Mouse models of neuropsychiatric phenotypes

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Daniela Winkler
from Kassel

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DOCTORAL THESIS COMMITTEE

Prof. Dr. Dr. Hannelore Ehrenreich (Supervisor, First reviewer)
Clinical Neuroscience
Max Planck Institute of Experimental Medicine
Hermann-Rein-Straße 3
37075 Göttingen

Prof. Dr. Klaus-Armin Nave (Second reviewer)
Neurogenetics
Max Planck Institute of Experimental Medicine
Hermann-Rein-Straße 3
37075 Göttingen

Prof. Dr. Uwe-Karsten Hanisch
Neuropathology
University Medical Center Göttingen
Robert-Koch-Straße 40
37075 Göttingen

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Declaration

I hereby declare that this thesis has been written independently and with no other sources and aids than quoted.

Daniela Winkler Göttingen, 27. August 2014

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1. Introduction and focus of the present thesis work

This cumulative thesis includes two original first author publications, which focus on two mouse models of neuropsychiatric phenotypes. In the first publication we developed an autism severity score for mice using *Nlgn4* null mutant mice as a construct-valid model of heritable monogenic autism (El-Kordi, Winkler et al., 2013). The second publication describes that *Gpm6b* deficiency impairs sensorimotor gating and modulates the behavioral response to a 5-HT2A/C receptor agonist in mice (Dere, Winkler et al. 2014).

1.1 Introduction to neuropsychiatric disorders

Neuropsychiatric disorders are highly heterogeneous with a plethora of symptoms, ranging from mild manifestations to severely hampering conditions that interfere with everyday life. Classification is difficult, as symptom clusters associated with disease entities frequently overlap. Defined diseases are rare and genetic components are hard to delineate from symptom complexes (Sullivan et al., 2012). Even if classification into a distinct disorder is possible, patients with the same diagnosis, of schizophrenia for example, are very heterogeneous. Some symptoms range from absent to severely abnormal in patients with the same diagnosis. It is important to note that psychiatric syndromes are referred to as disorders, a term defined as illnesses that disrupt normal functioning. The pathophysiology or structural pathology is not known in most cases, therefore use of the term 'disease' is unsupported (Sullivan et al., 2012). Further studies are needed to delineate the molecular pathophysiology of neuropsychiatric disorders and facilitate new treatment approaches.

The causes of many neuropsychiatric disorders vary from one disorder to another, but in some cases the mechanisms or triggers are unclear. The research focus of my work lies on neuropsychiatric phenotypes, mainly associated with autism spectrum disorders (ASD) and depression, sharing common features, such as increasing prevalence in recent years (Compton et al., 2006; Matson et al., 2011), patients with young age (for depression only in some patients), high disease

burden, lifelong illness and disturbance of social life. Additionally, treatment approaches are limited. There is no specific treatment available to treat the core symptoms of ASD (Kumar et al., 2012), while in depression antidepressant medications are used to treat certain symptom clusters; however, treatments can take several weeks to have an effect (Wong et al., 2004), and such effects are seen only in about 50% of depressed patients (Fava, 2003). Further symptoms commonly seen in neuropsychiatric disorders (e.g. ASD and depression) include lifelong cognitive impairments, hampered development and impeded learning. Psychiatric disorders are often accompanied by changes in affective state, which are part of the diagnosis; however, lifelong cognitive impairments, which can hamper development and learning, are equally cumbering but have been comparatively neglected (Millan et al., 2012). Antidepressant drugs, which are able to alleviate some symptoms, such as depression and anxiety, often worsen or have no effect on the cognitive impairments (Hill et al., 2010). Successful treatment of these impairments is not possible without first understanding them, which requires further studies (Millan et al., 2012).

Since society is aging (Murray et al., 1997) and number of patients affected by neuropsychiatric conditions are increasing (World Health Organization, 2008), observation and study of these disorders should become a global health priority (Ferrari et al., 2013). Considering the fact that there is so far no cure for most neuropsychiatric disorders, one possible target would be improvement of the cognitive abilities of psychiatric patients. For this endeavor, there is still need for valid animal models that cover a broad variety of symptoms observed in affected humans (Cryan & Holmes, 2005). Feasible animal models would facilitate pharmacological studies for the development of treatments in this field. In the last decade, there has been growing interest in the investigation, modeling and treatment of the cognitive dysfunctions involved in neuropsychiatric disorders (Brigman et al., 2010).

1.2 Animal models of neuropsychiatric conditions with a focus on mice

The need for animal models of human disorders has always been present. In the past, the rat became the favored rodent for studying behavior, especially for the examination of cognitive abilities and behavioral pharmacology. Rats are intelligent and more robust when it comes to invasive techniques than smaller rodents. Only the development of molecular technologies, which enabled researchers to target and alter specific genes in mice (Capecchi, 2005), indirectly reduced the impact of rats in the scientific field. Since the successful sequencing of the human genome (Venter et al., 2001), the mouse became the second most preferred mammal to investigate, due to ease of handling, space- and cost-effectiveness and short breeding times. Complete sequencing of the mouse genome (Waterston et al., 2002) made it possible to assess target genes for neuropsychiatric disorders in knockout mouse models.

To understand neuropsychiatric conditions, complex syndromes must be approached by investigation of animal models with the highest validity for the relevant symptoms. Furthermore, several models can be used in an additive fashion to understand the whole picture (Kalueff et al., 2007). The lack of universally accepted animal models for neuropsychiatric disorders has limited the development of new pharmacological treatments. Accepting the fact that the disorders cannot be modeled by one animal model alone, these single models can be used to dissect specific biological features, which can then be targeted by new compound classes addressing these features (Wong et al., 2004).

1.3 Introduction to autism spectrum disorders and the *Nlgn4* null mutant mouse model

Autism spectrum disorder (ASD) is an umbrella term for a group of disorders sharing common features. They are characterized by symptoms like deficits in social interaction, impairments in language acquisition and communication and repetitive behaviors, as well as restricted interests (American Psychiatric Association, 2013). ASD is of special significance, because it affects brain functions that are highly sophisticated: social awareness and communication. It is so far incurable, with a high socioeconomic burden and a profound impact on life of the patients and their families.

Some individuals with the diagnosis for ASD show mild symptoms, while others have more severe symptoms (Murcia et al., 2005), even diminishing their everyday functioning and quality of life. The wide range of observed symptoms is referred to when describing any of such disorders as 'Autism Spectrum Disorder' (Persico et al., 2006).

As defined in the *Diagnostic and Statistical Manual of Mental Disorders 4th edition* (DSM-IV), observation of a total of six items, composed of two items of qualitative impairment in social interaction, at least one item of qualitative impairment of social communication, and one item of restricted repetitive and stereotyped pattern, justifies the diagnosis of an autistic disorder (American Psychiatric Association, 2000).

It has been observed that autistic individuals can additionally show a range of symptom features, which diverge from the typical symptomatic picture for ASD. For example, Courchesne and coworkers found an increase in brain volume in the frontal lobes and the anterior temporal regions in ASD patients (Courchesne et al., 2007). Other examples of atypical symptoms include disturbed sensory responsiveness, abnormal motor activity, disrupted sleep and associated seizures (Lord et al., 2000; Newschaffer et al., 2007). The characteristics of these patients are grouped under the label of 'Pervasive Developmental Disorders'. The complexity of the heterogeneity becomes even more obvious when one considers

the latest version of the *Diagnostic and Statistical Manual of Mental Disorders V* (American Psychiatric Association, 2013) in which the definition of autism has been changed. With this iteration of the DSM, the category of ASD has officially been recognized. This umbrella term now replaces the group of 'Pervasive Developmental Disorders', including 'Autistic Disorders', 'Asperger's Disorders' and 'Pervasive Developmental Disorder not otherwise specified (including atypical autism)'. Changes to diagnostic criteria are still under consideration, pending new advances in understanding this disorder, indicating that autism is not a single disease entity, but rather shows a combination of clinical symptoms.

In 2008 the overall estimated prevalence of ASD was 11.3 per 1,000 American children at the age of eight year (Baio, 2012). However, sex dependence is clear as boys are roughly four times more often affected than girls (estimated prevalence from 2008: 18.4 per 1,000 in males, 4.0 per 1,000 in females) (Baio, 2012). In the last years, the prevalence of autism has increased for unknown reasons. This increase could be partially explained by the increased awareness of the disease (Rice, 2011). Diagnosis is purely based on the examination of patients and their behavior, since there are no biomarkers or parameters available to verify the disease type or state (Voineagu et al., 2013). As such, diagnosis numbers and types can vary substantially, strongly dependent on observer knowledge and training (Matson et al., 2011). In particular, cognitive symptoms might be judged subjectively when results of examinations are not easily categorized, especially in nonverbal and young children (Manning-Courtney et al., 2013).

In the case of monozygotic twins, concordance rates vary from 60-95%, indicating a robust genetic component of the disorder (Murcia et al., 2005). Different screening methods and diagnostic criteria lead to a wider range of estimates in heritability (Murcia et al., 2005). Although heritability is high in monozygotic twins, indicating the genetic contribution, finding the causative mutations or aberrations is not straightforward. Epidemiological studies have attempted to find a genetic basis for ASD resulting in a plethora of candidate genes probably involved in the development of the disease (Risch et al., 1999). However, the inheritance of ASD is far more complex than basic Mendelian genetics, suggesting contributions from multiple genes and from external factors, such as the environment (e.g. Gadad et

al., 2013). In some cases, single gene mutations have been linked to ASD; however, these genes are interestingly always synaptic genes and it is therefore unsurprising that abnormal synaptic homeostasis has been suggested as a risk factor for ASD (Bourgeron, 2009).

Though the etiology of ASD remains unknown, some monogenic heritable forms have been observed, a number of which were found to be linked to genes on the X-chromosome (for review see Provenzano et al., 2012). Of these, the most important and relevant for ASD are the Fragile Mental Retardation locus, the methyl-CpG binding protein 2 locus, the TSC locus, and the loci encoding NLGN3, NLGN4, NRXN1, SHANK2 and SHANK3.

Alteration of the X-linked Fragile Mental Retardation locus (*FMR1*) leads to Fragile X Syndrome, which is characterized by mental retardation and autistic features. *Fmr1* knockout mice show increased activity and attention dysfunctions, though the results of social tests vary and require further investigation (Mineur et al., 2002; for review see Bernardet et al., 2006).

Mutations in the gene encoding methyl-CpG binding protein 2 (MECP2) lead to Rett Syndrome (RTT), an X-linked developmental disorder. Mouse models with dysfunctional MeCp2 show symptoms similar to RTT (Chen et al., 2001) and exhibit enhanced anxiety and disturbed social interaction (Chahrour et al., 2007).
Tuberous sclerosis is a genetic disease showing some symptoms reminiscent of ASD. The proteins TSC1 and TSC2 inhibit the mammalian target of rampamycin (mTOR) in the phosphoinositide 3-kinase (PI3K) signalling pathway (Yates, 2006).

Loss-of-function mutations in the genes of *NLGN3*, *NLGN4* (Jamain et al., 2003), *NRXN1* (Etherton et al., 2009), *SHANK2* (Berkel et al., 2010) or *SHANK3* (Durand et al., 2007) have been perceived to cause monogenic heritable forms of ASDs, as well as point mutations of neuroligin-3 (homolog of neuroligin-4). NL-3 deficient mice show reduced ultrasound vocalizations and a lack of preference for social novelty (Radyushkin et al., 2009). *NRXN1*, *SHANK2* and *SHANK3,* though important for ASD, lie outside the scope of this thesis and will therefore not be further discussed.

In humans there are five Neuroligin (NL) isoforms: NL1-3, NL4X and NL4Y (Jamain et al., 2003). The distribution of the proteins is not limited to one specific brain area, they are essentially expressed all over the central nervous system, with NL4 being consistently localized to glycinergic postsynapses in the brainstem and spinal cord of mice (Hoon et al., 2011).

Altered synaptic gene expression can therefore be directly associated with autism spectrum disorders, indicating that synaptic integrity is of great importance for mental health, especially during early brain development (Currenti, 2010). Disruption of proteins involved in the regulation of synaptic protein synthesis can lead to aberrant transduction signaling in affected patients (Jamain et al., 2003). These familial monogenic heritable forms of autism could therefore be a possible basis for studying ASD and have led to the development of animal models with a nonsyndromic character.

Based on the fact that the criteria for autism diagnoses are purely based on behavior, it is important to consider that a valid animal model should cover the three main symptom clusters (Crawley, 2012). Most animal models for ASD show and are limited to face validity, because they model only the characteristic human symptoms. Recent animal models additionally show construct validity, by presenting also the underlying mechanism (e.g. animals modeling monogenic heritable forms of autism). Optimal animal models would simultaneously fulfill predictive validity; however, since there is no effective treatment available for autism (Kumar et al., 2012), it is not currently possible to test the predictive validity.

Animal models that are able to solidly model facets of the disease should replicate a subset of the reported pathologies (Murcia et al., 2005). ASD affects the social and communicative functioning of patients, some features of which are difficult to replicate in a rodent model of this disorder. To meet the criteria of being an actual animal model for ASD, the model has to cover the three main features of the disorder: social interaction deficits, communication deficits and restricted, repetitive and stereotyped patterns of behavior (Crawley, 2012). In former times, experimentally induced abnormalities produced behavioral changes reminiscent of

autistic characteristics. Lesion studies of the amygdala pointed to behavioral abnormalities that could be studied (Daenen et al., 2002); however, these changes were not based on genetic abnormalities or aberrant developmental pathways. Infectious agents and chemicals were also used to induce alterations in the global brain (Murcia et al., 2005).

ASD has been recognized as a comorbid feature of more than a hundred genetic and genomic disorders (Betancur, 2011). However, ASD mutations with high penetrance are rare, like the Rett's syndrome mutations in MECP2. Estimates from exonic de novo mutations suggest that ASD is highly polygenic (Sanders et al., 2011), particularly involving synaptic regions, which supports the previously mentioned hypothesis.

1.4 Introduction to depression and the *Gpm6b* null mutant mouse model

According to the World Health Organization, depression (short for major depressive disorder) will be the second leading cause of disability by 2020 (Murray et al., 1997) and is associated with a high risk for suicide (Lesage et al., 1994). Suicides are the second leading cause of death among young people between the age of 15-24 and 25-34 (Heron, 2013), indicating a global health priority for depressive disorders (Ferrari et al., 2013). The World Health Organization is classifying severe depression as a "severe" disability (equivalent level to blindness, Down syndrome or active psychosis), which accounted for one third of all disabilities worldwide in 2008 (World Health Organization, 2008). The financial burden for the society is immensely high, estimated at, for example $83.1 billion for the year 2000 in the United States (Greenberg et al., 2003).

The pathophysiology of depression is still not completely understood; however there are several mechanisms possibly involved (Femenia et al., 2012). In a great number of studies, it has been shown that there is a robust link between the short form of the serotonin transporter (SERT) polymorphism and depression (for review see Sharpley et al., 2014). In 1996 it was revealed that reduced SERT expression and altered SERT function (due to a repeat length variation in the SERT gene *SLC6A4*) are associated with anxiety and depression-related personality traits (Lesch et al., 1996). SERT actively transports serotonin from the synaptic cleft into the presynaptic neuron and it has been shown that M6b interacts with this transporter (Fjorback et al., 2009). The interaction between M6b and SERT has been hypothesized to mediate a down-regulation of SERT uptake, and could therefore be part of the regulatory mechanism of SERT trafficking (Fjorback et al., 2009). Alterations in SERT mediated serotonin reuptake and the accompanying effects on serotonergic neurotransmission have also been linked to psychiatric disorders (Murphy et al., 2004). Mouse models of altered SERT function have yielded observations of phenotypic changes including increased anxiety and stress-related behaviors (for review see Murphy et al., 2008).

M6A (Gpm6a) and M6B (Gpm6b) belong to a proteolipid family of neuronal tetraspan glycoproteins, which are expressed throughout the brain (Werner et al., 2001) and (Möbius et al., 2008). M6b was first described when Yan and coworkers utilized expression cloning to determine the sequence of M6 and its functional role. They found two cDNAs, which show a high homology to the oligodendroglial proteolipid protein PLP (Yan et al., 1993), which is a structural protein of the central nervous system (CNS) myelin (Möbius et al., 2008). Mutations in PLP have been linked to Pelizaeus-Merzbacher disease and spastic paraplegia type 2 (Saugier-Veber et al., 1994), indicating an important role of this protelipid family in health and disease.

More than 80 mutant animal models related to depression and anxiety have been described to date (Cryan & Holmes, 2005). In 1969, McKinney and Bunney suggested that the minimal requirements for the validity of an animal model for depression are:

- 'reasonably' analogous to the symptomatology of the disorder;
- behavioral changes can be objectively monitored;
- behavioral changes are reversed by the same pharmacological treatments as in humans; and
- reproducibility in different laboratory settings (McKinney et al., 1969).

This standard built the basis for today's judgement of animal model validity. According to these standards, we tested *Gpm6b* deficient mice in a number of paradigms, all important for the examination of depressive phenotypes. Since depression is often viewed as a manifestation of a disturbed reaction to stressful life events, behavioral paradigms have to account for stress. As such, the behavioral readouts of the depression assessment in rodents include exposure to stressful situations. One example of these experimental evaluations is the forced swim test (FST) for behavioral despair in mice, introduced by Porsolt in 1977 (Porsolt et al., 1977). A similar test is the tail suspension test (TST), in which mice are hung by their tails (Chermat et al., 1986; Cryan, Mombereau, et al., 2005). Both tests involve mice struggling to escape, however in both cases the mice will not be able to succeed. The time interval during which the mice keep exhibiting escape movements is measured, providing a feasible indicator for individual willingness to survive or surrender, making this test a very efficient tool for

depression like symptoms in mice and therefore the screening of new antidepressant drugs (Cryan & Holmes, 2005). Anhedonia, which is another important diagnostic criterion for depression, can be measured by sucrose preference in mice. Already in 1987 it was shown in rats, that stress induces anhedonia, which can be reversed by antidepressant treatment (Willner et al., 1987).

In my second project, we characterized a mouse lacking *Gpm6b*, to assess the impact of this deficiency on the behavioral phenotype of *Gpm6b* deficient mice and its impact on SERT signaling. Since it is well known that impairments in serotonin neurotransmission are involved in the symptoms associated with major depressive disorder, we studied in a series of experiments, whether Gpm6b-deficient mice (that might suffer from a lack of extracellular serotonin due to an increase in the cell-surface expression of the serotonin transporter) would consequently exhibit depression-like symptoms along with cognitive impairments (Dere, Winkler et al., 2014).

2. Aims of the present thesis work

The projects of my PhD thesis were mainly stimulated by the growing interest in animal models of neuropsychiatric diseases, such as autism-spectrum disorders, major depression, anxiety disorders and schizophrenia. Since valid animal models are needed for the assessment of higher brain functions and their variance, the present thesis work aims to investigate two models, one for ASD and one for depression.

2.1 Aim of Project 1

In this project, we specifically wanted to address autistic behavior in a construct valid animal model for ASD (*Nlgn4* knockout), extending previous findings (Jamain et al., 2008) to the extensive assessment of repetitive stereotypic behavior, and additionally to the female gender. Furthermore, we wanted to use the test readouts to generate an autism composite score, which would allow a sharp discrimination between non-autistic and autistic animals. This discrimination is crucial for the development of future therapeutics as well as treatment strategies and can therefore not be overestimated.

2.2 Aim of Project 2

In my second project, we wanted to explore the use of *Gpm6b* deficient mice as an animal model of depression. To pursue this question, we were interested in the assessment of the basic behavioral phenotype of *Gpm6b* deficient mice, since there was no information available at that time. We were specifically interested in the behavioral domains of sensory functions, motor functions, exploratory behavior, sociality, cognition and readouts for depression. Moreover, we wanted to reveal potential changes in 5-HT neurotransmission in the brain of *Gpm6b* deficient mice by using a 5-HT2A/C receptor agonist and comparing behavioral responses, between knockout and wildtype littermates.

3. Development of an autism severity score for mice using Nlgn4 null mutants as a construct-valid model of heritable monogenic autism

3.1 Overview of project 1

The term “Autism Spectrum Disorder” (ASD) is summarizing a group of pervasive developmental disorders characterized by difficulties in social interaction, communication and by the expression of stereotyped repetitive behaviors and restricted interests (American Psychiatric Association, 2013). Symptoms can vary from mild to very severe occurrence. The etiology of ASD is still unknown in most cases, but monogenic heritable forms exist that have provided insight into ASD pathogenesis and have led to the notion of autism as a ‘synapse disorder’ (El-Kordi et al., 2013). Several of these monogenic heritable ASD forms are caused by loss-of-function mutations in the *NLGN3*, *NLGN4*, *NRXN1*, or *SHANK2 and SHANK3* genes. The gene *NLGN4* encodes Neuroligin-4 (NL4), which is a cell adhesion protein at nerve cell synapses, and the current state of research indicates that aberrant signalling between nerve cells causes the ASD phenotype in affected patients (Jamain et al., 2003). Our group was able to show that *Nlgn4* null mutant (*Nlgn4* -/-) mice exhibit autistic-like behavior, including disturbed social interaction and compromised ultrasound vocalization. Therefore these animals can be used as a genetic animal model for ASD, due to their construct and face validity (Jamain et al., 2008).

The number of valid animal models for autism is still limited, and so far little is known about sex differences in these animal models (for review, see Kokras et al., 2014). Therefore, in my project, we wanted to extend the characterization of *Nlgn4* null mutant mice from males to females in order to delineate fine sex differences. Additionally, we included a more comprehensive set of ASD relevant readouts, especially extending to repetitive stereotyped behaviors.

For this project, we used male and female *Nlgn4* null mutant mice and their wildtype littermates. The animals underwent basic behavioral characterization including elevated plus maze, open field, hole board, rota-rod and prepulse inhibition of the startle response to measure general activity, anxiety levels, motor performance, exploratory behavior and sensorimotor gating, respectively. The resulting findings show that the basic behavior is not affected in *Nlgn4* null mutant mice. However, we found significant differences in *Nlgn4* null mutant social behavior and communication compared to wildtype animals in both genders. Social interaction time was significantly decreased in both male and female *Nlgn4* null mutants (see Fig. 1 in the publication). In terms of vocalization, the numbers of calls were significantly reduced in both genders, compared to their wildtype littermates. We additionally addressed nest-building abilities in these animals, however only the male *Nlgn4* null mutant mice showed significantly reduced nest-building scores. Female mice showed the same tendency, but their performance did not reach statistical significance compared to wildtype females.

The results of all tests relevant for ASD showed that *Nlgn4* null mutant mice of both genders exhibit a syndrome covering the three main domains: disturbed social interaction, compromised communication and expression of repetitive behaviors. From the results of the behavioral tests, we were able to derive an autism severity composite score, composed of seven autism-relevant behavioral readouts for mice, as an indicator of overall syndrome severity. With this score, we are able to predict the genotype with a 100% accuracy in males and with an accuracy of 83% in females. The development of the autism composite score in *Nlgn4* null mutant mice has helped to alleviate the variance in observed behaviors in mice and might therefore be an important tool for the evaluation of new pharmacological treatment approaches targeting NLGN4 function (El-Kordi et al., 2013; Wöhr et al., 2013). Although there is a huge variety of symptoms caused by varying expression levels of a single gene, there would optimally be one treatment for all the symptoms caused by a particular genetic modification. The autism score has already been recognized and appreciated in the field, and may help to establish a “one mutation - one drug” approach (Wöhr et al., 2013).

3.2 Original publication

El-Kordi A[1], Winkler D[1], Hammerschmidt K, Kästner A, Krueger D, Ronnenberg A, Ritter C, Jatho J, Radyushkin K, Bourgeron T, Fischer J, Brose N, Ehrenreich H: Development of an autism severity score for mice using Nlgn4 null mutants as a construct-valid model of heritable monogenic autism. Behavioral Brain Research 2013; 251(8):41-9.

[1]Equal contribution

Own contribution: I was responsible for planning and conducting the behavioral experiments of this study (elevated plus maze, open field, hole board, rotarod, prepulse inhibition, social interaction, LABORAS), including the establishment of a new grooming test (according to Kalueff et al. Nature Protocols 2007) in our facility. Together with Ahmed El-Kordi, I analyzed the data statistically, interpreted the findings and generated the graphical illustrations of the data (Figures 1 and 2) that were used for this publication. Furthermore, I was involved in the design, writing, revision and submission of the manuscript.

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Research report

Development of an autism severity score for mice using *Nlgn4* null mutants as a construct-valid model of heritable monogenic autism

Ahmed El-Kordi[a,b,1], Daniela Winkler[a,1], Kurt Hammerschmidt[c], Anne Kästner[a], Dilja Krueger[d], Anja Ronnenberg[a], Caroline Ritter[a], Jasmin Jatho[a], Konstantin Radyushkin[a,b], Thomas Bourgeron[e,f,g], Julia Fischer[c], Nils Brose[b,d,2], Hannelore Ehrenreich[a,b,*]

[a] *Division of Clinical Neuroscience, Max Planck Institute of Experimental Medicine, Göttingen, Germany*
[b] *DFG Center for Molecular Physiology of the Brain (CMPB), Göttingen, Germany*
[c] *Cognitive Ethology Laboratory, German Primate Center, Göttingen, Germany*
[d] *Department of Molecular Neurobiology, Max Planck Institute of Experimental Medicine, Hermann-Rein-Str. 3, 37075 Göttingen, Germany*
[e] *Human Genetics and Cognitive Functions, Paris, France*
[f] *CNRS URA 2182 'Genes, synapses and cognition', Institut Pasteur, Paris, France*
[g] *University Paris Diderot, Sorbonne Paris Cité, Human Genetics and Cognitive Functions, Paris, France*

HIGHLIGHTS

- Loss-of-function mutations of *NLGN4X* are the most frequent monogenic autism cause.
- *Nlgn4*KO mice show reduced social functions/communication and increased stereotypies.
- Females exhibit a slightly milder phenotype.
- For the first time a gender-specific autism severity composite score is presented.
- These data favour *Nlgn4* mutant mice as an ASD model with construct and face validity.

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ABSTRACT

Autism is the short name of a complex and heterogeneous group of disorders (autism spectrum disorders, ASD) with several lead symptoms required for classification, including compromised social interaction, reduced verbal communication and stereotyped repetitive behaviors/restricted interests. The etiology of ASD is still unknown in most cases but monogenic heritable forms exist that have provided insights into ASD pathogenesis and have led to the notion of autism as a 'synapse disorder'. Among the most frequent monogenic causes of autism are *loss-of-function* mutations of the *NLGN4X* gene which encodes the synaptic cell adhesion protein neuroligin-4X (NLGN4X). We previously described autism-like behaviors in male *Nlgn4* null mutant mice, including reduced social interaction and ultrasonic communication. Here, we extend the phenotypical characterization of *Nlgn4* null mutant mice to both genders and add a series of additional autism-relevant behavioral readouts. We now report similar social interaction and ultrasonic communication deficits in females as in males. Furthermore, aggression, nest-building parameters, as well as self-grooming and circling as indicators of repetitive behaviors/stereotypies were explored in both genders. The construction of a gender-specific autism severity composite score for *Nlgn4* mutant mice markedly diminishes population/sample heterogeneity typically obtained for single tests, resulting in p values of <0.00001 and a genotype predictability of 100% for male and of >83% for female mice. Taken together, these data underscore the similarity of phenotypical consequences of *Nlgn4/NLGN4X loss-of-function* in mouse and man, and emphasize the high relevance of *Nlgn4* null mutant mice as an ASD model with both construct and face validity.

