neuroinflammation (**Figure 5.1**). In the case of active demyelination, myelin debris activates microglia and macrophages, thus inducing the production of pro-inflammatory cytokines (Murugaiyan et al., 2020). Necroptotic death of axons due to impaired axon-myelin interaction also induces a pro-inflammatory response (Murugaiyan et al., 2020). Last but not least, astrocytes may provide an important interface between neuroinflammation and myelination, as astrocyte-secreted factors regulate oligodendroglia and promote neuronal survival, and, simultaneously, astrocytes regulate microglia and release cytokines and chemokines affecting inflammation (Kiray et al., 2016; Vainchtein and Molofsky, 2020). Astrocytes were shown to be neuronal activity-dependent, and changes in neuronal activity due to altered myelination may affect the above-mentioned astrocyte function.

Figure 5.1. Pathological pathways in G×E models of ADHD and ASD. G×E interaction in our models may lead to both neuroinflammation and dysmyelination, which are known to be interrelated. Impairments in myelination may cause neuroinflammation: myelin debris, products of neuron elimination due to necrosis and apoptosis, and dysregulation of activity-dependent astrocytes may activate microglia and macrophages. Pro-inflammatory cytokines, GSK-3β, and dysregulation of glia by inflammation negatively affect myelination. Aberrant signal propagation, axon degradation, and neuronal death due to lack of metabolic support from myelin sheaths disrupt brain connectivity. Additionally, neuroinflammation contributes to alterations in monoaminergic transmission and neural plasticity. Together, these factors lead to behavioral abnormalities resembling endophenotypes of neurodevelopmental disorders, i.e., ADHD and ASD (see the text).

Overall, the complex interplay between neuroinflammation and myelination may affect neuron function and brain functional connectivity both during development and in adult age. Genetic and

environmental factors and their combination, as in models used in our work, may affect those processes leading to behavioral consequences such as aggression and maladaptive social behavior.

5.5 Conclusion

In our work we identified signs of putative common interrelated mechanisms of stress-related aggression in two genetic animal models of neurodevelopmental disorders. Dysregulated immune function and inflammation, neuroinflammation in particular, were shown to play a role in ASD and ADHD (Dunn et al., 2019; Ye et al., 2021), as well as myelination deficits (Gozzi et al., 2012; Lesch, 2019; Onnink et al., 2015).

In a recent analysis of genome-wide association studies of eight psychiatric disorders performed by Cross-Disorder Group of the Psychiatric Genomics Consortium (2019), a high level of pleiotropy was found across multiple genes linking them to two or more psychiatric disorders. Notably, modelling of genetic correlations identified three groups among the eight studied disorders based on their shared genetics. One of them comprises ADHD, ASD, depression, and Tourette's syndrome, that is early-onset neurodevelopmental disorders and disorders with a substantial neurodevelopmental component. Such results led to an idea that multiple pleiotropic genes, acting from early in ontogenesis, contribute to the risk of numerous neurodevelopmental disorders. The authors assumed that alterations in such pleiotropic genes may be translated to discrete psychiatric syndromes by mechanisms involving G×E interactions.

These ideas are consistent with the hypothesis stating that common nonspecific mechanisms confer broad liability to neurodevelopmental disorders, including ADHD and ASD. These mechanisms may include but are not limited to dysregulated neuroinflammation and aberrant myelination. Notably, the gene for which the most highly pleiotropic locus was found, is Deleted in Colorectal Carcinoma (*DCC*) gene. This gene was significantly associated with all eight disorders and was shown to be crucial for myelination in the brain (Ye et al., 2021). Null knockout of *Dcc* in mice is lethal, and conditional knockout in oligodendrocytes leads to disruption in white matter ultrastructure, significant decrease in the protein content of MBP and MAG, and a trend to such decrease in the PLP1 expression (Bull et al., 2014). This finding further links myelination abnormalities with a broad spectrum of neurodevelopmental disorders and underlines the need for additional investigation into the role of myelination, as well as its interaction with neuroinflammation, in pathogenesis of these conditions.

