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# Přenos emocí a neurostrukturální změny u myších modelů autismu

# Emotional transition and neurostructural changes in mouse models of autism

Diploma thesis

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**Prohlášení**

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V Praze, 1. 8. 2022 ...……

Bc. Rozálie Nováková

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**Abstrakt**

Poruchy autistického spektra jsou skupinou neurovývojových poruch, které se projevují stereotypním chováním a poruchou v sociálním chování a komunikaci. Celková etologická heterogenita zatím stále nebyla plně objasněna. Je proto žádoucí se zaměřit na experimentální výzkum, zejména s využitím animálních modelů, s jejichž pomocí lze vyvíjet léčiva či hledat cesty k plnému uzdravení. V této práci proto navrhuji novou modifikaci testu pro evaluaci přenosu emoční informace mezi myšmi, která by znatelně rozšířila množství informace o sociálním chování myší získaném behaviorálními testy. Protokol podobného testu byl již nedávno publikován, nicméně se zatím stále ještě nezařadil do baterie běžných behaviorálních testů pro myši. Výsledky potvrzují možnost měření přenosu strachu mezi myšmi, ovšem pouze během jejich přímé komunikace s pomocí etologické evaluace a nikoli následovně skrze standardizovaný test měřící úroveň úzkosti. Self-grooming, tedy péče o srst, se ukázala být jediným etologickým parametrem natolik citlivým, aby ukázal signifikantní rozdíl mezi stresovanou a kontrolní skupinou, může být proto považován za nejvhodnější parametr pro danou evaluaci. Nicméně, statistické výsledky byly značně ovlivněné vysokou variabilitou individuálního chování jednotlivých myší. Behaviorální test přenosu emoční informace mezi myšmi by našel užitečné uplatnění zejména v experimentech s animálními modely Poruchy autistického spektra, například u myšího modelu 16p11.2 delece, který je aktuálně nejběžnějším myším experimentálním modelem tohoto onemocnění. V propojení s neurostrukturálními změnami typickými pro tento model bychom se dostali ještě blíže k pochopení komplikované podstaty autismu.

**Klíčová slova**

Poruchy autistického spektra, myší model, přenos emocionální informace, behaviorální test

**Abstract**

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterised by repetitive behaviour and impairments in social behaviour and social communication. The whole aetiological heterogeneity is still not fully elucidated. It is then very important to focus on experimental research, especially on animal models, to help with drug development and recovery. To broaden the variability of focus of behavioural tests on mouse sociability, a new modification of a test to assess transfer of emotional information was proposed. A similar test was published recently for the first time, but it is still not common to use it in mice. Results show that it is possible to measure transfer of fear between conspecifics only during their immediate direct encounter through behavioural evaluation, but not in further standardised anxiety-evaluating tests. Self-grooming behaviour was the only parameter significantly affected by transferred anxiety in the experimental setup used, and therefore should be considered as the most sensitive behavioural parameter describing animal emotional state. However, the variability in individual animal behaviour is still considerably large, which confounds the results to a great degree. Such a behavioural test for transfer of emotional information may be especially useful in experiments with genetic models of ASD, for example the 16p11.2del mouse model, currently the most commonly used model of ASD. Together with a link to neurostructural changes in this model, it would bring us closer to understanding the nature of autism pathology.

**Keywords**

Autism Spectrum Disorder, mouse model, transfer of emotional information, behavioural test

# Table of Contents

# List of abbreviations

| | |
|---|---|
| ACC | Anterior cingulate cortex |
| AD | Autistic Disorder |
| ASD | Autism Spectrum Disorder |
| ASR | Acoustic Startle Reflex test |
| BOLA2 | BolA-like protein 2 |
| BP | Breakpoint |
| CC | Corpus callosum |
| CDH8 | Chromodomain-helicase-DNA-binding protein 8 |
| CHL1 | Cell-adhesion molecule L1-like |
| CNTN | Contactin |
| CNTNAP2 | CNTN-associated protein-2 |
| CNVs | Copy Number Variants |
| COPDD | Childhood Onset Pervasive Developmental Disorders |
| DF | Degrees of freedom |
| Dim | Dimension |
| DSM | Diagnostic and Statistical Manual of Mental Disorders |
| EPM | Elevated Plus Maze |
| FAMD | Factor Analysis of Mixed Data |
| FI | Fronto-insular cortex |
| ITI | Inter-trial interval |
| KCTD13 | Potassium Channel Tetramerization Domain Containing 13 |
| LDB | Light/Dark box |

| | |
|---|---|
| MFA | Multi Factorial Analysis |
| MVP | Major vault protein |
| PBS | Phosphate-buffer solution |
| PD | Postnatal day |
| PDD-NOS | Pervasive Developmental Disorder Not Otherwise Specified |
| PFA | Paraformaldehyde |
| PI3K | Phosphoinositide 3-kinase |
| PTEN | Phosphatase and tensin homolog |
| RBFOX | RNA binding protein, fox-1 homolog |
| RT | Room temperature |
| SFARI | Simons Foundation Autism Research Initiative |
| Shank | SH3 and multiple ankyrin repeat domains protein |
| VEN | Von Economo neurons |

# 1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder that affects 1 in 100 individuals worldwide (Zeidan et al., 2022). This condition is characterised by two core symptoms: repetitive behaviour and impaired social behaviour and social communication. Additionally, around 70 % of patients suffer from one or more comorbid diseases, e.g. Attention-Deficit Hyperactivity Disorder, intellectual developmental disorder, anxiety, gastro-intestinal problems and epilepsy (APA, 2013). The whole aetiological heterogeneity is still not fully elucidated. Both preclinical and clinical research is therefore crucial to elucidate the complex nature of this mental illness and lay the groundwork for drug development. Animal models play an essential role in understanding etiopathogenesis and pathophysiology of psychiatric diseases. Currently, there is more than two thousand known ASD mouse models (*SFARI Gene*, 2022). Animal models demand not only resemblance to pathological changes observed in patients but also reproducibility of human symptomatology. However, due to the multifactorial nature of psychiatric disorders, their heterogeneity in etiological mechanisms and broad symptomatology many animal models mimic a limited number of specific disease traits (Victor Nani et al., 2020). Such endophenotypes are still very important and useful in preclinical studies.

Impairments in social behaviour have a very wide diversity and there are many evaluative approaches to choose from. In animal research social preference and social dominance are measured most commonly, but this do not cover the whole variety of animal sociability and is biased by subject's anxiety level (Kondrakiewicz, Kostecki, et al., 2019). That is why I proposed a modification of emotional transfer test for mice, to evaluate transfer of emotional information between mice, an ability analogous to human empathy. In this thesis, I will therefore present validation of a protocol for transfer of negative emotional information, which is one method of rodent communication beside direct and other well described methods such as scent or aggression. Transfer of emotional information is also a relevant feature in autism models.

A successfully validated protocol was planned to be used in a 16p11.2del mouse model of ASD alongside standardised behavioural tests to show its potential in autism research. The 16p11.2del mouse model is currently the most common mouse model of ASD, which reflects one of the most common causes of ASD in humans, genetic variants in the 16p11.2 region. Results will point on important features of animal behaviour serving as a possible measurable indices of transfer of emotional information.

# 2. Autism Spectrum Disorder

The term autism was used for the first time as one of the symptoms of schizophrenia. It was defined in 1911 by Eugen Bleuler as an aberrant relation to reality in terms of an overly dominant inner life to external world (Bleuler, 1911, transl. Zinkin, 1950). However in 1943 psychotherapist Leo Kanner officially described autism as a distinctive disorder, today known as Autism Spectrum Disorder (ASD) (Kanner, 1943).

What is today considered as Autism Spectrum Disorder is a continuum of Leo Kanner's Infantile Autism. The phenotype, which he described in the original work "Autistic Disturbances of Affective Contact" (Kanner, 1943), mostly mirrors the current diagnostic approach of Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5) (presented in Table 1), despite the fact that autism definition and diagnostic approach were put through extensive modifications throughout the time. During the time of autism's initial recognition, two diseases were termed: Kanner's Infantile Autism and Childhood Onset Pervasive Developmental Disorders (COPDD) (APA, 1987). Infantile Autism was then renamed to Autistic Disorder (AD); and COPPD to Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS) in 1994 (APA, 1994). Other than renaming, symptomatology definition remained almost unchanged. A very important feature of this symptomatology was language impairment. Until 1994, muteness or language aberrance were an essential symptom of autism. However, from this year autistic children with typically developed language skills were diagnosed with Asperger's disorder (APA, 1994). As a result, autism was differentiated into three separated conditions namely Autistic Disorder, COPPD and Asperger's syndrome. It was these three disorders together with Childhood disintegrative disorder, a rare disease characterised by a late-onset sudden reversal of language, mental and motor skills, that were in 2013 combined into one Autism Spectrum Disorder that we know today (APA, 2013). These dynamic changes in autism symptomatology thorough years complicates today's research, since former findings were based on a different diagnostic criteria (Hansen et al., 2015). First of all, previous observations became invalid in current viewpoint and secondly, it complicates large-scale, time dependent meta-analysis and estimation of prevalence.

Numerous separate mental conditions recognised in the precedent years led to a currently unified mental illness that distinguish only three levels of severity, i.e. requiring support, requiring substantial support and requiring very substantial support (APA, 2013). Today, ASD is recognized as a pervasive neurodevelopmental disorder that affects 1 in 100 individuals worldwide (Zeidan et al., 2022). There are two core symptoms of ASD: restricted repetitive behaviour; and impaired social interaction and social communication. Nevertheless, the symptomatic range of ASD is usually much wider as one or more comorbid mental illnesses such as intellectual disability.

| Kanner 1943 | | DSM-5 (2013) | |
|---|---|---|---|
| Social skills | Inability to relate themselves to other people and situations, good relation to stable objects with minimal relation to people | Deficits in social-emotional reciprocity (reduced sharing, emotions, affect) | Social communication and social interaction |
| | Extreme autistic aloneness | Deficit in developing, maintaining and understanding relationships | |
| | Failure to assume at any time an anticipatory posture | | |
| Language | Mute or limited only to parrot-like repeating | Deficit in nonverbal communication | |
| | Literalness of words with only a fixative meaning | | |
| Stereotypy | Monotonously repetitious noises and motions | Stereotyped or repetitive motor movements, use of objects, or speech | Restricted, repetitive patterns of behaviour, interests, or activities |
| | Anxiously obsessive desire for the maintenance of sameness | Insistence on sameness, inflexible adherence to routines, or ritualized patterns of verbal or nonverbal behaviour | |
| | Marked limitation in spontaneous activities | | |
| | | Highly restricted, fixated interests that are abnormal in intensity or focus | |
| Hypersensitivity | Intrusion from loud noises and moving objects | Hyper- or hyporeactivity to sensory input or unusual interest in sensory aspects of the environment | |
| Memory | Good cognition potentialities with excellent rote memory | | |

*Table 1* ***Symptoms of Autism Spectrum Disorder***
*Comparison between listed symptoms in Kanner´s original work (Kanner, 1943) and current symptoms from DSM-5 (APA, 2013)*

Attention Deficit Hyperactive Disorder, structural language disorder, sleep disturbances and developmental coordination disorder usually accompany this illness as well (APA, 2013). Even though there has been an immense amount of effort and energy spent on experimental and clinical research since Leo Kanner first described it, the exact pathophysiological mechanisms are still unclear. That is why the importance of animal models in preclinical research cannot be underestimated. Animal models that meet criteria of construct, face and predictive validity aim to reveal the underlying biological basis of the disorder and serve as a tool for more effective drug development that would help with more efficient ASD patients' treatment.

## 2.1. Comorbidities

Comorbidity of ASD with other psychiatric condition is highly frequent. In about 70 % of cases, ASD is accompanied by one comorbid disorder, in about 40 % with two or even more comorbid disorders (APA, 2013). Intellectual developmental disorder is frequently concurrent with ASD (APA, 2013). In the United States, intellectual developmental disorder coincided with ASD in 31 % (IQ ≤ 70), and 25 % (IQ 71-85) of eight-year old diagnosed children (Baio et al., 2018). Prevalence of other, similarly or less frequent, ASD comorbid mental health diagnoses reaches the following levels: attention-deficit hyperactivity disorder (28 %), anxiety disorders (20 %), sleep-wake disorders (13 %), disruptive, impulse-control and conduct disorders (12 %), depressive disorders (11 %), obsessive-compulsive and related disorders (9 %), bipolar and related disorders (5 %) and schizophrenia spectrum and psychotic disorders (4 %). ASD also often co-occurs with non-psychiatric diseases, e.g. gastro-intestinal problems, asthma, allergies, epilepsy, or immunodeficiency, which are all more prevalent in ASD than in the general population and which reflect heterogeneity of ASD etiopathology (Muskens et al., 2017). 12% of ASD diagnosed children also show speech abilities below the normal range (Cleland et al., 2010), especially in receptive but not expressive language (Koning & Magill-Evans, 2001).

## 2.2. Prevalence

ASD prevalence estimation was highly confounded by numerous changes in diagnostic criteria. Throughout the history and different parts of the world, prevalence has ranged extremely – from 0.02 % (2/10 000) in China before 1980 (Sun & Allison, 2010) to 3.13 % (313/10 000) in Iceland in 2015 (Delobel-Ayoub et al., 2020). Obviously, the growth of prevalence cannot be easily explained by simple increase of the disorder occurrence. As the years passed by, the diagnostic criteria changed several times and so did the socioeconomic situation of many developing regions allowing people to afford healthcare, therefore getting a chance to be diagnosed. Also, autism was highly popularised through mass-media, cinematic production and literature. The chance of parental ASD recognition in their children has therefore risen considerably.

Numerous changes in diagnostic criteria confound studies and complicate meta-analyses. One study found that the 33 % increase in prevalence can be explained by the change of criteria itself, a 42 % increase by the inclusion of outpatient data, giving 60 % increase in prevalence due to both change in diagnostic criteria and case-reporting practices (Hansen et al., 2015). To give an example, prevalence in a Swedish city Göteborg in 1997 was estimated to be 31/10 000 for Autistic Disorder and 46/10 000 for Autistic Disorder and PDD NOS combined when DSM-4 criteria were used. However, under Kanner's criteria only a 10/10 000 prevalence was measured (Ardvisson et al., 1997). Continuous broadening of diagnostic criteria, e.g. inclusion of autistic children without language impairments or with milder phenotypes is one of the important reasons for this dissimilarity of prevalence. In conclusion, in studies different diagnostic criteria have to be considered particularly carefully.

However, several long-term studies showed that an increase in prevalence cannot be explained only by changes in criteria. Increases in ASD prevalence, yet lacking symptom criteria influence, can be demonstrated by studies conducted in the same area and by the same investigator. Such research has shown that in Sweden, prevalence rose 3.5 fold from 0.42 % in 2001 to 1.44 % in 2011 (Idring et al., 2015). A similar result was found in Catalonia, where the increase was 3.3 fold, from 0.07 % in 2009 to 0.23 % in 2017 (Pérez-Crespo et al., 2019). Meta-analysis of global ASD prevalence show a 1.6 fold increase from 62/10 000 in 2012 to 100/10 000 in 2022 (Elsabbagh et al., 2012; Zeidan et al., 2022). Improvement in the early-diagnosis, especially in areas like Middle-East and Africa, which were not previously represented, is in part responsible for higher rates of prevalence over time. As of today, 100/10 000 is the most reliable estimate we have.

## 2.3. Etiology

The amount of available information about the possible etiology of Autism Spectrum Disorder highlights the heterogeneity of this disease. To give an example, SFARI gene (Simons Foundation Autism Research Initiative), a systematic database for ASD research, currently recognizes more than 1 089 human genes, 2 290 copy number variants, 1 279 protein interactions and 2 296 animal models, all potentially linked to ASD (*SFARI Gene*, 2022).

### 2.3.1. Genetic liability

Autism Spectrum Disorder is a genetically heterogeneous disease caused by a combination of inherited and *de novo* genetic and epigenetic alternations (Pinto et al., 2014). Moreover, environmental factors increase the severity of symptoms and probability of onset.

Common Copy Number Variants (CNVs), variations in the number of chromosomal regions between individuals with up to a 50 % heritability, cause 8 – 11 % of all cases of ASD (Gaugler et al., 2014;

(Kushima et al., 2018). An additional 2.6 – 4.7 % of cases of ASD is caused by rare and *de novo* CNVs (Gaugler et al., 2014). ASD may also develop as additional condition to already existing one. Such syndromic autism, which develops as a secondary disease, affects 60 % of individuals with Fragile X syndrome (Harris et al., 2008; Roberts et al., 2020). Additionally, syndromic ASD often occurs in tuberous sclerosis(Vignoli et al., 2015), phenylketonuria (Yoldaş et al., 2021), and Rett syndrome (Percy, 2011).

ASD-linked CNVs and mutations are found on all chromosomes including X and Y. However, several regions bear higher risk for ASD than others. Deletions in 7q, 22q13, 2q37, 18q, and X regions (Vorstman et al., 2006), duplication in 15q11q13 region, known for its connection to Angelman syndrome, have the higher risk for developing ASD (Baker et al., 2020; Kim et al., 2008; Puffenberger et al., 2012). Currently, the most ASD linked risk region is 16p11.2, found in 1 % of human patients with ASD. It was indicated as the most common autism CNV, and genetic manipulation in this region are widely used to create mouse model of this disorder (Marshall et al., 2008).