* Corresponding author at: Division of Clinical Neuroscience, Max Planck Institute of Experimental Medicine, Hermann-Rein Str. 3, 37075 Göttingen, Germany. Tel.: +49 551 3899 628; fax: +49 551 3899 670.
E-mail addresses: brose@em.mpg.de (N. Brose), ehrenreich@em.mpg.de (H. Ehrenreich).
[1] Equal contribution.
[2] Tel.: +49 551 3899 727; fax: +49 551 3899 715.

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1. Introduction

The term autism spectrum disorders (ASD) describes a group of etiologically heterogeneous conditions, forced under a common label according to presently used clinical classification systems of psychiatric diseases. The purely phenotypical diagnosis – with all its pitfalls and differential diagnoses on top – blinds out the tremendous heterogeneity of ASD and likely also accounts for the low interrater reliability achieved in the clinical diagnosis of adult autism [1,2]. The discovery of monogenic heritable forms of the disorder involving mutations in genes encoding neuroligin-4X (NLGN4X), neurexin-1 (NRXN1), neuroligin-3 (NLGN3), SHANK2, and SHANK3, has not only strengthened the notion that autism might be a disease of the synapse but also led to the development of urgently needed mouse models with proven construct validity [3–9]. In the past, only animal models with some face validity could be used to study ASD and to search for therapeutic strategies [10] but no effective treatment for humans is available to date that would measurably improve core symptoms of ASD. More adequate animal models are expected to improve this situation.

Strikingly, several monogenic mouse models with disruption of only one synaptic gene display an array of entirely different symptoms, reminiscent of autism, i.e. compromised verbal and non-verbal communication, disturbed social interaction and social skills, narrowed interests and stereotypical repetitive behaviors ('routines'), impulsivity and altered aggression (e.g. for review, see [11–13]). Comparable to humans, however, the presentation of symptoms with respect to their severity varies considerably among individual mice even if they carry the same ASD-related mutation. Therefore, an autism severity composite score rather than the selection of single readouts might better assist in the search for new treatments addressing the core features of the disorder.

The present study has been designed (1) to comprehensively characterize the behavior of *Nlgn4* null mutant mice, a construct-valid model of monogenic heritable autism, extending previous work [4] to a greater variety of symptoms, particularly to repetitive stereotyped behaviors, and to the female gender which has never been studied; (2) to develop an autism composite score with highest power of contrasting between autistic and non-autistic individuals as an important basis for future therapeutic strategies.

2. Materials and methods

2.1. Mice

For all experiments reported here, male and female *Nlgn4* null mutant (*Nlgn4−/−*) and wildtype (WT) C57BL/6J mice (littermates) were used. They were derived from revitalized frozen embryos that originated from a C57BL/6J-SV129 mosaic *Nlgn4−/−* line that had been backcrossed into C57BL/6J for 6 generations [4]. After revitalization, *Nlgn4−/+* mice were bred with C57BL/6J for 2 further generations, and then *Nlgn4−/+* mice were interbred for colony expansion to generate mice for experiments. The revitalization was initiated since there had been concerns raised by others regarding a potential loss of phenotypical changes in these mice with increasing numbers of generations ('phenotype bleaching'). See also the paper by [14]. These concerns even led us to prematurely unblind an 8-arm preclinical treatment study with *Nlgn4−/−* versus WT mice (littermates) of generation 13, which had been running in our lab. Due to the still small number of untreated (control) mice available for behavioral analysis at the time point of unblinding this study, we cannot make any firm conclusions regarding reduction or stability of the originally reported phenotype [4]. The tendencies obtained, however, clearly do not support a loss of phenotypic changes in *Nlgn4−/−* (see Supplementary Figures 1–3). Nevertheless, such gradual 'bleaching' of phenotypic changes over generations with respect to higher brain functions may well happen, as illustrated by our follow-up studies of another mouse strain, cEPOR transgenic mice, where the transgene expression remained stable over the respective generations, but the initially superior spatial learning and memory, reversal learning/cognitive flexibility and activity gradually returned to control levels ([15]; Supplementary Figure 4).

Mouse genotyping: WT and *Nlgn4−/−* littermates were obtained from *Nlgn4+/−* heterozygous breeding pairs. Genotypes of the offspring were analyzed by PCR of tail genomic DNA using the following primers: Forward primer 5′-CTTCCTATCCTGTTACTCTCAC-3′, WT reverse primer 5′-TAGGGAAAGCGGAATTGAGTGTAAC-3′ (yielding a 475 bp product) and KO reverse primer 5′-ACACTCCAACCTCCGCAAACTCCT-3′ (yielding a 183 bp product). PCR amplification of the DNA was carried out with the following conditions: 5 min, 94 °C (1 cycle); 30 s, 94 °C; 30 s, 64 °C; 1 min, 72 °C (30 cycles), followed by final extension at 72 °C for 7 min.

2.2. Behavioral testing

All experiments were approved by the local Animal Care and Use Committee in accordance with the German Animal Protection Law. For behavioral testing, mice were housed in groups of 3–5 (except where otherwise specified) in standard plastic cages, with food and water ad libitum. The temperature in the colony room was maintained at 20–22 °C, with a 12 h light–dark cycle (light on at 7:00 am). All behavioral experiments were conducted by investigators, unaware of the genotype ('blinded'), during the light phase of the day (between 8:00 am and 5:00 pm). Basic behavioral functions were assessed in 2 large consecutive cohorts of male and female mice (genders tested separately) in the following order: elevated plus maze, open field, hole board, rota-rod, pre-pulse inhibition of the startle response (PPI), social interaction in pairs, ultrasound vocalization, and LABORAS spontaneous home cage behavioral assessment. The second cohort (again genders tested separately) was additionally evaluated in LABORAS, in a modified version of social interaction in the tripartite chamber [16], olfaction, marble burying, nest building, induced self-grooming in LABORAS as well as in enriched environment, and finally aggression, using the resident-intruder paradigm. The age of mice at the beginning of testing was 11–12 weeks. Inter-test interval varied depending on the degree of 'test invasiveness' but was at least 1 day.

Elevated Plus Maze Test: Individual animals were placed on the central platform facing an open arm of the plus-maze (made of gray Perspex with a 5 cm × 5 cm central platform, 2 open arms of 30 cm × 5 cm, and 2 closed arms of 30 cm × 5 cm × 15 cm, with overall illumination at 135 lx). Behavior was recorded for 5 min by an overhead video camera and a computer equipped with Viewer 2 software (BIOBSERVE GmbH, St. Augustin, Germany) to calculate the time each animal spent in open or closed arms. The proportion of time spent in open arms was used for the estimation of open arm aversion, which is an indicator of fear.

Open Field Test: Spontaneous activity in the open field was tested in a gray circular Perspex arena (120 cm in diameter, 25 cm high). Individual animals were placed in the center of the open field and were allowed to explore it for 7 min. The behavior was recorded by a computer-linked overhead video camera. Viewer 2 software (BIOBSERVE GmbH) was used to calculate the distance traveled and the time spent in the central, intermediate, and peripheral zones of the open field.

Hole Board Test: Individual mice were placed in the center of the hole board (transparent Perspex chamber (50 cm × 50 cm × 36 cm), with a non-transparent floor raised 3 cm above the bottom of the chamber with 16 equally spaced holes of 2.2 cm diameter), and allowed to explore the chamber for 5 min. The number of holes explored (head dips) was monitored by 2 layers of infrared photo beams connected to a computer with the AKS software (TSE Systems GmbH, Bad Homburg, Germany).

Rota-Rod Test: The rota-rod (Ugo Basile Srl, Comerio, Italy) comprised a rotating drum which was accelerated from 4 to 40 rpm over the course of 5 min. Individual mice were placed on the drum, and once they were balanced, the drum was accelerated. The time in seconds at which the respective animal fell from the drum was recorded using a trip switch. Each animal went through 3 consecutive trials, one trial per day.

Pre-Pulse Inhibition Test: Individual mice were placed in small metal cages (82 mm × 40 mm × 40 mm) to restrict major movements and exploratory behavior. The cages were equipped with a movable platform floor attached to a sensor that recorded vertical movements of the floor. The cages were placed in 4 sound-attenuating isolation cabinets (TSE Systems GmbH). Startle reflexes were evoked by acoustic stimuli delivered from a loudspeaker that was suspended above the cage and connected to an acoustic generator. The startle reaction to an acoustic stimulus, which evokes a movement of the platform and a transient force resulting from this movement of the platform, was recorded with a computer during a recording window of 260 ms and saved for further evaluation. The recording window was defined from the onset of the acoustic stimulus. An experimental session consisted of a 2 min habituation to 65 dB background white noise (continuous throughout the session), followed by a baseline recording for 1 min at background noise. After baseline recording, 6 pulse-alone trials using startle stimuli of 120 dB intensity and 40 ms duration were applied to decrease influence of within-session habituation. These data were not included in the 120 dB/40 ms analysis of the pre-pulse inhibition. For tests of pre-pulse inhibition, the startle pulse was applied either alone or preceded by a pre-pulse stimulus of 70 dB, 75 dB, or 80 dB intensity and 20 ms duration. An interval of 100 ms with background white noise was used between each pre-pulse and pulse stimulus. The trials were presented in a pseudorandom order with an interval ranging from 8 to 22 s. Amplitude of the startle response (expressed in arbitrary units) was defined as a difference between the maximum force detected during a recording window and the force measured immediately before the stimulus onset. Amplitudes were averaged for each individual animal, separately for both types of trials (i.e. stimulus alone or stimulus preceded by a pre-pulse). Pre-pulse inhibition was calculated as a percentage of the startle response using the following

formula: % pre-pulse inhibition = 100 – [(startle amplitude after pre-pulse)/(startle amplitude after pulse only) × 100].

Sociability and Social Memory Tests: Sociability and social memory were tested as described [16] with small modifications. The social testing arena was a rectangular, 3-chambered box. Each chamber was 40 cm × 20 cm × 22 cm in size. Dividing walls were made from clear Plexiglas, with rectangular openings (35 mm × 220 mm) allowing access into each chamber. The chambers of the arena were cleaned and fresh wood-chip bedding was added between trials. The test mouse was first placed in the middle chamber and allowed to explore for 5 min. The openings into the 2 side chambers were obstructed by plastic boxes during this habituation phase. After the habituation period, a C57BL/6N male mouse of the same age (stranger 1) without prior contact with the test mouse was placed in one of the side chambers. The location of stranger 1 in the left versus right side chamber was systematically alternated between trials. The stranger mouse was enclosed in a small (140 mm × 75 mm × 60 mm), rectangular wire cage, which allowed nose contact through the bars but prevented fighting. The animals serving as strangers had previously been habituated to placement in the small cage. An identical empty wire cage was placed in the opposite chamber. A weighted cup was placed on the top of the small wire cages to prevent climbing by the test mice. Both openings to the side chambers were then unblocked and the subject mouse was allowed to explore the entire social test arena for a 10 min session. The amount of time spent in each chamber and the number of entries into each chamber were recorded by the video-tracking system Viewer 2 (BIOBSERVE GmbH). An entry was defined as all 4 paws in one chamber. At the end of the first 10 min, each mouse was tested in a second 10 min session where stranger 1 changed the side to avoid bias by side preference. At the end of this session, mice were tested in a third 10 min episode to quantify social preference for a new stranger. A second, unfamiliar mouse of the same age (stranger 2) was placed into the previously empty wire cage. The test mouse had a choice between the already familiar stranger 1, and the novel unfamiliar stranger 2. As described above, time spent in each chamber and the number of transitions between chambers of the apparatus during the first (social preference) and third 10 min session (social memory) were analyzed. Based on the amount of time spent in each chamber, a 'sociability index' and a 'social memory index' were calculated according to the following formulas:

$$\text{sociability index} = \frac{\text{time}_{\text{stranger}}}{\text{time}_{\text{stranger}} + \text{time}_{\text{empty}}} \times 100$$

$$\text{memory index} = \frac{\text{time}_{\text{novel}}}{\text{time}_{\text{novel}} + \text{time}_{\text{familiar}}} \times 100$$

Test of Social Interaction in Pairs, i.e. 'Social Approach': The social interaction test was performed in a neutral cage (gray Plexiglas box, 30 cm × 30 cm × 30 cm). During 2 consecutive days, each individual mouse went through one 10 min session in the neutral cage to habituate to the testing conditions. On day 3 (test day), pairs of unfamiliar mice of the same genotype were placed into the neutral cage for 10 min. Behavior of mice was recorded by a computer and the video-tracking system Viewer 2 (BIOBSERVE GmbH). The time spent in social interaction (defined as staying in close contact) was registered.

Buried Food Finding Test: Starting 6 days before testing, mice were habituated to clear cages (29.5 cm × 18.5 cm × 13 cm) for 20 min, 4× per day. Starting 4 days before testing, mice received a piece of chocolate cookie (1.6 g) with water ad libitum at each habituation. Additionally, mice received 3–5 cookies in their home cage over night which they consumed within 24 h. When mice consumed also the cookies during the habituation phase, testing was performed on the next day. Starting 12 h before testing, mice were deprived of food with water ad libitum. For testing, individual mice were placed into clear cages, in which a piece of chocolate cookie was hidden under 1.5 cm standard bedding at the end of the cage. The mouse was positioned in the right corner at the opposite end of the cage, and the food finding time, i.e. the time from the moment the mouse was placed into the cage to the time it located the cookie and initiated burrowing, was recorded. As soon as the cookie was detected, the mouse was removed from the cage. Fresh bedding was used for each trial; all mice underwent identical testing procedures.

Resident-Intruder Test: Inter-male aggression was studied using the resident–intruder paradigm (e.g. [17,4]). Previously group housed male mice were separated and housed individually for 21 days before testing. As standard opponent males, we used group-caged males of the same age (4 months) from the C57BL/6N strain (Charles River, Sulzfeld, Germany). A standard opponent was introduced into the cage of the tested resident male and observation started when a tested resident male sniffed the opponent for the first time. The observation was stopped immediately after the first attack (an attack being defined as a bite) to prevent wounding, but lasted 10 min if no attack occurred. The latency to attack was recorded by a stop watch.

Ultrasound Vocalization Analysis: We recorded ultrasonic vocalizations (USVs) of male mice using the recording software Avisoft Recorder 4.2 at a sampling frequency of 300 kHz. The microphone (UltraSoundGate CM16) was connected to a preamplifier (UltraSoundGate 116), which was connected to a computer (all sound recording hardware and software was from Avisoft Bioacoustics, Berlin, Germany). For the recording, resident mice (males and females) were housed in single cages. At the day of the recording, mice in their own home cage were placed on the desk in the recording room for 60 s. Subsequently, the intruder mouse was put into the home cage of the resident, and the vocalization behavior was recorded for 3 min. We recorded male mice with unfamiliar female mice in estrous. Female mice were tested with anaesthetized unfamiliar females (anesthetic: intraperitoneal injection of 0.25% tribromoethanol, 0.1 ml/10 g body weight). We counted the number of calls per recording session and separated USVs from other sounds using the whistles detection algorithm of AVISOFT RECORDER 4.2 with following selection criteria: Possible changes per step = 4 (4687 Hz), minimal continuity = 8 ms, possible frequency range = 35–150 kHz. These criteria had been tested in former studies of mouse USVs [4,18].

LABORAS: The LABORAS™ system (Metris b.v., Hoofddorp, The Netherlands) consists of a triangular shaped sensor platform (Carbon Fiber Plate 1000 mm × 700 mm × 700 mm × 30 mm, Metris b.v.), positioned on 2 orthogonally placed force transducers (Single Point Load Cells) and a third fixed point attached to a heavy bottom plate (Corian Plate 980 mm × 695 mm × 695 mm × 48 mm, Metris b.v.). The whole structure stands on 3 spikes, which are adjustable in height and absorb external vibrations. Mice are housed in clear polycarbonate cages (Makrolon type II cage, 22 cm × 16 cm × 14 cm) with a wood-chip bedding covered floor. The cage is placed directly onto the sensing platform, with the upper part of the cage (including the top, food hopper and drinking bottle) suspended in a height-adjustable frame separate from the sensing platform. Resultant electrical signals caused by the mechanical vibrations of the movement of the animal are transformed by each force transducer, amplified to a fixed signal range, filtered to eliminate noise, digitized and then stored on a computer. The computer then processes the stored data using several signal analysis techniques to classify the signals into the behavioral categories of eating, drinking, scratching, circling, climbing, immobility, locomotor activity and grooming (for details see [19]). The behavior which dominates is scored. Spontaneous mouse behavior was assessed from 5:00 pm until 9:00 am, with 1 h habituation to the cages before the initiation of recording. Behaviors during the light as well as the dark cycle were analyzed separately.

Marble Burying Test: This test is used to assess stereotypies and obsessive–compulsive behaviors in mice [20]. Mice were tested in plastic cages (34.5 cm × 56.5 cm × 18 cm) filled with 5 cm deep wood-chip bedding. Evenly spaced (4 cm apart) 24 glass marbles were placed on the surface. Individual mice were put in the cage and left there for 30 min. Illumination was dimmed (6 lx). The number of buried marbles (to 2/3 their depth) during this time was counted. Two groups of mice with different housing conditions were tested. Mice were single housed for at least 10 days. Another group was left in the group housing cages.

Nest Building: Nest construction is an important indicator of social and reproductive behavior [21,22]. The nest building test was conducted according to a previously reported protocol [21]. Approximately 1 h before the dark phase, group housed mice were transferred to single housing cages with wood-chip bedding and nesting towels, but no environmental enrichment items. After 2 nights of habituation, nesting towels were removed and nestlets (pressed cotton squares, weighing ~3 g) placed in the single housing cages. Nest building was assessed on the next morning using a rating scale where high scores indicate an almost perfect nest.

Induced Self-Grooming in LABORAS and Enriched Environment: Grooming is a common and robust behavior in rodents. Excessive grooming has been observed in animal models of obsessive-compulsive disorders as well as ASD [23,8]. The natural tendency to groom might be amplified by various experimental procedures [24]. We used water induced self-grooming according to a previously described protocol [24] with slight modifications. Mice were misted with water and placed on the LABORAS platforms (see above) under red light for a 30 min recording session. Behavior was simultaneously video-recorded. The misting procedure was as follows: Mice were taken by their tail and lifted over a table, then misted with a spray bottle filled with water at room temperature. The misting pattern was 3× on the back (distance 30 cm), 3× on the abdomen and again 3× on the back. The analysis of LABORAS data was done as described above. Video-recorded grooming duration was estimated by 2 independent raters, unaware of the genotype. After an interval of 2 weeks, animals were placed into cages with enriched environment (with paper towel, toilet rolls, running wheel) and kept there for 72 h. Then grooming was induced again as described above and behavior was recorded (under red light in the enriched environment cages) with a video camera. Video ratings were conducted in the same manner as described above.

Statistical Analysis: Unless stated otherwise, the data given in figures and text are expressed as mean ± SEM, and were compared by 2-way analysis of variance (ANOVA) with post hoc planned comparisons, or by ANOVA for repeated measurements, Mann–Whitney U and χ^2 test where appropriate, using SPSS v.17 Software, San Diego, USA. A p value below 0.05 was considered to indicate significance.

Autism Composite Score: For the autism composite score, selected single readouts were z-standardized and presented such that higher values represent higher symptom severity. Relevant items were selected based on significant Mann–Whitney U test results. To build the scores on complete data sets, for each behavioral test, a linear regression based multiple imputations (10 iterations) model of missing data (males: vocal calls 17.6%, nesting score 23.5%, social approach 11.8%, marble burying 23.5%, circling 35.3%, aggression 32.4%; females: vocal calls 2.8%, novelty index 2.8%, social approach 11.1%, marble burying 16.7%, circling 52.8%) was applied to the z-standardized single tests for male and female mice separately. A total of 17 male mice (N = 13 WT and N = 4 KO) had to be excluded prior to imputation because more than

3 out of 6 readouts were missing. Altogether 4 female mice ($N = 4$ WT) were excluded because more than 3 out of 5 readouts were lacking. Thus, the imputation models were based on 34 ($N = 16$ WT and $N = 18$ KO) male and 36 ($N = 21$ WT and $N = 15$ KO) female individuals. Note that missing values are mainly due to test logistics (limitations of mouse numbers running in a certain test); there was no other systematic reason for exclusion of animals from particular tests, and exclusions were all random. Gender specific composite scores were calculated by integrating the means of the imputation matrices of all behavioral readouts. Genotype dependent group comparisons were conducted by Mann–Whitney U tests. Intercorrelation patterns (pairwise Pearson correlations) were based on available sets of z-standardized raw scores.

3. Results

3.1. Basic behaviors are normal in male and in female Nlgn4 null mutant mice

Basic behavioral test results for activity, anxiety, motor performance, exploratory behavior and sensorimotor gating of both male and female *Nlgn4* null mutant mice are shown separately in Supplementary Figures 5 and 6. Genotypes did not differ in elevated plus maze, open field, rota-rod, hole board, startle response and PPI performance.

3.2. Nlgn4 null mutants of both genders display broadly impaired social behaviors and communication

To comprehensively characterize the social phenotype in adult *Nlgn4* null mutants of both genders, we assessed social approach behavior, vocalization, aggression and nest building (Fig. 1). Consistent with our previously reported findings in male *Nlgn4* null mutants only [4], we again saw significantly reduced interaction time in males (Fig. 1A; $p = 0.0006$). This observation was now also made independently in female mutants (Fig. 1B; $p = 0.015$). Additionally, the number of calls was significantly reduced in both genders of *Nlgn4* null mutants (Fig. 1C, D; $p = 0.006$ and $p = 0.0097$, respectively). Again, as shown earlier [4] male *Nlgn4* null mutants had a higher attack latency in the resident-intruder paradigm (Fig. 1E; $p = 0.025$). In a newly performed test, nest building, only male mutants had a higher proportion of untouched nesting material (Fig. 1F, inset, $p = 0.049$) and built significantly less functional nests (Fig. 1G; $p = 0.006$). Female mutants showed the same tendency as the males. Their potential performance deficits, however, did not reach statistical significance (Fig. 1F, H).

3.3. Deficits in social functions of Nlgn4 null mutants as determined in the tripartite chamber are mild and look different dependent on gender

As reported previously [4], male *Nlgn4* null mutants showed a deficit in the first trial of social interaction in the tripartite chamber (Supplementary Figure 7A; 2-way ANOVA RM, $F_{\text{interaction}(1,39)} = 8.935$, $p = 0.0048$; $F_{\text{compartment}(1,39)} = 94.45$, $p < 0.0001$; $F_{\text{genotype}(1,39)} = 0.6493$, $p = 0.4253$). Post hoc single comparisons revealed that mutant mice spent less time in the compartment with the stranger mouse, and more time in the empty compartment (mouse compartment: $p = 0.018$; empty compartment: $p = 0.02$). Consequently, male mutant mice had a significantly lower sociability index as compared to WT animals (Supplementary Figure 7C; $p = 0.0058$). In contrast, in the present series of tests, social novelty was indistinguishable between WT and mutant males (Supplementary Figure 7E,G). On the other hand, sociability was not affected in female *Nlgn4* null mutants (Supplementary Figure 7B,D) which, however, showed a deficit in social memory (Supplementary Figure 7F; 2-way ANOVA RM, $F_{\text{interaction}(1,37)} = 6.444$, $p = 0.0155$; $F_{\text{compartment}(1,37)} = 3.163$, $p = 0.0835$; $F_{\text{genotype}(1,37)} = 3.826$, $p = 0.0581$). Post hoc single comparisons revealed a significantly reduced time spent with the new mouse in the female mutant group (Supplementary Figure 7F, $p = 0.015$) and thus a reduction in their novelty index (Supplementary Figure 7H; $p = 0.036$).

3.4. Stereotyped repetitive behaviors are detectable in Nlgn4 null mutant mice of both genders

Since stereotypies are an important feature of ASD, we decided to study this trait in *Nlgn4* null mutants and their WT littermates in more detail. To this end, we assessed marble burying both in single and group housed mice. Single housing has previously been used as an environmental stressor leading to subtle alterations in behavior [25]. Interestingly, only single housed null mutants of both genders had an increased number of buried marbles (Fig. 2A, B, left panels; Mann–Whitney U tests, $p = 0.0281$ and 0.0093 respectively). Group housed mice did not demonstrate a genotype effect (Fig. 2A, B, right panels). Spontaneous homecage behavior assessed via LABORAS revealed other compulsive features: *Nlgn4* null mutants of both genders had more circling episodes (Fig. 2C, D, left panels; Mann–Whitney U tests, $p = 0.0244$ and $p = 0.0424$ respectively), with general locomotion being unaffected (Fig. 2C, D, right panels).

Grooming is considered to be an ecologically relevant behavioral trait in mice [26]. Excessive grooming has been reported in various mouse lines [23,27,28] and is regarded to be analogous to human obsessive–compulsive behaviors (e.g. [29]). To analyze grooming under standardized conditions, we misted mice with tap water and recorded self-grooming for 30 min under standard single housing conditions which revealed a significant interaction effect in female mice (Fig. 2F, left panel: $F_{\text{interaction}(2,54)} = 5.888$, $p = 0.0049$; $F_{\text{time}(2,54)} = 303.8$, $p < 0.0001$; $F_{\text{genotype}(1,27)} = 0.5509$, $p = 0.4644$). There were no significant differences between male *Nlgn4* null mutants and WT littermates (Fig. 2E, left panel: $F_{\text{interaction}(2,48)} = 0.3685$, $p = 0.6937$; $F_{\text{time}(2,54)} = 254.9$, $p < 0.0001$; $F_{\text{genotype}(1,24)} = 0.9074$, $p = 0.3503$). Post hoc single comparison detected a significant difference between female mutants and WT in the second 10 min interval (Fig. 2F, left panel; $p = 0.009$), indicating increased grooming time in the mutant group. Since autism is also characterized by reduced interests and higher resistance towards environmental distractors, we tested whether mice generally groomed less in a more attractive and stimulating environment – perhaps paying more attention to the environmental enrichment instead of compulsive self-grooming. Surprisingly, however, induced self-grooming under enriched conditions was even more pronounced and did not differ between genotypes in any of the genders (Fig. 2E, right panel; $F_{\text{interaction}(2,46)} = 1.621$, $p = 0.2088$; $F_{\text{time}(2,46)} = 53.59$, $p < 0.0001$; $F_{\text{genotype}(1,23)} = 1.422$, $p = 0.2453$. Fig. 2F, right panel; $F_{\text{interaction}(2,46)} = 0.123$, $p = 0.8847$; $F_{\text{time}(2,46)} = 96.26$, $p < 0.0001$; $F_{\text{genotype}(1,24)} = 0.1202$, $p = 0.7318$).