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Summary

In our work, we investigated the mechanisms underlying human neurodevelopmental brain pathology associated with abnormal aggression and accompanying emotional abnormalities that are related to endophenotypes of developmental neuropsychiatric disorders. Specifically, we used genetically modified mice deficient for tryptophan hydroxylase-2 (TPH2), the rate-limiting enzyme of neuronal serotonin (5-HT) synthesis, and mice deficient in lactosylceramide alpha-2,3-sialyltransferase (ST3GAL5), also called GM3 synthase, which synthesizes ganglioside GM3, the precursor of major brain gangliosides.

Genetic variation in the *TPH2* gene and *ST3GAL5* gene-dependent alterations in ganglioside synthesis and catabolism in humans were shown to contribute to neurodevelopmental disorders. Both TPH2- and ST3GAL5-deficient mice share features of neurodevelopmental and psychiatric disorders, including attention-deficit/hyperactivity disorder (ADHD) and autism spectrum disorders (ASD), such as hyperactivity as well as increased impulsivity and aggression. In addition, they display compromised cognitive control and signs of impaired brain myelination. Apart from genetic predisposition, environmental challenges, such as emotional stress or inflammatory stress caused by infectious diseases, are known to contribute to the pathogenesis of these disorders.

To investigate the contribution of environmental challenges in these experimental mouse models of disease, we used exposure to a predator or induction of inflammatory stress. In light of current views suggesting that genetic influence on psychiatric disorders involves genes

acting at early stages of neurodevelopment and the establishment of brain circuitry and, thus, conferring broad liability to psychiatric disorders, we sought to investigate two genetic models exhibiting partly similar behavioral phenotypes in order to facilitate identification of putative common neurobiological mechanisms leading to behavioral alterations, including increased aggression.

First, we used mice with a partial genetic deficiency of *Tph2*. In line with previously demonstrated abnormal aggression in male heterozygous *Tph2* (*Tph2*^{+/-}) mice subjected to environmental stressors, we showed increased aggression and dominant behavior, as well as deficits in neutral social behavior in both male and female mutants following rat exposure stress. Naïve *Tph2*^{+/-} male mice challenged with novelty exhibited increased exploratory activity and less behavioral despair in the modified swim test than wildtype (wt) control mice. Decreased floating was also found in naïve *Tph2*^{+/-} female mice, indicating stress resilience and locomotor hyperactivity which are a prominent feature of neurodevelopmental disorders. Thus, in both sexes in *Tph2*^{+/-} mice we observed increased stress resilience and stress-induced aggression.

We suggest similarities in neurobiological mechanisms of behavioral changes in male and female mice. Following previous research that found alterations in 5-HT metabolism, we used male mice for high-performance liquid chromatography (HPLC) assay of brain dopamine and norepinephrine. Stress-induced changes of dopamine and norepinephrine metabolism were most pronounced in the amygdala,

where dopamine concentration was drastically reduced and its turnover was heightened in stressed *Tph2*^{+/-} mice compared to wt controls and non-stressed mutants. Norepinephrine turnover in the amygdala was reduced in mutant mice. In the PFC of stressed *Tph2*^{+/-} mice we found strongly decreased dopamine concentration and elevated dopamine turnover compared to wt control mice, which may alter PFC activity and, thus, affect top-down control of impulsive aggression. We speculate that the observed neurochemical differences between wt controls and *Tph2*^{+/-} mice are implicated in the deficits of cognitive control dependent on the functional interplay between the PFC and amygdala.

RT-qPCR showed increased expression of 5-HT_{2A} in the PFC accompanying behavioral changes in stressed female *Tph2*^{+/-} mice of both genotypes. In *Tph2*^{+/-} female mice, 5-HT_{1A} receptor expression was downregulated. Together, these findings suggest stress × genotype interaction affecting the 5-HT system of the brain, which is essential for cognitive control. Expression of glycogen synthase kinase 3 beta gene (*Gsk-3β*) in the PFC was decreased in non-stressed *Tph2*^{+/-} female mice. GSK-3β is considered a molecular hub of distress and is a well-established physiological mediator of elevated synthesis of pro-inflammatory cytokines. Together with decreased behavioral despair in the modified swim test, lowered *Gsk-3β* expression may indicate reduced susceptibility of *Tph2*^{+/-} mutants to stress.