Affected genes are usually involved in chromatin remodelling, gene transcription and splicing but also in synapse function (de Rubeis et al., 2014). In ASD, chromatin remodelling is primarily epigenetically driven and CHD8 or RBFOX genes are mainly affected. Mutations in a gene encoding chromodomain-helicase-DNA-binding protein 8 (*CHD8*), a protein that recognises methylated lysine, leads to chromatin remodelling malfunction and has been linked to autism (de Rubeis et al., 2014; Wilkinson et al., 2015). Aberrances in the CHD8 gene are also often found in ASD individuals with macrocephaly (Wu et al., 2020). A tissue-specific splicing regulator *RBFOX* (RNA binding protein, fox-1 homolog) is also often mutated in ASD. Depletion of Rbfox protein leads to impaired axon initial segment assembly, cytoskeletal abnormalities and immature electrophysiological activity (Jacko et al., 2018). The results of their dysfunction nicely mirror common anomalies found in ASD.

Additionally, two important groups of proteins are often affected in ASD: cell-adhesion molecules and scaffolding proteins, both crucial for the development and proper function of the nervous system. The first large group of cell-adhesion molecules are the neuroligins, responsible for synaptic assembling, remodelling and activity (Ali et al., 2020; Gomez et al., 2021). The most often affected are neuroligin 3 and neuroligin 4X (Jamain et al., 2003). The second group are contactins (Zhao et al., 2021), especially proteins encoded by *CNTNAP2* (CNTN-associated protein-2) (D. Li et al., 2021; Penagarikano & Geschwind, 2012) and *CHL1* (Cell-adhesion molecule L1-like) (C. Li et al., 2016). And the last group of cell-adhesion proteins often linked to ASD are cadherins 9 and 10 and protocadherin 10 (Morrow et al., 2008; K. Wang et al., 2009). Shank1 and Shank3 (SH3 and multiple ankyrin repeat domains protein) are the most affected scaffolding proteins in ASD (Arons et al., 2012; Sato et al., 2012). Shank together with another ASD-risk protein Homer are both are responsible for postsynaptic density formation (X. Wang et al., 2016).

#### 2.3.1.1. 16p11.2 deletion mouse model

16p11.2 is a region that was originally linked with the microdeletion syndrome in humans. Apart from severe anatomical and morphological malformations, this syndrome also causes profound language delay, low IQ, anxiety, sleep problems and congenital analgesia (Ballif et al., 2007). Interestingly, these symptoms mirror numerous symptoms of Autism Spectrum Disorder. Further disease gene identification recognized this locus as the most profound CNV present in Autism Spectrum Disorder at approximately 1 % frequency of occurrence (Marshall et al., 2008). Since then, the region became the most frequently used mouse model of ASD.

Human 11.2 locus is located on the proximal short arm of 16$^{th}$ chromosome (BP4-BP5) and consists of 29 genes (Morson et al., 2021; USCS Genome Browser, 2022). In mice, this region clusters on the 7$^{th}$ chromosome (7F3) with the deletion usually positioned between *Giyd2* and *Sept1* genes, about 390 kb in size (Horev et al., 2011) (Figure 1).

Structural variations of this locus are linked to many neurodevelopmental disorders besides ASD. To name a few: intellectual developmental disorder, motor and developmental delay, epilepsy, schizophrenia, and obesity (Maillard et al., 2015), often present as comorbid ASD diseases. However, there is a causative relationship between particular structural anomalies and observed symptoms. While 16p11.2 inversions mostly result in asthma and obesity susceptibility (González et al., 2014, 2020), duplications have a very wide range of neurodevelopmental outcomes with the strongest link to schizophrenia (Niarchou et al., 2019; Posar & Visconti, 2020). And finally, deletions in this region has the strongest association to ASD (Crespi & Crofts, 2012).

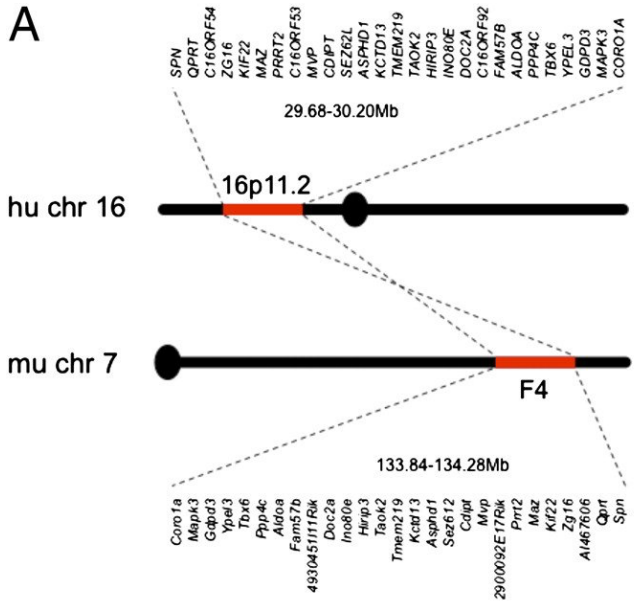



*Figure 1* ***Genes mapping to human 16p11.2 and mouse 7F4***
*Adapted from (Horev et al., 2011)*

Mouse models of 16p11.2 deletion show a high range of phenotypes, which vary depending on particular strain but also research centre (mostly C57Bl/6J mixed with 129S1/SvlmJ, C57Bl6N, B6/129S, and B6129SF1/J). However, some findings occur systematically, i.e. hyperactivity, lower anxiety, reduced sleep, reduced weight, diminished pup vocalisation with a disordered syllable pattern, worsened cognitive skills and memory (Agarwalla et al., 2020; Angelakos et al., 2017; Brunner et al., 2015; Lu et al., 2019; Lynch et al., 2020; Menzies et al., 2021; Mitchell et al., 2020; Nakamura et al., 2021; Portmann et al., 2014; Pucilowska et al., 2015, 2018; Tian et al., 2015; Yang et al., 2015).

The number of CNVs between ASD subjects differs extensively, however 5 genes were highlighted with the most contributory role in 16p11.2 deletion: *CDIPT1, SEZ6L2, ASPHD1* and most importantly *KCTD13* and *MVP*. Deletion of *KCTD13* (Potassium Channel Tetramerization Domain Containing 13) mirrors 16p11.2del phenotype, overexpression causes microcephaly and is commonly modified in ASD patients (Golzio et al., 2012). *MVP* (Major vault protein) closely interacts with *PTEN* regulating its $Ca^{2+}$-dependent nuclear translocation (Minaguchi et al., 2006; Yu et al., 2002). *PTEN* (Phosphatase and tensin homolog) is mutated in 17 % of ASD cases, especially in autistic individuals with macrocephaly (Tilot et al., 2015).

Additionally, one especially intriguing study put another gene from this region in the spotlight: *BOLA2* (BolA-like protein 2), which is involved in the maturation of cytosolic iron-sulfur proteins (Giannuzzi et al., 2019). Nuttle and colleagues found that this gene was duplicated exclusively in the *Homo sapiens* species early in the human lineage. This duplication is the reason of 16p11.2 region inclination to CNVs, because all 96 % of total human breakpoints happen exactly in the 95 kbp large *BOLA2* segment (Nuttle et al., 2016).

### 2.3.2. Neuronal morphology abnormalities in autism

The most prominent morphological and anatomical changes found in patients with ASD are altered whole-brain and regional volumes, density of neurons, changed arborisation and functional connectivity.

Unusual brain volume is a common abnormality found in children with ASD, but direction of pathological brain volumes seems to depend on the nature of ASD etiology. Newborns with ASD have significantly higher variability in occipital frontal circumference, with higher incidence of both microcephaly and macrocephaly (Crucitti et al., 2020). Several studies also described an abnormal development of brain volume in autistic children in comparison to typical children. Often, smaller to normal brain volume at birth is followed by unusually rapid growth resulting in enlarged brain volume in infants and then stabilizes around 2 years of age on either standard or enlarged volume (Bloss & Courchesne, 2007; Courchesne, 2003; Gillberg & de Souza, 2002; Sparks et al., 2002). Interestingly, the intensity of rapid

growth correlates with the severity of autistic social incapability (Hazlett et al., 2005). Additionally, while the number of genomic copies in 16p11.2 negatively correlates with brain volume, deletions in this locus leads to brain expansion (Martin-Brevet et al., 2018; Sønderby et al., 2020). Such causative relationship and its mechanism has been demonstrated in brain organoids. Urresti with colleagues studied RhoA, a member of the Rho protein family of small GTPases. They have shown that the number of RhoA replicates in the aforementioned 16p11.2 region negatively correlates with organoids volume (Urresti et al., 2021). Supplementary, a deletion of *RAB39b*, an ASD risk X-linked gene interacting with phosphoinositide 3-kinase (PI3K) in the PI3K-AKT-mTOR pathway, leads to over-proliferation and impaired differentiation of neural progenitor cells of human cerebral organoids, while in knockout mice macrocephaly and social memory deficits is observed (W. Zhang et al., 2020).

There is no consensus if adults with ASD have a larger overall brain size. Some studies found no difference between ASD patients and controls (Aylward et al., 2002; Hardan et al., 2003) while other confirmed enlargement in ASD patients, which again can be a result of heterogeneous etiology of ASD (Denier et al., 2022).

Macroscopic changes of the central nervous system reflect abnormalities at microscopic level. Axonal number, diameter, orientation and myelination are often altered in the cerebral cortex of ASD individuals (Beaulieu, 2002; Sundaram et al., 2008). Increased density is commonly found both in cortical axons and dendrites (Matsuoka et al., 2020). These changes influence cortical minicolumnar structure, neuronal functional units of prefrontal cortex. In children with autism, frontal minicolums are smaller and have lower cell-packing density due to lower size of somas in comparison with typically developing children (Casanova et al., 2002). Alterations are also found in fronto-insular (FI) and anterior cingulate (ACC) cortices, both involved in the formation of social bonds, anticipation of reward and punishment, and also in cognitive uncertainty (Critchley et al., 2001; Insel & Young, 2001; Sokoloff & Schwartz, 2002). Von Economo neurons (VENs), also called spindle cells, are so far proved to be in the V layer of FI and ACC. They are predominantly present in humans and in great apes and they project to numerous brain structures controlling autonomic functions, motor functions, emotions, and higher-level processes such as attention or risk assessment (Critchley et al., 2001; Nimchinsky et al., 1995, 1999). In Autism Spectrum Disorder, ACC and FI often have increased number of neurons, mostly VENs, also with decreased cell size and packing density (Santos et al., 2011; Simms et al., 2009). A positive correlation was found between the number of pyramidal and von Economo neurons and the severity of social interaction score in young individuals with ASD (Uppal et al., 2014). Additionally, impairments in ACC were observed in the earlier mentioned Shank3 mouse model of ASD. These mice also showed severe social deficit (Guo et al., 2019).

These results suggest that VENs and structural aberrances in ACC and FI may play a prominent role in the social deficit of ASD.

The limbic system is involved in many functions including memory, emotions and motivation brain functions altered in ASD (Guo et al., 2019). Structures of the limbic system that were consistently found altered in ASD are the amygdala and hippocampus, both involved in processing of emotions. It has been shown that patients with ASD often have enlarged volume of amygdalar neuronal cell bodies (Groen et al., 2010; Seguin et al., 2021; Xu et al., 2020). The amygdala of ASD individuals also exhibits reduced functional connectivity with the ventrolateral prefrontal cortex (Ibrahim et al., 2019), higher diffusivity of tracts in white matter and reduced branching of tracts in the temporal cortex (Gibbard et al., 2018). Moreover, the amygdala often possesses greater serotonergic axon density (Lew et al., 2020). Elevated level of serotonin, called hyperserotonemia, is a common biomarker of ASD (Muller et al., 2016). Similar to the amygdala, the hippocampus is also enlarged in autistic individuals (Groen et al., 2010; Xu et al., 2020). The reason for this could be connected to a higher number and denser packing of parvalbumin positive interneurons in the CA1 and CA3 areas of hippocampal Amon's horn (Lawrence et al., 2010).

Corpus callosum (CC), the axonal bridge between brain hemispheres, has a very intriguing role in ASD research. A congenital condition called Agenesis of corpus callosum, partial or complete absence of this brain structure, causes a variety of symptoms that noticeably overlap with ASD, mainly in social reasoning, imagination, communication and repetitive behaviour (Lau et al., 2013; Wolff et al., 2015). In both Agenesis and ASD, a considerable number of CC fibres is decreased (Frazier & Hardan, 2009; Guadarrama-Ortiz et al., 2020; Sui et al., 2018; Taylor & David, 1998). When Agenesis of corpus callosum was closely inspected for a possible misdiagnosis of ASD, it was confirmed that these two are separate conditions and that only a few percent of people with Agenesis meet the full criteria for ASD (Paul et al., 2014). However, Agenesis could be possibly a comorbid disorder with ASD and CC impairment can potentially explain some of the ASD symptoms.

The cerebellum is not only a sensorimotor and one of the vestibular centres, but to some extent a centre of cognition and emotion (Ashida et al., 2019; Schmahmann, 2019). It plays a very significant role in autism. There was even a cerebellar theory of autism proposed, where early-life cerebellar dysfunction could possibly cause severe subsequent abnormalities of structures that receive cerebellar projections. As an example may serve an incomplete neuronal pruning in prefrontal cortex resulting in an autistic phenotype (Wang et al., 2014). Additionally, an altered connectivity between the cerebellum and the prefrontal cortex and within the cerebellum itself was found as well in patients with ASD (Haghighat et al., 2021; Smith et al., 2019).

Similar to other brain regions, the cerebellum is enlarged in ASD too (Traut et al., 2018) and it has a reduced number, density, and soma size of Purkinje cells (Fatemi et al., 2002; Wegiel et al., 2014) accompanied by decreases in parvalbumin synthesis (Soghomonian et al., 2017).

# 3. Emotional transfer test

Human patients with ASD express a variety of social impairments, from social anhedonia (Chevallier et al., 2012), a lack of social orienting to social cues (e.g. eye gaze), altered social rank and dominance recognition (Dawson et al., 1998; Klin et al., 2009; Ogawa et al., 2019), to lowered empathy (Baron-Cohen & Wheelwright, 2004; Rueda et al., 2015; Shah et al., 2019). Accordingly, a variety of social impairments is found in mouse models of ASD as well, e.g. in social interactions (sniffing, following, crawling), social approach, pro-social interest, and social memory (Bolivar et al., 2007; Jamain et al., 2008; Silverman et al., 2010; Yang et al., 2011). Since the complexity and nature of social behaviours is different between humans and mice, assessing and interpreting social behaviour in mice must be adapted to the specificity of animal behaviour.

Social behaviour is tightly intertwined with communication. Rodent communication is commonly assessed with ultrasonic vocalisation evaluation. In the past, mouse vocalisation was ascribed only to non-aggressive interactions, such as mating (Gourbal et al., 2004). On the contrary, current research describes much wider variety of different calls used intentionally in different social and non-social situations, developmental stages and contexts, e.g. presence/absence of a conspecific, social isolation and mate selection (Lefebvre et al., 2020; Nomoto et al., 2018). Other rodent communication tools include visual and olfactory cues. Fear and panic are recognised by another mouse through a conspecific jumping, moving around or freezing behaviour (Kavaliers et al., 2001). Concurrently, the anxious or fearful mouse also releases stress olfactory signals. These are especially useful since they serve as warnings for the conspecific even in an absence of signs maker (Brechbühl et al., 2013).

Recognition of pain, stress or fear in other organisms is an essential evolutional tool. For example, mice prefer to eat food already eaten by other familiar mice and avoid areas where their conspecific got hurt (Baptista-de-Souza et al., 2015; Watanabe, 2012; Wrenn, 2004). This cognitive process of learning from the experience of others is called social learning and passes information about imminent threat. It tremendously increases the chances of survival, both in individuals and eventually a whole species. It was also proposed that ASD patients have altered social learning abilities (DeMayo et al., 2019; Espinosa et al., 2020).

Evaluation of information transfer about imminent threat is not a part of standard battery of tests in mice yet. Instead, research focuses on a broader understanding of evaluation of social interaction. The most

popular test used in studies of mice social interaction is three-chamber sociability test that evaluates social preference in rodents (Kaidanovich-Beilin et al., 2011; Moy et al., 2004; Reppucci et al., 2020; Yang et al., 2011). It is also common to assess social dominance with a tube test or a food competition test (Brodkin et al., 2014; Fan et al., 2019; Kraeuter et al., 2019; Lindzey et al., 1961; Merlot et al., 2004). These tests use a set up where the novelty environment of the maze may substantially bias the result. This confound is elegantly avoided in home-cage testing systems, where mice are observed in a group and safe environment. However, these systems are unaffordable for many laboratories (Jhuang et al., 2010; Kiryk et al., 2020; Puścian et al., 2016). Laborious and time demanding evaluation of ethological parameters, e.g. pro-social sniffing, following or crawling and anxiety-related rearing, digging and self-grooming is rarely used to describe mouse behaviour during social interaction (An et al., 2011).