3.5. Development of an autism severity composite score for mice

Comparable to a known form of a human monogenic heritable autism, where mutations of the *NLGN4X* gene can lead to a variety of ASD typical symptoms, we found readouts of social interaction, ultrasound communication, and stereotyped behaviors also altered in *Nlgn4* null mutant mice. Thus, disruption of only one single gene results in a diverse array of behavioral abnormalities. To account for individuality of discrete symptom severity in the autistic syndrome as a whole as well as for gender differences, we created an autism severity score, separately for male and female mice. For this autism composite score, selected single symptom readouts were z-standardized and genotype groups were contrasted by Mann–Whitney U tests (Fig. 3A, B). Behavioral tests with a clear genotype dependent dissociation of means

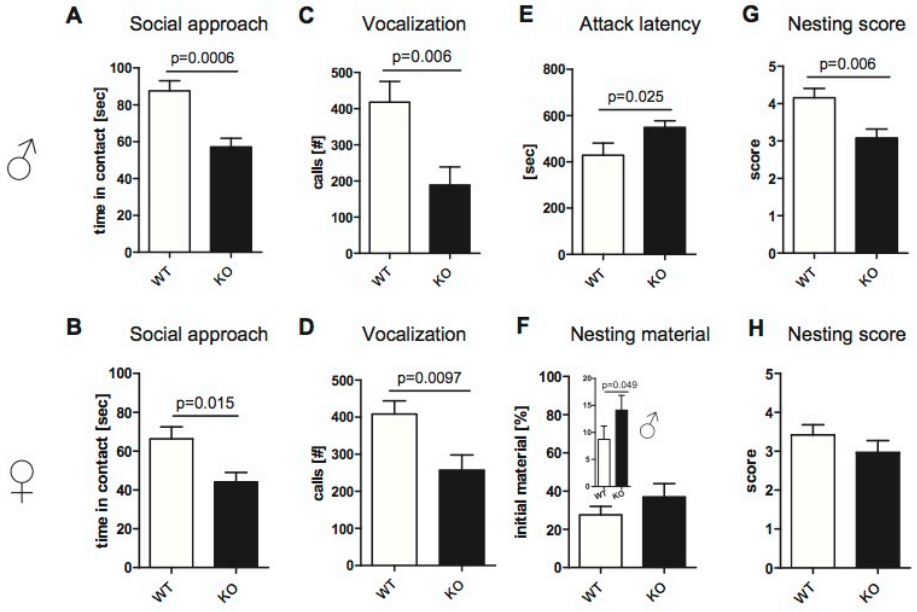

Fig. 1. Diverse features of social behaviors are affected in *Nlgn4* null mutants (KO) of both genders as compared to wildtype (WT) mice. (A, B) Male as well as female *Nlgn4* null mutant mice spent less time in direct social contact with a stranger mouse from the same genotype and gender (♂: WT = 36; KO = 26; ♀: WT = 30; KO = 22). (C, D) Null mutants had significantly fewer calls in the ultrasound vocalization paradigm (♂: WT = 29; KO = 24; ♀: WT = 36; KO = 23). (E) Male *Nlgn4* KO mice had longer attack latency in the resident-intruder paradigm, consistent with reduced social aggression (♂: WT = 12; KO = 11). (F, inset, and G) Only male mutants left a higher proportion of initial nesting material untouched and built significantly less functional nests (♂: WT = 13; KO = 13). (F, H) Nest building readouts in female *Nlgn4* KO mice show only a tendency of a deficit, without reaching significance (♀: WT = 24; KO = 15). Data presented as mean ± SEM.

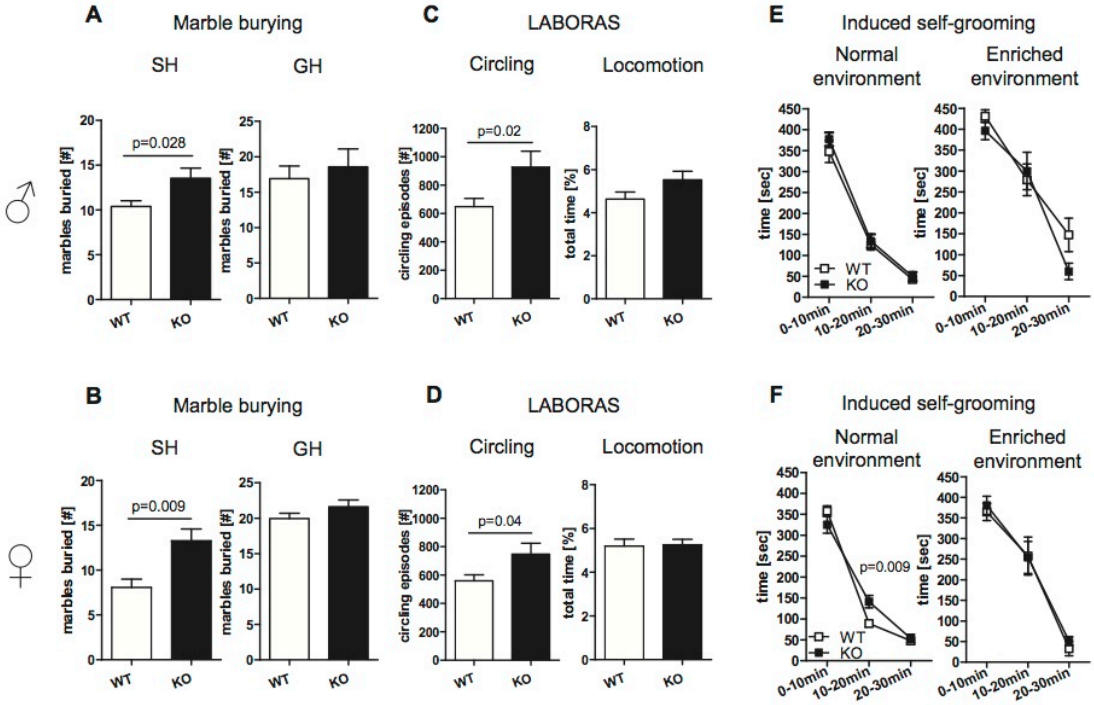

Fig. 2. *Nlgn4* null mutant mice demonstrate increased stereotyped behaviors (A, B) Both male and female *Nlgn4* mutants–when single housed (left panel: ♂: WT = 13; KO = 13; ♀: WT = 15; KO = 15) – buried more marbles in contrast to WT mice. This significant difference disappeared upon group housing (right panel: ♂: WT = 13; KO = 7; ♀: WT = 24; KO = 13). (C, D) Spontaneous circling behavior (left) in LABORAS was increased in *Nlgn4* mutants of both genders without alterations in general locomotor activity (right) (♂: WT = 17; KO = 16; ♀: WT = 14; KO = 15). (E, F) Water misting induced self-grooming, which showed an increase in female, but not in male *Nlgn4* KO mice as compared to WT (left: normal environment: ♂: WT = 13; KO = 13; ♀: WT = 15; KO = 14; right: enriched environment, ♂: WT = 12; KO = 13; ♀: WT = 13; KO = 13). Mean ± SEM presented.

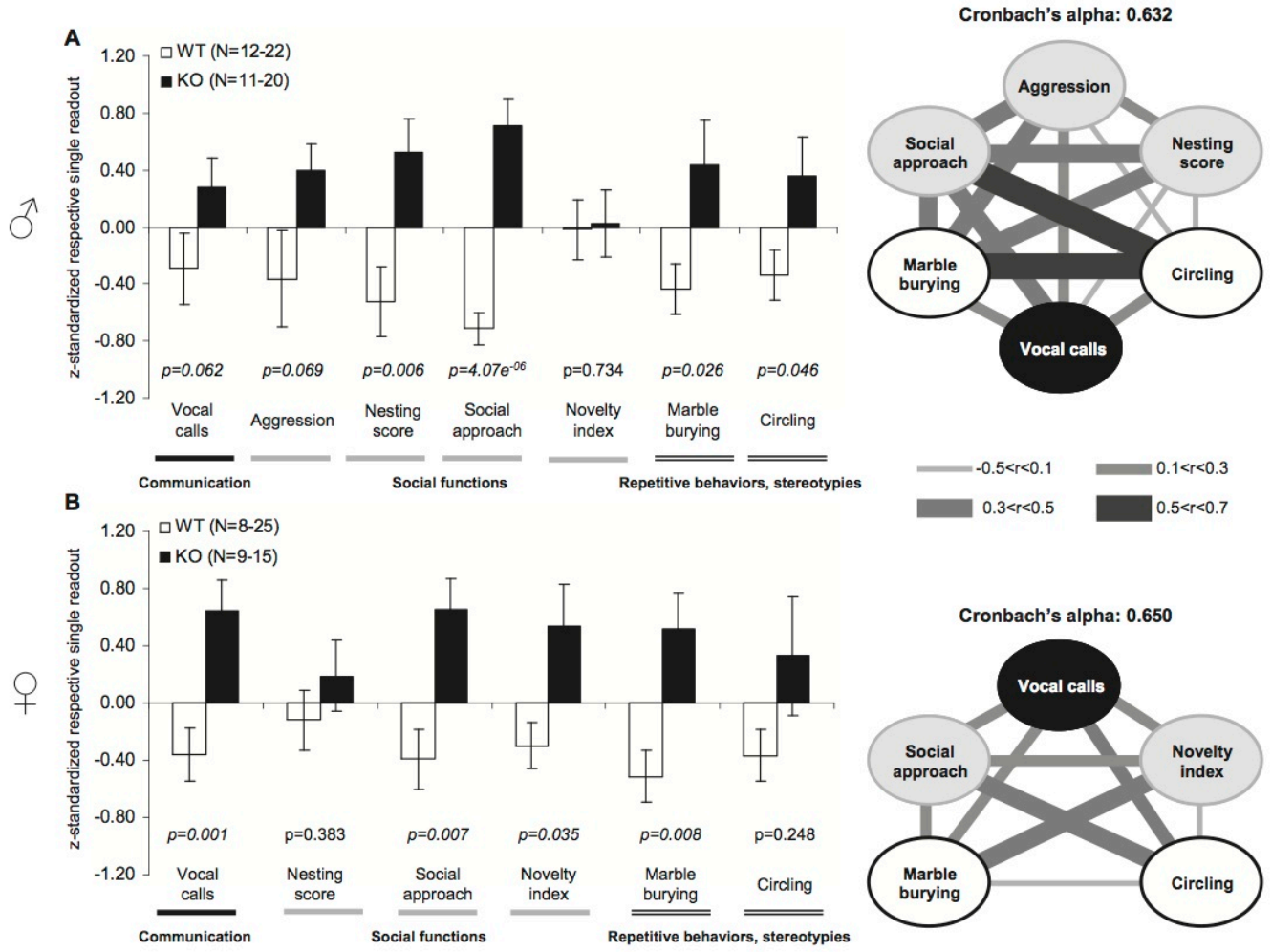

Fig. 3. Transformation and preparation of data for the development of an autism severity composite score (A) Male mice: Contrasting of 7 autism-relevant single behavioral readouts (mean ± SEM) following z-standardization allows all but one (novelty index) to be integrated into the composite score based on a very good Cronbach's alpha of 0.6 This value supports a high internal consistency of the data substantiating the integration of all readouts into one score. Intercorrelations are displayed in the construct, consisting of all 3 lead domains of ASD (B) Female mice: Contrasting of 6 autism-relevant single behavioral readouts (mean ± SEM) following z-standardization allows all but one (nesting score) to be integrated into the composite score based on a very good Cronbach's alpha of >0.6. This value supports a high internal consistency of the data substantiating the integration of all readouts into one score. Intercorrelations are displayed in the construct, consisting of all 3 lead domains of ASD.

(group comparison significant or at least showing a strong tendency) were selected with the aim of building gender specific composite scores for each individual mouse, assuring coverage of all 3 diagnostic domains of ASD (i.e. communication, social functions, repetitive behaviors/stereotypies). These composite scores should reflect the overall severity of autistic behaviors in a continuous fashion with higher values indicating higher severity of autistic behaviors. For male mice, the composite score was based on vocal calls, aggression, nesting, social approach, marble burying and circling (resulting in a Cronbach's $\alpha = 0.632$). For female mice, vocal calls, social approach, novelty index, marble burying, and circling (Cronbach's $\alpha = 0.650$) were included in the composite score. Intercorrelations between single score items are displayed in Fig. 3A, B. Gender specific autism severity composite scores, calculated by integrating the means of all behavioral readouts (following imputations as described in materials) yielded highly significant results ($p < 0.0001$) for both genders for contrasting between mutant and WT mice (Fig. 4A, B). The relative frequency distribution of the autism severity composite score discriminates well between WT and null mutant mice, with a slightly higher overlap seen for female as compared to male subjects (Fig. 4C, D). Individual animals can be assigned to the correct genotype with nearly 100% prediction accuracy in males (75% of phenotypic variance explained; calculations based on a binary logistic regression model using the imputed complete data set). In female mice, 54% of phenotypic variance is explained by the respective model and >83% of predictions of genotype status are correct (73% of mutants correctly identified as such and 91% of WT correctly assigned to the WT group by the model) (Fig. 4E, F).

4. Discussion

The present study provides a comprehensive phenotypical characterization of *Nlgn4* null mutant mice, thereby essentially reproducing our own previous work on *Nlgn4*−/− males [4] and extending it – independently – also to females and, notably, to a number of new ASD relevant tests, most of which turned out to be informative. The results show that both genders of *Nlgn4* null mutants display a multifaceted autistic-like syndrome consisting of distinctive symptoms for all 3 lead features of the disorder: Disturbed social interactions, compromised verbal (ultrasound) communication and repetitive behaviors/restricted interests. Most importantly, based on these typical behavioral symptoms (as evaluated by the most informative tests), we have been able to

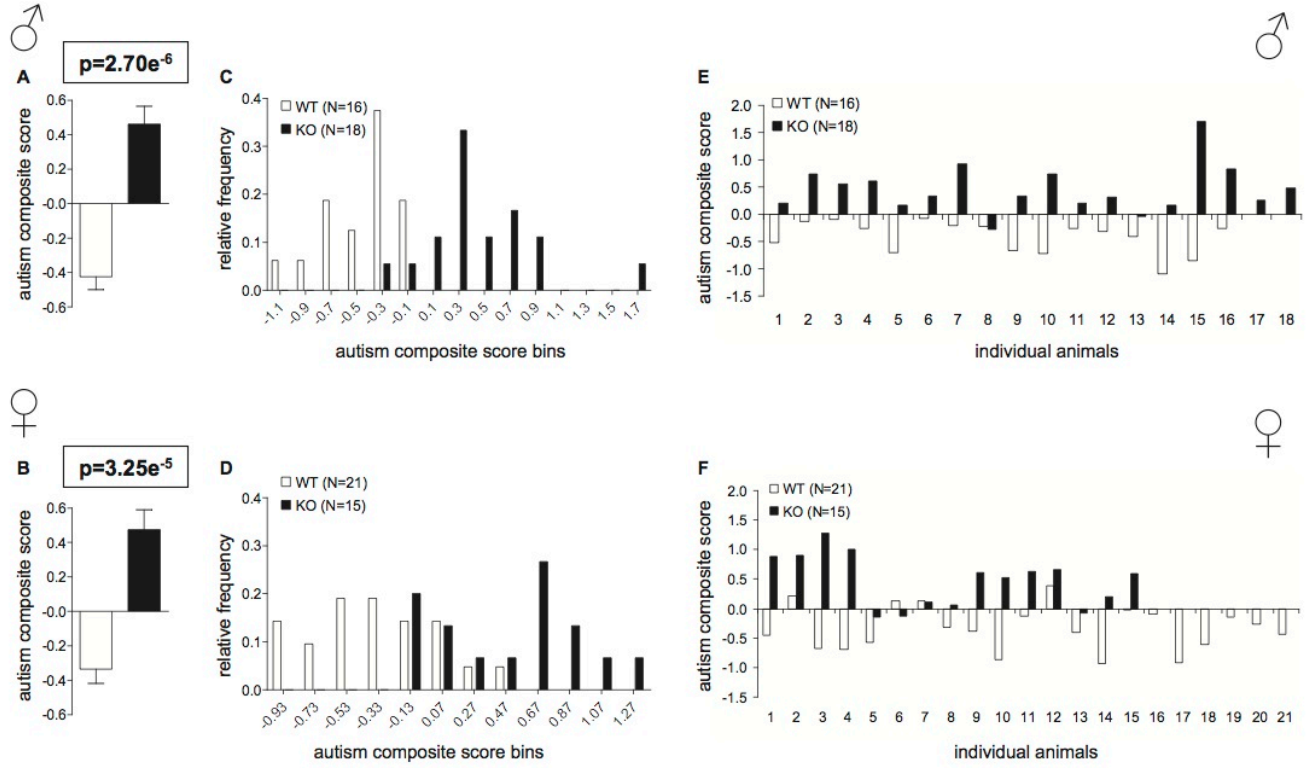

Fig. 4. Presentation of the first autism severity composite score for mice (A, B) The newly developed autism severity composite score (higher values represent higher symptom severity) based on the *z*-transformed raw data of selected tests with significant difference between genotypes (Fig. 3) and imputed values for missing data (as explained in materials), gives highest significance levels for both genders. (C, D) Relative frequency distribution of autism composite score bins separates WT and mutants, with less overlap in males than in females. (E, F) The autism score of individual mice strikingly discriminates between genotypes.

develop for the first time an autism severity composite score for mice. This score allows a highly significant discrimination between autistic and non-autistic *Nlgn4−/−* mice, with an accuracy of 100% for males and of >83% for females to predict the genotype status from the behavioral readouts. In the meanwhile, this same score has already been successfully applied to other genetic mouse models of autism analyzed in our lab (manuscript in preparation).

Compared to the other tests of social behavior with their consistently robust findings (social approach behavior, vocalization, aggression and nest building), demonstrated again in the present work, the discriminatory power of the tripartite chamber tests [16] remains delicate and difficult to reproduce, at least in our hands. In fact, we made similar observations with these tests in various different mouse lines that we have analyzed over the years. Lab-to-lab variations including differences in breeding, upbringing and maintenance or even differential effects of modifier genes may explain at least part of these difficulties. In the present work, these problems of the tripartite chamber tests are reflected by the partly divergent results in male mice (i.e. in social memory) when comparing the previously published [4] and the present cohort. The reason for this discrepancy remains unknown but all other findings of the previous study were entirely reproducible.

The fact that the prediction of genotype from behavior is less accurate for female mice reflects the overall milder and slightly different ASD-like phenotype found here in female as compared to male mice, despite the use of mostly higher *n*-numbers for testing of females. It has to be mentioned, however, that due to space, personnel and logistic limitations, male and female mice were not strictly tested in parallel. Therefore, a direct statistical comparison is not justified. The comparison provided here is descriptive and looks separately at the test results obtained with both genders.

Particularly in nesting but also in induced self-grooming and social interaction readouts, genders differ clearly. Of note, gender-specific findings have been reported in several other mouse models of autism (e.g. [30,9]) and, also in humans, autistic symptom severity and distribution show gender differences [31–36].

Moreover, individuality is an important issue in ASD. Despite having the same mutation, persons affected by the disorder can show different severity and different lead symptom distribution [37–43]. This observation may partly be explained by genetic and environmental modulation of the syndrome. Interestingly, however, the same still holds true for mice where almost identical environmental conditions (cage, group, food, etc.) on top of the highly inbred background tremendously diminish variability as compared to humans.

Nevertheless, a fascinating example of how environment can modify behavior is shown by our results on marble burying behavior in both genders. Here, group housing alleviates the compulsive phenotype. Interestingly, social enrichment and environmental complexity have been reported to improve the autistic phenotype in e.g. fragile X mice [44] as well as in humans [45,46], supporting the important role of the social context and of behavioral psychotherapy in autistic individuals [47–49].

As shown here, the development of an autism severity score has delivered a more robust ground for future treatment studies than single readouts with their higher heterogeneity in the population and their smaller effect sizes. The score provides a powerful and unitary measure for the overall severity of autistic symptoms, covering all ASD relevant behavioral domains. In fact, testing new

compounds for the treatment of autism should always happen in both genders and across a wide range of autism-relevant tests. Applying integrative gender-specific scores will largely simplify the quantification and validation of treatment outcomes in both male and female experimental groups.

Since autism is usually diagnosed in children before the age of 3 years, assessment of neurobehavioral developmental milestones in neonatal mice up to the age of 3–4 weeks will be interesting for further characterization of the *Nlgn4*−/− mouse model of autism. In the present work, we did not yet address these questions. Moreover, developmental disturbances (e.g. seizures, learning and memory impairments) will have to be assessed. The good construct and face validity of the present mouse model may now encourage in depth studies on the issue of predictive validity e.g. by using pharmacological agents or genetic rescue of the phenotype. In the case studied here, one gene aberration causes the syndrome. Therefore, ideally, a causal treatment should also combat all features of the disorder. In the present study, group in contrast to single housing of *Nlgn4* null mutants obviously attenuated selected compulsive/stereotypic traits, indicating a social component of symptom severity. Further work will have to elucidate the effect of potential modifying environmental factors, e.g. environmental complexity and social enrichment, in the *Nlgn4* null mutant model.

Acknowledgements

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Appendix A. Supplementary data

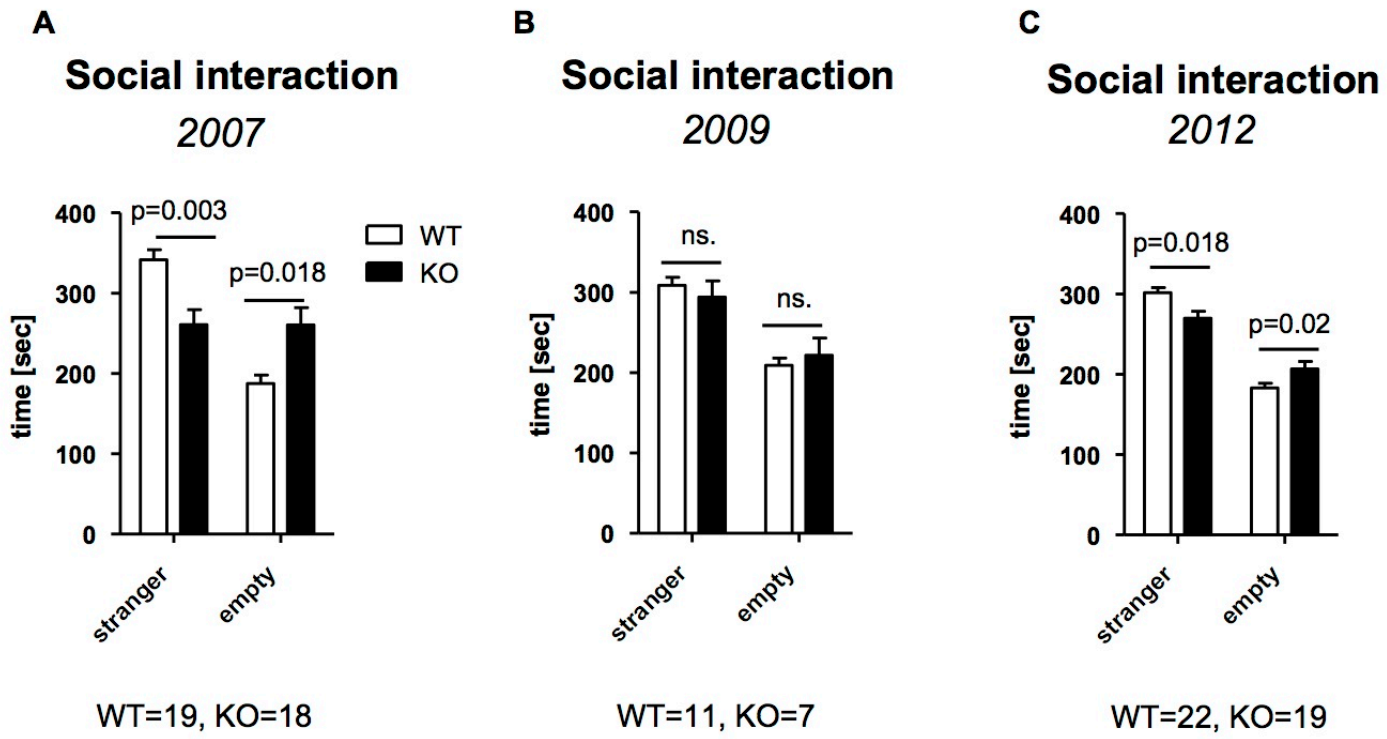
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2012.11.016.