For the first time, we found elevated mRNA expression of four major myelin structural proteins: myelin basic protein (MBP), proteolipid protein 1 (PLP1), myelin-associated glycoprotein (MAG), and myelin

oligodendrocyte glycoprotein (MOG), in the PFC of non-stressed *Tph2*^{+/-} female mice. Stress lowered the expression level of these proteins in *Tph2*^{+/-} mice. Changes in the expression of myelination-related peptides were reciprocal to the changes in the *Gsk-3β* expression, which is in accordance with the literature. In non-stressed male *Tph2*^{+/-} mice we found lowered expression of *Plp1* and *Mbp* in the hippocampus. Taken together, these changes indicate the possibility of compromised myelination of neurons, which may contribute to the observed behavioral alterations, as dysregulated myelin formation disrupts conductivity and, thus, connectivity in the brain.

Overall, in *Tph2*^{+/-} mice of both sexes, we found a stress-induced increase in aggression, as well as decreased stress susceptibility and anxiety in naïve mice, the behavioral features similar to those observed in *Tph2* knockout mice (*Tph2*^{-/-}). We also showed signs of altered myelination and altered catecholamine metabolism in the brain regions implicated in aggression and emotional regulation.

Secondly, we used mice of both sexes with GM3 synthase knockout (*St3gal5*^{-/-}), which are lacking major brain gangliosides. Disruption in ganglioside metabolism in humans leads to devastating neurological impairments, but more subtle alterations may contribute to an increased risk of neurodevelopmental disorders, including ADHD. However, the role of ST3GAL5 in endophenotypes of ADHD and ASD has been poorly investigated and no data are available in the corresponding knockout mouse model. Our studies demonstrated elevated measures of aggressive and dominant behavior in mutants of both sexes. Notably, we

found decreased expression of PLP1 in the cerebral cortex of *St3gal5*^{-/-} mice at both gene and protein levels, suggesting compromised myelination. As there is an established link between neurodevelopmental disorders and infectious diseases suffered in a broad time window from the prenatal period to adolescence, we challenged *St3gal5*^{-/-} mice with a model of infectious disease causing inflammatory stress. It led to an aggravation of changes in the behavior of *St3gal5*^{-/-} mice, and an aberrant inflammatory response to systemic inflammation was found in the CNS and in the periphery. Altogether, our findings support the view that ganglioside deficiency, dysmyelination, and neuroinflammation are interrelated, which may underlie neurodevelopmental disorders of various genetic origins.

Thus, in both *Tph2* and *St3gal5* mouse lines, we found signs of inflammation and altered myelination. Our data has led us to hypothesize that impaired myelination and neuroinflammation may be sharing mechanisms leading to disrupted brain connectivity and subsequent aggressiveness, hyperactivity, and other behavioral alterations that are characteristic of ADHD- and ASD-like features. Based on our results, we speculate that these mechanisms are common for different genetic aberrations. Our results further support the findings from recent genome-wide association studies, suggesting that variation in multiple pleiotropic genes interacting with environmental challenges may cause similar dimensions of symptoms in neurodevelopmental psychiatric disorders.

Samenvatting

In ons werk onderzochten we de mechanismen die ten grondslag liggen aan menselijke neurologische ontwikkelingsstoornissen die gekenmerkt worden door abnormale agressie en emotionele afwijkingen, gerelateerd aan endofenotypen voor neuropsychiatrische ontwikkelingsstoornissen. Hiervoor zijn genetisch gemodificeerde muizen gebruikt met een gebrek aan tryptofaanhydroxylase-2 (Tph2), betrokken bij de synthese van serotonine (5-HT), en muizen met een gebrek aan lactosylceramide-alfa-2,3-sialyltransferase (ST3GAL5, ookwel bekend als GM3-synthase), verantwoordelijk voor de synthese van de precursor van de belangrijkste gangliosiden in de hersenen, GM3. Genetische variatie in het *TPH2*-gen en veranderingen in de synthese en afbraak van gangliosiden door het *ST3GAL5*-gen dragen bij aan neurologische ontwikkelingsstoornissen bij mensen. Zowel *TPH2*- als *ST3GAL5*-deficiënte muizen vertonen kenmerken van neurologische en psychiatrische ontwikkelingsstoornissen, zoals aandachtstekort-hyperactiviteitstoornis (attention-deficit hyperactivity disorder [ADHD]) en autismespectrumstoornissen (ASS), met kenmerken als hyperactiviteit, verhoogde impulsiviteit en agressie. Daarbij vertonen zij verminderde executieve functionaliteit en zijn er aanwijzingen voor achterblijvende myelinisatie van de hersenen. Naast genetische aanleg kunnen omgevingsfactoren, zoals emotionele stress en inflammatoire stress door infecties, bijdragen aan de pathogenese van deze stoornissen.