Researchers also study direct information transfer of emotional value additionally to characteristics of rodent social interaction obtained by versatile techniques. In these, their attention is focused on either transfer of information about an imminent or remote threat (Ito et al., 2015; Jeon & Shin, 2011; Keum et al., 2016). In imminent threat information transfer, freezing is detected as a reaction of the mouse that *witnesses* a conspecific to suffer and serves as a measurement of efficacy of social learning. Assessing the transfer of pure emotional information connected to remote threat was proposed only recently. The team of Knapska was the first to publish a protocol for remote transfer of fear both for rats and mice. In the mentioned protocol, a demonstrator mouse is stressed in a distant, isolated area, usually with a series of electric shocks. Afterwards, the mouse is put back into its home cage to the naïve cage-mate called an acceptor mouse. Acceptor mouse behaviour and optionally both mice's ultrasonic vocalisation are measured subsequently. Rearing, self-grooming, sniffing to nose, anogenital area and body of the demonstrator, allo-grooming, digging of bedding, following the demonstrator, any physical contact (e.g. sleeping huddled together) and submitting or evading proposed contact are all evaluated in the frequency of occurrence and total duration of time (Kondrakiewicz, Rokosz-Andraka, et al., 2019). This protocol is still not extensively exploited, but some data already looks promising. Knapska's team successfully used this test to measure social deficit in the BTBR inbred mouse model (K. Meyza et al., 2015). BTBR T+ tf/J mice show a strong autism-like anatomical, morphological and behavioural phenotype including reduced corpus callosum and diminished social behaviour (McFarlane et al., 2008; K. Z. Meyza et al., 2013; Wahlsten et al., 2003). This protocol therefore shows a major importance for ASD research. Altered empathy and communication are core features of this disorder and it is then necessary to be able to assess these abilities in mouse models as well.

# 4. Aims of the thesis

The aim of this thesis was to modify behavioural test to assess the ability of mice to transfer emotional information (stress, fear) between conspecifics. Next, a 16p11.2del mouse model of Autism Spectrum Disorder will be developed. Consequently, its behavioural phenotype together with neurostructural changes will be evaluated.

# 5. Materials and Methods

## 5.1. Animals

I used 82 C57Bl/6N mice between 6 - 15 weeks of age for behavioural testing. Animals were bred in the animal facility of Czech Centre for Phenogenomics of the Institute of Molecular Genetics of the Czech Academy of Sciences in BIOCEV. Additionally, 16p11.2del mouse model was produced in Czech Centre for Phenogenomics at C57Bl/6J and at C57Bl/6JN background. Mutant sperm of this model was generously offered by Pavel Osten laboratory in Cold Spring Harbor (Horev et al., 2011) and then used to create experimental cohorts with in-vitro fertilisation by transgenic laboratory team. Male mice with one copy of 440 kbp region of chromosome 7 (which corresponds to human 520 kbp region of 16p11.2) were afterwards bred with wild type female mice of C57Bl/6J line. Their offspring between 1 to 13 weeks of age was used in behavioural experiments. All mice were housed under specific-pathogen-free conditions in individually ventilated cages (Techniplast) at 22 ± 2 °C with 12-hour light/dark cycle. Access to food and water was *ad libitum*.

All experiments were performed according to the European directive 2010/63/EU and were approved by the Czech Central Commission for Animal Welfare.

## 5.2. The Emotional Transfer Test

Three different protocols were used during the development and validation of emotional transfer test. All protocols were divided into four steps: (1) acclimatisation to the experimental conditions, (2) demonstrator stress induction, (3) transfer of emotional information and (4) ultimate test (Table 2). Each sex was equally divided into two groups: sham (demonstrator separation without stressing during second step) and stressed (demonstrator separation with delivery of electric shocks during second step). Within each of these groups, mice were randomly divided to a group of mice demonstrating their emotional state (demonstrator mice) and a group of mice receiving this emotional information (acceptor mice).

(1) Acclimatisation: on the day of the test, mice were brought to the testing room one hour prior to test to habituate to the room. From a pair of mice, one was randomly selected as the 'demonstrator mice' and the other as the 'acceptor mouse'. Acceptor mouse had its tail marked with a black permanent marker.

(2) Demonstrator stress induction: during this step demonstrator mouse was put in a plastic transparent chamber with wire mesh floor that was enclosed in a soundproof ventilated box with a camera hanging above (Ugo Basile srl). The mouse spent 7 minutes under visible light (40 lux) and with 2 kHz background noise. Stressed mice received electric shocks of 0.6 mA current magnitude and 1 second duration in amount specific for each protocol, while sham group stayed in the chamber for 7 minutes without any shocks.

(3) Transfer of emotional information: during this phase, demonstrator mouse was returned to the cage with acceptor cagemate and left there to move freely. Additionally, a video of animal behaviour was recorded in the second protocol.

(4) Acceptor testing: effectiveness of transfer of negative emotion between animals was validated with a given behavioural test specific for each protocol (Elevated Plus Maze, Light Dark box or Acoustic Startle test).

After accomplishing of the whole procedure, mice were returned to their homecages. Conditions of particular protocols are presented in Table 2. All equipment was cleaned with 70 % isopropanol before the first and after each animal to remove olfactory traces.

| | *First protocol* | *Second protocol* | *Third protocol* |
|---|---|---|---|
| *Prior to testing* | - | Handling, separation by pairs | Handling, separation by pairs |
| *1. Acclimatisation* | A new cage | Homecage | Homecage |
| *2. Demonstrator stress induction* | 3 electric shocks | 10 electric shocks | 6 electric shocks |
| *3. Transfer of emotional information* | The new cage | Homecage in darkness, ethological parameters evaluation | Homecage |
| *4. Acceptor testing* | Elevated Plus Maze | Light Dark Box | Startle response |

*Table 2* ***Scheme of three different protocols for Emotional transfer test***

### 5.2.1. First protocol – pilot experiment

In the first protocol, all mice were housed in groups and were separated into pairs only for the time of experiment. First and third phases were therefore conducted in a clean cage with fresh bedding and a handful of bedding from their homecage. Only male mice were used for the pilot experiment.

In the stress induction phase demonstrator mouse received three electric shocks with 30 second long inter-trial interval (ITI).

Transfer of the negative emotion between animals was validated on Elevated Plus Maze (Viewer, Biobserve). Elevated Plus Maze (EPM) is a standard tool for rodent anxiety level evaluation (Komada et al., 2008). Well lit cross-shaped maze raised about 50 cm above the floor is divided into two enclosed and two open arms. Naturally mice tend to avoid unprotected, well-lit areas i.e. open arms of EPM, however at the same time have a strong tendency to explore novelty. This so called approach-avoidance conflict can be decreased by anxiolytic drugs, which is reflected by increased number of visits and time spent in open arms (Rodgers & Dalvi, 1997). Thus, EPM serves as a tool for rodent anxiety level estimation. Each mouse was allowed to explore the maze freely for 5 minutes under 54 lux light intensity at the maze centre. The trajectory of mouse movement was recorded and its distance was automatically analysed by a tracking system software (Biobserve). The total time spent in each part of the maze (open arms, closed arms and centre) as well as walked distance, velocity and number of entries to each arm was computed automatically and further used for statistical analysis.

### 5.2.2. Second protocol

During the second protocol all mice were habituated to the handling for four consecutive days prior to experiment to minimise animals' stress induced by human during testing procedure. On the first day of handling each mouse was, as gently as possible, moved twice from its homecage to a clean cage and back. On the second day, each mouse was gently picked up and held in hands while the experimenter walked to another room and back. This procedure was repeated after a one-hour delay. On the third and fourth day, each mouse was picked up, carried around and pat, also twice a day.

At least three days prior to test, mice were separated to be kept in pairs per cage. Pairs were of the same sex and age. Mice were assigned to demonstrator and acceptor as previously described.

In the acclimatisation phase, the homecage was put in a separated room. Food pellets, water and nest building material were removed from the cage and a pair of mice was let to acclimatize for 30 minutes.

In the demonstrator stress induction phase, stressed mouse received ten shocks every 30 seconds.

Afterwards, demonstrator mouse was returned to its cagemate for further observation performed in a red light condition. A 5 minutes long video of the mice behaviour was recorded (IC Capture) and later analysed manually with the help of Behaview software. Time and frequency of rearing, self-grooming, digging behaviour of acceptor mouse as well as acceptor following and sniffing (nose to nose, nose to body and nose to anus) of the demonstrator animal was measured.

Next, the acceptor mouse was moved to Light Dark box maze (Viewer, Biobserve) for 5 minutes to evaluate animal anxiety level. LDB is another standardised tool for rodent anxiety level evaluation. In a squared shaped box divided into enclosed dark side and well lit (200 lux) open side, mouse exploration of each side is recorded and analysed. Mice have an innate aversion to brightly lit areas, but also very actively explore novelty. Exploration of the light zone is therefore conflicting and is highly affected by its anxiety-level. Similar to EPM, LDB also uses approach-avoidance conflict and also here anxiolytic drug administration increases the exploration of the light side (Bourin & Hascoët, 2003). In our experiment, the mouse was put in the middle of the dark zone at the beginning of the 5 minutes long test. The movement of the animal was video recorded and analysed by Biobserve software Viewer. A total distance walked, number of transfers between zones, latency to enter light zone and activity in each compartment was automatically calculated.

In oppose to the first protocol, naïve mice awaiting the test were spatially separated from animals already tested or being tested.

### 5.2.3. Third protocol

The habituation to experimenter and the acclimatization phase were identical with the second protocol. However, in the demonstrator stress induction phase, the stressed mouse received six electric shocks. Transfer of emotional information stage was performed under standard, dimmed lights (30 lux).

Efficacy of emotional information transfer was evaluated with Acoustic Startle Reflex test (ASR). ASR is a subject to non-associative learning i.e. habituation (to repeated acoustic stimuli) and sensitisation (by aversive stimulus) or associative learning i.e. attenuation (by conditioned appetitive stimulus), and fear potentiation (by conditioned aversive stimulus). Since the reaction of mice in this test is affected by fear, sensitisation to acoustic stimuli can be used as an animal emotional state measuring tool (Plappert & Pilz, 2001). During the ASR test, mice were enclosed in a holder with a wire floor inside a soundproof box (Med Associates). The protocol started with 5 minutes of acclimation to the experimental conditions with 65 dB background noise, followed by acoustic startle protocol. Ten acoustic startle stimuli of white noise (110 dB, 50 ms duration) were used at random length of inter-trial interval ranged between 9 and 52 seconds. Stimuli delivery was made with the constant background noise of 65dB. The amplitude of animal physical

response to the acoustic stimuli was detected automatically (Med Associates, Inc. software). All ten responses were averaged for each animal for further statistical comparison.

## 5.3. Mice genotyping

To assess the genotype of 16p11.2del mice, Polymerase Chain Reaction (PCR) assay was used followed by gel electrophoresis.

A tip of a tail was collected from each pup. The tissue was lysed in a solution of 100 µl DirectPCR (Tail) lysis reagent (Viagen, #102-T) mixed with 0.5 µl Proteinase K (P-LAB, #R 75282; 10mg of lyophilised Proteinase K dissolved in 1 ml $H_2O$) for 4 hours in 55 °C. Then, the lysate was diluted adding 400 µl of $H_2O$.

A PCR Master Mix was mixed in the order and volume as stated in Table 3. For this genotypization, two different commercial polymerases were tested: DreamTaq™ (Thermo Fisher Scientific) and GoTaq™ (Promega). Only DreamTaq™ worked, while GoTaq™ did not bring any results (data not shown) and therefore was not used further. Deoxynucleotide mix (dNTPs) was of 10 mM concentration (Sigma-Aldrich, #D7295). Forward and reversed primers were generously offered by Pavel Osten laboratory (Table 3) (Horev et al., 2011). In each PCR tube, 1µl of tissue lysate was mixed with 19 µl of Master Mix, then vortexed and quickly centrifuged. The PCR reaction was carried out in BIO-RAD T100™ Thermal Cycler according to the procedure in Table 4.

| Solution | Volume [µl] |
|---|---|
| *Sterile $H_2O$* | 15.65 |
| *dNTPs* | 0.5 |
| *Forward primer* | 0.5 |
| *Reverse primer* | 0.5 |
| *DreamTaq buffer* | 2 |
| *DreamTaq polymerase* | 0.15 |
| Total | 19.3 |

| Target loci | Size of PCR product | Forward sequence | Reverse sequence |
|---|---|---|---|
| *135k15* | 2.2 kb | 5'-CCTCATGGACTAATTATGGAC-3' | 5'-CCAGTTTCACTAATGACACA-3' |

*Table 3* ***Master Mix solution and used primers for Polymerase Chain Reaction***.

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Temperature [°C] | 94 °C | 94 °C | 55 °C | 72 °C | 72 °C | 12°C |
| Time [min:sec] | 4:00 | 0:30 | 1:00 | 2:00 | 5:00 | Forever |
| | | REPEAT 34x | | | | |

*Table 4* ***Protocol for PCR reaction in Thermal Cycler***

Electrophoresis was run in 1 % agarose gel prepared from SeaKem® LE Agarose (Lonza, #50004) dissolved in TAE buffer (40mM Tris, 20nM acetic acid, 1mM EDTA) for 30 minutes under 95 V current. Approximately 6 µl of each sample was used together with 6 µl GeneRuler 1 kb Plus (Thermo Scientific™, #SM1331) ladder used as a reference tool. Afterwards, the gel was visualised in ChemiDoc™ MP Imaging System (Bio-Rad, #1708280). Product size after deletion assay was ~2.2 kb large and the genotype was then assigned accordingly (Figure 2).

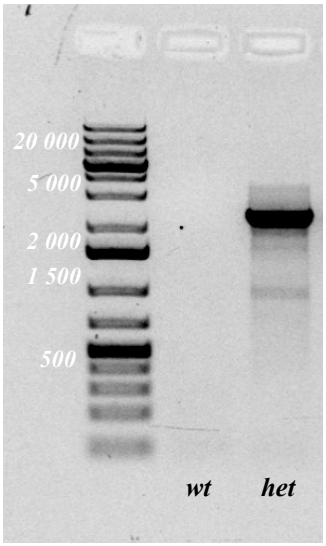



*Figure 2* ***Agarose separation of PCR products***
*Product of 16p11.2del$^{+/-}$ (het) with 2.2 kb weight in comparison to wild types*

## 5.4. Behavioural, morphological and physiological evaluation of 16p11.2del mouse model

To monitor mice early life development, body weight and eye opening was evaluated. Also, nestlet shredding, olfactory and thermal nociception tests were conducted. Before every behavioural test, mice

were let to acclimatize to the room one hour prior to testing. Also, before the first animal and after each animal, all used equipment was cleaned with 70 % isopropanol to remove any remaining odours.

### 5.4.1. Body weight

Mouse pups were weighted immediately after birth and then on postnatal days (PD) PD7, PD14 and PD21 (the day of weaning). Body weight curves were then compared between animals.

### 5.4.2. Eye opening

Every day between PD12 and PD16, the opening of the eyes was observed in the morning. A value was given to each eye separately depending of the state of opening: 0 (closed), 1 (half open), 2 (fully open). Values from left and right eye were then summed up. The value for each day was therefore ranging from 0 (both eyes closed) to 4 (both eyes fully open). The development of eye opening between mice was compared.

### 5.4.3. Nestlet shredding

Prior to test, cotton Nestlets (square 5x5 cm, Datesand Ltd.) were weighted. The Nestlets were then placed in the middle of clean cages with bedding, one Nestlet in each cage. Five weeks old mice were moved to assigned cages (one mouse per cage) and let for 30 minutes under dimmed lights. Afterwards, mice were moved back to their homecages and remaining, not shredded, Nestlet material was weighted again. The difference of Nestlet weight before and after test was calculated and results were compared between animals.

### 5.4.4. Olfactory discrimination test

Three solutions were prepared in 50 ml plastic falcons: pure distilled $H_2O$ and 1 % solutions of two isomers of Limonene (R enantiomer and L enantiomer) (Sigma-Aldrich). Solutions were prepared in another room in a laminar flow hood, so that the mice do not smell the odour of the concentrated Limonenes in advance of the test.

Mice were single-housed at least three days prior to test. A standard housing cage, where testing took place, was inserted in a laminar flow hood. Water bottle, top cover and nest building material were removed from the cage; wire mesh with food pellets was exchanged for a clean and empty one. Mouse was inserted into the cage and presented with a cotton swab soaked in a specific solution according to a test schedule (Table 5). Cotton swabs were attached to a holder and then placed on the wire mesh, so the soaked part was in the height of mouse's nose. Each presentation lasted 1 minute and was followed by 2 minutes break, when the cotton swab was removed from the cage. The cotton swab was remerged before every presentation to keep the odour fresh and intensive. Half of the animals were presented with the R enantiomer

first, the second half with the L enantiomer first. For each 1-minute presentation, time spent sniffing towards the swab was measured by the experimenter.

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| R enantiomer group | $dH_2O$ | - | $dH_2O$ | - | $dH_2O$ | - | $dH_2O$ | - | R | - | R | - | R | - | R | - | L |
| L enantiomer group | $dH_2O$ | - | $dH_2O$ | - | $dH_2O$ | - | $dH_2O$ | - | L | - | L | - | L | - | L | - | R |

*Table 5* ***Test schedule of Olfactory discrimination test***

### 5.4.5. Thermal nociception test

Before the experiment, all mice were habituated to restraining procedure. Mice were placed on a small piece of cotton towel (approximately 15 x 15 cm) with their body on the towel and uncovered tail three times in total. The towel was then folded in half over their head and body and folded more to fix the mouse in position with only its tail protruding. About a third of the tail was next submerged into a beaker with room-temperature water.