References

[1] Campbell JM. Diagnostic assessment of Asperger's disorder: a review of five third-party rating scales. Journal of Autism and Developmental Disorders 2005;35:25–35.
[2] Piven J, Rabins P. Autism spectrum disorders in older adults: toward defining a research agenda. Journal of American Geriatrics Society 2011;59:2151–5.
[3] Tabuchi K, Blundell J, Etherton MR, Hammer RE, Liu X, Powell CM, et al. A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. Science 2007;318:71–6.
[4] Jamain S, Radyushkin K, Hammerschmidt K, Granon S, Boretius S, Varoqueaux F, et al. Reduced social interaction and ultrasonic communication in a mouse model of monogenic heritable autism. Proceedings of the National Academy of Sciences of the United States of America 2008;105:1710–5.
[5] Etherton MR, Blaiss CA, Powell CM, Sudhof TC. Mouse neurexin-1alpha deletion causes correlated electrophysiological and behavioral changes consistent with cognitive impairments. Proceedings of the National Academy of Sciences of the United States of America 2009;106:17998–8003.
[6] Radyushkin K, Hammerschmidt K, Boretius S, Varoqueaux F, El-Kordi A, Ronnenberg A, et al. Neuroligin-3-deficient mice: model of a monogenic heritable form of autism with an olfactory deficit. Genes, Brain and Behavior 2009;8:416–25.
[7] Berkel S, Marshall CR, Weiss B, Howe J, Roeth R, Moog U, et al. Mutations in the SHANK2 synaptic scaffolding gene in autism spectrum disorder and mental retardation. Nature Genetics 2010;42:489–91.
[8] Peca J, Feliciano C, Ting JT, Wang W, Wells MF, Venkatraman TN, et al. Shank3 mutant mice display autistic-like behaviours and striatal dysfunction. Nature 2011;472:437–42.
[9] Schmeisser MJ, Ey E, Wegener S, Bockmann J, Stempel AV, Kuebler A, et al. Autistic-like behaviours and hyperactivity in mice lacking ProSAP1/Shank2. Nature 2012;486:256–60.
[10] Murcia CL, Gulden F, Herrup K. A question of balance: a proposal for new mouse models of autism. International Journal of Developmental Neuroscience 2005;23:265–75.
[11] Silverman JL, Yang M, Lord C, Crawley JN. Behavioural phenotyping assays for mouse models of autism. Nature Review Neuroscience 2010;11:490–502.
[12] Ey E, Leblond CS, Bourgeron T. Behavioral profiles of mouse models for autism spectrum disorders. Autism Research 2011;4:5–16.
[13] Fischer J, Hammerschmidt K. Ultrasonic vocalizations in mouse models for speech and socio-cognitive disorders: insights into the evolution of vocal communication. Genes, Brain and Behavior 2011;10:17–27.
[14] Ey E, Yang M, Katz AM, Woldeyohannes L, Silverman JL, Leblond CS, et al. Lack of deficits in social behaviors and ultrasonic vocalizations in later generations of mice lacking neuroligin 4 [in press].
[15] Sargin D, El-Kordi A, Agarwal A, Muller M, Wojcik SM, Hassouna I, et al. Expression of constitutively active erythropoietin receptor in pyramidal neurons of cortex and hippocampus boosts higher cognitive functions in mice. BMC Biology 2011;9:27.
[16] Moy SS, Nadler JJ, Perez A, Barbaro RP, Johns JM, Magnuson TR, et al. Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. Genes, Brain and Behavior 2004;3:287–302.
[17] Winslow JT, Miczek KA. Habituation of aggression in mice: pharmacological evidence of catecholaminergic and serotonergic mediation. Psychopharmacology 1983;81:286–91.
[18] Hammerschmidt K, Radyushkin K, Ehrenreich H, Fischer J. The structure and usage of female and male mouse ultrasonic vocalizations reveal only minor differences. PLoS One 2012;7:e41133.
[19] Van de Weerd HA, Bulthuis RJ, Bergman AF, Schlingmann F, Tolboom J, Van Loo PL, et al. Validation of a new system for the automatic registration of behaviour in mice and rats. Behavioural Processes 2001;53:11–20.
[20] Deacon RM. Digging and marble burying in mice: simple methods for in vivo identification of biological impacts. Nature Protocols 2006;1:122–4.
[21] Deacon RM. Assessing nest building in mice. Nat Protocols 2006;1:1117–9.
[22] Satoh Y, Endo S, Nakata T, Kobayashi Y, Yamada K, Ikeda T, et al. ERK2 contributes to the control of social behaviors in mice. Journal of Neuroscience 2011;31:11953–67.
[23] Welch JM, Lu J, Rodriguiz RM, Trotta NC, Peca J, Ding JD, et al. Cortico-striatal synaptic defects and OCD-like behaviours in Sapap3-mutant mice. Nature 2007;448:894–900.
[24] Kalueff AV, Aldridge JW, LaPorte JL, Murphy DL, Tuohimaa P. Analyzing grooming microstructure in neurobehavioral experiments. Nature Protocols 2007;2:2538–44.
[25] Adamcio B, Havemann-Reinecke U, Ehrenreich H. Chronic psychosocial stress in the absence of social support induces pathological pre-pulse inhibition in mice. Behavioural Brain Research 2009;204:246–9.
[26] Spruijt BM, van Hooff JA, Gispen WH. Ethology and neurobiology of grooming behavior. Physiological Reviews 1992;72:825–52.
[27] Chen SK, Tvrdik P, Peden E, Cho S, Wu S, Spangrude G, et al. Hematopoietic origin of pathological grooming in Hoxb8 mutant mice. Cell 2010;141:775–85.
[28] Yang XW, Lu XH. Molecular and cellular basis of obsessive-compulsive disorder-like behaviors: emerging view from mouse models. Current Opinion in Neurology 2011;24:114–8.
[29] Shmelkov SV, Hormigo A, Jing D, Proenca CC, Bath KG, Milde T, et al. Slitrk5 deficiency impairs corticostriatal circuitry and leads to obsessive-compulsive-like behaviors in mice. Nature Medicine 2010;16:598–602, 591 pp. following 602.
[30] Laarakker MC, Reinders NR, Bruining H, Ophoff RA, Kas MJ. Sex-dependent novelty response in neurexin-1alpha mutant mice. PLoS One 2012;7:e31503.
[31] Hartley SL, Sikora DM, 2009. Sex differences in autism spectrum disorder: an examination of developmental functioning, autistic symptoms, and coexisting behavior problems in toddlers. Journal of Autism and Developmental Disorders.
[32] Baron-Cohen S, Lombardo MV, Auyeung B, Ashwin E, Chakrabarti B, Knickmeyer R. Why are autism spectrum conditions more prevalent in males? PLoS Biology 2011;9:e1001081.
[33] Bolte S, Duketis E, Poustka F, Holtmann M. Sex differences in cognitive domains and their clinical correlates in higher-functioning autism spectrum disorders. Autism 2011;15:497–511.
[34] Rinehart NJ, Cornish KM, Tonge BJ. Gender differences in neurodevelopmental disorders: autism and fragile × syndrome. Current Topics in Behavioral Neurosciences 2011;8:209–29.
[35] Beacher FD, Radulescu E, Minati L, Baron-Cohen S, Lombardo MV, Lai MC, et al. Sex Differences and Autism: Brain Function during Verbal Fluency and Mental Rotation. PLoS One 2012;7:e38355.
[36] Zwaigenbaum L, Bryson SE, Szatmari P, Brian J, Smith IM, Roberts W, et al. Sex differences in children with autism spectrum disorder identified within a high-risk infant cohort. Journal of Autism and Developmental Disorders 2012.
[37] Laumonnier F, Bonnet-Brilhault F, Gomot M, Blanc R, David A, Moizard MP, et al. X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family. American Journal of Human Genetics 2004;74:552–7.
[38] Talebizadeh Z, Bittel DC, Veatch OJ, Butler MG, Takahashi TN, Miles JH. Do known mutations in neuroligin genes (NLGN3 and NLGN4) cause autism? Journal of Autism and Developmental Disorders 2004;34:735–6.

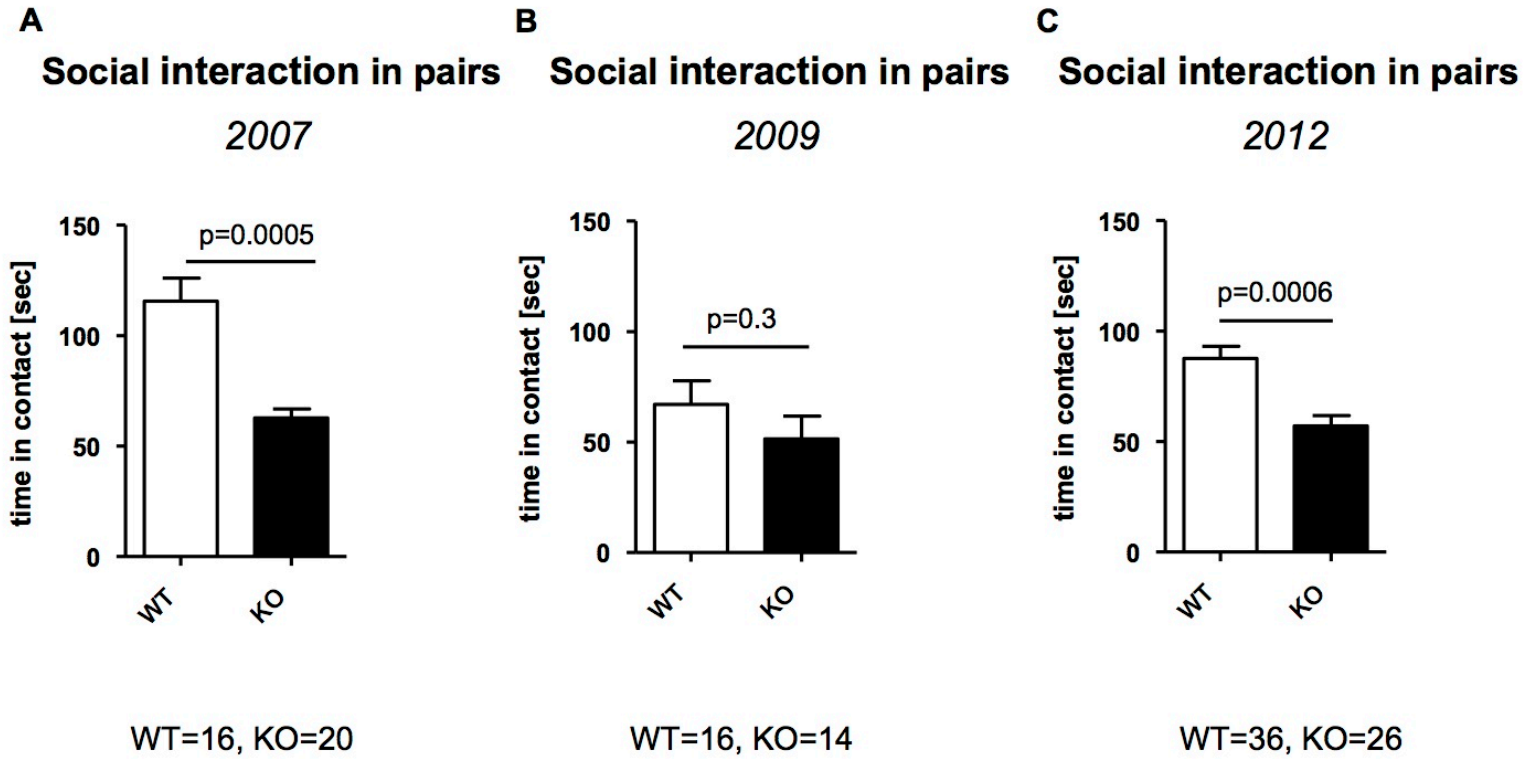
[39] Talebizadeh Z, Lam DY, Theodoro MF, Bittel DC, Lushington GH, Butler MG. Novel splice isoforms for NLGN3 and NLGN4 with possible implications in autism. Journal of Medical Genetetics 2006;43:e21.
[40] Moessner R, Marshall CR, Sutcliffe JS, Skaug J, Pinto D, Vincent J, et al. Contribution of SHANK3 mutations to autism spectrum disorder. American Journal of Human Genetics 2007;81:1289–97.
[41] Lawson-Yuen A, Saldivar JS, Sommer S, Picker J. Familial deletion within NLGN4 associated with autism and Tourette syndrome. European Journal of Human Genetics 2008;16:614–8.
[42] Yan J, Feng J, Schroer R, Li W, Skinner C, Schwartz CE, et al. Analysis of the neuroligin 4Y gene in patients with autism. Psychiatric Genetics 2008;18:204–7.
[43] Hanson E, Nasir RH, Fong A, Lian A, Hundley R, Shen Y, et al. Cognitive and behavioral characterization of 16p11.2 deletion syndrome. Journal of Developmental & Behavioral Pediatrics 2010;31:649–57.
[44] Restivo L, Ferrari F, Passino E, Sgobio C, Bock J, Oostra BA, et al. Enriched environment promotes behavioral and morphological recovery in a mouse model for the fragile X syndrome. Proceedings of the National Academy of Sciences of the United States of America 2005;102:11557–62.
[45] Lewis MH. Environmental complexity and central nervous system development and function. Mental Retardation and Developmental Disabilities Research Reviews 2004;10:91–5.
[46] Dawson G. Early behavioral intervention, brain plasticity, and the prevention of autism spectrum disorder. Development and Psychopathology 2008;20:775–803.
[47] Williams White S, Keonig K, Scahill L. Social skills development in children with autism spectrum disorders: a review of the intervention research. Journal of Autism and Developmental Disorders 2007;37:1858–68.
[48] Cotugno AJ. Social competence and social skills training and intervention for children with Autism Spectrum Disorders. Journal of Autism and Developmental Disorders 2009;39:1268–77.
[49] Bohlander AJ, Orlich F, Varley CK. Social skills training for children with autism. Pediatric Clinics of North America 2012;59:165–74, xii.

El-Kordi et al Supplementary Figure 1



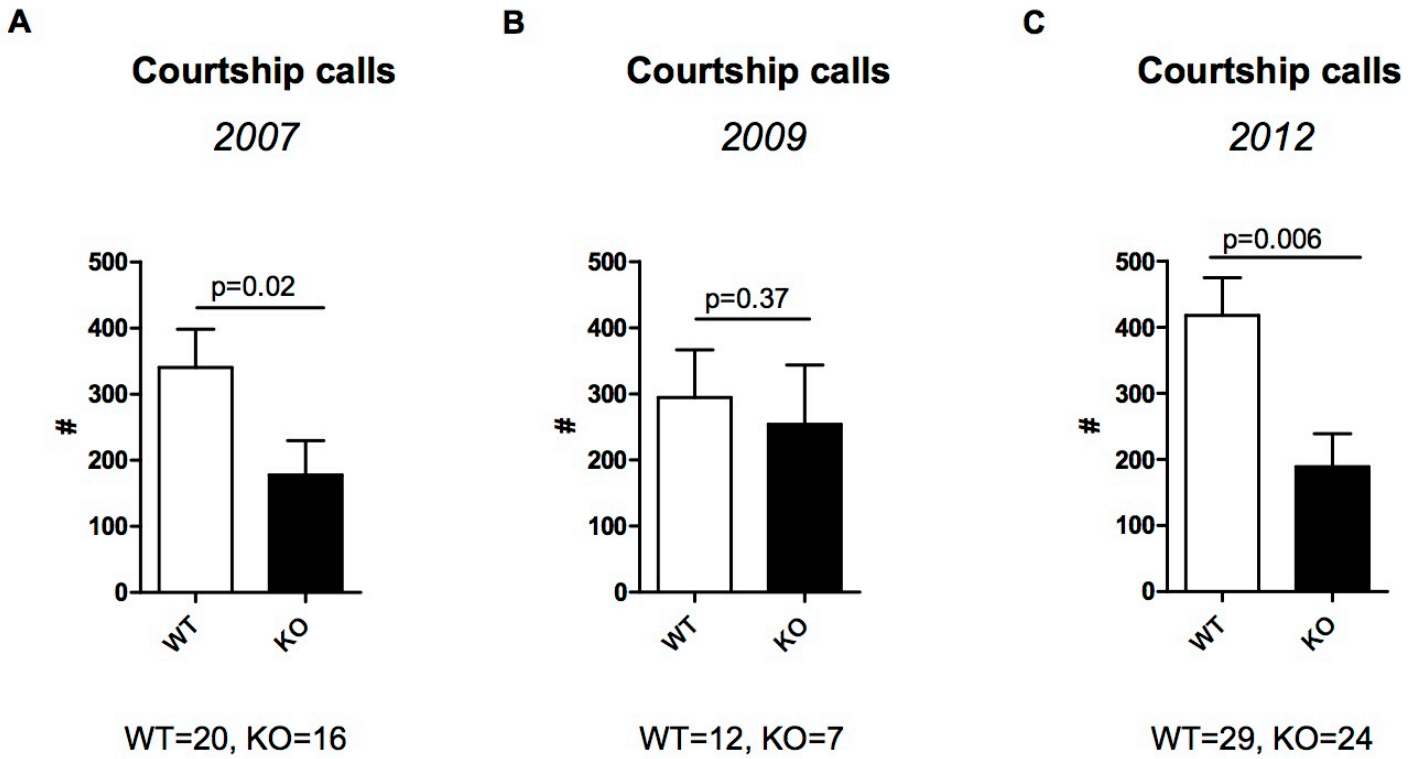
Comparative presentation of social interaction & sociability results (tripartite chamber) of 3 different generations of male mice. Tests were performed in the years 2007 (**A**, generation 6-7; WT=17, KO=17), 2009 (**B**, generation 13; WT=11, KO=7) and 2012 (**C**, revitalized embryos - generation 7-8; WT=22, KO=19). Premature unblinding (low n-numbers) rather than 'bleaching' of the phenotype may explain the non-significant results obtained with generation 13 in 2009. Mean±SEM presented.

El-Kordi et al Supplementary Figure 2



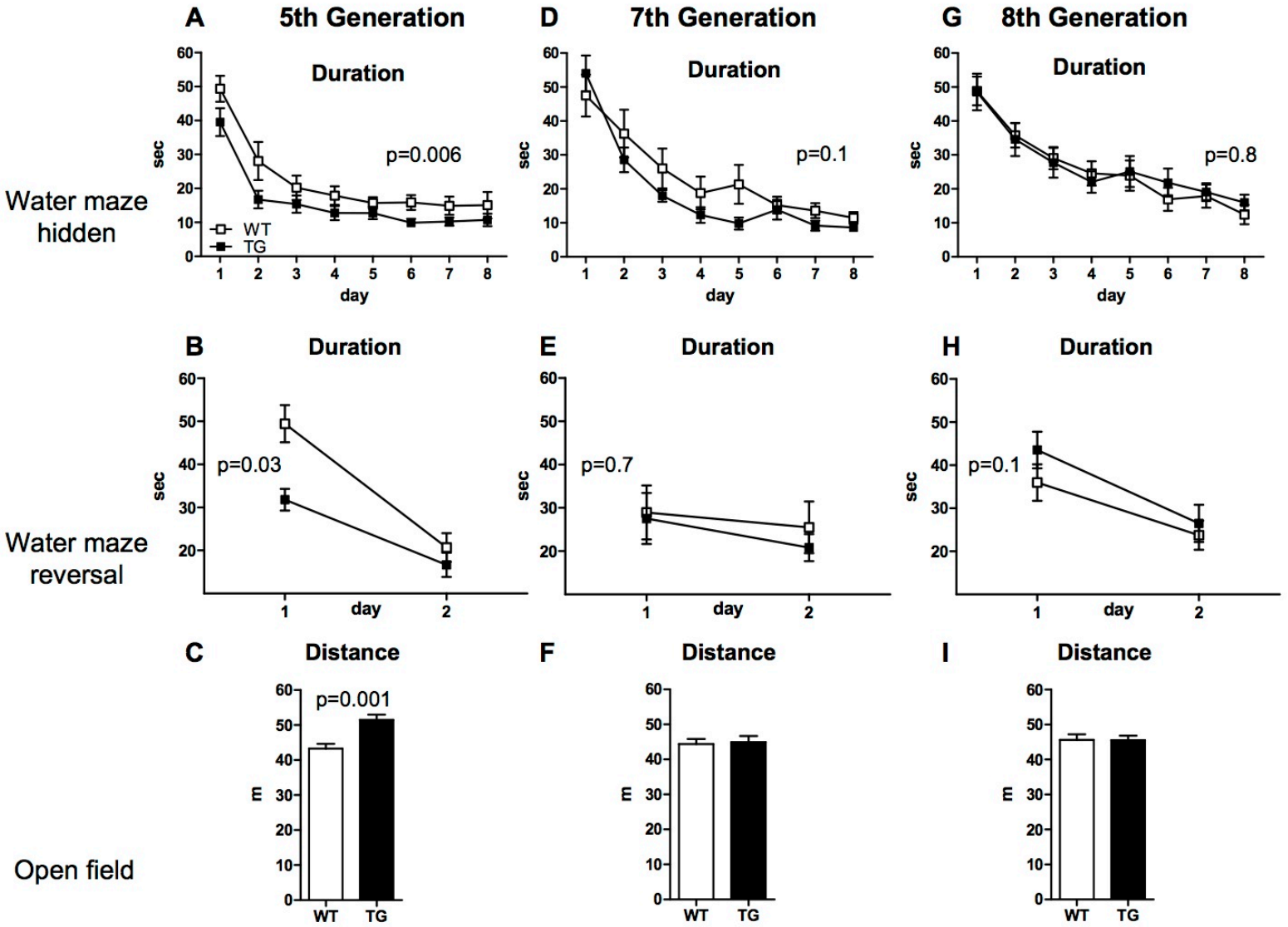
Comparative presentation of results obtained from social interaction in pairs of 3 different generations of male mice. Tests were performed in the years 2007 (**A**, generation 6-7; WT=16, KO=20), 2009 (**B**, generation 13; WT=16, KO=14) and 2012 (**C**, revitalized embryos - generation 7-8; WT=36, KO=26). Premature unblinding (low n-numbers) rather than 'bleaching' of the phenotype may explain the non-significant results obtained with generation 13 in 2009. Mean±SEM presented.

El-Kordi et al Supplementary Figure 3



Comparative presentation of results obtained from ultrasound communications of 3 different generations of male mice. Tests were performed in the years 2007 (**A**, generation 6-7; WT=20, KO=16), 2009 (**B**, generation 13; WT=12, KO=7) and 2012 (**C**, revitalized embryos - generation 7-8; WT=29, KO=24). Premature unblinding (low n-numbers) rather than 'bleaching' of the phenotype may explain the non-significant results obtained with generation 13 in 2009. Mean±SEM presented.

El-Kordi et al Supplementary Figure 4

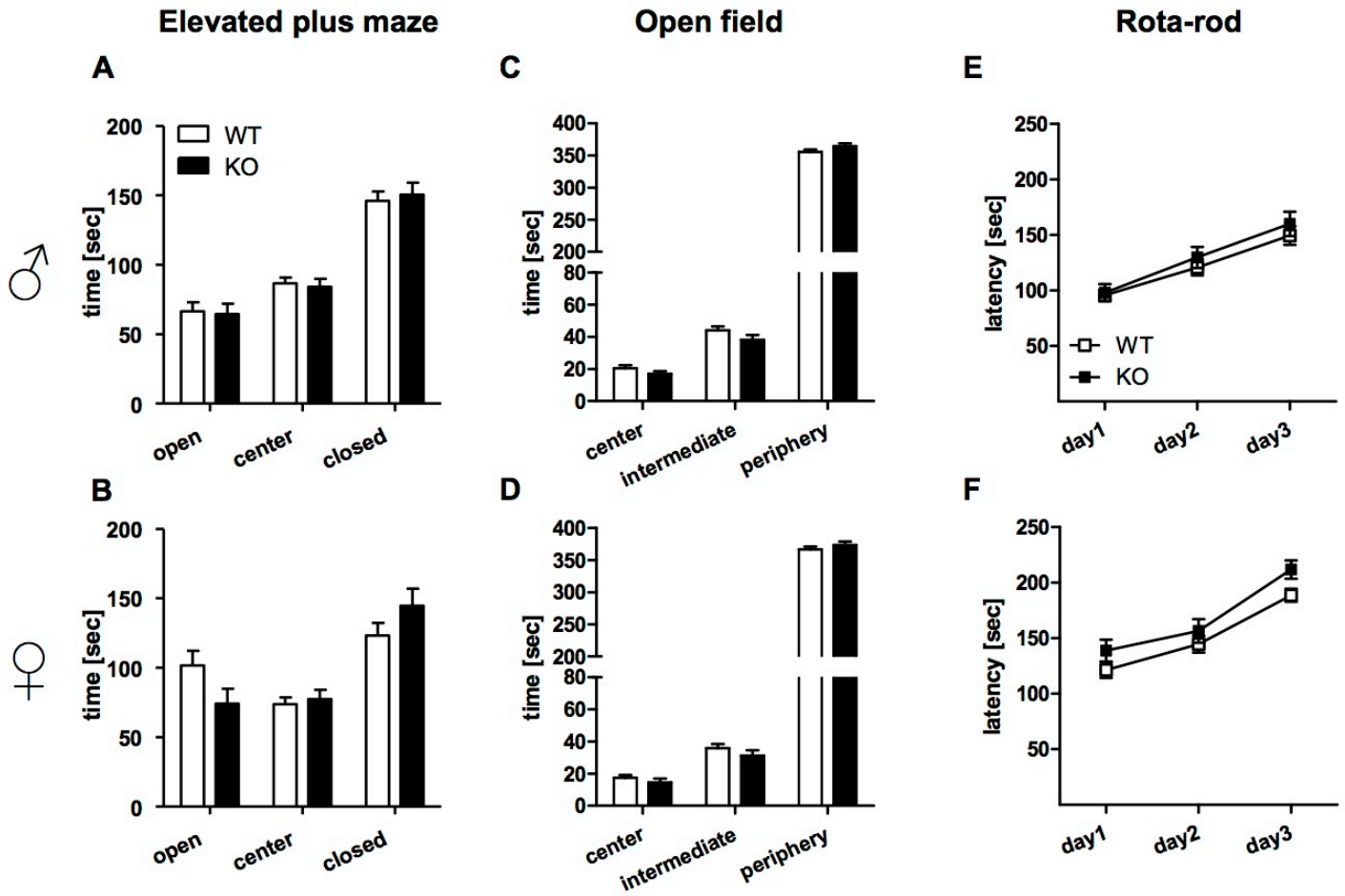


Legend to Supplementary Figure 4:

Example of a 'true phenotype bleaching', i.e. successive loss of phenotype in male cEPOR transgenic mice upon testing of 3 different generations.

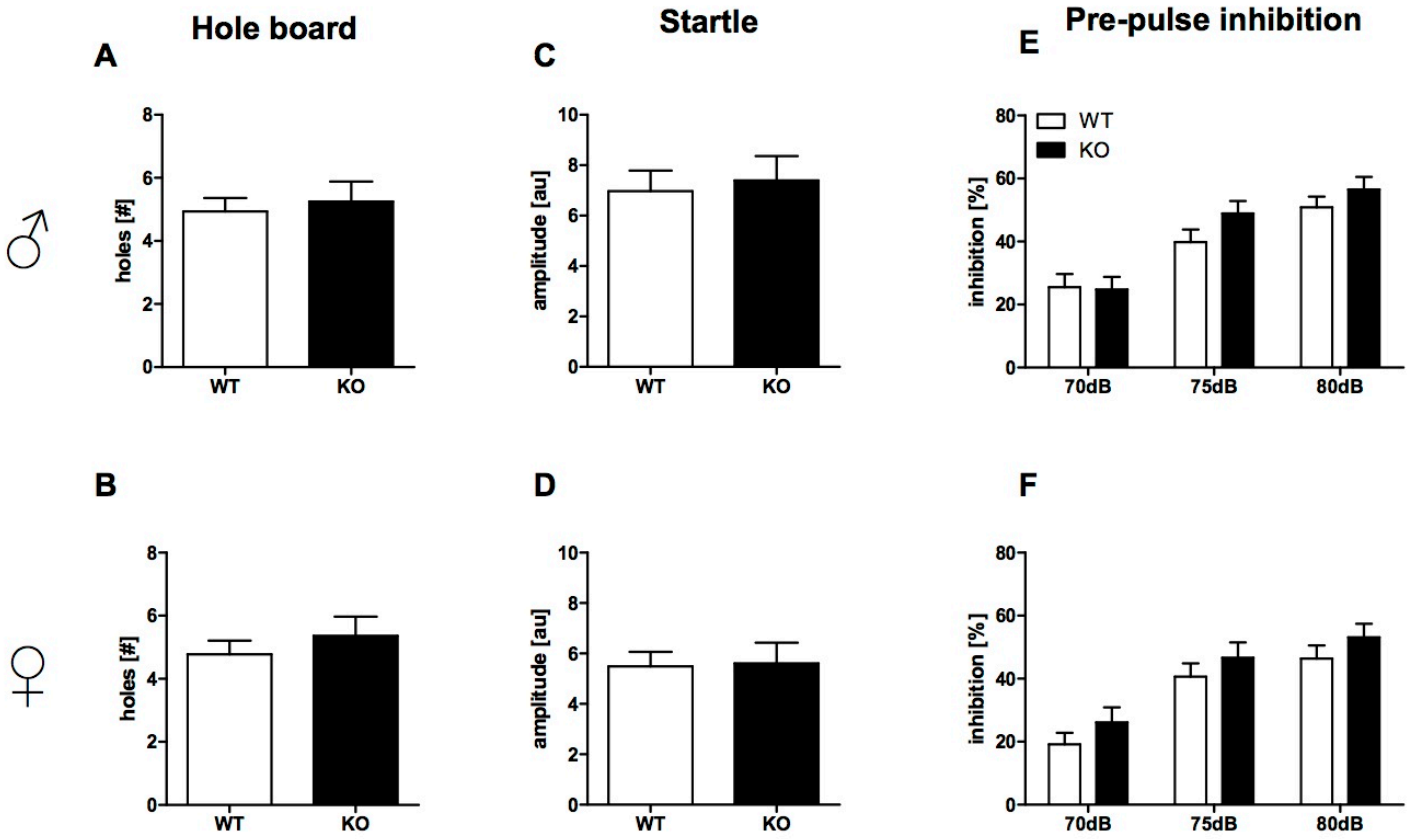
Cognitive performance measured in Morris water maze (spatial learning and memory as well as reversal learning/flexibility) and hyperactivity determined in the open field show significant enhancement in cEPOR mice that is stepwise lost upon testing of subsequent generations. This observation was made by chance in a transgenic mouse line expressing a constitutively active form of erythropoietin receptor (cEPOR) under control of the α-CaMKII promoter which restricts expression of cEPOR to forebrain pyramidal neurons of postnatal mice (for mouse line description, see ***Sargin et al, 2011***). Transgene expression remained unaltered over the respective generations. **(A)** Generation 5: WT=18, TG=13; **(D)** generation 7: WT=10, TG=14; **(G)** generation 8: WT=29, TG=16. Mean±SEM presented.