Om de bijdrage van omgevingsfactoren in experimentele muismodellen te onderzoeken, is gebruik gemaakt van blootstelling aan

een roofdier en geïnduceerde inflammatoire stress. De genetische invloed op psychiatrische stoornissen betreft volgens de huidige opvattingen genen die betrokken zijn bij de vroege neurologische ontwikkeling en de opbouw van hersencircuits, die bij deze processen een belangrijke rol spelen in de ontwikkeling van psychiatrische stoornissen. In dit licht hebben we twee genetische modellen gebruikt die gedeeltelijk vergelijkbare gedragsfenotypen vertonen, om de mogelijk gemeenschappelijke neurobiologische mechanismen te identificeren die leiden tot afwijkend gedrag, zoals verhoogde agressie.

Ten eerste hebben we muizen met een genetische gedeeltelijke deficiëntie van *Tph2* gebruikt. In overeenstemming met eerder aangetoonde abnormale agressie bij mannelijke heterozygote *Tph2* (*Tph2*^{+/-}) muizen die onderworpen werden aan omgevingsstressoren, vonden we na stressvolle blootstelling aan ratten zowel bij mannelijke als vrouwelijke mutanten een toename van agressie en dominant gedrag, en een gebrek aan neutraal sociaal gedrag. Naïeve mannelijke *Tph2*^{+/-}-muizen die werden uitgedaagd met onbekende omstandigheden vertoonden verhoogde exploratieve activiteit en minder hulpeloos gedrag dan *wildtype* (wt) controle-muizen in een aangepaste Porsolt zwemtest. Verminderde immobiliteit werd ook waargenomen bij naïeve vrouwelijke *Tph2*^{+/-}-muizen, hetgeen duidt op verminderde stressgevoeligheid en locomotorische hyperactiviteit. Dit is een belangrijk kenmerk van neurologische ontwikkelingsstoornissen. Bij beide geslachten van *Tph2*^{+/-}-muizen werden dus verhoogde stressbestendigheid en stress-geïnduceerde agressie waargenomen.

We suggereren overeenkomsten in de neurobiologische mechanismen achter gedragsveranderingen bij mannelijke en vrouwelijke muizen. In navolging van eerder onderzoek dat veranderingen in het 5-HT-metabolisme aantoonde, gebruikten we *high-performance liquid chromatography* (HPLC) om het gehalte van dopamine en noradrenaline in de hersenen van mannelijke muizen te bepalen. Stress-geïnduceerde veranderingen in het dopamine- en noradrenaline-metabolisme waren het meest uitgesproken in de amygdala. Daar waren bij gestreste *Tph2*^{+/-}-muizen de dopamineconcentraties drastisch verlaagd en de dopamineturnover verhoogd, in tegenstelling tot wt-controles en niet-gestreste mutanten. De turnover van noradrenaline in de amygdala was juist verlaagd bij de mutant-muizen. In de prefrontale cortex (PFC) van de gestreste *Tph2*^{+/-}-muizen vonden we een sterk verlaagde dopamineconcentratie en een verhoogde turnover, in vergelijking met wt-controlemuizen. Deze veranderingen zouden de activiteit van de PFC kunnen veranderen en zo de top-down controle van impulsieve agressie kunnen beïnvloeden. We speculeren dat de waargenomen neurochemische verschillen tussen wt-controlemuizen en *Tph2*^{+/-}-muizen betrokken zijn bij de gebreken in executieve functionaliteit, die afhankelijk is van de functionele interactie tussen de PFC en de amygdala bij mutanten.