During the experiment, the exact steps were repeated, only with 55 °C hot water. A latency to reflexive twitch of a tail was measured. Submersion was repeated three times with 15 minutes intra-trial interval.

### 5.4.6. Free-float immunohistochemical labelling of brain slices

For evaluation of neurostructural changes in brains of 16p11.2del mice, I implemented a new methodology into our lab based on a protocol that I learned on my internship in the Helsinki Institute of Life Sciences. Fixated brains sectioned on vibratome were stained with specific antibodies using free-float approach and then visualised with a fluorescent microscope.

#### 5.4.6.1. Brain fixation and sectioning

Two C57Bl6 mice were deeply anesthetised with intraperitoneal administration of ketamine (100 mg/kg) and xylazine (20 mg/kg) mixture. During anaesthesia, mice were given a trans-cardiac perfusion of 4% paraformaldehyde (PFA) to remove residual blood and initiate brain tissue fixation process. Brains were then carefully removed and left overnight in 4 degrees in 4% PFA.

The next day, brain were washed three times for 10 minutes in phosphate-buffer solution (PBS) on nutating shaker. One hemisphere was cut with a blade to mark right-left orientation of slices. Whole brains were next sectioned on vibratome with focus on hippocampus and cerebellum. Slices (40 or 70 µm thick) were moved into 24-well plates (Baria) following a specific order. First six slices were one after another placed into wells 1-6 (first row) filled with PBS. Afterwards the second six slices were again placed in these

wells 1-6, as well as the third, fourth, fifth and sixth group of slices. As a result, each well contained 6 slices from different brain parts. This way it was ensured that slices were distinguishable from each other and the slice order was kept intact in further analysis.

### 5.4.7. Free-floating immunostaining

Brain slices in well plates were washed three times, 10 minutes each, in PBS (~ 2.5 ml) on a shaker (200 rpm). To exchange PBS for a fresh one, slices were moved into new wells with fresh PBS using a small paint brush. Following, brains were blocked against non-specific binding in wells with 1 ml of blocking solution (Table 6) for 1 hour, shaking (150 rpm).

| Blocking solution | Carrier solution | |
|---|---|---|
| 6 g | 2 g | Bovine Serum Albumine (Sigma-Aldrich, # A9418) |
| 6 ml | 6 ml | 10% Triton X-100 solution (Milipore, # 648463) |
| 20 ml | 2 ml | Normal Goat serum (Biotech, # GO-605/500) |
| 2 ml | 2 ml | 10% Sodium Azide (Sigma-Aldrich, # S2002-25G) |
| 200 ml | 200 ml | PBS |

*Table 6* ***Blocking and Carrier solutions***

Following, brain slices were moved into wells with 1 ml primary antibody solution (primary antibody diluted in the goat carrier solution in a concentration advised by seller, Table 7) and left shaking (150 rpm) overnight at RT in dark (primary antibody solution was reused up to three times). The next day, brain slices were washed three times for 10 minutes in PBS shaking in dark (200 rpm) and subsequently washed in 1 ml secondary antibody solution (secondary antibody diluted in the goat carrier solution in a concentration advised by seller, Table 7) for 2 hours at RT in dark (150 rpm). And finally, slices were washed three times in PBS as previous and stained with DAPI (Roche) diluted in PBS (1:1000) for 10 minutes shaking in dark. This was followed by the last wash in PBS three times in the dark.

| Primary antibody (Abcam) | Dilution | Secondary antibody (Invitrogen) | Dilution |
|---|---|---|---|
| Anti-Parvalbumin (ab277625) | 1:1000 | Goat anti-rabbit 594 (A32740) | 1:1000 |
| Anti-Myelin Basic protein (ab218011) | 1:2000 | Goat anti-mouse 488 (A32723) | 1:1000 |

*Table 7* ***Antibodies and their respective dilutions used in free-float immunohistochemistry***
*Primary and secondary antibodies in the same row are respective to each other.*

#### 5.4.7.1. Mounting and imaging

Brain slices from one well were moved into a Petri dish filled with PBS placed on a black surface. Slices were ordered in rostro-caudal direction and turned over into the same dorso-ventral orientation. Using a small paint brush, brain slices were placed in their order on a coated microscope slide and left to dry out in dark (Bamed). Microscope slides were marked with the well number alongside other detailed information about brain slices. Two drops of fluorescence mounting solution (Dako) were instilled on dry microscope slide, which was then carefully covered with a cover slide. Using a light press on the cover slide, all bubbles were removed. Covered slides were left for a few hours in dark to dry out. Afterwards, the edges of cover slides were fixed with transparent nail polish. With the dried he nail polish, slides were stored in dark until imaging. Slides were imaged on Zeiss Axio Imager microscope and analysed using ZEN blue edition software.

### 5.4.8. Statistical analysis

Statistical analysis was done using either GraphPad Prism (version 9.3.1) or R software. D'Agostino-Pearson test (GraphPad Prism) or Shapiro normality test (R software) were used to assess normality of data, unpaired t-test and Mann-Whitney U test when comparing two groups (depending on the normality of data distribution). To analyse effects of sex and treatment two-way ANOVA (GraphPad Prism) or Analysis of Variance of Aligned Rank Transformed Data test (R software), depending on data distribution were used. Evaluation of the ethological parameters during social interaction in third step of protocol collects multiple dependent variables that characterise either animal stress level (grooming, digging, and rearing), direct exploration of conspecific (sniffing nose, anus or body) or interest in conspecific (following). To handle with repeated measurement of the same subject and find correlation between parameters grouped into mentioned three categories Factor Analysis of Mixed Data (FAMD) and Multi Factor Analysis (MFA) were used for statistical analysis respectively (R software, (Abdi et al., 2013)). All graphs were plotted in GraphPad, with the exception of MFA and FAMD when R software was used. In 4.2.0 R version, stat version 4.2.0, ARTool version 0.11.1, FactoMineR version 2.4, factoextra version 1.0.7 packages were used.

# 6. Results

## 6.1. Emotional Transfer Test

### 6.1.1. First protocol - Elevated Plus-Maze

In the first protocol efficiency of transfer of emotional information was measured in acceptor mice on EPM. All EPM parameters were compared between group of animals that was in the contact with stressed mouse, labelled as "Stressed" (n = 9), and group of animals that was in the contact with non-stressed conspecific, labelled as "Sham" (n = 9). Neither time spent in each zone (Figure 3A), distance travelled in opened and closed arms (Figure 3B) nor latency to visit open arm (Figure 3D) differ between compared groups. Only a slight and not significant trend was seen in the number of visits into the open and closed zones (Figure 3C). It was then concluded that EPM will not be used in the subsequent protocols.

| | Source of variation | DF | F | *p* – value |
|---|---|---|---|---|
| Time spent in each zone | Zones | 2 | 47.97 | 0.7302 |
| | Treatment | 1 | 0.5654 | 0.4637 |
| | Zone/treatment | 2 | 0.3178 | 0.7302 |
| | Residual | 30 | | |
| Distance travelled in each zone | Zones | 1 | 64.03 | <0.0001 |
| | Treatment | 1 | 0.03285 | 0.8586 |
| | Zone/treatment | 1 | 0.7591 | 0.3973 |
| | Residual | 15 | | |
| Number of visits in each zone | Zones | 1 | 26.67 | <0.0001 |
| | Treatment | 1 | 0.5780 | 0.4582 |
| | Zone/treatment | 1 | 3.916 | 0.0653 |
| | Residual | 16 | | |
| Latency to visit open zone | | 1 | 1.630 | 0.2903 |

*Table 8* ***Results from Elevated Plus Maze***
*Unpaired t-test was used for latency enter to the light zone, the rest of parameters were analysed with two-way ANOVA with dependant measurements.*

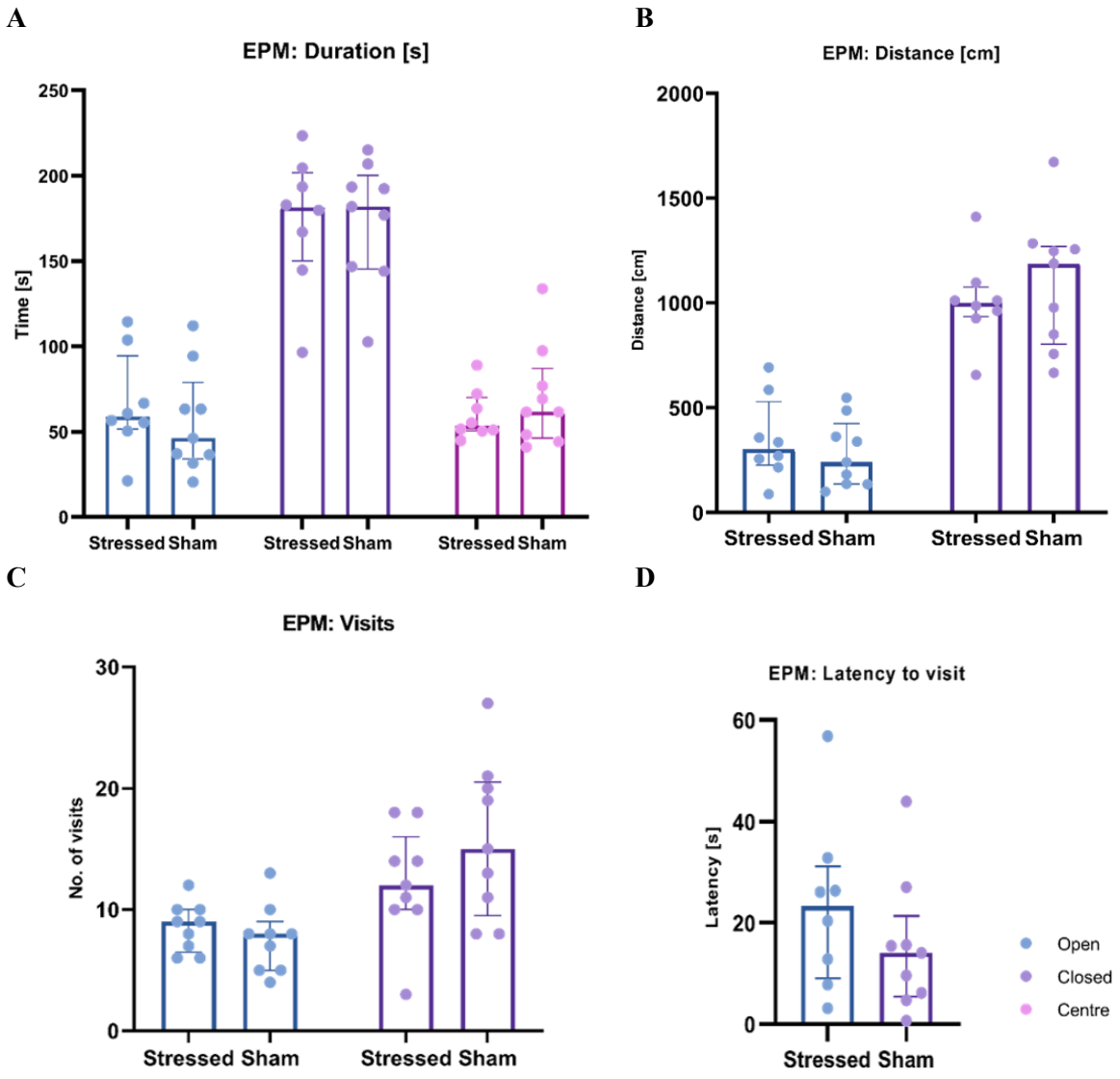



*Figure 3* ***Elevated Plus Maze***
*A) Duration spent in each zone of the maze B) total distance travelled, C) number of visits into maze arms, and D) latency to enter open zone. Raw data are shown with bars that represent median with interquartile range.*

### 6.1.2. Second protocol - FAMD & MFA analysis of animal behaviour

FAMD analysis revealed that the first two dimensions explain 44 % of data set variation. Contributions of behaviours, sex and treatment to each dimension are shown in Table 9 and portrayed in Figure 4A. The biggest contribution to the first dimension was following and sniffing to anus. Sex and treatment had negligible contribution to the first dimension. Interestingly, sex was a source of a much more pronounced variability than treatment to the second dimension however its influence was not bigger or comparable to individual behaviours (sniffing duration to nose, frequency of rearing). Altogether these data suggest that the highest source of variability is not treatment but rather individual differences in animal behaviour.

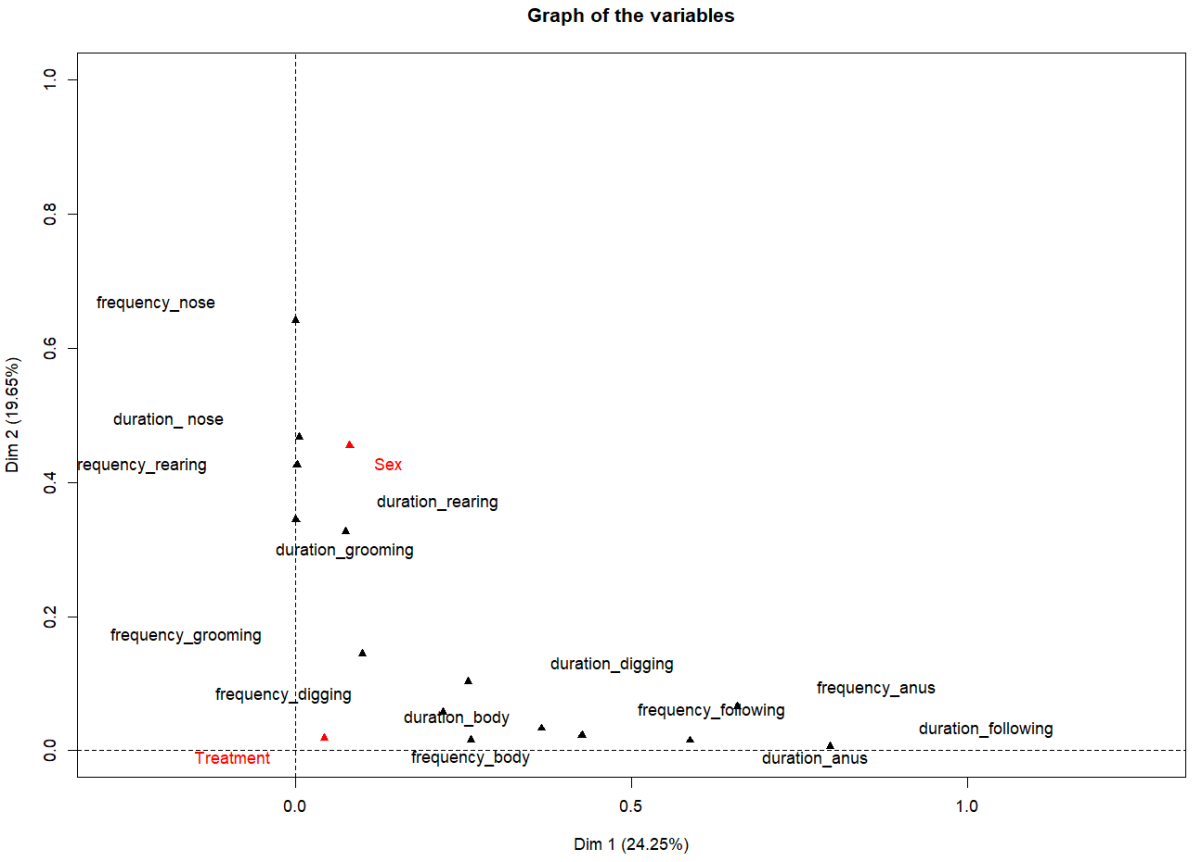
Visualisation of qualitative variables at the FAMD graph of categories (Figure 4B) indicates that both dimensions separate sex and treatment however, contribution to dimensions are much greater for sex than treatment. This observation is confirmed on the individual factor map, where male and female individuals are clearly separated (Figure 4C, sex panel). However, treatment is not that clearly separated (Figure 4C, treatment panel). Due to clear male and female separation I conducted further comparisons separately for each sex.

MFA analysis again confirmed the lesser contribution of treatment to first two dimensions than behavioural categories (Figure 4A,C males, 5B,D females). In males, the first dimension markedly separates the group of anxiety-related behaviours from a group of direct exploration of a conspecific. The second dimension also detaches digging behaviour from the rest of anxiety-related behaviours. This division is also present in females, already split in the first dimension. In females there is no clear differentiation between groups of behaviours. In both sexes, sniffing to nose and digging shows the lowest power of correlation with all the other parameters.