El-Kordi et al Supplementary Figure 5



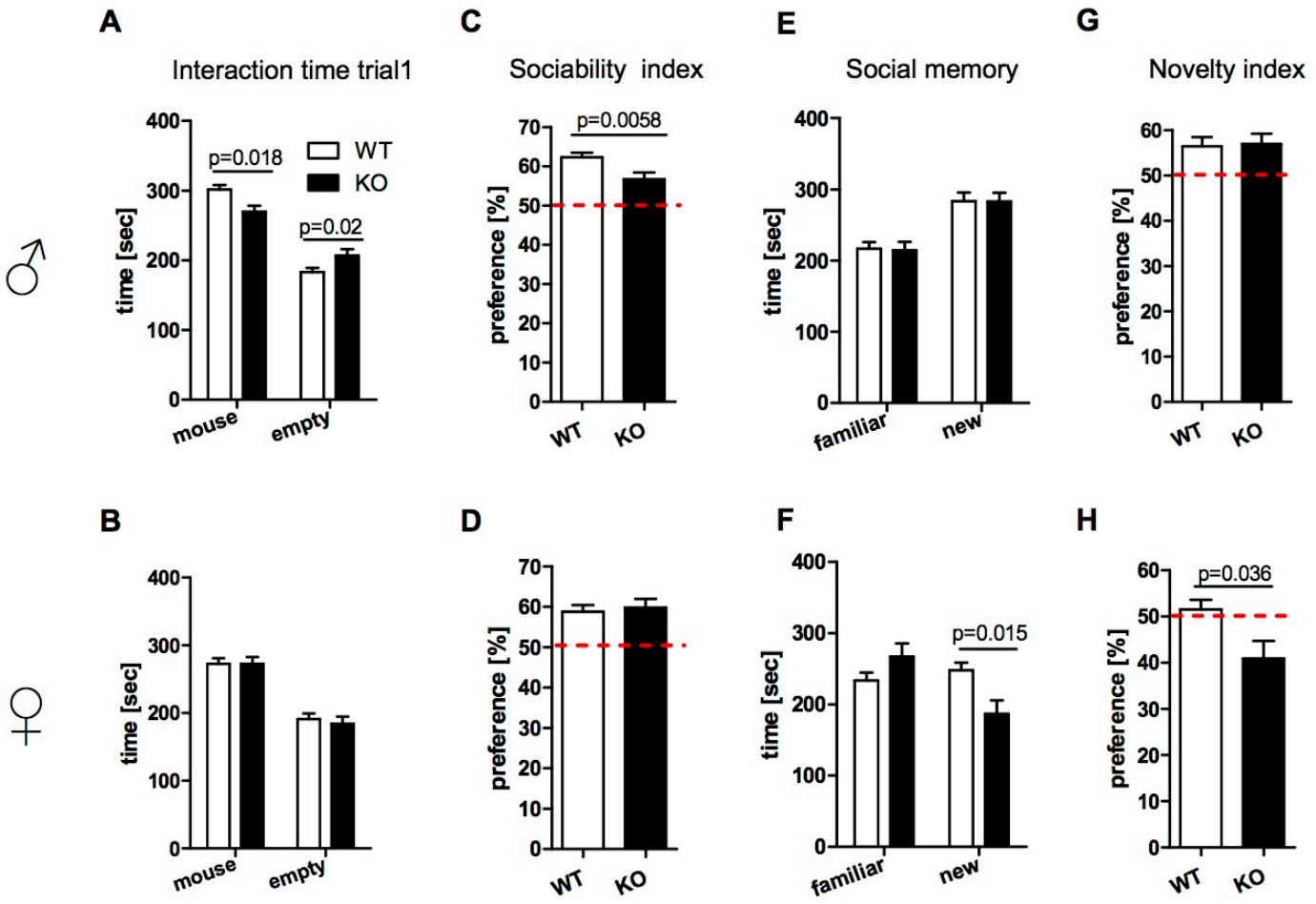
***Nlgn4* null mutant mice of both genders exhibit normal locomotor behavior and anxiety.**
Genotypes of both genders did not differ in basic behavior, i.e. in elevated plus maze **(A,B)**, open field **(C,D)** and rota-rod **(E,F).**
♂: WT=49; KO=30; ♀: WT=38; KO=27. Mean±SEM presented.

El-Kordi et al Supplementary Figure 6



***Nlgn4* null mutant mice of both genders show unaltered exploratory activity and sensorimotor gating.**
Exploratory activity **(A,B)**, startle response **(C,D)** as well as pre-pulse inhibition **(E,F)** are normal in *Nlgn4* KO mice of both genders. **A,B**: WT=49; KO=30; ♀: WT=38; KO=27. **C-F**: WT=48; KO=30; ♀: WT=38; KO=26. Mean±SEM presented.

El-Kordi et al Supplementary Figure 7



Nlgn4 null mutant mice exhibit gender-specific social deficits in the tripartite chamber

Male *Nlgn4* KO mice show less social interest in social interaction in the 3-partite chamber **(A,C)** but normal social memory capabilities **(E,G).** In contrast, female *Nlgn4* KO mice demonstrate no alterations in social interaction **(B,D)** but reduction of social memory **(F,H).** Data presented as mean±SEM; ♂: WT=22; KO=19; ♀: WT=25; KO=14.

4. Gpm6b deficiency impairs sensorimotor gating and modulates the behavioral response to a 5-HT2A/C receptor agonist

4.1 Overview of project 2

Depression (major depressive disorder) is the leading cause of disability worldwide (Ferrari et al., 2013) and is associated with a high risk for suicide (Lesage et al., 1994). Suicides are the second leading cause of death among young people between the age of 15-24 and 25-34 (Heron, 2013), indicating a global health priority for depressive disorders (Ferrari et al., 2013).

The pathophysiology of depression is still not completely understood, though several mechanisms are possibly involved (Femenia et al., 2012). In a great number of studies, it has been shown that there is a robust link between the short form of the serotonin transporter polymorphism and depression (for review see Sharpley et al., 2014). The serotonin transporter (SERT) actively transports serotonin from the synaptic cleft in the presynaptic neuron and it has been shown that M6b interacts with this transporter (Fjorback et al., 2009). In my second project, we addressed a mouse model deficient of *Gpm6b*, to assess the impact of this deficiency on the behavioral phenotype and on SERT signaling.

To elucidate the role of *Gpm6b* on behavior, we used *Gpm6b* null mutant (*Gpm6b*-/-) male and female mice. A comprehensive battery of behavioral approaches was utilized to test for sensory and motor functions, cognition, social behavior, as well as anxiety. *Gpm6b* null mutant mice displayed completely normal basic behavior, indistinguishable from their wildtype littermates. Only the home-cage observation with LABORAS indicated a slightly increased locomotion (indicated by greater distance travelled) and therefore reduced immobility (time) in male *Gpm6b* null mutant mice compared to their wildtype littermates. From our data, one can also derive a general sex difference, regardless of the genotype. This is of importance due to the recent increase in interest in sex differences among animal models for psychiatric conditions (Kokras et al., 2014) and the sex

differences observed in humans under neuropsychiatric conditions, e.g. females have a higher lifetime prevalence of depression (Rafful et al., 2012).

As mentioned above, impairments in serotonin neurotransmission are involved in the symptoms associated with major depressive disorder. Therefore, in another series of experiments, we wondered whether *Gpm6b*-deficient mice (that might suffer from a lack of extracellular serotonin due to an increase in the cell-surface expression of the serotonin transporter) would consequently exhibit depression-like symptoms along with cognitive impairments. Although we suspected a depressive phenotype in *Gpm6b* null mutant mice, we found all our tests for depression unaffected. The results of the forced swim test, tail suspension, sucrose preference and the chimney test were comparable between the *Gpm6b* null mutant mice of both genders and their wildtype littermates.

Cognitive performance was assessed with novel object recognition and Morris water maze. Neither immediate, nor delayed object recognition yielded a difference in performance by genotype. The spatial learning task, as well as its reversal (as a measure of cognitive flexibility), remained unaffected by the *Gpm6b* deficiency in mice.

In the prepulse inhibition paradigm (PPI) we were able to explore the impact of *Gpm6b*-deficiency on sensorimotor gating, which resulted in a decreased response compared to wildtype animals.

In the brains of suicide completers, the mRNA levels of GPM6B were found to be reduced. As a result we assessed the behavioral response of *Gpm6b* null mutant mice to DOI (5-HT2A/C receptor agonist). Our data indicated impaired receptor signalling as an effect of the absence of *Gpm6b*, leading to a blunted behavioral response to the 5-HT2A/C receptor agonist.

4.2 Original publication

Dere E[1], Winkler D[1], Ritter C, Ronnenberg A, Poggi G, Patzig J, Gernert M, Müller C, Nave KA, Ehrenreich H, Werner HB: Gpm6b deficiency impairs sensorimotor gating and modulates the behavioral response to a 5-HT2A/C receptor agonist. Behavioral Brain Research 2014; (in press).

[1]Equal contribution

Own contribution: I was responsible for conducting the behavioral experiments (elevated plus maze, open field, rota-rod, hole board, social interaction, marble burying, novel object recognition and LABORAS). Together with Ekrem Dere, I analyzed and discussed the data and designed the graphs and figures implemented in the publication. Furthermore, I was involved in the design, writing, drafting, revision and submission of the manuscript.

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Research report

Gpm6b deficiency impairs sensorimotor gating and modulates the behavioral response to a 5-HT2A/C receptor agonist

Ekrem Dere[a,b,1], Daniela Winkler[a,1], Caroline Ritter[a], Anja Ronnenberg[a], Giulia Poggi[a], Julia Patzig[c], Manuela Gernert[d], Christian Müller[e], Klaus-Armin Nave[b,c], Hannelore Ehrenreich[a,b,*], Hauke B. Werner[c]

[a] *Clinical Neuroscience, Max Planck Institute of Experimental Medicine, Göttingen, Germany*
[b] *DFG Center for Nanoscale Microscopy & Molecular Physiology of the Brain (CNMPB), Göttingen, Germany*
[c] *Department of Neurogenetics, Max Planck Institute of Experimental Medicine, Göttingen, Germany*
[d] *Department of Pharmacology, Toxicology and Pharmacy, University of Veterinary Medicine, Hannover, Germany*
[e] *Department of Psychiatry & Psychotherapy, University of Erlangen, Germany*

HIGHLIGHTS

- *Gpm6b*$^{-/-}$ mice of both genders and their wild-type (WT, *Gpm6b*$^{+/+}$) littermates were tested in an extensive behavioral test battery.
- *Gpm6b*$^{-/-}$ mice display completely normal sensory and motor functions, cognition, as well as social and emotionality-like (anxiety, depression) behaviors.
- *Gpm6b*$^{-/-}$ mice of both genders exhibit a selective impairment in prepulse inhibition of the acoustic startle response.
- *Gpm6b*$^{-/-}$ mice demonstrate a blunted behavioral response to a 5-HT2A/C receptor agonist.
- In conclusion, *Gpm6b* deficiency impairs sensorimotor gating and modulates the behavioral response to a serotonergic challenge.

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ABSTRACT

The neuronal tetraspan proteins, M6A (Gpm6a) and M6B (Gpm6b), belong to the family of proteolipids that are widely expressed in the brain. We recently reported *Gpm6a* deficiency as a monogenetic cause of claustrophobia in mice. Its homolog proteolipid, Gpm6b, is ubiquitously expressed in neurons and oligodendrocytes. Gpm6b is involved in neuronal differentiation and myelination. It interacts with the N-terminal domain of the serotonin transporter (SERT) and decreases cell-surface expression of SERT. In the present study, we employed *Gpm6b* null mutant mice (*Gpm6b*$^{-/-}$) to search for behavioral functions of Gpm6b. We studied male and female *Gpm6b*$^{-/-}$ mice and their wild-type (WT, *Gpm6b*$^{+/+}$) littermates in an extensive behavioral test battery. Additionally, we investigated whether *Gpm6b*$^{-/-}$ mice exhibit changes in the behavioral response to a 5-HT2A/C receptor agonist. We found that *Gpm6b*$^{-/-}$ mice display completely normal sensory and motor functions, cognition, as well as social and emotionality-like (anxiety, depression) behaviors. On top of this inconspicuous behavioral profile, *Gpm6b*$^{-/-}$ mice of both genders exhibit a selective impairment in prepulse inhibition of the acoustic startle response. Furthermore, in contrast to WT mice that show the typical locomotion suppression and increase in grooming activity after intraperitoneal administration of DOI [(±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride], *Gpm6b*$^{-/-}$ mice demonstrate a blunted behavioral response to this 5-HT2A/C receptor agonist. To conclude, *Gpm6b* deficiency impairs sensorimotor gating and modulates the behavioral response to a serotonergic challenge.

1. Introduction

The neuronal tetraspan glycoproteins, M6A (Gpm6a) and M6B (Gpm6b), are members of the proteolipid family and expressed throughout the brain. A closely related family member is the oligodendroglial proteolipid protein (PLP1), a structural protein of central nervous system (CNS) myelin [1]. Mutations in the

* Corresponding author at: Clinical Neuroscience, Max Planck Institute of Experimental Medicine, Göttingen, Germany. Tel.: +49 551 3899615; fax: +49 551 3899670.
E-mail address: ehrenreich@em.mpg.de (H. Ehrenreich).
[1] These authors contributed equally to this work.

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corresponding gene have been associated with Pelizaeus-Merzbacher disease and spastic paraplegia type2 [2]. The phenotypical impairments caused by PLP-mutations in humans can be partly modeled in PLP-deficient mice [3,4]. Until now, however, the functions of M6/Gpm6 proteins are not too well understood. *Gpm6a* expression is downregulated by behavioral stress in mice [5] and by cortisol in tree shrews [6]. Using *Gpm6a* null mutant mice, we recently provided behavioral evidence of *Gpm6a* deficiency as a monogenetic cause of claustrophobia [7]. Additional studies in humans suggested that an alteration in the dynamic regulation of *GPM6A* expression, caused by a single nucleotide polymorphism in the 3′UTR of the gene, might increase the risk for developing claustrophobia [7].

The X-chromosomal [8] *Gpm6b* encodes a protein, Gpm6b, the sequence of which is >50% homologous to both Gpm6a and PLP1 [1]. Gpm6b is widely expressed in neurons and oligodendrocytes in the CNS [9,10], and in a subset of activated astrocytes [11]. However, its mRNA has also been detected in peripheral tissues including the testes, lung, heart, and spleen [12]. Gpm6b seems to play a role in neuronal and osteoblast differentiation and myelination [13,14], and interacts with the N-terminal domain of the serotonin transporter, SERT (gene name *Slc6a4*). Transfection studies in which Gpm6b and SERT were co-expressed in HEK-MSR-293 cells yielded a 50% reduction of the cell-surface expression of SERT concomitant with a reduction of extracellular 5-HT uptake [15]. In line with the important role of serotonin (5-HT) neurotransmission for affective disorders, *GPM6B* was found downregulated in the brains of suicide completers who had suffered from these disorders [16]. In consequence, reduced abundance of *GPM6B* should be associated with an increase in SERT cell-surface expression. This increase in turn could lead to enhanced cleavage of 5-HT from the extracellular space causing reduced activation of post- and extra-synaptic 5-HT receptors in the brain. The latter has been implicated as a key mechanism in the development of mood disorders including major depression [17].

In oligodendrocytes, PLP1 is required to establish the abundance in myelin of sirtuin-2 [18] and the membrane lipid cholesterol [13], while neuronal GPM6A [19] affects the subcellular sorting of the μ-opioid receptor [20,21]. Together, these data are in agreement with the concept that all members of the proteolipid protein family may mediate the subcellular trafficking of their respective partner molecules [1].

To our knowledge, there is virtually no information available on the behavioral functions of Gpm6b. In the present study, we asked (1) whether male and/or female *Gpm6b*$^{-/-}$ mice would under baseline conditions show any measurable alterations in a wide spectrum of behavioral domains including sensory, motor and exploration performance, social readouts, cognition, and measures of emotionality. (2) In an approach to uncover changes in 5-HT neurotransmission in the brain, we monitored the behavioral response of *Gpm6b*$^{-/-}$ versus WT mice of both genders to a serotonergic challenge using the 5-HT2A/C receptor agonist DOI [(±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride] [22].

The selection of DOI was based on the following considerations: alterations in the expression of SERT in SERT$^{-/-}$ and hSERT overexpressing mice have been reported to be associated with bi-directional changes in the expression of 5-HT2A and 5-HT2C receptors [23–25]. Furthermore, it has been shown that SERT$^{-/-}$ mice show diminished 5-HT2A and 5-HT2C receptor signaling as well as reduced behavioral response to the 5-HT2A/2C agonist DOI [26]. Moreover, hSERT overexpressing mice exhibit an alteration of cerebral metabolic responses after DOI challenge [27]. Together, these studies suggest that changes in the expression level of SERT modulate quantity of 5-HT2A and 2C receptors. Therefore, we hypothesized that DOI would induce a differential behavioral response in Gpm6b deficient mice as compared to their WT littermates.

2. Materials and methods

2.1. Generation and genotyping of *Gpm6b*$^{-/-}$ mice

The generation and genotyping of male (−/y) and female (−/−) *Gpm6b* null mutant mice (for simplicity in the text together referred to as *Gpm6b*$^{-/-}$) have been described elsewhere [13]. The *Gpm6b*$^{-/-}$ mouse line has been generated via homologous recombination in embryonic stem cells derived from 129SV mice and thus had initially a mixed 129SVxC57BL/6J genetic background. Therefore, the genetic background was homogenized by backcrossing to the C57BL/6J strain for 10 generations before breeding the experimental animals used in the present study.

2.2. Animals and housing conditions

All experiments were approved by the local Animal Care and Use Committee in accordance with the German Animal Protection Law. For behavioral testing, mice were housed in groups of 4–6 in standard plastic cages, with food and water ad libitum, a temperature maintained at 20–22 °C, and a 12 h light–dark cycle (lights on at 7:00 am).

2.3. Behavioral testing

Behavioral and pharmacological experiments were performed during the light phase of the day (8:00 am and 5:00 pm; except for LABORAS home cage observation) by experimenters unaware of genotype or treatment ('blinded'). Results of individual tests represent data obtained from testing either one or more independent cohorts. For each cohort of mice tested, the order of tests was arranged in a way that the less aversive tests were always performed prior to the more aversive ones.

2.3.1. Basic behavioral characterization

Male and female *Gpm6b*$^{-/-}$ mice and their WT littermates were tested in 5 and 2 consecutive cohorts, respectively. Testing was started at the age of 10–14 weeks. Mice were subjected to a basic behavioral characterization including tests for sensory function (visual cliff test, hearing assessment using the acoustic startle reflex, buried food finding test for measuring olfaction, hot plate test to determine pain threshold), motor and exploratory behavior (rotarod, open field, hole board), anxiety and depression (elevated plus-maze, forced swim, tail suspension, sucrose preference, chimney test), social and stereotypic behavior (social preference and memory test, social interaction in pairs, marble burying test), and cognition (novel object recognition, Morris water maze). A detailed description of these behavioral tests has been published elsewhere [7,28–33].

2.3.2. Prepulse inhibition of the acoustic startle reflex (PPI)

Animals were placed in small metal cages (82 mm × 40 mm × 40 mm) to restrict major movements and exploratory behavior. The cages were equipped with a movable platform floor attached to a sensor that recorded vertical movements of the floor. The cages were placed in 4 sound attenuating cabinets (TSE Systems, Germany). Startle reflexes were evoked by acoustic stimuli delivered by a loudspeaker that was suspended above the cage and connected to an acoustic generator. The startle reaction to an acoustic stimulus that induces a movement of a force-sensitive platform was recorded over a period of 260 ms beginning with the onset of the pulse. An experimental session consisted of a 2 min habituation to a 65 dB background white noise (continuous throughout

the session), followed by a baseline recording for 1 min at background noise. After baseline recording, 6 pulse-alone trials using startle stimuli of 120 dB intensity and 40 ms duration were applied to decrease the influence of within-session habituation. These data were not included in the 120 dB/40 ms analysis of the PPI. For tests of PPI, the startle pulse was applied either alone or preceded by a prepulse stimulus of 70-, 75-, or 80 dB intensity and 20 ms duration. A delay of 100 ms with background noise was interposed between the presentation of the prepulse and pulse stimulus. The trials were presented in a pseudorandom order with a variable interval ranging from 8 to 22 s. The amplitude of the startle response (expressed in arbitrary units) was defined as the difference between the maximum force detected during the recording window and the force measured immediately before the stimulus onset. For each animal, the amplitudes were averaged separately for the 2 types of trials (i.e. stimulus alone or stimulus preceded by a prepulse). PPI was calculated as the percentage of the startle response using the following formula: PPI% = 100 − [(startle amplitude after prepulse)/(startle amplitude after pulse only) × 100].

2.3.3. LABORAS

The LABORAS home cage behavior monitoring system (Metris b.v., Hoofddorp, The Netherlands) consists of a triangular shaped sensor platform (Carbon Fiber Plate 1000 mm × 700 mm × 700 mm × 30 mm), positioned on 2 orthogonally placed force transducers (Single Point Load Cells) and a third fixed point attached to a heavy bottom plate (Corian Plate 980 mm × 695 mm × 695 mm × 48 mm). The apparatus is mounted on 3 spikes, which are adjustable in height and absorb external vibrations. Mice are housed in transparent polycarbonate cages (Makrolon type II cage, 22 cm × 16 cm × 14 cm) with a floor covered with wood-chip bedding. The cage is placed directly onto the sensor platform, with the upper part of the cage (including the top, food hopper and drinking bottle) suspended in a height-adjustable frame separate from the sensing platform. Mechanical vibrations caused by the movement of the animal are transformed by force transducers into electrical signals, amplified to a fixed signal range, filtered to eliminate noise, digitized and then stored on a computer. The computer then processes the stored data using several signal analysis techniques to classify the signals into specific behavioral categories including eating, drinking, scratching, circling, climbing, immobility, locomotor activity and grooming. The behavior that dominates at a certain time point is scored by the LABORAS software. For each behavioral category, the time spent performing this particular behavior (counted in seconds), as well as its frequency, is measured. In order to ensure that the LABORAS software indeed provides a reliable measure of grooming behavior, we correlated manual scorings of grooming time (10 min observation time) for 18 randomly selected mice that had received an injection of DOI (see below) with the measures provided by the LABORAS software. We found a highly significant correlation between manual scorings and the LABORAS measurement of grooming behavior ($r = 0.87$, Pearson bivariate correlation; linear regression $p < 0.0001$) (Fig. 1).

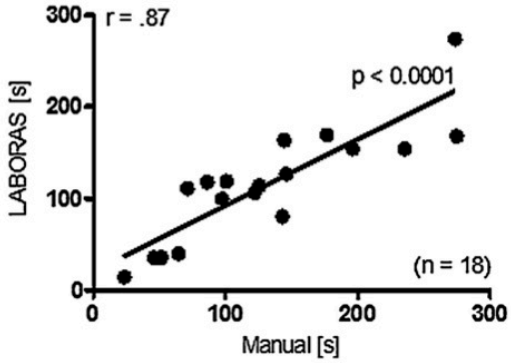

Fig. 1. Highly significant correlation between manual and automated measurement of grooming behavior. The time spent with grooming behavior as obtained from the LABORAS system was cross-validated with manual scoring of grooming time. The correlation ($r = .87$; Pearson) indicates that the LABORAS system provides a valid measurement of grooming.

3. Pharmacological challenge of the serotonin system in the LABORAS setting

The behavioral effects of a pharmacological challenge of the serotonergic system in *Gpm6b*$^{-/-}$ and WT mice were monitored using the LABORAS home cage observation system (described above). The 5-HT2A/C receptor agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI; Sigma-Aldrich, Germany) was freshly dissolved in 0.9% sodium chloride solution.

3.1. Pilot study

The optimal dose of DOI that would suppress locomotion and increase grooming behavior was determined in a pilot study, performed with 10 male WT mice. Here, one mouse at a time was removed from its home cage, gently weighed and placed in a LABORAS cage. After baseline recording of 30 min, the mouse was injected intraperitoneally with a dose of 0.5 or 0.75 mg/kg DOI and thereafter observed for another 30 min in the LABORAS cage. The results of this pilot experiment suggested that a dose of 0.5 mg/kg DOI reliably suppressed locomotion and increased grooming behavior without inducing sedation or motor impairments. Moreover, the results showed that head twitches as an additionally explored behavioral readout of DOI challenge [34] were highly variable in our hands with the 0.5 mg/kg dose and could not be reliably documented by LABORAS. This is why we restricted our measurements to locomotion and grooming. The time spent grooming is an important internal control to adequately interpret the behavioral effects of DOI and to exclude major side effects. The simultaneous registration of grooming and locomotion behavior allows determining whether DOI has suppressed locomotion due to motor impairments or even sedation. A mouse with a motor impairment or sedation would probably not be able to show increased grooming behavior. With these 2 readouts (locomotion and grooming) we were able to describe the behavioral effects of the DOI challenge in terms of decreases in one type of active behavior (locomotion) and a concurrent increase in another type of active behavior (grooming). Please note that DOI could also induce drowsiness or even sleep and thereby lead to a reduction in locomotor behavior as the consequence of a sedative side effect [35].

3.2. DOI-challenge in *Gpm6b*$^{-/-}$ and WT mice

For the actual experiment using male and female *Gpm6b*$^{-/-}$ mice and their WT littermates, individual mice were placed in LABORAS cages and the time spent with locomotion and grooming was recorded for 30 min. These 2 behavioral readouts were selected because they are mutually exclusive and can be used to evaluate the effect of DOI on 2 behavioral readouts simultaneously. Thereafter, the mouse received an intraperitoneal injection of DOI (0.5 mg/kg dissolved in physiological saline; injected in a volume of 0.1 ml/10 g body weight). Immediately thereafter, locomotion and grooming were measured in the LABORAS cage for another 30 min.

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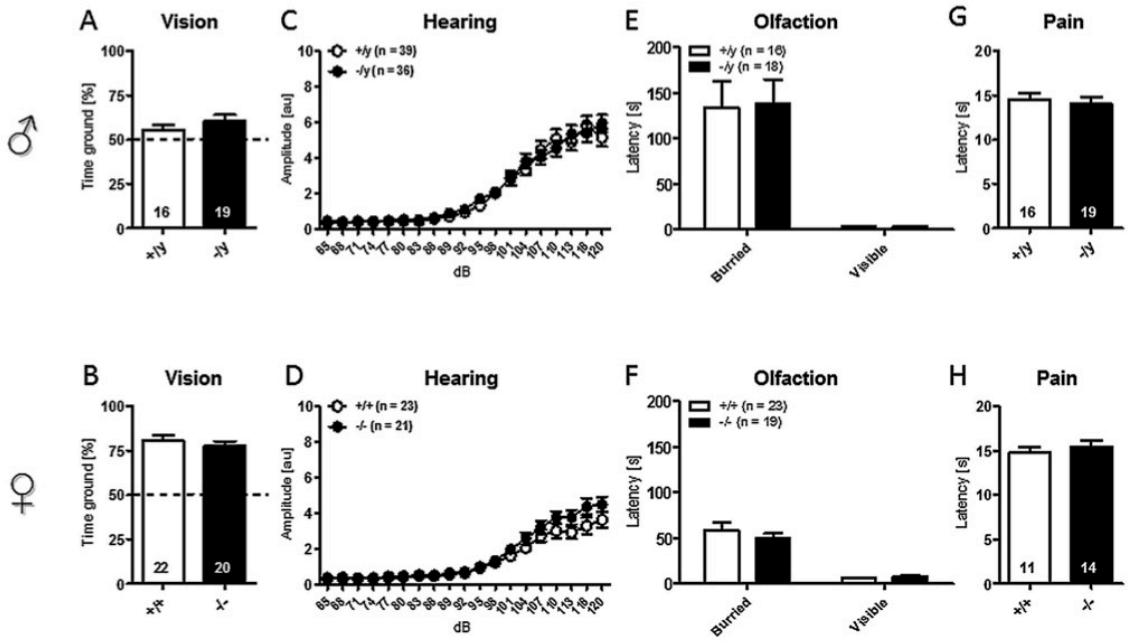

Fig. 2. Sensory functions are not altered in male or female *Gpm6b*$^{-/-}$ mice. The upper row presents results for male, the lower row for female mice. (A and B) visual cliff test; (C and D) hearing curve; (E and F) buried food finding and (G and H) hot plate test. Mean ± sem presented; respective sample sizes are indicated in the figures.