Bij gestreste vrouwelijke *Tph2*-muizen van beide genotypen, toonde RT-qPCR een verhoogde expressie van 5-HT_{2A} in de PFC die gepaard ging met gedragsveranderingen. Bij vrouwelijke *Tph2*^{+/-}-muizen was de expressie van de 5-HT_{1A}-receptor gedownreguleerd. Samen

suggereren deze twee bevindingen een interactie tussen stress en genotype, die het 5-HT-systeem in de hersenen beïnvloedt, wat gevolgen heeft voor de executieve functionaliteit. De expressie van glycogeensynthase-kinase-3-beta (*Gsk-3 β*) in de PFC was verlaagd bij niet-gestreste vrouwelijke *Tph2^{+/-}*-muizen. GSK-3 β wordt beschouwd als een moleculair knooppunt van angst en nood, en is een bekende fysiologische mediator van verhoogde synthese van pro-inflammatoire cytokines. Samen met de verminderde hulpeloosheid in de gemodificeerde zwemtest, kan de verlaagde *Gsk-3 β* wijzen op een verminderde gevoeligheid van *Tph2^{+/-}*-mutanten voor stress.

Voor het eerst vonden we in de PFC van niet-gestreste vrouwelijke *Tph2^{+/-}*-muizen een verhoogde expressie van vier belangrijke eiwitten voor de myelineductuur: basisch myeline-eiwit (MBP), proteolipide-eiwit 1 (PLP1), myeline-geassocieerd glycoproteïne (MAG) en myeline-oligodendrocyt glycoproteïne (MOG). Stress verlaagde het expressieniveau van deze eiwitten in *Tph2^{+/-}*-muizen. In overeenstemming met de literatuur was er een verband tussen veranderingen in de expressie van myelinisatie-gerelateerde peptiden en veranderingen in de *Gsk-3 β* -expressie. In niet-gestreste mannelijke *Tph2^{+/-}*-muizen vonden we een verlaagde expressie van *Plp1* en *Mbp* in de hippocampus. Samen wijzen deze bevindingen op de mogelijkheid van gecompromitteerde myelinisatie van neuronen. Dit kan bijdragen aan de waargenomen gedragsveranderingen, aangezien ontregelde myelinevorming de geleiding, en dus de connectiviteit, in de hersenen verstoort.

Over het geheel vonden we bij *Tph2*^{+/-}-muizen van beide geslachten een stress-geïnduceerde toename van agressie, en een afname van stressgevoeligheid en angst bij naïeve muizen. Deze gedragskenmerken zijn vergelijkbaar met het waargenomen gedrag van *Tph2*-knockout-muizen (*Tph2*^{-/-}). Daarnaast vonden we aanwijzingen voor veranderingen in de myelinisatie en het catecholamine-metabolisme, in de hersengebieden die betrokken zijn bij agressie en emotieregulatie.

Ten tweede hebben we muizen van beide geslachten gebruikt met GM3-synthase-knockout (*St3gal5*^{-/-}), die daardoor belangrijke hersengangliosiden missen. Verstoring van het ganglioside-metabolisme bij mensen leidt tot verwoestende neurologische stoornissen, maar subtielere veranderingen kunnen bijdragen aan een verhoogd risico op neurologische ontwikkelingsstoornissen, waaronder ADHD. Echter, de rol van ST3GAL5 in endofenotypen van ADHD en ASS is slecht onderzocht en er zijn geen gegevens beschikbaar van het corresponderende knockout-muismodel. Onze studies toonden verhoogde maten van agressief en dominant gedrag aan bij mutanten van beide geslachten. In het bijzonder vonden we een verminderde expressie van PLP1 in de hersenschors van *St3gal5*^{-/-}-muizen op zowel gen- als eiwitniveau, hetgeen wijst op een verminderde myelinisatie. Omdat er een verband bestaat tussen neurologische ontwikkelingsstoornissen en infectieziekten optredend tussen de prenatale periode tot aan adolescentie, hebben we *St3gal5*^{-/-}-muizen geconfronteerd met een modelziekte waardoor inflammatoire stress werd veroorzaakt. Dit leidde

tot een verergering van het afwijkende gedrag van *St3gal5*^{-/-}-muizen. Een afwijkende ontstekingsreactie op systemische ontstekingen werd gevonden in het centraal zenuwstelsel en in de periferie. Onze bevindingen ondersteunen de opvatting dat ganglioside-deficiëntie, dysmyelinisatie en neuro-inflammatie met elkaar in verband staan, wat ten grondslag kan liggen aan neurologische ontwikkelingsstoornissen van verschillende genetische oorsprong.