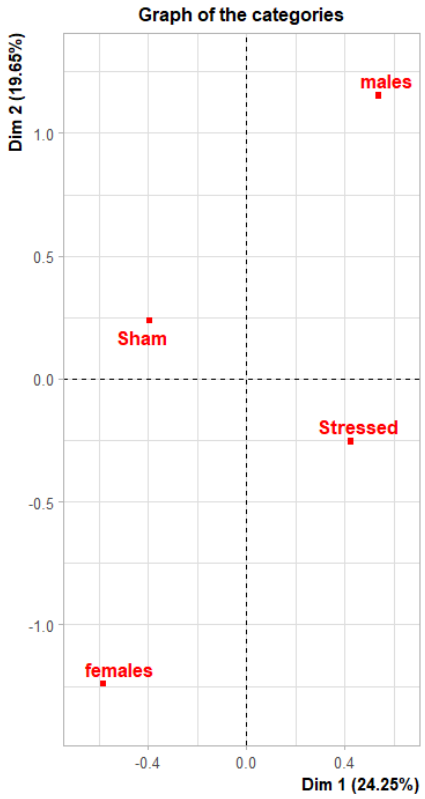
| Parameter | Dim.1 | Dim.2 | Dim.3 | Dim.4 | Dim.5 |
|---|---|---|---|---|---|
| duration_following | **0.7957** | 0.0067 | 0.1180 | 0.0020 | 0.0037 |
| frequency_anus | **0.6578** | 0.0658 | 0.0042 | 0.0233 | 0.1756 |
| duration_anus | 0.5873 | 0.0152 | 0.0951 | 0.0170 | 0.1588 |
| duration_body | 0.4265 | 0.0229 | 0.0975 | 0.0196 | 0.1885 |
| frequency_following | 0.3660 | 0.0331 | 0.0041 | 0.2608 | 0.0440 |
| frequency_body | 0.2611 | 0.0162 | 0.1092 | 0.0105 | 0.0300 |
| duration_digging | 0.2576 | 0.1026 | 0.2070 | 0.1853 | 0.0003 |
| frequency_digging | 0.2202 | 0.0573 | 0.1858 | 0.2664 | 0.0379 |
| frequency_grooming | 0.0995 | 0.1447 | 0.4930 | 0.0057 | 0.0260 |
| Sex | *0.0811* | 0.4555 | 0.0000 | 0.0199 | 0.1409 |
| duration_grooming | 0.0747 | 0.3264 | 0.2167 | 0.0811 | 0.0007 |
| Treatment | *0.0430* | 0.0191 | 0.2997 | 0.0135 | 0.2661 |
| duration_ nose | 0.0054 | 0.4676 | 0.0814 | 0.1661 | 0.0761 |
| frequency_rearing | 0.0025 | 0.4263 | 0.3194 | 0.1623 | 0.0062 |
| duration_rearing | 0.0011 | 0.3445 | 0.2938 | 0.3054 | 0.0081 |
| frequency_nose | 0.0008 | **0.6406** | 0.0978 | 0.0225 | 0.0301 |

*Table 9* ***Contribution of each parameter to all five dimensions in FAMD analysis***

**4A**



**4B**




**4C**

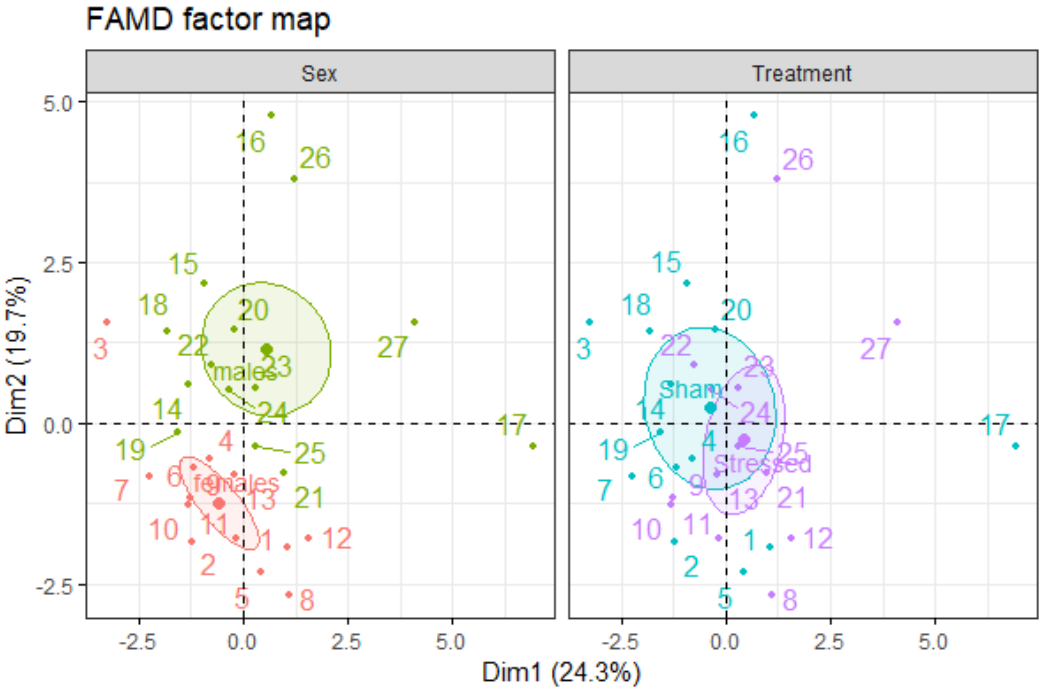
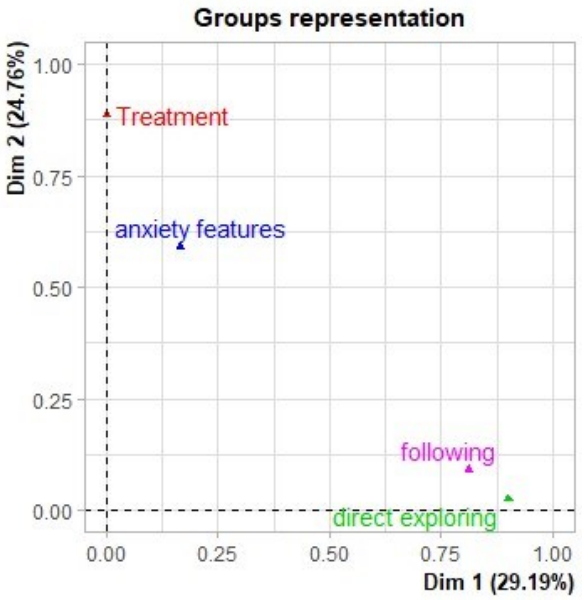
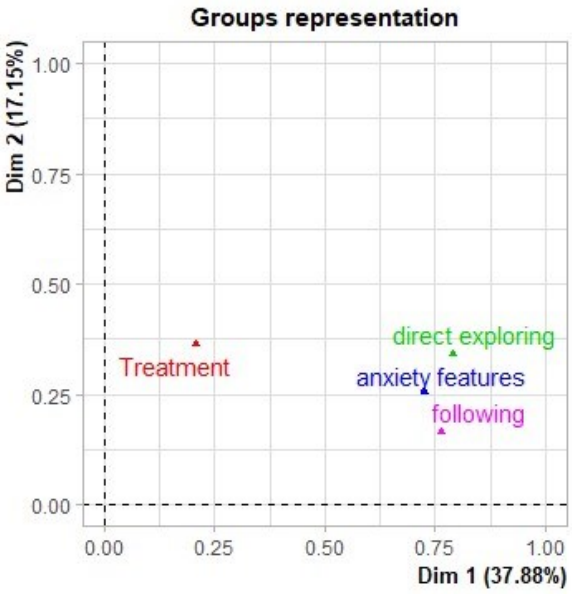



*Figure 4* ***Factor Analysis of Mixed Data of ethological parameters***
*(A) Contribution of qualitative and quantitative parameters to the first two dimensions (B) Contribution of qualitative parameters to the first two dimensions, (C) Factor map clustering of individuals of the same profiles.*

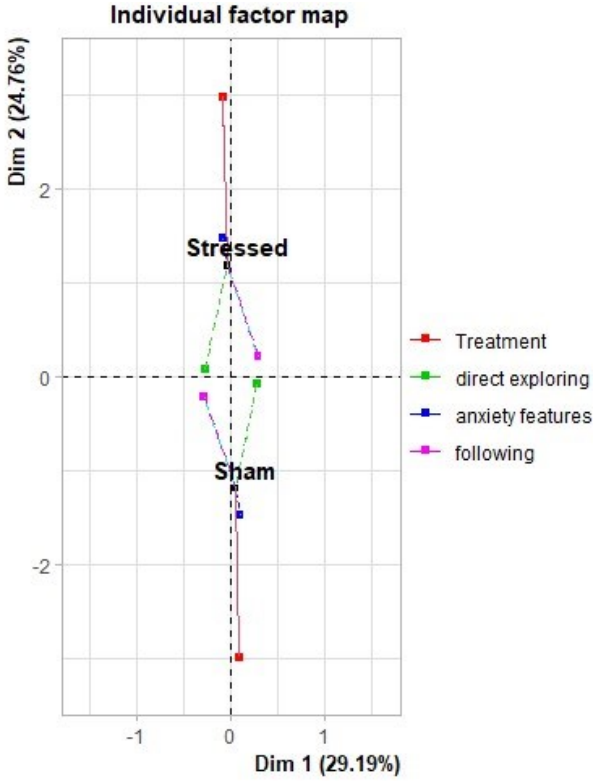
**5A males**



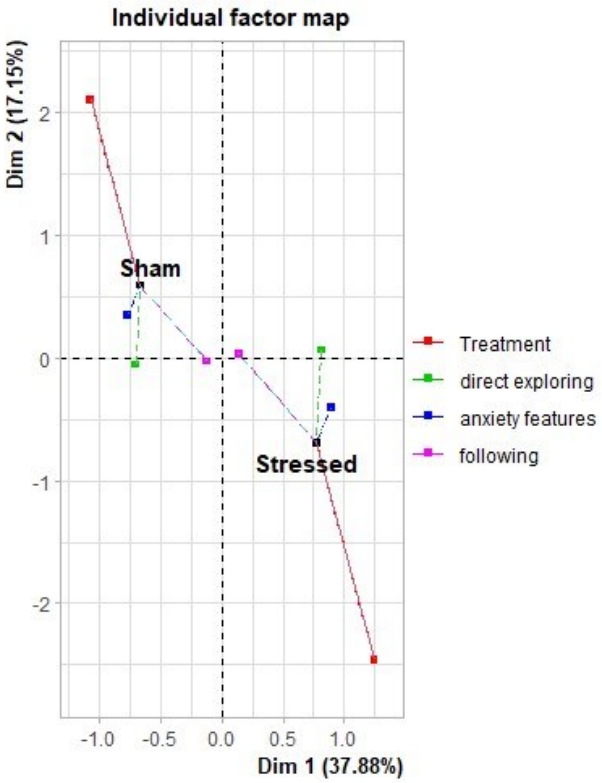

**5B females**



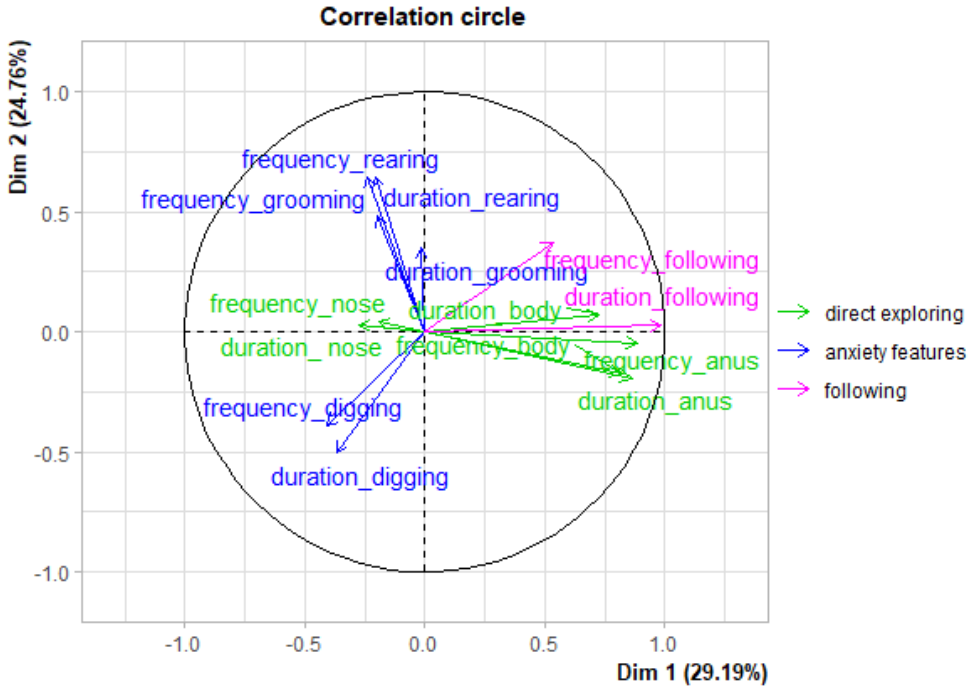

**5C males**




**5D females**



**5E**




**5F**

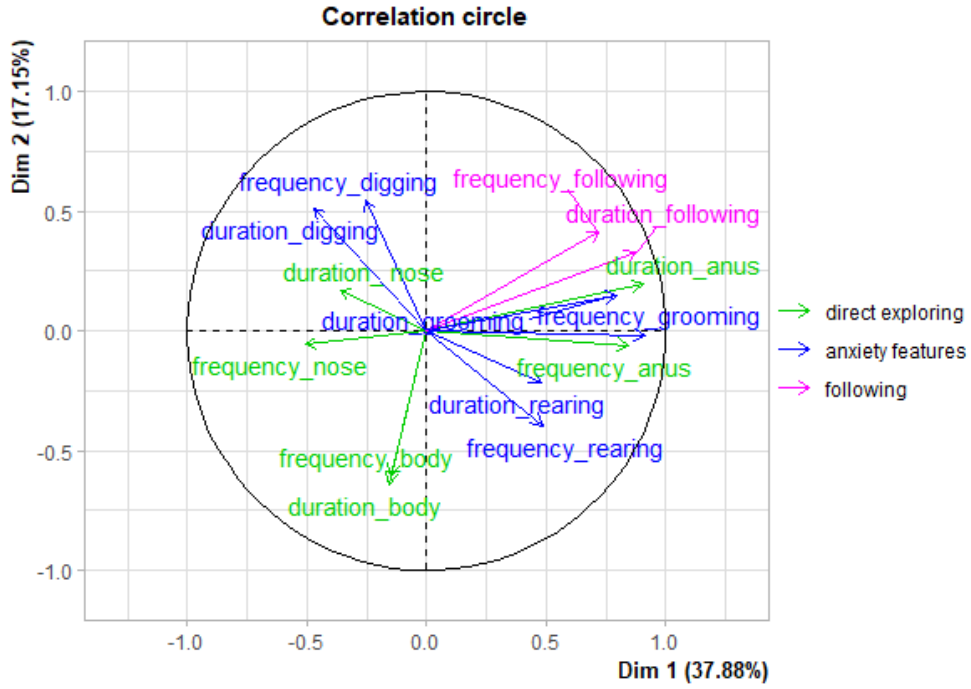


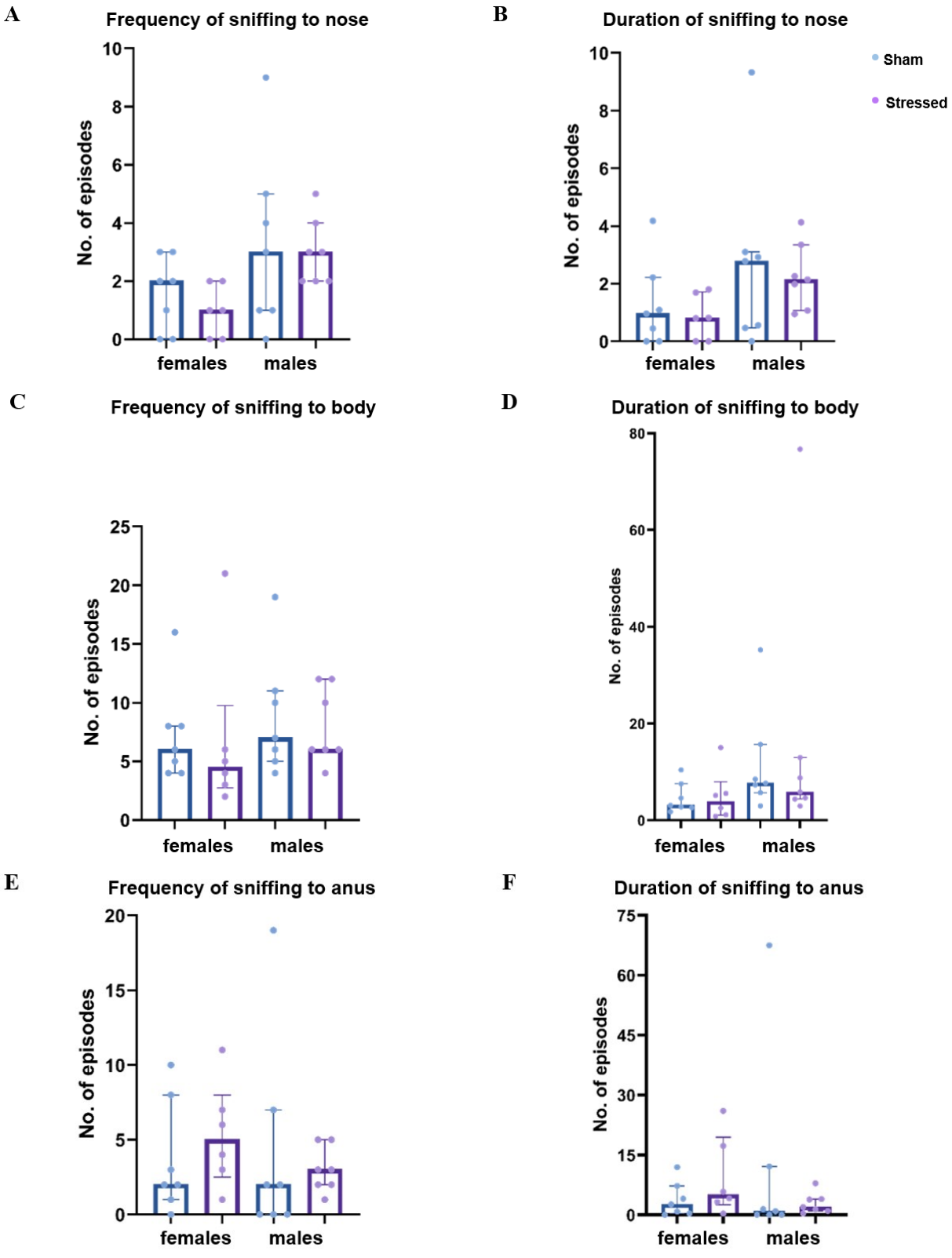

*Figure 4* ***Multi Factorial Analysis of mice behaviour***
*(A) for males (B) for females: contribution of qualitative and quantitative parameters to the first two dimensions (C) for males and (D) for females: correlation of quantitative parameters grouped by qualitative variable (E) for males and (F) for females: correlation of etiological parameters*