4. Statistical analysis

The data presented in figures and text are expressed as mean ± sem. Data were analyzed separately for males and females. Please note that the experiments were conducted with 5 (male) and 2 (female) cohorts. Not all tests were performed in all cohorts, leading to sometimes prominent differences in group sizes. Minor differing n-numbers of the groups are further due to cases where ratios could not be computed because of zero values. Also, exclusion of single outliers (after statistical testing for outlier status; see below) or missing values sometimes account for minor differences in *n*-numbers. Between-group comparisons were made by either one-way analysis of variance (ANOVA) with repeated measures or *t*-tests for independent samples. Within-group tests of chance level performance using ratio or percentage calculations were performed via single-group *t*-tests against a chance level of 50%. Mann–Whitney U and Wilcoxon tests were used where the normality assumption was violated as assessed by the Kolmogorov–Smirnov test. All statistics were performed using SPSS v.17 Software (San Diego, USA). Correlational analysis between 2 readouts was performed using Pearson bivariate correlation as well as linear regression analysis. The search and exclusion of significant outliers from single data sets (indicated from the visual inspection of the corresponding scatter plots) was performed using the Grubbs' test. Given p-values are 2-tailed and were considered significant if <0.05.

5. Results

5.1. Basic behaviors are unaffected in *Gpm6b*$^{-/-}$ mice

An extensive behavioral characterization of male and female *Gpm6b*$^{-/-}$ mice revealed that basic behaviors including sensory function (Fig. 2), general activity, exploratory behavior, motor performance and motor learning (Fig. 3), anxiety (Fig. 4) as well as social and stereotypic behaviors (Fig. 5) are indistinguishable between genotypes. The 15 h-overnight assessment of home-cage behavior in the LABORAS setting yielded a marginally increased locomotion and reduced immobility in male *Gpm6b*$^{-/-}$ versus WT mice (Fig. 6). We note that in a number of tests, there is – genotype-independently – a gender difference visible at first view.

5.2. *Gpm6b*$^{-/-}$ mice show no depression-like behavior or cognitive impairments

Gpm6b$^{-/-}$ mice of both genders performed comparable to their WT littermates in a test battery for depression-like behaviors (Fig. 7). Cognitive performance in terms of novel object recognition as well as spatial learning and reversal learning was basically identical between male *Gpm6b*$^{-/-}$ and WT mice (Fig. 8).

5.3. *Gpm6b*$^{-/-}$ mice exhibit decreased prepulse inhibition of the acoustic startle reflex

To explore whether *Gpm6b* deficiency has an impact on sensorimotor gating, male and female *Gpm6b*$^{-/-}$ and WT mice were tested in the prepulse inhibition (PPI) paradigm. Expectedly, PPI of the acoustic startle reflex increased across the 3 prepulse intensities in both male ($p < 0.001$; repeated measures ANOVA; Fig. 9A) and female mice ($p < 0.001$; Fig. 9B). *Gpm6b*$^{-/-}$ mice of both genders showed significantly reduced PPI as compared to WT mice (Fig. 9A and B). There was a trend for higher startle amplitude in male (Fig. 9C) and female (Fig. 9D) *Gpm6b*$^{-/-}$ relative to WT mice. This difference is unlikely to account for the reduction in PPI found in *Gpm6b*$^{-/-}$ mice, because an increased startle response should rather mask group differences than facilitate them. Also, by its incorporation into the PPI formula, the individual startle response is used for normalization of PPI (see methods section). No difference in body weight, neither among males nor females was noted (Fig. 9E and F), which could have confounded the measurement of

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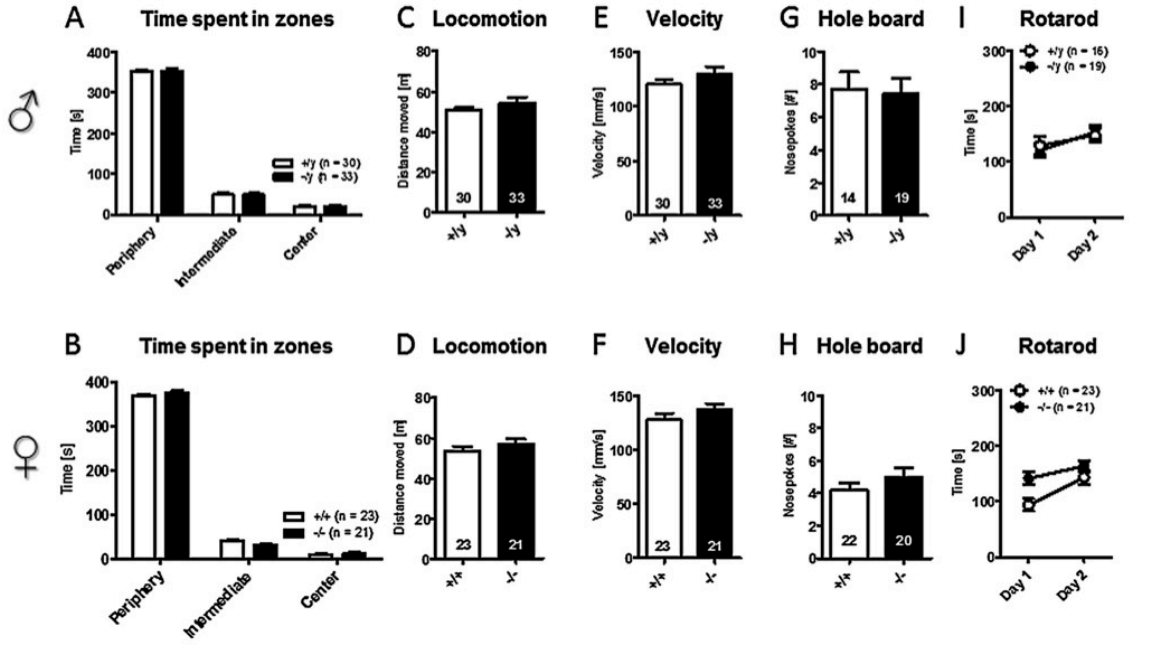

Fig. 3. Novelty-induced activity, exploration behavior and motor coordination are not affected in *Gpm6b*$^{-/-}$ mice. The upper row presents results for male, the lower row for female mice. (A–F) Open field readouts; (G and H) exploration in the hole board. (I and J) motor performance and motor learning. Mean ± sem presented; respective sample sizes are indicated in the figures.

PPI. These results indicate that *Gpm6b* deficiency impairs sensorimotor gating in the mouse.

5.4. *Gpm6b*$^{-/-}$ mice show blunted behavioral response to a 5-HT2A/C receptor agonist

To test whether *Gpm6b* deficiency has an impact on serotonergic mechanisms, we challenged *Gpm6b*$^{-/-}$ mice with the 5-HT2A/C receptor agonist DOI and measured locomotion and grooming behavior in the LABORAS home cage setting.

5.4.1. Males

Baseline performance of *Gpm6b*$^{-/-}$ and WT mice during the 30 min observation period, analyzed in 3 interval bins of 10 min, did not show any difference between genotypes, neither in locomotion nor grooming (Fig. 10A). Expectedly, overall locomotion decreased from the first to the third bin in the new environment. Therefore, for each mouse, the effect of DOI on locomotion and grooming behavior was assessed as change% from baseline, with the last 10 min interval of the baseline session set to 100%. DOI treatment decreased locomotion in both genotypes. In WT mice, the time spent with locomotion decreased to 17.42 ± 4.47% of baseline, whereas the maximal reduction in *Gpm6b*$^{-/-}$ mice reached only 42.12 ± 5.77% (between-group comparison of maximal reduction values of individual mice: $p = 0.002$; *t*-test for independent samples; repeated measures ANOVA in Fig. 10B). Conversely, DOI induced a dramatic increase in grooming over baseline levels. This increase reached up to 817.84 ± 236.40% in WT mice. Again, with an increase from baseline level of only 548.82 ± 83.33%, the DOI effect appeared less pronounced in *Gpm6b*$^{-/-}$ mice. However, neither a between-group comparison of the maximum increase values of individual mice, nor a repeated measures ANOVA on the 3 observation intervals reached significance ($p > 0.05$, Fig. 10C and D). Since locomotion and grooming are behaviors that are mutually exclusive, we also expressed the effects of DOI as ratio, hoping to get a clearer genotype contrasting: time spent with grooming[s]/time spent with locomotion[s]. The ratio between grooming and locomotion increased equally in both genotypes across the 3 time intervals of the baseline session ($p < 0.001$; Fig. 10E), likely due to behavioral habituation to the LABORAS cages leading to decreases in exploratory behaviors including locomotion and to a simultaneous increase in self-directed behaviors such as grooming. After treatment with

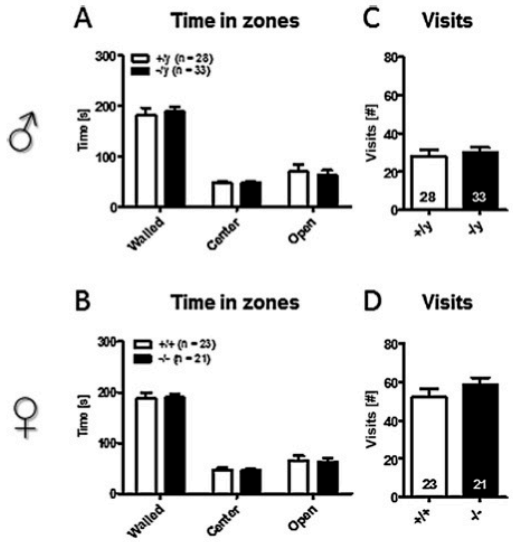

Fig. 4. Anxiety-like behavior is normal in male and female *Gpm6b*$^{-/-}$ mice. The upper row presents results for male, the lower row for female mice. (A–D) Elevated plus maze readouts. Mean ± sem presented; respective sample sizes are indicated in the figures.

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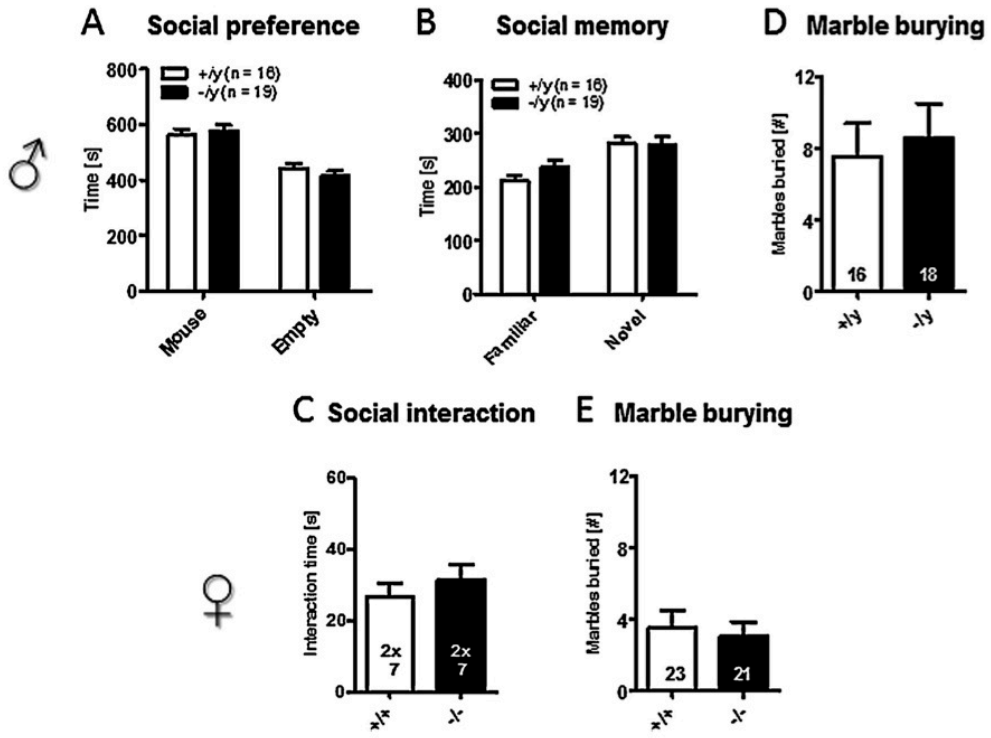

Fig. 5. Social and stereotypic behaviors are comparable to WT in male and female *Gpm6b*$^{-/-}$ mice. The upper row presents results for male, the lower row for female mice. (A and B) Social preference and memory tested in the tripartite chamber; (C) social interaction in pairs; (D and E) stereotypies/repetitive compulsive behaviors: Marble burying. Mean ± sem presented; respective sample sizes are indicated in the figures.

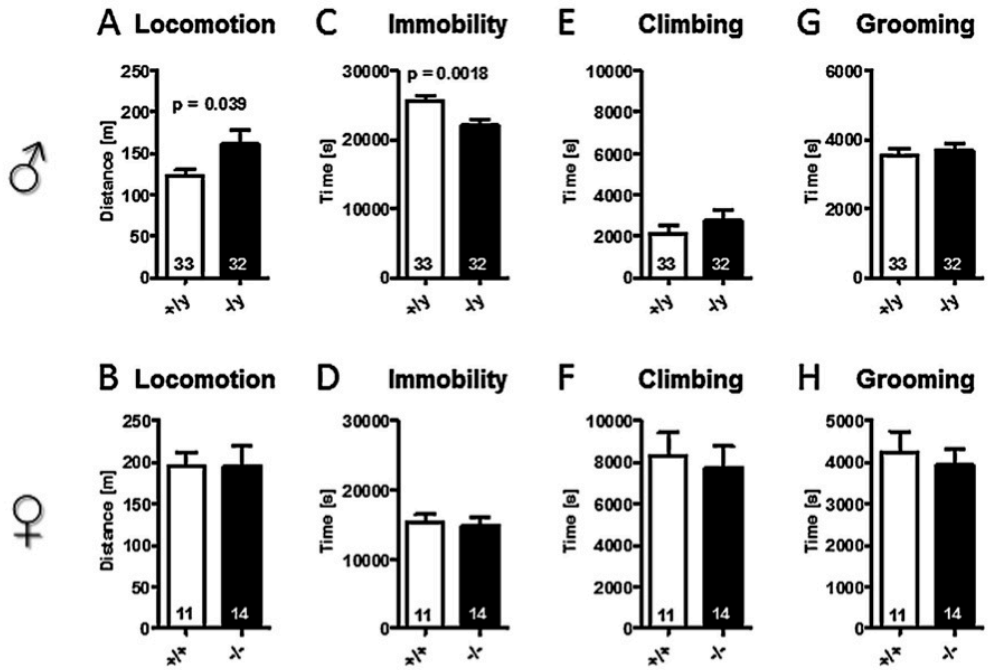

Fig. 6. Overnight 15 h home-cage assessment of spontaneous behavior. The upper row presents results for male, the lower row for female mice. (A–H) LABORAS standard readouts. Note that male *Gpm6b*$^{-/-}$ mice showed increased locomotion combined with decreased immobility as compared to WT mice (A and C). Mean ± sem presented; respective sample sizes are indicated in the figures.

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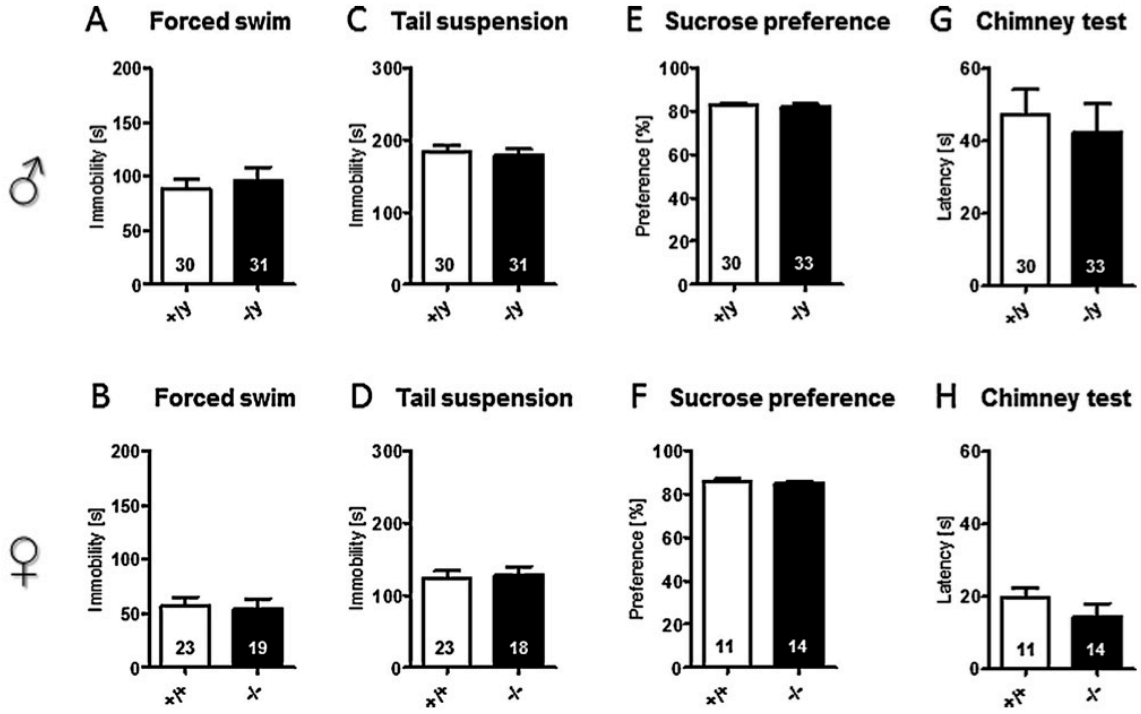

Fig. 7. No evidence of depression-like symptoms in *Gpm6b*$^{-/-}$ mice. The upper row presents results for male, the lower row for female mice. (A–D) Standard depression tests. (E and F) Test of anhedonia. (G and H) Test for motivation. Mean ± sem presented; respective sample sizes are indicated in the figures.

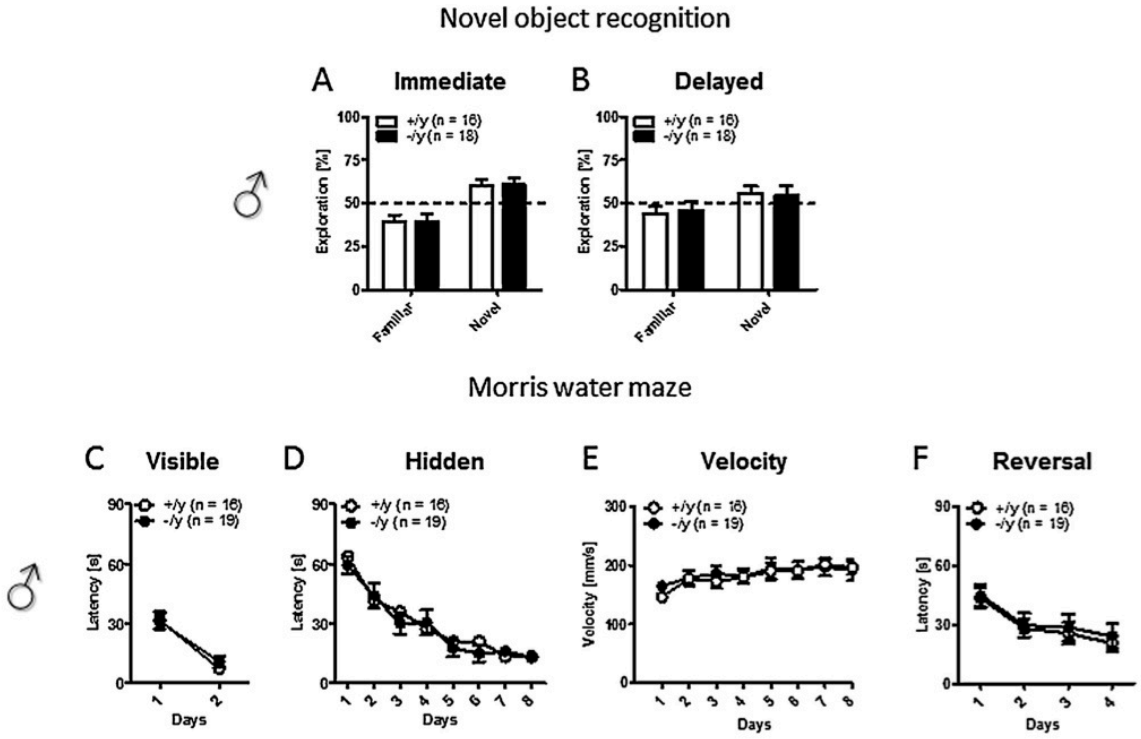

Fig. 8. Novel object recognition and Morris water maze performance of male *Gpm6b*$^{-/-}$ mice is unaffected. (A) Immediate and (B) delayed versions of the novel object recognition test. (C) Stimulus-response learning measured during the visible version of the Morris water maze task. (D–F) Spatial and reversal learning of the position of a hidden platform. Mean ± sem presented; respective sample sizes are indicated in the figures.

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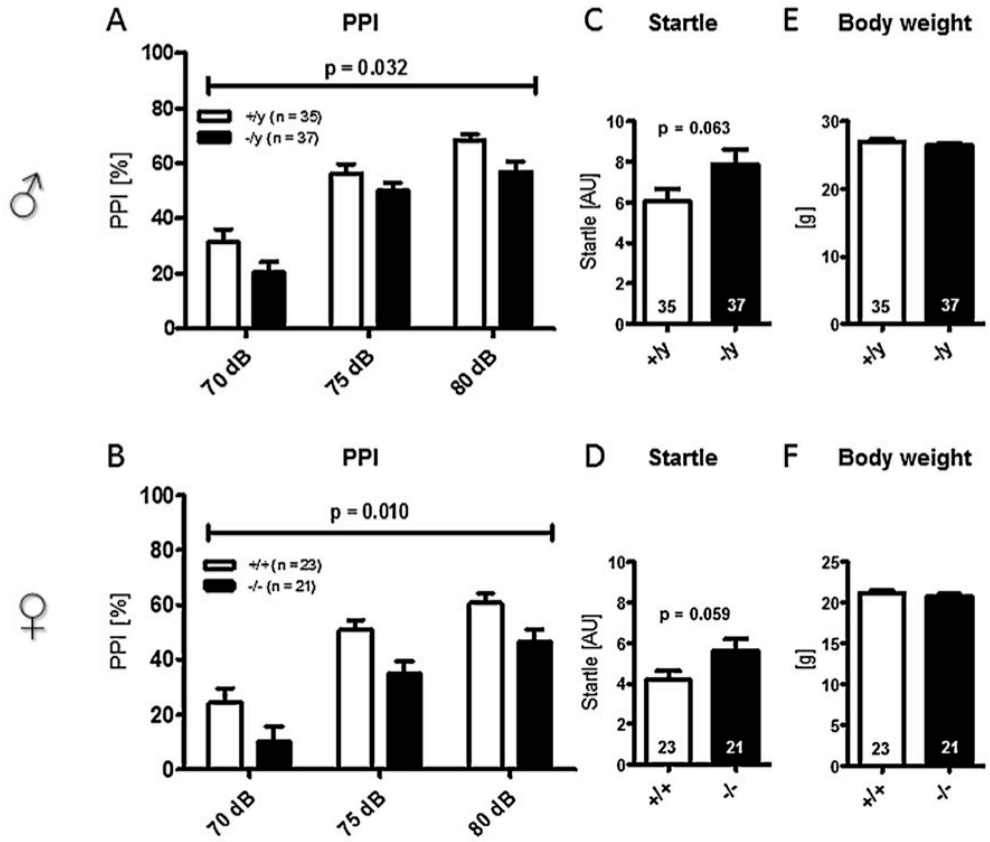

Fig. 9. Sensorimotor gating is impaired in male and female *Gpm6b*$^{-/-}$ mice. The upper row presents results for male, the lower row for female mice. (A and B) Prepulse inhibition (PPI) of the acoustic startle reflex. (C and D) Startle response to a 120 dB stimulus. (E and F) Body weight at the time of PPI testing. Mean ± sem presented; respective sample sizes are indicated in the figures.

DOI, WT mice showed a significantly higher overall ratio as compared to *Gpm6b*$^{-/-}$ mice (repeated measures ANOVA in Fig. 10F). In WT mice, the mean of the individual maximum ratios reached 19.14 ± 3.61, whereas it amounted to only 6.47 ± 1.65 in *Gpm6b*$^{-/-}$ mice (between-group comparison $p = 0.001$, Mann–Whitney U test). In sum, these results indicate that male *Gpm6b*$^{-/-}$ mice show a blunted behavioral response to challenge with a 5-HT2A/C receptor agonist.

5.4.2. Females

In contrast to males, female *Gpm6b*$^{-/-}$ mice showed already during the baseline session a trend for lower levels of locomotion (Fig. 10G) and for higher levels of grooming behavior (Fig. 10I). Also in females, DOI treatment induced a slight but significant decrease of locomotion relative to baseline in WT (reduction up to 67.21 ± 6.60%) and *Gpm6b*$^{-/-}$ mice (reduction up to 52.07 ± 4.90%; between-group comparison of maximal reduction values of individual mice: $p = 0.084$; t-test for independent samples, Fig. 10H), however, without significant main effect of genotype in repeated measures ANOVA. After DOI, WT and *Gpm6b*$^{-/-}$ mice responded with a clear increase in grooming. The change% relative to baseline during the first time interval reached 1190.82 ± 332.26% in WT mice and 732.83 ± 141.32% in *Gpm6b*$^{-/-}$ mice (between-group comparison of maximal increase values of individual mice: $p > 0.05$; t-test for independent samples). There was also no significant main effect of genotype (ANOVA, Fig. 10J). The genotype differences in baseline performance are reflected by a significantly higher baseline grooming/locomotion ratio of *Gpm6b*$^{-/-}$ as compared to WT mice (ANOVA, Fig. 10K). After DOI treatment, *Gpm6b*$^{-/-}$ mice kept their higher ratio as compared to WT (ANOVA, Fig. 10L). The maximal ratio values of WT mice reached only 1.56 ± 0.28, those of *Gpm6b*$^{-/-}$ mice amounted to 4.08 ± 0.81 (between-group comparison of maximal ratio values of individual mice: $p = 0.0012$; t-test for independent samples). Taken together, the effects of DOI on locomotion and grooming in female *Gpm6b*$^{-/-}$ and WT mice were somewhat weaker and slightly different as compared to those observed in males.