We vonden dus zowel bij *Tph2*- als bij *St3gal5*-muizenlijnen aanwijzingen voor inflammatie en veranderde myelinisatie. Onze gegevens hebben geleid tot de hypothese dat verminderde myelinisatie en neuro-inflammatie mechanismen kunnen delen die leiden tot verstoorde hersenconnectiviteit en daaropvolgend agressiviteit, hyperactiviteit en andere gedragsveranderingen die kenmerkend zijn voor ADHD- en ASS-achtige beelden. Op basis van onze resultaten speculeren wij dat gemeenschappelijke mechanismen betrokken zijn bij verschillende genetische afwijkingen. Onze resultaten ondersteunen verder de bevindingen van recente genomwijde associatiestudies, die suggereren dat een variatie van meerdere pleiotrope genen, in wisselwerking met omgevingsfactoren, vergelijkbare symptoomdimensies van neurologische ontwikkelingsstoornissen kan veroorzaken.

Impact

Neurodevelopmental disorders include, for example, attention-deficit/hyperactivity disorder (ADHD) with prevalence estimates ranging from 5 to 12% and autism spectrum disorders (ASD) with estimated prevalence from 0.6 to 2.3%. Marked aggression is one of the common symptoms: according to epidemiological research, the prevalence of abnormal aggression is estimated in the range from 5.7% in ADHD to about 68% in ASD. The World Health Organization categorizes aggression and violence among the top 20 causes of disability, so there is an evident need to understand the neurobiology of aggressive behavior with a view to developing new treatments.

In the present study, we aimed to investigate the impact of environmental adversities on neurobiological correlates of two experimental genetic animal models and study the validity of these models as models of gene $\times$ environment interaction in neurodevelopmental disorders. Genes affected in these models were shown to be the risk factors for neurodevelopmental disorders.

The interaction of an individual's genetic background with adverse environmental experiences (gene $\times$ environment interaction) is one of the main pathogenic factors for these disorders, but not many models are available to mimic this situation experimentally.

We used mice with a genetic decrease in neuronal serotonin, a model of gene $\times$ environment interaction in male aggression. Mutation in the gene, which is partially inactivated in these mice, was associated with neurodevelopmental disorders, including ADHD and ASD. We showed

that in these mice emotional stress-related aggression is accompanied by substantial changes in the brain neurochemistry of the regions crucial for aggressive, emotional, and social behavior. For these mice, no data on the behavior of females was previously available. Surprisingly, although aggression is thought to be outside of the natural behavioral repertoire of female mice, we found aggressive behavior in female mice with deficiency of brain serotonin after emotional stress.

This behavior was accompanied by prominent changes in brain molecular markers, which were previously shown to be involved in aggression and depressive behavior. One of these markers is also implicated in inflammation. Notably, in both male and female mice with a serotonin deficiency we found signs of alteration in myelination, a neurodevelopmental process ensuring proper signal propagation in the nervous system, and, thus, functional connectivity, which was shown to be affected in many psychiatric disorders.

Secondly, for the first time, we studied the social behavior of mice with a genetic deficit of major brain gangliosides — ubiquitous molecules playing various roles in the CNS. In humans, lack of brain gangliosides leads to devastating neurodevelopmental deficits, accompanied by severe intellectual disability, growth retardation, and seizures. These mice, however, do not mirror these deficits completely due to compensatory mechanisms. Thus, these mice are considered as a model of more subtle neurodevelopmental anomalies linked to brain gangliosides. Genetic variants of one of the genes involved in brain ganglioside synthesis were found to be associated with ADHD and ASD.

In ganglioside-deficient mice we have found aberrant social and dominant behaviors, as well as signs of repetitive behaviors, which are reminiscent of ASD-like syndrome. These findings were complemented by increased inflammatory markers, which are also known to be increased in patients with ASD. Additionally, as in the case with the first mouse model, we found signs of altered myelination in both sexes of ganglioside-deficient mice. We also found an altered response to inflammatory stress accompanied by increased aggression and aberrant social behavior in these mice, thus underpinning the importance of gene $\times$ environment interaction in the pathogenesis of excessive aggression.