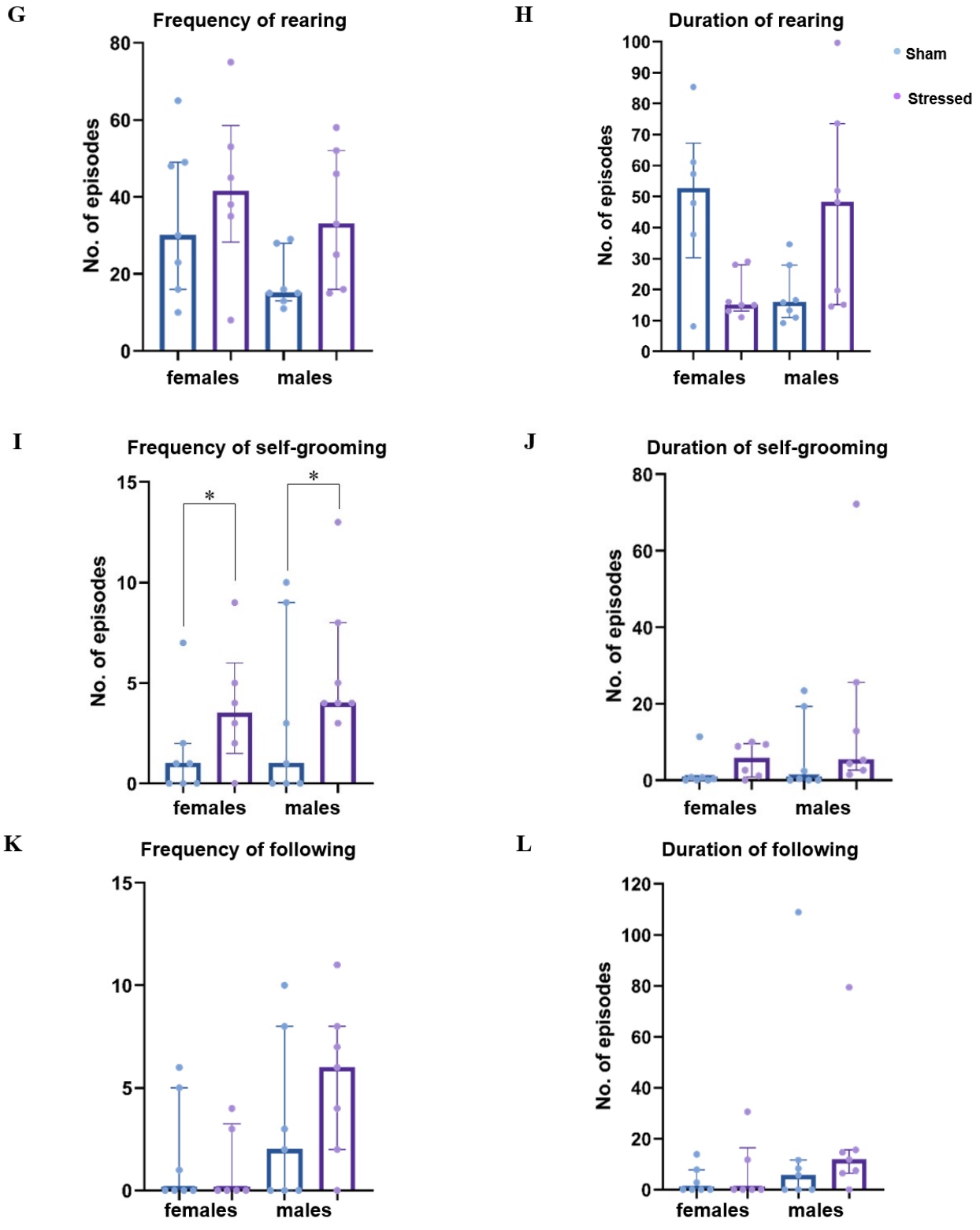
### 6.1.3. Statistical analysis of ethological parameters collected during transfer of emotional information

After data visualisation of relationship between parameters with MFA and FAMD, each behavioural parameter was also measured separately with statistical analysis (Analysis of Variance of Aligned Rank Transformed Data test). Despite significant differences between sex in sniffing to nose and body as well as following, the only significant influence of treatment was observed for both sexes in the self-grooming parameter (details in Table 10 and at Figure 5).

| Parameter | Duration | | | Frequency | | |
|---|---|---|---|---|---|---|
| | Source of Variation | F | p-value | Source of Variation | F | p-value |
| Sniffing to nose | Treatment | 0.017 | 0.898 | Treatment | 0.178 | 0.677 |
| | Sex | 4.448 | **0.046** | Sex | 4.637 | **0.042** |
| | Treatment:Sex | 0.333 | 0.569 | Treatment:Sex | 0.475 | 0.497 |
| Sniffing to anus | Treatment | 1.920 | 0.179 | Treatment | 2.252 | 0.147 |
| | Sex | 1.986 | 0.172 | Sex | 1.762 | 0.197 |
| | Treatment:Sex | 0.882 | 0.357 | Treatment:Sex | 0.392 | 0.538 |
| Sniffing to body | Treatment | 0.575 | 0.456 | Treatment | 0.334 | 0.569 |
| | Sex | 5.667 | **0.026** | Sex | 2.428 | 0.133 |
| | Treatment:Sex | 0.181 | 0.675 | Treatment:Sex | 0.334 | 0.569 |
| Digging | Treatment | 2.721 | 0.113 | Treatment | 1.186 | 0.287 |
| | Sex | 1.241 | 0.277 | Sex | 0.915 | 0.349 |
| | Treatment:Sex | 0.608 | 0.443 | Treatment:Sex | 0.102 | 0.753 |
| Following | Treatment | 1.666 | 0.210 | Treatment | 1.798 | 0.193 |
| | Sex | 3.386 | 0.079 | Sex | 6.029 | **0.022** |
| | Treatment:Sex | 1.537 | 0.228 | Treatment:Sex | 2.750 | 0.111 |
| Self-grooming | Treatment | 3.676 | 0.068 | Treatment | 9.535 | **0.005** |
| | Sex | 1.318 | 0.263 | Sex | 1.530 | 0.229 |
| | Treatment:Sex | 0.510 | 0.482 | Treatment:Sex | 0.235 | 0.632 |
| Rearing | Treatment | 2.019 | 0.169 | Treatment | 3.419 | 0.077 |
| | Sex | 2.165 | 0.155 | Sex | 2.856 | 0.105 |
| | Treatment:Sex | 0.470 | 0.500 | Treatment:Sex | 0.241 | 0.628 |

*Table 10* ***Analysis of Variance of Aligned Rank Transformed Data test of ethological parameters***
*Significant results (*$p < 0.05$*) are highlighted.*


A
Frequency of sniffing to nose
No. of episodes
females
males
B
Duration of sniffing to nose
Sham
Stressed
C
Frequency of sniffing to body
D
Duration of sniffing to body
E
Frequency of sniffing to anus
F
Duration of sniffing to anus


G
Frequency of rearing
No. of episodes
0
20
40
60
80
females
males
H
Duration of rearing
No. of episodes
0
10
20
30
40
50
60
70
80
90
100
females
males
Sham
Stressed
I
Frequency of self-grooming
No. of episodes
0
5
10
15
*
*
females
males
J
Duration of self-grooming
No. of episodes
0
20
40
60
80
females
males
K
Frequency of following
0
5
10
15
females
males
L
Duration of following
No. of episodes
0
20
40
60
80
100
120
females
males

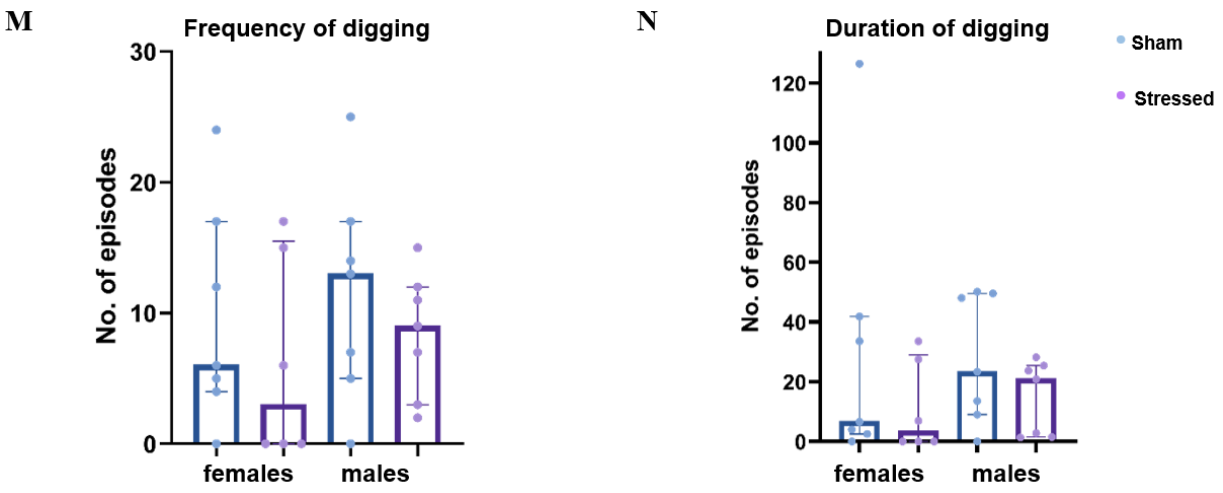
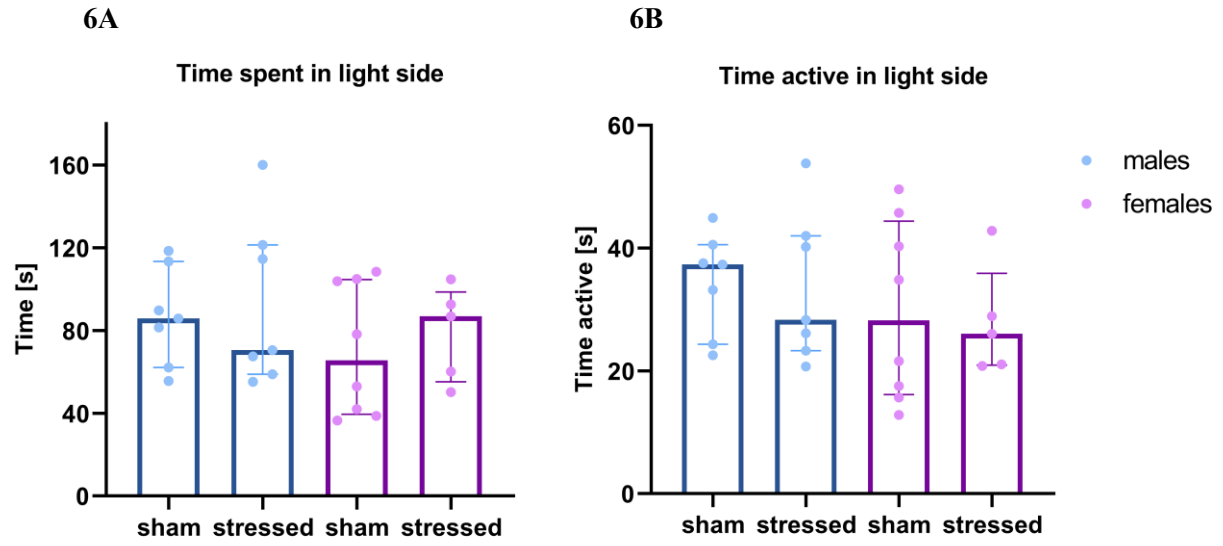



*Figure 5* ***Analysis of Variance of Aligned Rank Transformed Data test of ethological parameters***
*Frequency and duration of (A – B) sniffing to nose, (C – D) sniffing to body, (E – F) sniffing to anogenital area, (G – H) rearing, (I – J) self-grooming, (K – L) following, and (M – N) digging. Raw data with bars that represent median with interquartile range.*

### 6.1.4. Second protocol - Light/dark box

The light/dark box test was used to measure an efficiency of transfer of emotional information between demonstrator and acceptor mice. Time spent in light (Figure 6A), activity time in light side (Figure 6B) as well as latency to enter light side (Figure 6C) did not differ between groups nor sex. Results are shown in Table 11.



**6C**

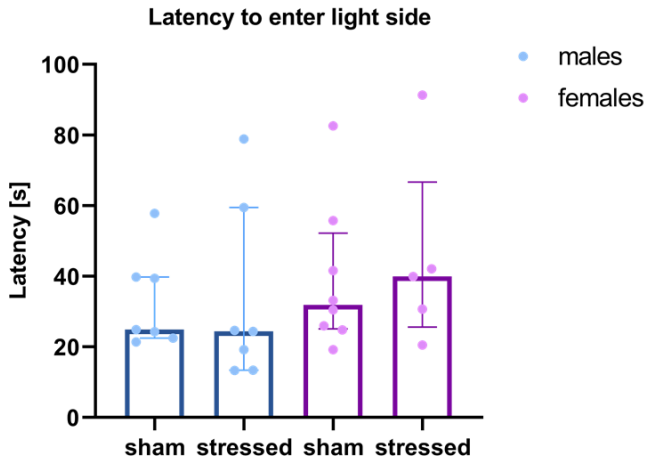



*Figure 6* ***Light Dark Box***
*Light Dark box. (A) Time spent in light side, (B) time active in light side (C) latency to enter light side. Raw data with bars that represent median with interquartile range.*

| Sex | Side | Parameter | n1 sham | n2 stressed | U value | *p* value |
|---|---|---|---|---|---|---|
| Males | Light | Time spent | 7 | 7 | 24 | > 0.999 |
| | | Time active | 7 | 7 | 23 | 0.902 |
| | Dark | Time spent | 7 | 7 | 24 | > 0.999 |
| | | Time active | 7 | 7 | 21 | 0.710 |
| | Dark → Light | Latency | 7 | 7 | 20 | 0.620 |
| Females | Light | Time spent | 8 | 5 | 17 | 0.724 |
| | | Time active | 8 | 5 | 20 | > 0.999 |
| | Dark | Time spent | 8 | 5 | 16 | 0.622 |
| | | Time active | 8 | 5 | 11 | 0.222 |
| | Dark → Light | Latency | 8 | 5 | 16 | 0.622 |

*Table 11* ***Light/Dark Box***
*Results of Mann-Whitney U test for latency and total time and active time spent in light and dark sides of the Light/Dark box.*

### 6.1.5. Third protocol – Acoustic Startle Reflex test

In this protocol evaluation of emotional information transfer efficacy was estimated by the magnitude of the startle reflex. Successful transfer would change acceptor animal emotional state that would result in potentiation of responses to the startle stimuli – fear potentiated startle. Neither parameter in this evaluation results were significantly different.

| | DF | F | *p* – value |
|---|---|---|---|
| Treatment | 1 | 1.911 | 0.174 |
| Sex | 1 | 2.143 | 0.153 |
| Treatment/sex | 1 | 0.111 | 0.741 |

*Table 12* ***Acoustic startle response***
*Startle response to acoustic stimulus analysed by Analysis of Variance of Aligned Rank Transformed Data test*

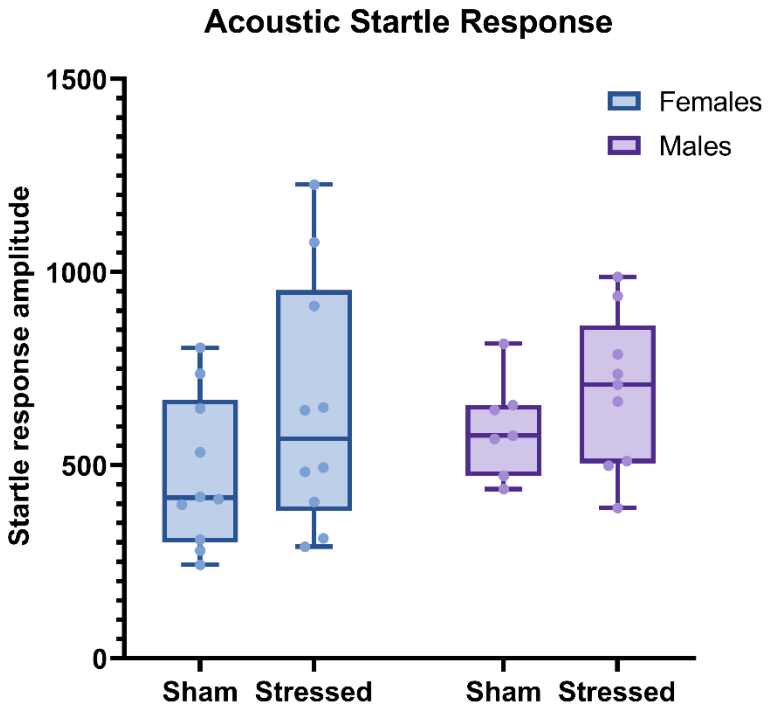



*Figure 7* ***Acoustic startle response***
*Box plot of acoustic startle response. Graph shows raw data, median with interquartile range, minimum and maximum values.*

## 6.2. 16p11.2 deletion mouse model

### 6.2.1. Reanimation of 16p11.2del mice

The success rate of in vitro fertilisation was only 18 % from 245 used eggs. Additionally, from these eggs only 13 mice were born, which lowers the success rate to only 5 % in total. Also, the heterozygote to wild type ratio was shifted (from expected 50:50 to 11.5 : 50). These results suggested possible sperm damage e.g. during transportation. To confirm this hypothesis, sperm was reanimated one more time but on a different mouse line. Instead of C57Bl/6J line, we tried C57Bl/6N. Unfortunately, the performance was even worse. Success rate of in vitro fertilisation declined to 7 % and overall success of reanimation reached 1.4 %.