6. Discussion

This is the first study that explored the behavioral consequences of Gpm6b deficiency in male and female *Gpm6b*$^{-/-}$ mice. Using a comprehensive battery of behavioral tests, we found – on top of an entirely normal basic behavior – a highly selective impairment in PPI of the acoustic startle reflex in both male and female *Gpm6b*$^{-/-}$ mice. Considering that astrogliosis, microgliosis and lymphocyte invasion are not a feature in the brains of *Gpm6b*$^{-/-}$ mice [13], the observed behavioral effects are probably a direct consequence of Gpm6b deficiency rather than of a secondary neuropathological reaction. Due to the reported reduced mRNA abundance of *GPM6B* in the brains of suicide completers [16] on one hand and the association of this protein with SERT on the other hand [15], we searched

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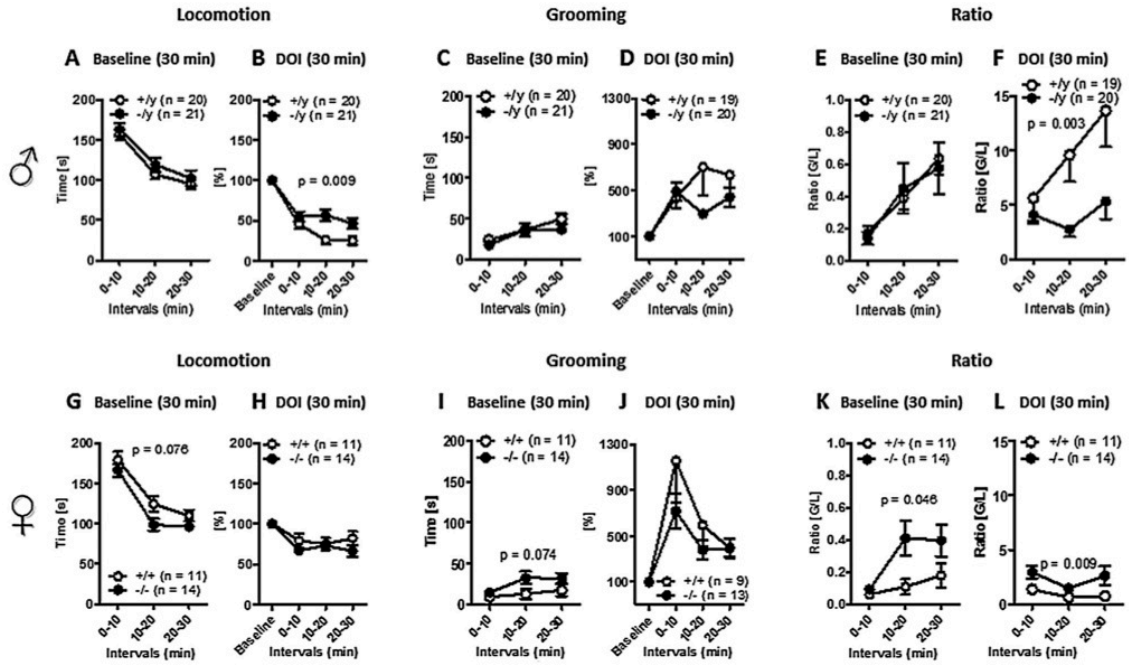

Fig. 10. Male and female *Gpm6b*$^{-/-}$ mice exhibit a blunted behavioral response to a 5-HT2A/C receptor agonist (acute injection of 0.5 mg/kg body weight DOI intraperitoneal). The upper row presents results for male, the lower row for female mice. (A and G) Baseline locomotion during a 30 min observation period (trend for a main effect of genotype in G). (B and H) Effects of an acute DOI treatment on locomotion. Shown is the %change from baseline (last 10-min interval). (C and I) Baseline grooming during a 30 min observation period (trend for a main effect of genotype in I). (D and J) Effects of an acute DOI treatment on grooming. Shown is the %change from baseline (last 10-min interval). (E and K) Grooming/locomotion ratio during the 30 min baseline observation period. (F and L) Grooming/locomotion ratio after the injection of DOI. Mean ± sem presented; respective sample sizes are indicated in the figures. Main genotype effects are given as p-values in the figures. Repeated measures ANOVA.

for first hints of an altered reaction of *Gpm6b*$^{-/-}$ mice to serotonergic stimulation. Indeed, we observed that male and to a minor extent also female *Gpm6b*$^{-/-}$ mice exhibited a diminished behavioral response to a 5-HT2A/C receptor agonist, DOI. These data indicate an impaired 5-HT2A/C receptor signaling as a consequence of the absence of *Gpm6b*.

However, it is difficult to conclude at this point that the PPI deficits are also a direct consequence of a decreased stimulation of postsynaptic 5-HT receptors with serotonin. PPI is a measure of sensorimotor gating and as such a model of network function in the brain. It is disrupted in a variety of neurologic and psychiatric disorders, ranging from trauma to inflammation and from schizophrenia to autism [36–39]. Animal studies have delineated a complex neuronal circuit including several brain regions that mediates PPI. These brain regions include the nucleus accumbens, hippocampus, colliculus superior, pedunculopontine tegmental nucleus and the brain stem [40,41]. It is also well known that the pharmacological modulation of monoamine neurotransmission (dopamine and 5-HT) modulates PPI in rats and mice [42–44].

Given that Gpm6b is a negative regulator of cell-surface expression of SERT [15], the PPI deficits of male and female *Gpm6b*$^{-/-}$ mice might be somehow related to regional changes in extracellular serotonin levels. We note, however, that changes in SERT expression can principally induce compensatory alterations at any level of the 5-HT system. For example, alterations in serotonin reuptake from the extracellular space can affect: (i) 5-HT synthesis in presynaptic neurons due to regulatory feedback mechanisms, (ii) the intra- and extracellular 5-HT metabolization rate, (iii) the expression, subtype and sensitivity of postsynaptic 5-HT receptors, as well as (iv) presynaptic serotonin autoreceptors that control 5-HT transmitter release [45]. A disruption of PPI can thus result from any of these changes that might be associated with an increase in SERT expression or activity.

As opposed to the findings with PPI and DOI, all basic behaviors were normal in *Gpm6b*$^{-/-}$ mice, with a minor exception of slightly increased locomotion (and subsequently decreased immobility) of male *Gpm6b*$^{-/-}$ mice in the overnight assessment of spontaneous home cage activity. In contrast, neither male nor female *Gpm6b*$^{-/-}$ mice showed changes in locomotion readouts of open field, hole board or elevated plus-maze. Therefore, it is unlikely that *Gpm6b* deficiency induces appreciable and consistent hyperactivity in the mouse.

The association of Gpm6b/GPM6B deficiency with consequences on the serotonergic system and with suicide [15,16] had initially motivated us to check *Gpm6b*$^{-/-}$ mice for potential signs of depressive-like behavior. There was, however, no depression-like phenotype observed in either gender under basal conditions. We cannot exclude that such phenotype would become obvious under conditions of e.g. severe stress, hormonal challenge or inflammation to just name a few known external inducers or co-factors of depression [46,47]. Interestingly, despite a potential reciprocal compensation of Gpm6a and Gpm6b for each other in some cellular functions [48,49], the behavioral consequences of their absence are distinct: Claustrophobia-like phenotype only in *Gpm6a* deficiency [7] and sensorimotor impairment only upon lack of *Gpm6b*.

To conclude, despite high homology and some functional overlap, the homolog proteins Gpm6a and Gpm6b fulfill distinct tasks in the brain extending from a considerable impact on the behavioral profile and network function to a possible modulation of neurotransmission. Even though an interaction of Gpm6b with the brain serotonin system is obvious, it still remains to be determined how exactly *Gpm6b* deficiency affects this system.

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Conflict of interest

None to report.

Author contributions

D.W., C.R., A.R., and G.P. performed all behavioral experiments. E.D. and H.E. performed the statistical analyses, designed the figures and interpreted the final data. J.P. and H.W. organized breeding and mouse colony maintenance. H.E., supported by E.D., H.W., M.G., C.M. and K.A.N., planned, supervised and coordinated the project. H.W., E.D., and H.E. wrote the manuscript. All authors contributed to the current version of the paper.

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References

[1] Mobius W, Patzig J, Nave KA, Werner HB. Phylogeny of proteolipid proteins: divergence, constraints, and the evolution of novel functions in myelination and neuroprotection. Neuron Glia Biol 2008;4(2):111–27.
[2] Saugier-Veber P, Munnich A, Bonneau D, Rozet JM, Le Merrer M, Gil R, et al. X-linked spastic paraplegia and Pelizaeus-Merzbacher disease are allelic disorders at the proteolipid protein locus. Nat Genet 1994;6(3):257–62.
[3] Petit B, Giraudet F, Bechon C, Bardin L, Avan P, Boespflug-Tanguy O, et al. Mice with a deletion of the major central myelin protein exhibit hypersensitivity to noxious thermal stimuli: involvement of central sensitization. Neurobiol Dis 2014;65C:55–68.
[4] Griffiths I, Klugmann M, Anderson T, Yool D, Thomson C, Schwab MH, et al. Axonal swellings and degeneration in mice lacking the major proteolipid of myelin. Science 1998;280(5369):1610–3.
[5] Alfonso J, Frick LR, Silberman DM, Palumbo ML, Genaro AM, Frasch AC. Regulation of hippocampal gene expression is conserved in two species subjected to different stressors and antidepressant treatments. Biol Psychiatry 2006;59(3):244–51.
[6] Alfonso J, Pollevick GD, Van Der Hart MG, Flugge G, Fuchs E, Frasch AC. Identification of genes regulated by chronic psychosocial stress and antidepressant treatment in the hippocampus. Eur J Neurosci 2004;19(3):659–66.
[7] El-Kordi A, Kastner A, Grube S, Klugmann M, Begemann M, Sperling S, et al. A single gene defect causing claustrophobia. Trans Psychiatry 2013;3:e254.
[8] Olinsky S, Loop BT, DeKosky A, Ripepi B, Weng W, Cummins J, et al. Chromosomal mapping of the human M6 genes. Genomics 1996;33(3):532–6.
[9] Yan Y, Lagenaur C, Narayanan V. Molecular cloning of M6: identification of a PLP/DM20 gene family. Neuron 1993;11(3):423–31.
[10] Yan Y, Narayanan V, Lagenaur C. Expression of members of the proteolipid protein gene family in the developing murine central nervous system. J Comp Neurol 1996;370(4):465–78.
[11] Choi KM, Kim JY, Kim Y. Distribution of the immunoreactivity for glycoprotein M6B in the neurogenic niche and reactive glia in the injury penumbra following traumatic brain injury in mice. Exp Neurobiol 2013;22(4):277–82.
[12] Werner H, Dimou L, Klugmann M, Pfeiffer S, Nave KA. Multiple splice isoforms of proteolipid M6B in neurons and oligodendrocytes. Mol Cell Neurosci 2001;18(6):593–605.
[13] Werner HB, Kramer-Albers EM, Strenzke N, Saher G, Tenzer S, Ohno-Iwashita Y, et al. A critical role for the cholesterol-associated proteolipids PLP and M6B in myelination of the central nervous system. Glia 2013;61(4):567–86.
[14] Drabek K, van de Peppel J, Eijken M, van Leeuwen JP. GPM6B regulates osteoblast function and induction of mineralization by controlling cytoskeleton and matrix vesicle release. J Bone Miner Res 2011;26(9):2045–51.
[15] Fjorback AW, Muller HK, Wiborg O. Membrane glycoprotein M6B interacts with the human serotonin transporter. J Mol Neurosci 2009;37(3):191–200.
[16] Fiori LM, Zouk H, Himmelman C, Turecki G. X chromosome and suicide. Mol Psychiatry 2011;16(2):216–26.
[17] Blier P, El Mansari M. Serotonin and beyond: therapeutics for major depression. Philos Trans R Soc Lond B Biol Sci 2013;368(1615):20120536.
[18] Werner HB, Kuhlmann K, Shen S, Uecker M, Schardt A, Dimova K, et al. Proteolipid protein is required for transport of sirtuin 2 into CNS myelin. J Neurosci 2007;27(29):7717–30.
[19] Sato Y, Mita S, Fukushima N, Fujisawa H, Saga Y, Hirata T. Induction of axon growth arrest without growth cone collapse through the N-terminal region of four-transmembrane glycoprotein M6a. Dev Neurobiol 2011;71(9):733–46.
[20] Wu DF, Koch T, Liang YJ, Stumm R, Schulz S, Schroder H, et al. Membrane glycoprotein M6a interacts with the micro-opioid receptor and facilitates receptor endocytosis and recycling. J Biol Chem 2007;282(30):22239–47.
[21] Liang YJ, Wu DF, Stumm R, Hollt V, Koch T. Membrane glycoprotein M6A promotes mu-opioid receptor endocytosis and facilitates receptor sorting into the recycling pathway. Cell Res 2008;18(7):768–79.
[22] Koshikawa N, Mocaer E, Stephenson JD. The effects of tianeptine on wet-dog shakes, fore-paw treading and a flexor reflex in rats are consistent with enhancement of 5-hydroxytryptamine uptake. Eur J Pharmacol 1991;198(1):51–7.
[23] Li Q, Wichems CH, Ma L, Van de Kar LD, Garcia F, Murphy DL. Brain region-specific alterations of 5-HT2A and 5-HT2C receptors in serotonin transporter knockout mice. J Neurochem 2003;84(6):1256–65.
[24] Rioux A, Fabre V, Lesch KP, Moessner R, Murphy DL, Lanfumey L, et al. Adaptive changes of serotonin 5-HT2A receptors in mice lacking the serotonin transporter. Neurosci Lett 1999;262(2):113–6.
[25] Roth BL, Willins DL, Kristiansen K, Kroeze WK. 5-Hydroxytryptamine2-family receptors (5-hydroxytryptamine2A,5-hydroxytryptamine2B,5-hydroxytryptamine2C): where structure meets function. Pharmacol Ther 1998;79(3):231–57.
[26] Qu Y, Villacreses N, Murphy DL, Rapoport SI. 5-HT2A/2C receptor signaling via phospholipase A2 and arachidonic acid is attenuated in mice lacking the serotonin reuptake transporter. Psychopharmacology (Berlin) 2005;180(1):12–20.
[27] Dawson N, Ferrington L, Lesch KP, Kelly PA. Cerebral metabolic responses to 5-HT2A/C receptor activation in mice with genetically modified serotonin transporter (SERT) expression. Eur Neuropsychopharmacol 2011;21(1):117–28.
[28] El-Kordi A, Winkler D, Hammerschmidt K, Kastner A, Krueger D, Ronnenberg A, et al. Development of an autism severity score for mice using Nlgn4 null mutants as a construct-valid model of heritable monogenic autism. Behav Brain Res 2013;251:41–9.
[29] Radyushkin K, El-Kordi A, Boretius S, Castaneda S, Ronnenberg A, Reim K, et al. Complexin2 null mutation requires a 'second hit' for induction of phenotypic changes relevant to schizophrenia. Genes Brain Behav 2010;9(6):592–602.
[30] Porsolt RD, Le Pichon M, Jalfre M. Depression: a new animal model sensitive to antidepressant treatments. Nature 1977;266(5604):730–2.
[31] Heinecke H. A comparison of two variants of the chimney behaviour test in mice. Z Versuchstierkd 1987;29(3/4):145–8.
[32] Reim K, Regus-Leidig H, Ammermuller J, El-Kordi A, Radyushkin K, Ehrenreich H, et al. Aberrant function and structure of retinal ribbon synapses in the absence of complexin 3 and complexin 4. J Cell Sci 2009;122(Pt 9):1352–61.
[33] Cryan JF, Mombereau C, Vassout A. The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. Neurosci Biobehav Rev 2005;29(4/5):571–625.
[34] Canal CE, Morgan D. Head-twitch response in rodents induced by the hallucinogen 2,5-dimethoxy-4-iodoamphetamine: a comprehensive history, a re-evaluation of mechanisms, and its utility as a model. Drug Test Anal 2012;4(7/8):556–76.
[35] Williams AA, Ingram WM, Levine S, Resnik J, Kamel CM, Lish JR, et al. Reduced levels of serotonin 2A receptors underlie resistance of Egr3-deficient mice to locomotor suppression by clozapine. Neuropsychopharmacology 2012;37(10):2285–98.
[36] Braff DL, Geyer MA, Swerdlow NR. Human studies of prepulse inhibition of startle: normal subjects, patient groups, and pharmacological studies. Psychopharmacology (Berlin) 2001;156(2/3):234–58.
[37] Kohl S, Heekeren K, Klosterkotter J, Kuhn J. Prepulse inhibition in psychiatric disorders – apart from schizophrenia. J Psychiatr Res 2013;47(4):445–52.
[38] Kumru H, Vidal J, Kofler M, Portell E, Valls-Sole J. Alterations in excitatory and inhibitory brainstem interneuronal circuits after severe spinal cord injury. J Neurotrauma 2010;27(4):721–8.
[39] Meyer U, Feldon J, Dammann O. Schizophrenia and autism: both shared and disorder-specific pathogenesis via perinatal inflammation? Pediatr Res 2011;69(5 (Pt 2)):26R–33R.
[40] Koch M. The neurobiology of startle. Prog Neurobiol 1999;59(2):107–28.
[41] Swerdlow NR, Caine SB, Braff DL, Geyer MA. The neural substrates of sensorimotor gating of the startle reflex: a review of recent findings and their implications. J Psychopharmacol 1992;6(2):176–90.
[42] Shanahan NA, Holick Pierz KA, Masten VL, Waeber C, Ansorge M, Gingrich JA, et al. Chronic reductions in serotonin transporter function prevent 5-HT1B-induced behavioral effects in mice. Biol Psychiatry 2009;65(5):401–8.
[43] Dulawa SC, Geyer MA. Effects of strain and serotonergic agents on prepulse inhibition and habituation in mice. Neuropharmacology 2000;39(11):2170–9.
[44] van den Buuse M. Modeling the positive symptoms of schizophrenia in genetically modified mice: pharmacology and methodology aspects. Schizophr Bull 2010;36(2):246–70.
[45] Meneses A, Perez-Garcia G, Ponce-Lopez T, Tellez R, Castillo C. Serotonin transporter and memory. Neuropharmacology 2011;61(3):355–63.
[46] Mahar I, Bambico FR, Mechawar N, Nobrega JN. Stress, serotonin, and hippocampal neurogenesis in relation to depression and antidepressant effects. Neurosci Biobehav Rev 2014;38:173–92.
[47] Rohleder N, Wolf JM, Wolf OT. Glucocorticoid sensitivity of cognitive and inflammatory processes in depression and posttraumatic stress disorder. Neurosci Biobehav Rev 2010;35(1):104–14.
[48] Fernandez ME, Alfonso J, Brocco MA, Frasch AC. Conserved cellular function and stress-mediated regulation among members of the proteolipid protein family. J Neurosci Res 2010;88(6):1298–308.
[49] Sato Y, Watanabe N, Fukushima N, Mita S, Hirata T. Actin-independent behavior and membrane deformation exhibited by the four-transmembrane protein M6a. PLoS One 2011;6(12):e26702.

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5. Summary and conclusions

In this cumulative thesis, two original first author publications have been included with the focus on two mouse models of neuropsychiatric phenotypes. In the first publication we created and refined an autism severity score for mice using *Nlgn4* null mutant mice as a construct-valid model of heritable monogenic autism (El-Kordi A, Winkler D et. al 2013). The second publication describes that *Gpm6b* deficiency impairs sensorimotor gating and modulates the behavioral response to a 5-HT2A/C receptor agonist in mice (Dere E, Winkler D et al. 2014).

5.1 Development of an autism severity score for mice using Nlgn4 null mutants as a construct-valid model of heritable monogenic autism

Autism spectrum disorder (ASD) is characterized by symptoms such as difficulties in social interaction, disturbed communication, and expression of stereotyped repetitive behaviors and restricted interests. Among patients, symptoms can vary noticeably from mild to very severe presentation. From this highly heterogeneous picture, conclusions about the origin of the disorder or possible genes involved are difficult to draw. In the last years the number of individuals receiving the diagnosis of autism spectrum disorder has increased for unknown reasons, though increased awareness may have played a role, since diagnosis depends entirely on observation and identification of unusual behavior in patients. Prevalence rates of the disorder continue to rise, indicating that fine-tuning of diagnosis methods is still necessary, as well as studying the molecular basis and influencing factors (e.g. environmental) of the disease. The interaction of multiple factors affecting the prevalence of ASD is still not fully understood and requires further investigation (Baio, 2012).

Since the etiology of ASD is still unknown in most cases, one possibility is to focus on the monogenic heritable forms that have been described, which are caused by loss-of-function mutations in synaptic genes. Aberrant signalling between nerve cells caused by mutations in *NLGN4*, results in the ASD phenotype in affected

patients (Jamain et al., 2003). Previously, our group was able to show that *Nlgn4* null mutant (*Nlgn4* -/-) mice exhibit autistic-like behavior, including disturbed social interaction and compromised ultrasonic vocalization. As such, these animals can be used as construct and face valid genetic animal model for ASD (Jamain et al., 2008). Since so far little is known about sex differences in these animal models (for review see Kokras et al., 2014), we extended the characterization of *Nlgn4* null mutant mice from males to females in order to delineate acute sex differences. Additionally, we included a more comprehensive set of ASD relevant readouts, especially extending to repetitive stereotyped behaviors. Male and female *Nlgn4* null mutant mice and their wildtype littermates underwent basic behavioral characterization including elevated plus maze, open field, hole board, rota-rod and prepulse inhibition of the startle response. The resulting findings show that the *Nlgn4* null mutant mice exhibit normal basic behavior. By examining ASD relevant readouts, we noticed significant differences in *Nlgn4* null mutant social behavior and communication compared to wildtype animals and both genders were affected. Although we were able to show the autistic phenotype in both genders, the results indicated a marginally milder phenotype in females with a mild deviant phenotype.

Our results align with the fact that human patients with the same mutation can show a different combination of symptoms with differing severities, and indicate that environmental influences or epigenetic modifications may be involved, although our animals were kept in a stable environment with defined housing conditions. The importance of the environment, namely the housing conditions, was clearly illustrated by the results of the marble burying test. In our experience, group housing mitigated the compulsive phenotype. Further work is needed to delineate the effects of environmental changes.

From the results of the behavioral tests, we were able to derive an autism severity composite score, as an indicator of overall syndrome severity. With this score, we are able to predict the genotype with a 100% accuracy in males and with an accuracy of 83% in females. The development of the autism composite score in *Nlgn4* null mutant mice has helped to limit the variance in observed behaviors in the mice, and could therefore be an important tool for the evaluation of new

pharmacological treatment approaches targeting NLGN4 function (El-Kordi et al., 2013; Wöhr et al., 2013). Although there is a huge variety of symptoms caused by varying expression levels of a single gene, there would optimally be one treatment for all the symptoms caused by a particular genetic modification.

Possibly, there are different mechanisms involved in the development of ASD, finally leading to the identification of various forms of ASD, which than would lead to a further refinement of diagnostic criteria. Some progress in identification of susceptibility genes is ongoing, while refinement of imaging techniques continues to improve definition rates of structural correlates and abnormalities in autism.

5.2 Gpm6b deficiency impairs sensorimotor gating and modulates the behavioral response to a 5-HT2A/C receptor agonist

Depression (major depressive disorder) is the leading cause of disability worldwide (Ferrari et al., 2013) and is associated with a high risk of suicide (Lesage et al., 1994), indicating a global health priority for depressive disorders (Ferrari et al., 2013). The pathophysiology of depression is still not completely understood, though there are several mechanisms possibly involved (Femenia et al., 2012). A number of studies show possible involvement of the serotonin transporter and disturbed serotonergic signaling in the emergence of depression (for review see Sharpley et al., 2014). The serotonin transporter (SERT) actively transports serotonin from the synaptic cleft in the presynaptic neuron, and M6b has been shown to interact with this transporter (Fjorback et al., 2009). In my second project, we addressed a *Gpm6b* deficient mouse model, to assess the impact of this deficiency on the behavioral phenotype of *Gpm6b* deficient mice and on SERT signaling; in order to obtain insight into the development of depressive phenotypes.

To elucidate the role of *Gpm6b* on behavior, we used *Gpm6b* null mutant (*Gpm6b*-/-) male and female mice and assessed motor functions, cognition, social behavior, as well as anxiety, and found *Gpm6b* null mutants indistinguishable from their wildtype littermates. However, a general sex difference was observed, independent of the genotype. Such sex differences have already been described among other animal models for psychiatric conditions (Kokras et al., 2014).

As mentioned above, impairments in serotonin neurotransmission are involved in the symptoms associated with major depressive disorder. Therefore, in another series of experiments, we wondered whether *Gpm6b*-deficient mice (that might suffer from a lack of extracellular serotonin due to an increase in the cell-surface expression of the serotonin transporter) would consequently exhibit depression-like symptoms along with cognitive impairments. Although we suspected a depressive phenotype in *Gpm6b* null mutant mice, we found all of our tests for depression unaffected. The results of the forced swim test, tail suspension,

sucrose preference and the chimney test were comparable between the *Gpm6b* null mutant mice of both genders and their wildtype littermates. Depression may have several forms (e.g. psychotic depression or melancholia) (Parker, 2005), and since the domains affected in depression are difficult to model in rodents, further work is needed to find animal models with high face, construct and predictive validity.

Cognitive performance was assessed with novel object recognition and Morris water maze. Neither immediate, nor delayed object recognition yielded to a difference in performance by genotype. The spatial learning, as well as its reversal (as a measure of cognitive flexibility), remained unaffected by the *Gpm6b* deficiency in mice, compared to their wildtype littermates.

In the prepulse inhibition (PPI) paradigm we were able to explore the impact of *Gpm6b*-deficiency on sensorimotor gating, which resulted in a decreased inhibition response of the sensory information compared to wildtype animals. This finding is coherent with the literature, since it was shown that severe depletion of brain serotonin leads to disrupted PPI of the acoustic startle response (Fletcher et al., 2001).

In the brains of suicide completers, mRNA levels of GPM6B were found to be reduced. Therefore we assessed the behavioral response of *Gpm6b* null mutant mice to DOI (5-HT2A/C receptor agonist). Our results indicated impaired receptor signalling as an effect of the absence of *Gpm6b*, leading to a blunted behavioral response to the 5-HT2A/C receptor agonist.

It has been noted recently that the test order can influence the results of specific tests (McIlwain et al., 2001). Ideally, for every test a new group of animals should be used; however, this is not possible according to animal welfare regulations and therefore it is possible that this effect led to the disappearance of the depressive phenotype in our animals.

6. Outlook

Finding a way to model complex human phenotypes in mice is always difficult, and mouse assays modelling disturbed social functions in humans are highly limited. Currently, the most common test is the three-chambered social approach paradigm from the lab of J. Crawley (Moy et al., 2004). Unfortunately, this test is sensitive to lab-to-lab variability, and results of mouse models for ASD are difficult to reproduce (Ey et al., 2012). There is even a study available indicating that C57Bl/6J animals fail to show a preference for social novelty in the automated three-chamber apparatus (Pearson et al., 2010). As such, we felt the need to develop a new social memory task, which considers the rich and complex social behavior mice can exhibit. So far, I have worked on a novel test chamber called *Sociobox*, which allows for the presentation of ultimately 10 strangers mice. With this version, we are aiming to improve the phenotyping of mouse models of ASD and to provide more robust results for potential pharmacological interventions.