Thus, in both mouse lines we found abnormal aggression and other alterations in social behavior as a consequence of environmental stressors, which suggests them as promising models for studies of the gene $\times$ environment interaction in ADHD and ASD.

Our work also offers a new model of female aggression. Such models are very scarce, and female aggression demonstrated in serotonin-deficient mice may also prove itself useful as a tool for studies of gene $\times$ environment interaction in female aggression.

We also are the first to report signs of alterations in myelination in these models. Myelination and neuroinflammation are interrelated processes, and with our results we may set the direction for further investigations, as the data on the role of alterations in myelination and brain connectivity in neurodevelopmental disorders such as ADHD and ASD are scarce. Overall, we assume that our work provides further addition to the idea that the interaction between various genetic and

environmental factors may affect converging mechanisms leading to neurodevelopmental pathologies such as ADHD and ASD.

We consider our primary target group to be researchers working in the field of neurobiology of aggression and neurodevelopmental disorders; however, our results are relevant also for the medical community, as they provide data for the development for new pharmacological treatments. The data will be published in peer-reviewed journals and presented at national and international conferences.

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About the author

Evgeniy Svirin was born on January 30th, 1991, in Moscow, USSR (now Russia). In 2010 – 2015 he studied physiology at the Biological Faculty of Lomonosov Moscow State University. During his undergraduate studies, he was involved in research on electroencephalographic features of schizophrenia under the supervision of Dr. Zhanna Garakh at the Laboratory of Human Higher Nervous Activity, Institute of Higher Nervous Activity and Neurophysiology of Russian Academy of Sciences, and later on EEG- and eye-tracking-based brain-computer interfaces at the Department of Cognitive Neurotechnologies, National Research Center “Kurchatov Institute”.

After graduating in 2015 with MS in physiology, Evgeniy studied magnetoencephalographic correlates of intention in a joint research project between Kurchatov Institute and Moscow State University of Psychology and Education under supervision of Dr. Sergei Shishkin. In October 2016 after finishing the project, he started to work as a junior researcher under the supervision of Prof. Tatyana Strekalova and Prof. Klaus-Peter Lesch at the Laboratory of Psychiatric Neurobiology in the I.M. Sechenov First Moscow State Medical University (Sechenov University). In 2017 he was appointed as an external Ph.D. student at the Department of Neuroscience, School for Mental Health and Neuroscience, Maastricht University, Netherlands. He was doing his PhD research on mouse models combining both genetic and environmental factors of pathogenesis of neurodevelopmental disorders and excessive aggression, under the immediate supervision of Prof. Tatyana Strekalova

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In 2016 – 2019, he undertook multiple travels to Germany, where he was trained in research methods at the Division of Molecular Psychiatry of Würzburg University Clinic with a grant support of German Academic Exchange Service (DAAD). During this traineeship, Evgeniy learned methods of immunohistochemical assays under supervision of Dr. Andrea Forero and Hsing Ping Ku, cell and slice electrophysiology under supervision of Prof. Erhard Wichmeyer and Dr. Sina Kollert, mammal cell cultures under supervision of Katharina Gerber, RNA extraction under supervision of Dr. Natalia Markova, RT-PCR under supervision of Gabriela Ortega. Evgeniy had an opportunity to undertake training on animal research and manipulations according to FELASA category B at the University of Lisbon and participated in workshops at the Würzburg University Graduate School of Life Sciences.

In between travels, Evgeniy participated in animal experiments in Moscow, gaining a sound experience in practical running of tests for anxiety and depressive-like behaviour, social, motor, and cognitive functions and stress models, brain dissection and surgery under supervision of Prof. Strekalova. He attended international and local conferences, such as FENS Forum of Neuroscience in Berlin, Germany; Göttingen Meeting of the German Neuroscience Society in Göttingen, Germany; Inflammation and oxidative stress in translational research workshop in Estoril, Portugal; and numerous other conferences, where he gave in total 4 oral presentations and presented 8 posters. He contributed

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Presentations at domestic and international conferences:

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- **Svirin E.**, Nuzhdin Y., Fedorova A. “A preliminary study of fixation-related potentials during gaze-fixation controlled gaming”, XVIII School and Conference of Young Scientists on Physiology of Higher Nervous Activity and Neurophysiology, Moscow, Russia, 29-30.10.2014. (oral presentation)

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