### 6.2.2. Breeding and survival rate

Only 40 % of heterozygote pups survived up until weaning (postnatal day 21) in comparison to 96.3 % of wild types (Figure 8). Also, breeding of these pups was complicated itself, since the parents (heterozygote males and wild type females) had troubles conceiving and females often experienced spontaneous abortions. An evidence for this problem is the resulting number of litters and pups after seven months of continuous breeding (January – July) where from 5 females, only nine litters were delivered with five alive pups per litter on average. Standard C57Bl/6 mice breeding of 5 pairs gives up to 25 litters during 7 months. Thanks to the combination of prolonged breeding and worsened survival rate, only 26 mice survived until adulthood, with only 4 male and 1 female heterozygotes (Table 13).

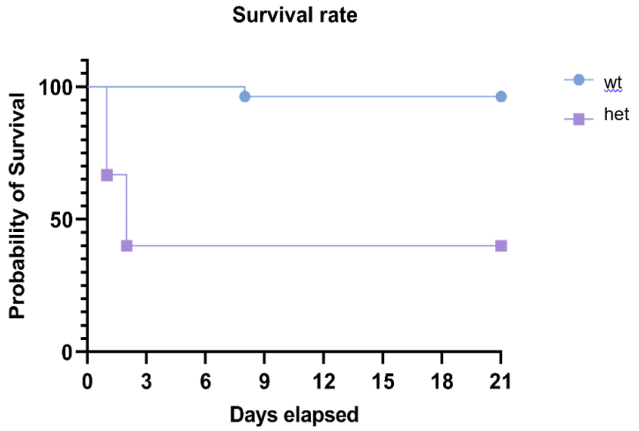



*Figure 8* ***Survival rate of 16p11.2del mouse pups***

*Table 13* ***Overview of all 16p11.2del mice that survived until adulthood***

| | wt | het |
|---|---|---|
| males | 13 | 4 |
| females | 8 | 1 |

Due to the low success rate of IVF and breeding and a presence of a lot of defects in born mice (alopecia, glaucoma), the used model was concluded to be aberrant. Most likely, the offered sperm got damaged during transportation. The original 16p11.2del mice model, provided by Pavel Osten's laboratory, does not show these severe problems either in breeding, survival rate or phenotypical defects.

### 6.2.3. Early-life development evaluation

Heterozygote pups showed a tendency for lower body weight on all postnatal days PD1 – PD21 (Figure 9) and normal progress of eye opening (Figure 10). Due to technical reasons, only 3 heterozygote pups were measured in weight gain and eye opening.

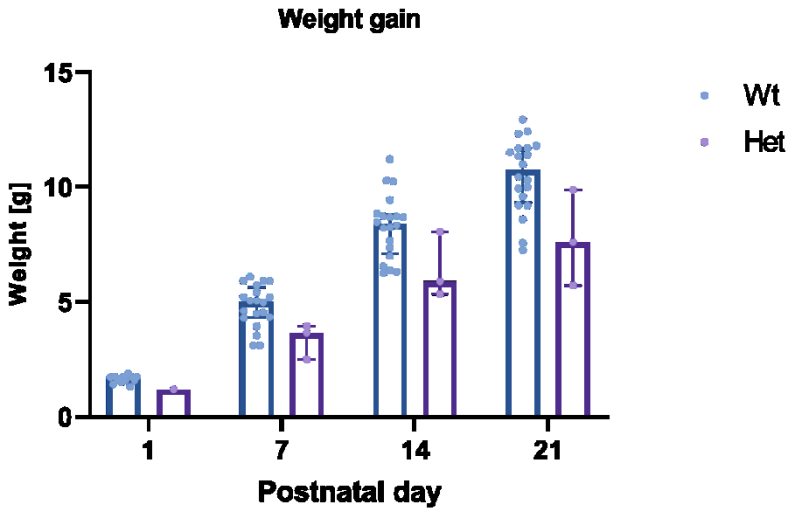



*Figure 9* ***Weight gain of 16p11.2del mouse pups***
*Bars show median with interquartile range.*

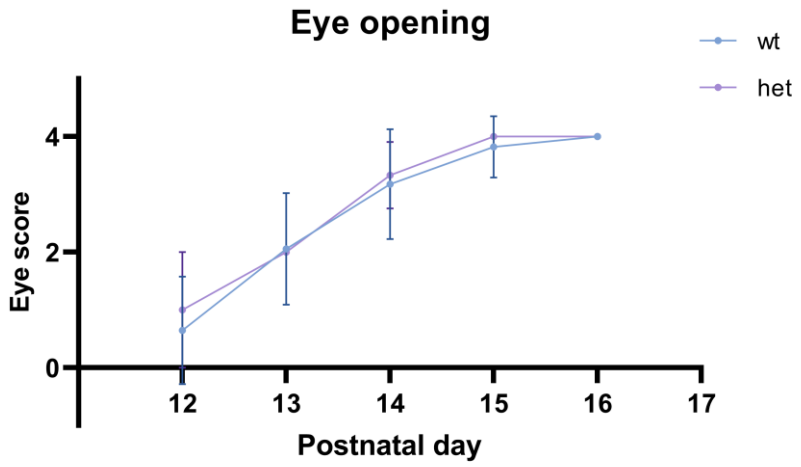



*Figure 10* ***Eye opening of 16p11.2del mouse pups***
*Eye opening score: (0 – closed, 1 – partially opened, 2 – opened) for each eye separately. 17 wild types and 3 heterozygotes were measured. Bars show mean and standard deviation.*

Also, an unusually high number of mice had unexpected phenotypical defects. Nine mice had partial alopecia around the second to third week of life. Alopecia affected mainly the back and belly of mice, leaving fur on head, legs and the root of the tail. After a few weeks, mice were fully covered with fur and no traces of alopecia were left. Additionally, one mouse pup was born without an eye and one other had severe glaucoma. None of these abnormalities can be attributed to their genotype, sex or parental lineage. It was therefore attributed to the sperm defect.

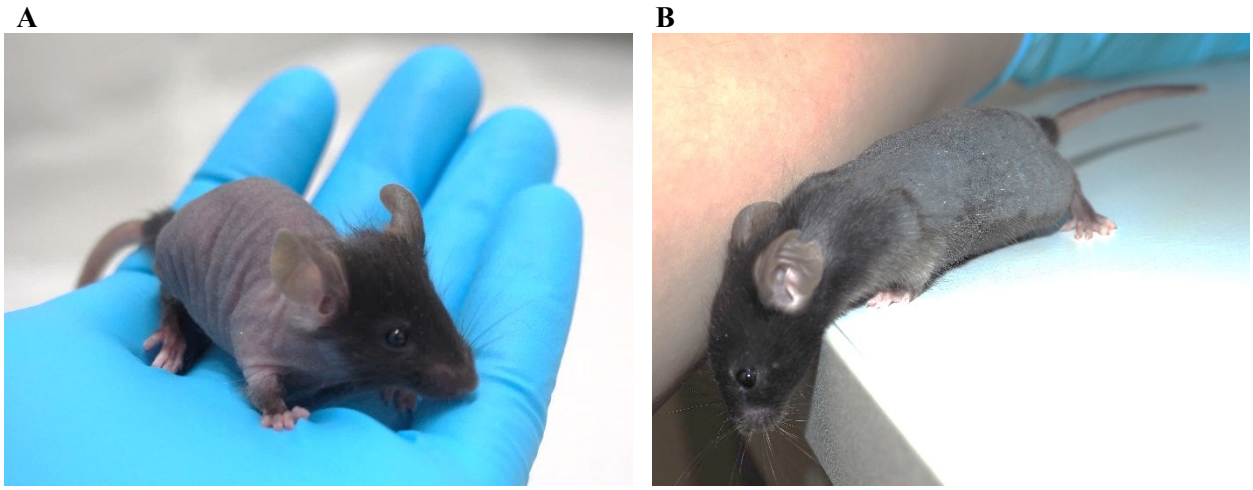



*Figure 11* ***Partial alopecia in a 16p11.2del mouse***
*Alopecia recovery, comparison between PD21 (A) and PD31 (B) in the same mouse. In (A), mouse has fur only on its head, root of the tail and legs. After ten days, fur have already begun to grow back (B).*

### 6.2.4. Nestlet shredding, olfactory and thermal nociception tests

No difference was found between heterozygote and wild type 16p11.2del mice in compulsive behaviour (Figure 12), olfactory sense (Figure 13) or nociception (Figure 14).

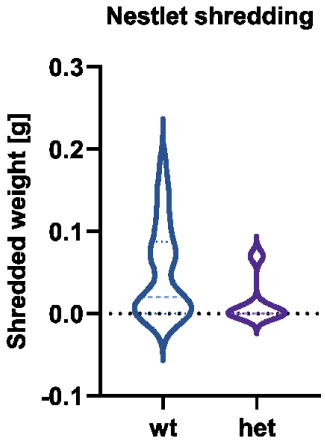



*Figure 12* ***Nestlet shredding in 16p11.2del mice***
*Comparison between wild type (n = 22) and heterozygote (n = 6) mice in the amount of shredded part of a cotton nestlet.*

In the olfactory discrimination test, all mice showed an expected pattern of sniffing duration. Duration of sniffing declined with every repeating presentation, but increased considerably with a presentation of a new odour. Interest was also more pronounced in the limonene odours than in water odour. Since heterozygotes followed the sniffing pattern correctly, it can be assumed that their olfactory sense was intact. However, a tendency appearance may be speculated. Heterozygotes overall spent slightly less time sniffing water than wild-types, yet then tended to spent more time sniffing both first and second enantiomer in comparison to wild types.

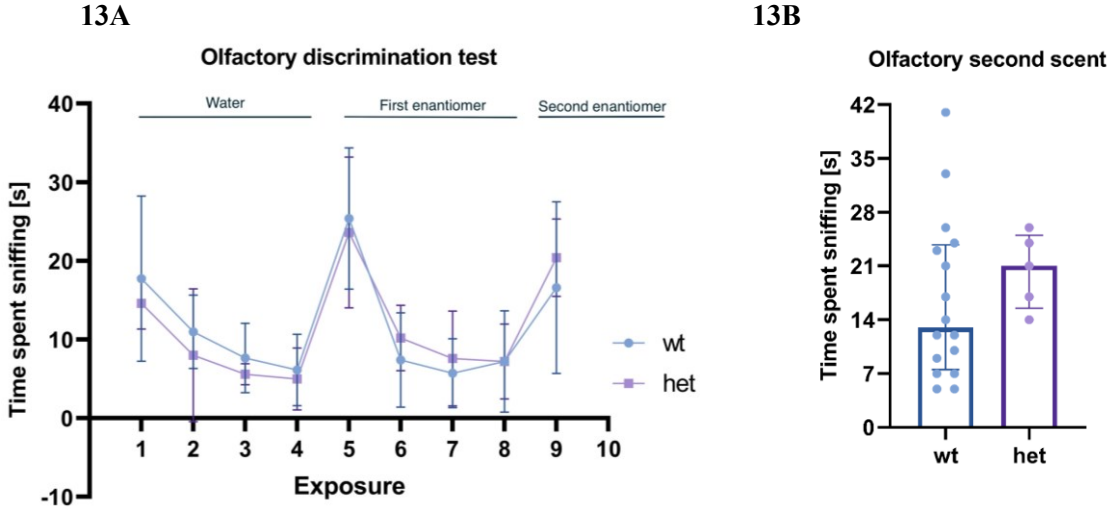



*Figure 13* ***Olfactory discrimination in 16p11.2del mice***
*(A) 1 – 4 presentation of water, 5 – 8 presentation of first enantiomer, 9 – 10 presentation of second enantiomer; (B) first presentation of the second enantiomer*

Results from the Thermal nociception test show no difference between treatment groups (Figure 14).

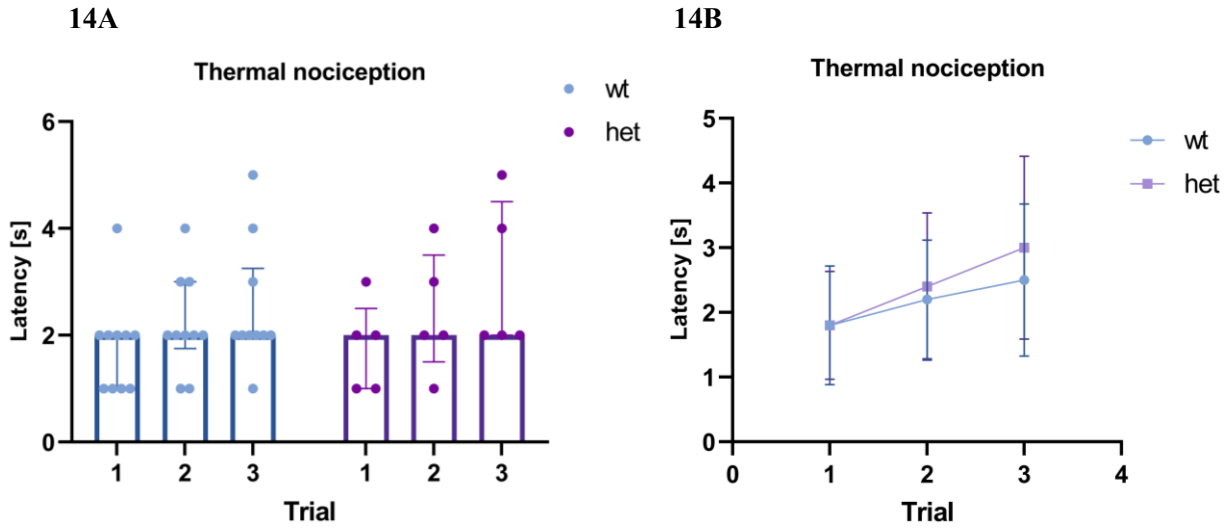



*Figure 14* ***Thermal nociception in 16p11.2del mice***
*(A) raw data and bars representing median with interquartile range (B) average latency per trial presented as mean with standard deviation.*

## 6.3. Brain imaging

The following data presents exemplar results of the establishment of free floating immunohistochemical labelling technique. Both 40 µm and 70 µm thick slices were fully stained with three different antibodies. Results suggest that 40 µm thick slices are more convenient for cell count or density evaluation, whereas 70 µm thick for further connectivity and arborisation evaluation. 70 µm slices were too thick for cell count thanks to numerous layers of cells, but this could be possibly resolved with imaging on an ApoTome or confocal microscope and working in a specific layer. Furthermore, the concentration of Anti-Myelin Basic Protein antibody was too low. Although the vendor advises 1:2000 dilution, this resulted in too low intensity of fluorescence. A 1:1000 dilution would be a preferable concentration. Additionally, for more specified staining of neuronal nuclei, anti-NeuN antibody would be a better option to DAPI. Parvalbumin-positive cells, myelin sheaths and cell nuclei were labelled in the hippocampus and cerebellum (details in Figure 16). This technique was prepared for further evaluation of samples of 16p11.2del mice model. Unfortunately, in 16p11.2del mice, neurostructural changes were not assessed since this model was concluded to be aberrant.