Given that cognitive deficits are frequently observed in neurodegenerative diseases including autism spectrum disorders, major depression, anxiety disorders and schizophrenia, we were also interested in the involvement of the endogenous erythropoietin (EPO)-system in various cognitive functions. It is well known that erythropoietin stimulates neurogenesis and can ameliorate cognitive symptoms in human neuropsychiatric diseases, including schizophrenia, as shown previously by our group (Ehrenreich et al., 2007). As such, we addressed the application of EPO as a possible treatment option for cognitive decline in disease and for cognitive improvement under wildtype conditions in mice. Mouse assays for the assessment of higher cognitive functions are anyhow limited, the improvement of these functions (e.g. cognitive flexibility) are rather difficult to measure with most of the paradigms available. For example in Morris water maze, a superior cognitive function is not possible to delineate by the basic paradigm protocol used in most of the laboratories, since the swimming speed is limited by the muscle capacities of each mouse and can not be further increased. Hence, the development of more sophisticated testing tools is mandatory, which will enable a more detailed analysis of the learning capacities of each mouse, and also allow the revelation of superior performance in higher cognitive functions.

Bussey and colleagues have adapted to mice a touchscreen-based operant learning paradigm, which is similar to the ones used in humans (Robbins et al., 1998) and in monkeys (Dias et al., 1996) and allows the assessment of sophisticated learning tasks, in which mice learn to respond to a touch-sensitive screen (Izquierdo et al., 2006). In this task, reversal learning functions can be used as a measure of cognitive flexibility. The subject first learns to associate a stimulus with a reward and then the reward contingency is reversed, overall taxing multiple executive functions, including attention, working memory and response inhibition (Brigman et al., 2010). I was able to establish the touchscreen-based visual discrimination and reversal task in our unit. The protocol I am using was adapted from Izquierdo and coworkers from 2006 (Izquierdo et al., 2006) and is comprised of several phases, starting with reward approach training, touch training, discrimination learning and the reversal of the discrimination learning.

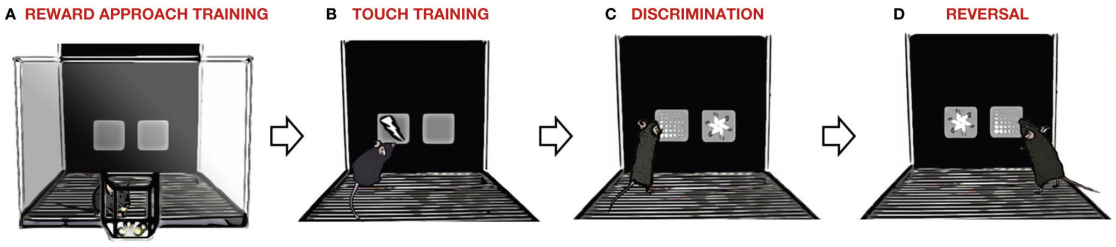

Figure 1 Illustration of the phases necessary for the assessment of visual discrimination and its reversal in a touchscreen based operant system (adapted from Brigman et al., 2010)

Since the touchscreen based visual discrimination and reversal paradigm fulfils the characteristics of a multifaceted learning task, after the successful establishment, we are currently assessing the effect of EPO application in mice on the learning performance in the touchscreen paradigm.

It has been described that people with ASD show difficulties in flexibly adjusting behavior according to environmental demands, and that these so-called inhibition problems are often observed in communication and social interactions. Thus, there is a general notion that inhibitory abilities underlie many of the atypical behaviors seen in people with ASD (de Vries et al., 2014). Since it is difficult to develop tests addressing the same executive functions seen in humans, no

studies so far have looked into the inhibition ability of animal models of autism. With the touchscreen-based operant system we will also be able to test for cognitive flexibility problems of ASD mouse models in the future.

7. References

American Psychiatric Association. (2000). *Diagnostic and statistical manual of mental disorders* (4th, text rev. ed.). Washington, DC: Author.

American Psychiatric Association. (2013). *Diagnostic and statistical manual of mental disorders* (5th ed.). Arlington: American Psychiatric Publishing, VA.

Baio. (2012). Prevalence of autism spectrum disorders--Autism and Developmental Disabilities Monitoring Network, 14 sites, United States, 2008. *Morbidity and mortality weekly report., 61*(3), 1-19.

Berkel, S., Marshall, C. R., Weiss, B., Howe, J., Roeth, R., Moog, U., Endris, V., Roberts, W., Szatmari, P., Pinto, D., Bonin, M., Riess, A., et al. (2010). Mutations in the SHANK2 synaptic scaffolding gene in autism spectrum disorder and mental retardation. *Nature genetics, 42*(6), 489-491.

Bernardet, M., & Crusio, W. E. (2006). Fmr1 KO mice as a possible model of autistic features. *ScientificWorldJournal, 6*, 1164-1176.

Betancur, C. (2011). Etiological heterogeneity in autism spectrum disorders: more than 100 genetic and genomic disorders and still counting. *Brain Res, 1380*, 42-77.

Bourgeron, T. (2009). A synaptic trek to autism. *Current opinion in neurobiology, 19*(2), 231-234.

Brigman, J. L., Graybeal, C., & Holmes, A. (2010). Predictably irrational: assaying cognitive inflexibility in mouse models of schizophrenia. *Frontiers in neuroscience, 4*.

Capecchi, M. R. (2005). Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. *Nature reviews. Genetics, 6*(6), 507-512.

Chahrour, M., & Zoghbi, H. Y. (2007). The story of Rett syndrome: from clinic to neurobiology. *Neuron, 56*(3), 422-437.

Chen, R. Z., Akbarian, S., Tudor, M., & Jaenisch, R. (2001). Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nature genetics, 27*(3), 327-331.

Chermat, R., Thierry, B., Mico, J. A., Steru, L., & Simon, P. (1986). Adaptation of the tail suspension test to the rat. *Journal de pharmacologie, 17*(3), 348-350.

Compton, W. M., Conway, K. P., Stinson, F. S., & Grant, B. F. (2006). Changes in the prevalence of major depression and comorbid substance use disorders in the United States between 1991-1992 and 2001-2002. *The American journal of psychiatry, 163*(12), 2141-2147.

Courchesne, E., Pierce, K., Schumann, C. M., Redcay, E., Buckwalter, J. A., Kennedy, D. P., & Morgan, J. (2007). Mapping early brain development in autism. *Neuron, 56*(2), 399-413.

Crawley, J. N. (2012). Translational animal models of autism and neurodevelopmental disorders. *Dialogues in clinical neuroscience, 14*(3), 293-305.

Cryan, J. F., & Holmes, A. (2005). The ascent of mouse: advances in modelling human depression and anxiety. *Nature reviews. Drug discovery, 4*(9), 775-790.

Cryan, J. F., Mombereau, C., & Vassout, A. (2005). The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. *Neuroscience and biobehavioral reviews, 29*(4-5), 571-625.

Currenti, S. A. (2010). Understanding and determining the etiology of autism. *Cellular and molecular neurobiology, 30*(2), 161-171.

Daenen, E. W., Wolterink, G., Gerrits, M. A., & Van Ree, J. M. (2002). The effects of neonatal lesions in the amygdala or ventral hippocampus on social behaviour later in life. *Behavioural brain research, 136*(2), 571-582.

de Vries, M., & Geurts, H. M. (2014). Beyond individual differences: are working memory and inhibition informative specifiers within ASD? *Journal of neural transmission*.

Dere, E., Winkler, D., Ritter, C., Ronnenberg, A., Poggi, G., Patzig, J., Gernert, M., Muller, C., Nave, K. A., Ehrenreich, H., & Werner, H. B. (2014). Gpm6b deficiency impairs sensorimotor gating and modulates the behavioral response to a 5-HT2A/C receptor agonist. *Behavioural brain research*.

Dias, R., Robbins, T. W., & Roberts, A. C. (1996). Primate analogue of the Wisconsin Card Sorting Test: effects of excitotoxic lesions of the prefrontal cortex in the marmoset. *Behavioral neuroscience, 110*(5), 872-886.

Durand, C. M., Betancur, C., Boeckers, T. M., Bockmann, J., Chaste, P., Fauchereau, F., Nygren, G., Rastam, M., Gillberg, I. C., Anckarsater, H., Sponheim, E., Goubran-Botros, H., et al. (2007). Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. *Nature genetics, 39*(1), 25-27.

Ehrenreich, H., Hinze-Selch, D., Stawicki, S., Aust, C., Knolle-Veentjer, S., Wilms, S., Heinz, G., Erdag, S., Jahn, H., Degner, D., Ritzen, M., Mohr, A., et al. (2007). Improvement of cognitive functions in chronic schizophrenic patients by recombinant human erythropoietin. *Molecular psychiatry, 12*(2), 206-220.

El-Kordi, A., Winkler, D., Hammerschmidt, K., Kastner, A., Krueger, D., Ronnenberg, A., Ritter, C., Jatho, J., Radyushkin, K., Bourgeron, T., Fischer, J., Brose, N., & Ehrenreich, H. (2013). Development of an autism severity score for mice using Nlgn4 null mutants as a construct-valid model of heritable monogenic autism. *Behavioural brain research, 251*, 41-49.

Etherton, M. R., Blaiss, C. A., Powell, C. M., & Sudhof, T. C. (2009). Mouse neurexin-1alpha deletion causes correlated electrophysiological and behavioral changes consistent with cognitive impairments. *Proceedings of the National Academy of Sciences USA, 106*(42), 17998-18003.

Ey, E., Yang, M., Katz, A. M., Woldeyohannes, L., Silverman, J. L., Leblond, C. S., Faure, P., Torquet, N., Le Sourd, A. M., Bourgeron, T., & Crawley, J. N. (2012). Absence of deficits in social behaviors and ultrasonic vocalizations in later generations of mice lacking neuroligin4. *Genes, brain, and behavior*, in press.

Fava, M. (2003). Diagnosis and definition of treatment-resistant depression. *Biological psychiatry, 53*(8), 649-659.

Femenia, T., Gomez-Galan, M., Lindskog, M., & Magara, S. (2012). Dysfunctional hippocampal activity affects emotion and cognition in mood disorders. *Brain research, 1476*, 58-70.

Ferrari, A. J., Charlson, F. J., Norman, R. E., Patten, S. B., Freedman, G., Murray, C. J., Vos, T., & Whiteford, H. A. (2013). Burden of depressive disorders by country, sex, age, and year: findings from the global burden of disease study 2010. *PLoS medicine, 10*(11), e1001547.

Fjorback, A. W., Muller, H. K., & Wiborg, O. (2009). Membrane glycoprotein M6B interacts with the human serotonin transporter. *Journal of molecular neuroscience, 37*(3), 191-200.

Fletcher, P. J., Selhi, Z. F., Azampanah, A., & Sills, T. L. (2001). Reduced brain serotonin activity disrupts prepulse inhibition of the acoustic startle reflex. Effects of 5,7-dihydroxytryptamine and p-chlorophenylalanine. *Neuropsychopharmacology, 24*(4), 399-409.

Gadad, B. S., Hewitson, L., Young, K. A., & German, D. C. (2013). Neuropathology and animal models of autism: genetic and environmental factors. *Autism research and treatment, 2013*, 731935.

Greenberg, P. E., Kessler, R. C., Birnbaum, H. G., Leong, S. A., Lowe, S. W., Berglund, P. A., & Corey-Lisle, P. K. (2003). The economic burden of depression in the United States: how did it change between 1990 and 2000? *The Journal of clinical psychiatry, 64*(12), 1465-1475.

Heron, M. (2013). Deaths: leading causes for 2010. *National vital statistics reports, 62*(6), 1-96.

Hill, S. K., Bishop, J. R., Palumbo, D., & Sweeney, J. A. (2010). Effect of second-generation antipsychotics on cognition: current issues and future challenges. *Expert review of neurotherapeutics, 10*(1), 43-57.

Hoon, M., Soykan, T., Falkenburger, B., Hammer, M., Patrizi, A., Schmidt, K. F., Sassoe-Pognetto, M., Lowel, S., Moser, T., Taschenberger, H., Brose, N., & Varoqueaux, F. (2011). Neuroligin-4 is localized to glycinergic postsynapses and regulates inhibition in the retina. *Proceedings of the National Academy of Sciences USA, 108*(7), 3053-3058.

Izquierdo, A., Wiedholz, L. M., Millstein, R. A., Yang, R. J., Bussey, T. J., Saksida, L. M., & Holmes, A. (2006). Genetic and dopaminergic modulation of reversal learning in a touchscreen-based operant procedure for mice. *Behavioural brain research, 171*(2), 181-188.

Jamain, S., Quach, H., Betancur, C., Rastam, M., Colineaux, C., Gillberg, I. C., Soderstrom, H., Giros, B., Leboyer, M., Gillberg, C., & Bourgeron, T. (2003). Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nature genetics, 34*(1), 27-29.

Jamain, S., Radyushkin, K., Hammerschmidt, K., Granon, S., Boretius, S., Varoqueaux, F., Ramanantsoa, N., Gallego, J., Ronnenberg, A., Winter, D., Frahm, J., Fischer, J., et al. (2008). Reduced social interaction and ultrasonic communication in a mouse model of monogenic heritable autism. *Proceedings of the National Academy of Sciences USA, 105*(5), 1710-1715.

Kalueff, A. V., Wheaton, M., & Murphy, D. L. (2007). What's wrong with my mouse model? Advances and strategies in animal modeling of anxiety and depression. *Behavioural brain research, 179*(1), 1-18.

Kokras, N., & Dalla, C. (2014). Sex differences in animal models of psychiatric disorders. *British journal of pharmacology*.

Kumar, B., Prakash, A., Sewal, R. K., Medhi, B., & Modi, M. (2012). Drug therapy in autism: a present and future perspective. *Pharmacological reports, 64*(6), 1291-1304.

Lesage, A. D., Boyer, R., Grunberg, F., Vanier, C., Morissette, R., Menard-Buteau, C., & Loyer, M. (1994). Suicide and mental disorders: a case-control study of young men. *The American journal of psychiatry, 151*(7), 1063-1068.

Lesch, K. P., Bengel, D., Heils, A., Sabol, S. Z., Greenberg, B. D., Petri, S., Benjamin, J., Muller, C. R., Hamer, D. H., & Murphy, D. L. (1996). Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science, 274*(5292), 1527-1531.

Lord, C., Cook, E. H., Leventhal, B. L., & Amaral, D. G. (2000). Autism spectrum disorders. *Neuron, 28*(2), 355-363.

Manning-Courtney, P., Murray, D., Currans, K., Johnson, H., Bing, N., Kroeger-Geoppinger, K., Sorensen, R., Bass, J., Reinhold, J., Johnson, A., & Messerschmidt, T. (2013). Autism spectrum disorders. *Current problems in pediatric and adolescent health care, 43*(1), 2-11.

Matson, J. L., & Kozlowski, A. M. (2011). The increasing prevalence of autism spectrum disorders. *Research in Autism Spectrum Disorders, 5*, 418-425.

McIlwain, K. L., Merriweather, M. Y., Yuva-Paylor, L. A., & Paylor, R. (2001). The use of behavioral test batteries: effects of training history. *Physiology & behavior, 73*(5), 705-717.

McKinney, W. T., Jr., & Bunney, W. E., Jr. (1969). Animal model of depression. I. Review of evidence: implications for research. *Archives of general psychiatry, 21*(2), 240-248.

Millan, M. J., Agid, Y., Brune, M., Bullmore, E. T., Carter, C. S., Clayton, N. S., Connor, R., Davis, S., Deakin, B., DeRubeis, R. J., Dubois, B., Geyer, M. A., et al. (2012). Cognitive dysfunction in psychiatric disorders: characteristics, causes and the quest for improved therapy. *Nature reviews. Drug discovery, 11*(2), 141-168.

Mineur, Y. S., Sluyter, F., de Wit, S., Oostra, B. A., & Crusio, W. E. (2002). Behavioral and neuroanatomical characterization of the Fmr1 knockout mouse. *Hippocampus, 12*(1), 39-46.

Möbius, W., Patzig, J., Nave, K. A., & Werner, H. B. (2008). Phylogeny of proteolipid proteins: divergence, constraints, and the evolution of novel functions in myelination and neuroprotection. *Neuron glia biology, 4*(2), 111-127.

Moy, S. S., Nadler, J. J., Perez, A., Barbaro, R. P., Johns, J. M., Magnuson, T. R., Piven, J., & Crawley, J. N. (2004). Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. *Genes, brain, and behavior, 3*(5), 287-302.

Murcia, C. L., Gulden, F., & Herrup, K. (2005). A question of balance: a proposal for new mouse models of autism. *International journal of developmental neuroscience, 23*(2-3), 265-275.

Murphy, D. L., Lerner, A., Rudnick, G., & Lesch, K. P. (2004). Serotonin transporter: gene, genetic disorders, and pharmacogenetics. *Molecular interventions, 4*(2), 109-123.

Murphy, D. L., & Lesch, K. P. (2008). Targeting the murine serotonin transporter: insights into human neurobiology. *Nature reviews. Neuroscience, 9*(2), 85-96.

Murray, C. J., & Lopez, A. D. (1997). Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *Lancet, 349*(9064), 1498-1504.

Newschaffer, C. J., Croen, L. A., Daniels, J., Giarelli, E., Grether, J. K., Levy, S. E., Mandell, D. S., Miller, L. A., Pinto-Martin, J., Reaven, J., Reynolds, A. M., Rice, C. E., et al. (2007). The epidemiology of autism spectrum disorders. *Annual review of public health, 28*, 235-258.

Parker, G. (2005). Beyond major depression. *Psychological medicine, 35*(4), 467-474.

Pearson, B. L., Defensor, E. B., Blanchard, D. C., & Blanchard, R. J. (2010). C57BL/6J mice fail to exhibit preference for social novelty in the three-chamber apparatus. *Behavioural brain research, 213*(2), 189-194.

Persico, A. M., & Bourgeron, T. (2006). Searching for ways out of the autism maze: genetic, epigenetic and environmental clues. *Trends in neurosciences, 29*(7), 349-358.

Porsolt, R. D., Bertin, A., & Jalfre, M. (1977). Behavioral despair in mice: a primary screening test for antidepressants. *Archives internationales de pharmacodynamie et de therapie, 229*(2), 327-336.

Provenzano, G., Zunino, G., Genovesi, S., Sgado, P., & Bozzi, Y. (2012). Mutant mouse models of autism spectrum disorders. *Disease markers, 33*(5), 225-239.

Radyushkin, K., Hammerschmidt, K., Boretius, S., Varoqueaux, F., El-Kordi, A., Ronnenberg, A., Winter, D., Frahm, J., Fischer, J., Brose, N., & Ehrenreich, H. (2009). Neuroligin-3-deficient mice: model of a monogenic heritable form of autism with an olfactory deficit. *Genes, brain, and behavior, 8*(4), 416-425.

Rafful, C., Medina-Mora, M. E., Borges, G., Benjet, C., & Orozco, R. (2012). Depression, gender, and the treatment gap in Mexico. *Journal of affective disorders, 138*(1-2), 165-169.

Rice, C. E. (2011). The changing prevalence of the autism spectrum disorders. *American family physician, 83*(5), 515-520.

Risch, N., Spiker, D., Lotspeich, L., Nouri, N., Hinds, D., Hallmayer, J., Kalaydjieva, L., McCague, P., Dimiceli, S., Pitts, T., Nguyen, L., Yang, J., et al. (1999). A genomic screen of autism: evidence for a multilocus etiology. *American journal of human genetics, 65*(2), 493-507.

Robbins, T. W., James, M., Owen, A. M., Sahakian, B. J., Lawrence, A. D., McInnes, L., & Rabbitt, P. M. (1998). A study of performance on tests from the CANTAB battery sensitive to frontal lobe dysfunction in a large sample of normal volunteers: implications for theories of executive functioning and cognitive aging. Cambridge Neuropsychological Test Automated Battery. *Journal of the International Neuropsychological Society, 4*(5), 474-490.

Sanders, S. J., Ercan-Sencicek, A. G., Hus, V., Luo, R., Murtha, M. T., Moreno-De-Luca, D., Chu, S. H., Moreau, M. P., Gupta, A. R., Thomson, S. A., Mason, C. E., Bilguvar, K., et al. (2011). Multiple recurrent de novo CNVs, including duplications of the 7q11.23 Williams syndrome region, are strongly associated with autism. *Neuron, 70*(5), 863-885.

Saugier-Veber, P., Munnich, A., Bonneau, D., Rozet, J. M., Le Merrer, M., Gil, R., & Boespflug-Tanguy, O. (1994). X-linked spastic paraplegia and Pelizaeus-Merzbacher disease are allelic disorders at the proteolipid protein locus. *Nature genetics, 6*(3), 257-262.

Sharpley, C. F., Palanisamy, S. K., Glyde, N. S., Dillingham, P. W., & Agnew, L. L. (2014). An update on the interaction between the serotonin transporter promoter variant (5-HTTLPR), stress and depression, plus an exploration of non-confirming findings. *Behavioural brain research, 273c*, 89-105.

Sullivan, P. F., Daly, M. J., & O'Donovan, M. (2012). Genetic architectures of psychiatric disorders: the emerging picture and its implications. *Nature reviews. Genetics, 13*(8), 537-551.

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P., et al. (2001). The sequence of the human genome. *Science, 291*(5507), 1304-1351.

Voineagu, I., & Yoo, H. J. (2013). Current progress and challenges in the search for autism biomarkers. *Disease markers, 35*(1), 55-65.

Waterston, R. H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J. F., Agarwal, P., Agarwala, R., Ainscough, R., Alexandersson, M., An, P., Antonarakis, S. E., Attwood, J., et al. (2002). Initial sequencing and comparative analysis of the mouse genome. *Nature, 420*(6915), 520-562.

Werner, H., Dimou, L., Klugmann, M., Pfeiffer, S., & Nave, K. A. (2001). Multiple splice isoforms of proteolipid M6B in neurons and oligodendrocytes. *Molecular and cellular neurosciences, 18*(6), 593-605.

Willner, P., Towell, A., Sampson, D., Sophokleous, S., & Muscat, R. (1987). Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. *Psychopharmacology, 93*(3), 358-364.

Wöhr, M., & Scattoni, M. L. (2013). Behavioural methods used in rodent models of autism spectrum disorders: current standards and new developments. *Behavioural brain research, 251*, 5-17.

Wong, M. L., & Licinio, J. (2004). From monoamines to genomic targets: a paradigm shift for drug discovery in depression. *Nature reviews. Drug discovery, 3*(2), 136-151.

World Health Organization. (2008). *The global burden of disease: 2004 update*. Geneva: World Health Organisation Press.

Yan, Y., Lagenaur, C., & Narayanan, V. (1993). Molecular cloning of M6: identification of a PLP/DM20 gene family. *Neuron, 11*(3), 423-431.

Yates, J. R. (2006). Tuberous sclerosis. *European journal of human genetics, 14*(10), 1065-1073.

8. Appendix

8.1 Co-author publications

Tantra T, Kröcher T, Papiol S, Winkler D, Röckle I, Jatho J, Burkhardt H, Ronnenberg A, Gerardy-Schahn R, Ehrenreich H, Hildebrandt H: St8sia2 deficiency plus juvenile cannabis exposure in mice synergistically affect higher cognition in adulthood. Submitted to Behavioral Brain Research.

Rao Netrakanti P, Dere E, Poggi G, Winkler D, Cooper B, Brose N, Ehrenreich H: Comprehensive behavioral and cognitive characterization of male and female Munc13-3 deficient mice reveals only a mild cerebellar phenotype. Manuscript in preparation.

8.2 Curriculum vitae

Personal Data	
Name	Daniela Winkler
Date of birth	25.06.1983
Place of birth	Kassel, Germany
Nationality	German
E-mail	dwinkler@em.mpg.de
Education	
since June 2010	Doctoral candidate in the PhD program “Systems Neuroscience“ at the Göttingen Graduate School for Neurosciences, Biophysics and Molecular Biosciences (GGNB), Georg August University of Göttingen, Germany
	Doctoral candidate, Clinical Neuroscience, Max Planck Institute of Experimental Medicine, Göttingen, Germany Thesis: “Mouse models of neuropsychiatric phenotypes” Supervisor: Prof. Dr. Dr. Hannelore Ehrenreich, Max Planck Institute of Experimental Medicine, Göttingen, Germany
2002-2010	Diploma in Biology Main focus on genetics, microbiology and cell biology Thesis: “Analysis of individual molecular genetic tumor markers in astrocytotic and oligodendroglial primary brain tumors” Supervisor: Prof. Dr. Dr. Gerhard-Franz Walter, Institut für Pathologie, Klinikum Kassel
Areas of Expertise	Behavioral characterization of genetic mouse models targeting genes relevant to cognitive functions and neuropsychiatric disorders (especially ASD). Development of more sophisticated testing tools for higher cognitive functions and complex behavioral traits in mice.

Research Experience

2010 -Present	Doctoral student, Clinical Neuroscience, Max Planck Institute of Experimental Medicine, Göttingen, Germany
08/2008 - 10/2008	Internship: Klinikum Kassel, Zentrallabor (Bakteriologie) Preparation and testing of patient samples, bacterial culture, microscopy, PCR
12/2007 - 01/2008	Internship: Klinikum Kassel, Institut for Pathology Preparation and testing of patient tissue samples, histology, microscopy, PCR, FISH, immunhistochemistry

Teaching Experience

Lecturer in yearly interdisciplinary workshops:

2011 - Present	Translational Neuroscience Block 'Schizophrenia' Lecture on 'Testing schizophrenia-related functions in mice' and accompanying practical session, Max Planck Institute of Experimental Medicine, Göttingen, Germany
2010 - 2014	Translational Neuroscience Block 'Multiple Sclerosis' Lecture on 'Testing MS-relevant functions in mice' and accompanying practical session, Max Planck Institute of Experimental Medicine, Göttingen, Germany

8.3 List of publications

El-Kordi A[1], Winkler D[1], Hammerschmidt K, Kästner A, Krueger D, Ronnenberg A, Ritter C, Jatho J, Radyushkin K, Bourgeron T, Fischer J, Brose N, Ehrenreich H: Development of an autism severity score for mice using Nlgn4 null mutants as a construct-valid model of heritable monogenic autism. Behavioral Brain Research 2013; 251(8):41-9.

[1]Equal contribution

Dere E[1], Winkler D[1], Ritter C, Ronnenberg A, Poggi G, Patzig J, Gernert M, Müller C, Nave KA, Ehrenreich H, Werner HB: Gpm6b deficiency impairs sensorimotor gating and modulates the behavioral response to a 5-HT2A/C receptor agonist. Behavioral Brain Research 2014; (in press).

[1]Equal contribution

Tantra T, Kröcher T, Papiol S, Winkler D, Röckle I, Jatho J, Burkhardt H, Ronnenberg A, Gerardy-Schahn R, Ehrenreich H, Hildebrandt H: St8sia2 deficiency plus juvenile cannabis exposure in mice synergistically affect higher cognition in adulthood. Submitted to Behavioral Brain Research.

Rao Netrakanti P, Dere E, Poggi G, Winkler D, Cooper B, Brose N, Ehrenreich H: Comprehensive behavioral and cognitive characterization of male and female Munc13-3 deficient mice reveals only a mild cerebellar phenotype. Manuscript in preparation.