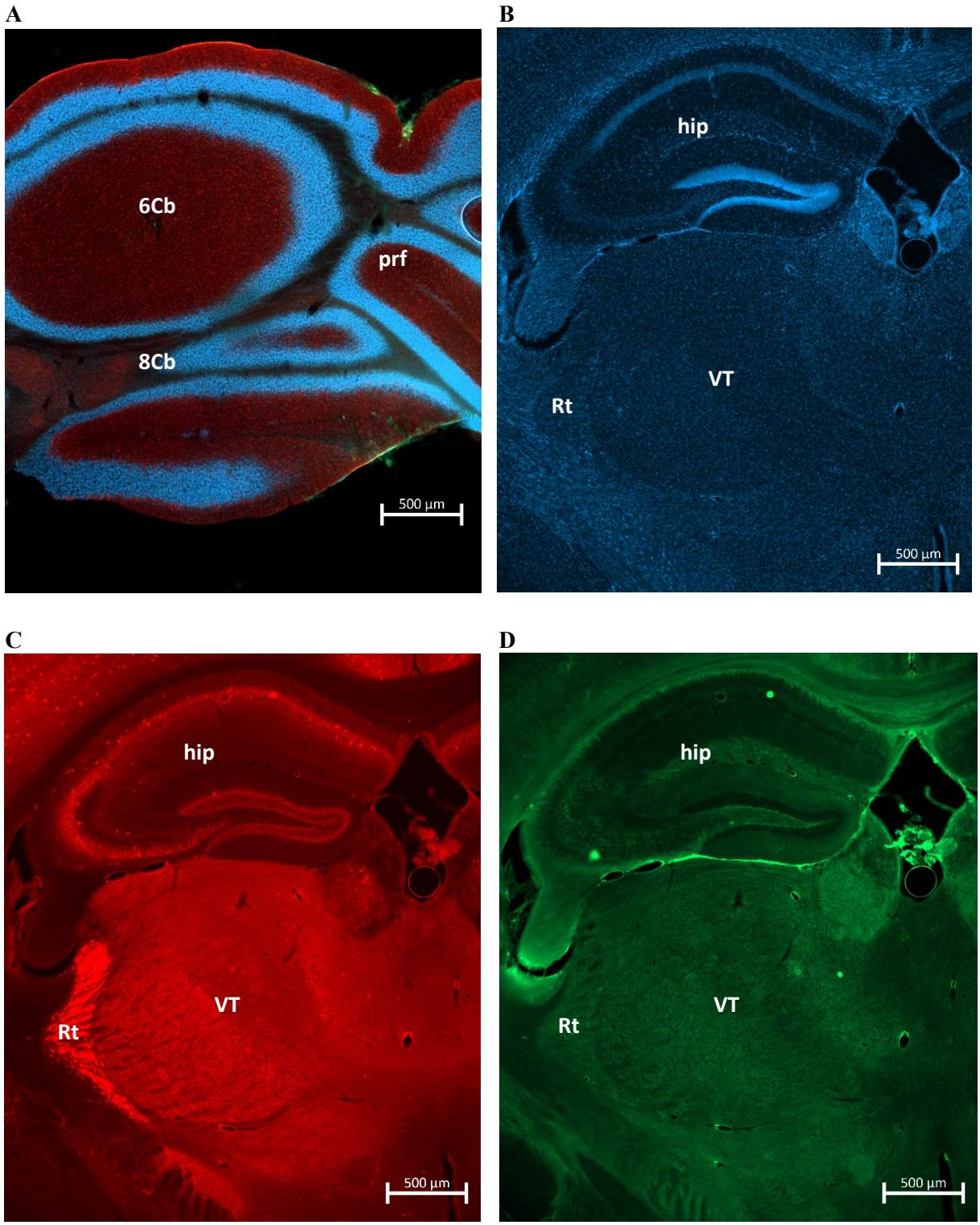



*Figure 15* ***Immunohistological labelling of hippocampus and cerebellum***
*(A) Cerebellum (40 µm slice, 20x focus), bregma -6.95, lobules 6 (6Cb) and 8 (8Cb) of cerebellar vermis and prepyramidal fissure (prf), (B-D) hippocampus (hip), thalamic reticular nucleus (Rt) and ventral thalamus (VT) (40 µm slice, 5x focus), channels showed separately; channels: red (parvalbumin-positive cells), green (myelin sheath), blue (cell nuclei)*

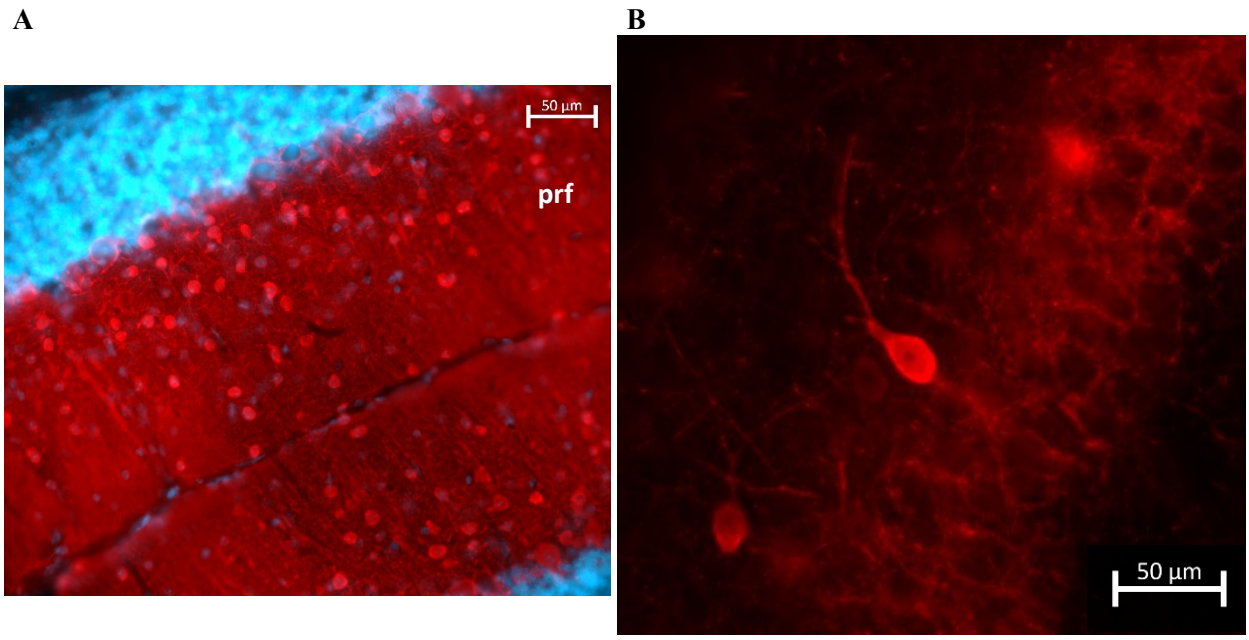



*Figure 16* ***Immunohistological labelling of hippocampus and cerebellum***
*(A) cerebellum (40 µm brain slice, 40x focus), bregma -5.91, primary fissure, (B) hippocampal parvalbumin-positive neuron (40 µm slice, 40x focus); channels: red (parvalbumin-positive cells), blue (cell nuclei)*

# 7. Discussion

## 7.1. Emotional transfer test validation

The aim of using different approaches for an emotional transfer test protocol was to establish proper conditions for an evaluation of a transfer of information carrying emotional load between mice. Presented data support a presence of such transfer, measurable during a direct communication of conspecifics. While data showed that neither former preparation of animals aiming to reduce stress through handling nor extent of stress put on the demonstrator mouse influenced the successfulness of the ultimate test (EPM, LDB, AR), the present data marks a necessity to adjust social tests for each sex individually, since the expression of emotion and social interaction significantly differs. Similar differences are observed in empathic consolation and general social behaviour, where male mice are more prone to participate in allo-grooming, allo-sniffing and allo-licking its conspecific in pain than females (Du et al., 2020). Also the general level of social play and social investigation in healthy mice markedly varies between sexes and is regulated by interaction of sex chromosome genes with gonadal hormones (Cox & Rissman, 2011a).

After the failure of the first protocol, it was speculated that the level of induced stress in the donor mouse could have been insufficient to influence the following emotional state of acceptor mouse. Although presentation of even one foot-shock is enough to induce freezing behaviour and fear in mice (Dvorakova et

al., 2021), direct observation of mice behaviour to the three foot-shocks delivery revealed only a little reaction and stressful acting of demonstrator mice. Following the published protocol by Knapska with colleagues (Kondrakiewicz, Kostecki, et al., 2019), the number of foot-shocks was increased to ten in the second protocol, however mice reacted extremely fearfully, which is why the number was thereafter adjusted to six. Combined with an additional statistical comparison of freezing behaviour between different protocols (data not shown), a six shock protocol showed the best potential for stress induction and it will be therefore used in further experiments.

Overall, data evaluation showed minimal difference between treatment groups. However, FAMD analysis revealed a very strong confound factor: sex. When results were reanalysed with MFA separately for each sex, a small yet noticeable effect of treatment was found. While this effect was less pronounced in females, in males it showed a considerable contribution to the second dimension. These obvious differences in results of both sexes are most probably caused by the already mentioned distinct tendencies of social behaviour in mice males and females (Cox & Rissman, 2011a). Since in males both the first and second dimensions explained comparable amount of variation, the effect of treatment on the second dimension is definitely non-negligible. In both sexes there was found a marked negative correlation between parameters describing animal emotional state (so called anxiety-related parameters) and parameters of direct social exploration, without any connection to treatment group. Interestingly, behavioural studies show that male mice of different mouse strains have diverse levels of social behaviour and react differently when encountering a stressed conspecific. Mice with normal level of social behaviour, e.g. C57Bl/6J, increase pro-social behaviour, mainly in nose-to-nose and nose-to-anogenital sniffing, when exposed to a stressed conspecific. However, transgenic male mice with decreased pro-social behaviour (e.g. BTBR T+ Itpr3tf/J or Fmr1KO(FVB)) withdraw from social interaction even more and show an increase in self-grooming ( Meyza et al., 2015). In my experiment, the division in behaviours may be therefore suggesting a lower pro-social tendency of C57Bl/6N mouse line.

Sniffing to nose showed a very small variation and minimal correlation with other parameters, it may be therefore skipped in further experiments for such limited informative potential. Digging showed a very small correlation with other parameters and a strong correlation with the rest of anxiety-related behaviours. The results suggest that during the short 5 minutes of observation, anxious mice spent most time rearing and self-grooming and therefore may not have enough time to dig. In my setup, digging is thus less sensitive to mouse anxiety than other parameters. Following showed a strong sexual dimorphism. This, not only occurred very seldom in females in opposition to some males which spent almost the whole emotional transfer phase following their partner. Moreover, its presence can be explained more by their emotional state in females while in males more by their preferred type of social interaction. Comparably,

sniffing to anus shows a bigger connection to emotional state in females than in males, but this phenomenon may be explained by a wrong approach to ethological scoring. When mice followed their conspecific, they also usually sniffed their anus or body. Originally, I scored both following and sniffing even during their co-occurrence. Since females followed conspecifics much less than males, sniffing to anus was less correlated with following and showed a stronger correlation with emotional state. However, in males the presence of sniffing was only correlated with following. This strong positive correlation between following and sniffing to anus brought a small confound to data analysis and complicated result interpretation. In future, sniffing to anus/body and following should be scored only separately.

The effect of treatment found with MFA was further confirmed with statistical analysis of each parameter separately, where significant difference between treatment groups was found in self-grooming and strong tendency in increased rearing frequency in stressed group was present. Self-grooming is an innate behaviour involved in hygiene maintenance, social communication and stress with a very rigid cephalo-caudal movement order (Tikhonova et al., 2011; van den Boom et al., 2017; Y.-F. Zhang et al., 2022). The intensity of self-grooming increases both in relaxed state in a safe environment and anxious state in a novel environment (Kalueff & Tuohimaa, 2004). However, in the latter one, the rigid pattern of rostro-caudal order of grooming is often discontinuous with a higher number of incorrect transitions between different grooming stages. It results in a consequent increase in the number of grooming bouts and duration of self-grooming (Kalueff & Tuohimaa, 2004). In a novel environment, self-grooming therefore serves as a useful anxiety-related behavioural parameter. Here I also show that self-grooming may be the most sensitive parameter to measure transfer of negative emotional information between mice.

Comparisons of individual parameters showed not significant, yet visible tendency of increased rearing frequency and duration, another anxiety parameter. However, differences in parameters describing social behaviour were not significant and moreover not always consisted between their frequency and duration. It could be explained by small sample size of used experimental groups that was not sufficient to overcome individual variability. Individual differences rise from innate predisposition, preceding social encounter and hierarchy position of both acceptor mice and demonstrator mice. In my setup there were at least three known pre-existing sources of variability that would influence extent of data variation in the last test, i.e. EPM, LDB and ASR. First of all, different individual sensitivity of demonstrator mouse to electric shocks would result in a different stress level. Secondly, various acceptor mouse ability to perceive emotional state of the demonstrator. And at last, distinct individual acceptor performance in either EPM, LDB or during ASR. It would be therefore very useful to evaluate anxiety level and social behaviour of all mice prior to testing and correlate it with the rest of obtained data.

In summary, the results suggest that male mice respond less variably to a stressed conspecific than females and that in both sexes, self-grooming is the most sensitive ethological parameter for a transfer of emotional information evaluation. Furthermore, this transfer is detectable during the direct encounter but not further in the ultimate test possibly due to high individual variability and small samples size. It is also possible that the novel environment of the maze of EPM, LDB or the holder of ASR combined with recurrent manipulation might outweigh the transferred fear and simply refocus the mice towards a new stressor equally in the sham and the stressed group. Possibility of not sufficient sample size is concordant with results of transfer of emotional information in rats (Knapska et al., 2006). In this paper authors showed changed exploratory behaviour in acceptor animals and higher startle response, however they used 16 animals per group for behavioural experiments.

Increasing animal number in such studies remains questionable due to financial and space requirements for this behavioural test. First, in this protocol a lot of different equipment is used, preferably all in separate rooms. I usually used one room for each: naïve mice, donor receiving foot-shocks, transfer of emotions, acceptor mouse in EPM, LDB or ASR, and already tested mice. This is unfulfillable for many laboratories. Additionally, mice are housed in pairs, which raises space requirement also in the animal housing area. Moreover, separation or pooling mice to live in pairs is also problematic. While pairing mice works smoothly in females, in males it causes problems. Often males harmed or killed one another after pairing, even though the pairs were always combined from siblings. Often, this situation resulted in an undesirable low number of male pairs available for the experiment. To resolve this problem, more mice should be used for pairing prior to experiment. Yet still, it raises financial and space requirements, which remains a negative aspect of this behavioural test.

## 7.2. 16p11.2del mouse model of ASD

Evaluation of developmental differences between 16p11.2 deletion mice and wild type were performed on insufficient samples size due to technical obstacles and that is why no data suitable for discussion were acquired. The mouse sperm that we obtained from Cold Spring Harbor Laboratory was most likely damaged during the transport, which affected reanimation and breeding to a sufficient extent. Nevertheless, according to the scarce data that I collected, mice mirror the general phenotype of 16p11.2del mice described in publications in lower body weight and normal pup development, nociception, olfactory sense and nestlet shredding.

Speculatively, mice also showed a tendency to an elevated basal level of anxiety showed in the olfactory discrimination test. In mice, higher anxiety shortens novel object exploration while lower anxiety prolongs it (Cox & Rissman, 2011b). Because of that, heterozygotes could explore the cotton bud less in

the first presentations, but once they habituated to it, their exploration lengthened. Anxiety was not assessed with other behavioural tests, therefore this theory cannot be confirmed. However, 16p11.2del mice usually express altered anxiety, thus similar alteration can be expected in my mice as well (Lynch et al., 2020; Mitchell et al., 2020; Pucilowska et al., 2015, 2018).

# 8. Conclusion

This study presents a behavioural paradigm to study a transfer of negative emotional information in mice. Presented data show a very pronounces sexual dimorphism and individual variability confounding the results. Male mice appear to be more suitable for this protocol as their behaviour was more specifically affected by treatment. Innate and momentary individual pro-social tendency of male mice resulted in a strong differentiation in the type of reaction to a stressed conspecific, where some increased the direct inspection and pro-social behaviour while other withdrew from social interaction and showed increased anxiety-related behaviour. Nonetheless, in both sexes the anxiety-related self-grooming behaviour was the most sensitive to detect a transfer of negative emotion between conspecifics. Usage of a larger amount of subjects might help overcoming the inter-individual variability and possibly obtain significant results in other relevant parameters.

Additionally, this study presents a partial evaluation of 16p11.2 deletion mouse model of ASD, mostly mirroring published findings such as lower body weight and normal pup development, no sensory issues and typical nestlet shredding behaviour. However, presented data were not suitable for any statistical evaluation because the offered mouse sperm was damaged during transport which resulted in a reanimation and breeding issues together with a high amount of phenotypical defects. Although I was able to successfully implement a new method of free floating immunohistochemistry staining into our lab, it was not used for neurostructural evaluation of 16p11.2del mice since our model was concluded to be aberrant. Hopefully, this method will be used in a different model, since neurostructural evaluation together with validation of transfer of negative emotion may push us way forward in autism research.

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