# University of Veterinary Medicine Hannover

## Comprehensive behavioral characterization of Gpm6b null mutants as a mouse model of depression

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*To my parents*

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# 1 Introduction

Depression, also known as major depressive disorder, major depression, unipolar depression or clinical depression, is one of the most serious disorders in today's society. With a lifetime prevalence of approximately 20% in the United States (Kessler et al., 2005) and by affecting not only the individuals but also their families and surrounding social environments, this illness cannot be overestimated.

Depression is currently among the three leading contributors to the global disease burden (Collins et al., 2011) and is associated with high levels of morbidity and mortality (Evans et al., 2005). As the most prominent risk factor for suicide, and therefore a leading cause of death worldwide (Saveanu and Nemeroff, 2012), depression results each year in the loss of about 850 000 lives worldwide (Chung et al., 2011). Furthermore, affected patients are at higher risk of developing physical health problems, namely diabetes (Knol et al., 2006) or coronary artery disease (Baune et al., 2012) and additionally the prognosis of other medical conditions is aggravated when a comorbidity with depression is present (Evans et al., 2005).

A major problem in the diagnosis and treatment of depression is that the mechanisms of the disease are not yet fully understood and therefore the possibilities of curing affected patients are still unsatisfactory (Pollak et al., 2010). The range of available therapeutic options for affected persons is narrow and standard pharmacological treatment plans are only successful in 30% - 40% (Trivedi, 2006) and often result in serious side effects (Gibiino and Serretti, 2012).

Despite the alarming numbers in terms of prevalence and possible negative consequences, research is still a long way from understanding the underlying mechanisms that may lead to this heterogeneous disease. Further research is required to investigate the interaction between different pathological processes as well as provide new etiological hypotheses.

The aim of this thesis is to contribute to the growing knowledge of depression by providing a comprehensive characterization of a mouse model that may be utilized as an animal model of depression. It is essential to continue research for the comprehension of this complex disorder in order to develop new approaches that may help to overcome it and it is undeniable that

animal models must have a crucial contribution in present and future research. Therefore, an entirely new mouse strain is brought into focus that will participate to better understand depression and its contributing factors. New insights obtained by this means could possibly lead to the development of novel targets for pharmacological treatment.

# 2 Literature

## 2.1 Depression in humans

### 2.1.1 Etiology

Looking at the etiology of depression, meta-analyses revealed that approximately 30% - 40% of disease developing risk is heritable (Sullivan et al., 2000), with women suffering twice as often as men (Weissman et al., 1996). Several specific genes are thought to be associated with depression. Probably the most extensively studied gene is the promoter region of the serotonin transporter gene (5-HTTLPR), although many studies demonstrated the involvement of various other genes in humans (for review see Saveanu and Nemeroff, 2012).

Despite genetic factors, environmental challenges play a crucial role in the risk of developing a depression. A number of studies have shown that onset of depression is undoubtedly influenced by stressful life events during childhood, such as sexual or physical abuse as well as neglect (e.g. Zlotnick et al., 1995, Mullen et al., 1996). Caspi et al. (2003) could even reveal a significant association between 5-HTTLPR, stress and depression. Depending on the genotype individuals were more or less vulnerable to the depressogenic effects of stressful life events.

### 2.1.2 Symptoms

One of the major problems that occurs when dealing with depression is the lack of objective diagnostic tests. Consequently, diagnoses are based on subjective judgement of physicians or investigators with the focus on symptoms, signs and course of illness.

In order to provide a coherent standard for the classification of this disease, the criteria for major depressive episode - contained in the Diagnostic and Statistical Manual of Mental Disorders, 4$^{th}$ edition, text revision (DSM-IV-TR) - are used (American Psychiatric Association., 2000). In this manual typical symptoms of depression are conceived (Table 1).

**DSM criteria for major depressive episode**

A. At least five of the following symptoms are present simultaneously for at least 2 weeks (symptom 1 or 2 is necessary):

1. Depressed or irritable mood
2. Markedly diminished interest or pleasure in all, or almost all, daily activities
3. Substantial weight loss or weight gain
4. Insomnia or hypersomnia nearly every day
5. Psychomotor agitation or retardation nearly every day
6. Fatigue or loss of energy nearly every day
7. Feelings of worthlessness or inappropriate guilt nearly every day
8. Diminished ability to think or concentrate nearly every day
9. Recurrent thoughts of death or suicide

B. It cannot be established that an organic factor is the cause and the disturbance is not a normal reaction to the death of a loved one (abstracted from DSM-IV-TR (American Psychiatric Association., 2000))

**Table 1:** DSM-IV-TR criteria for major depressive disorder
- mod. from Nestler et al. (2010)

### 2.1.3 Pathophysiology

Although the pathophysiology of depression is poorly understood, there are several mechanisms thought to be associated with the pathogenesis of this disorder.

Different non-physiological observations have been reported in depressed patients, but often it cannot be clearly determined if the changes are indeed causing the outbreak, if they are one of many contributing factors or if they are secondary findings. The observations reported with regard to depression include a reduced activity in neurotransmission, a dysregulation of the hypothalamo-pituitary-adrenal (HPA) axis, a reduction in brain neurotrophins (for review see

Palazidou, 2012) as well as elevated levels of pro-inflammatory cytokines (e.g. Pollak and Yirmiya, 2002, Lotrich et al., 2007). These observed changes are often accompanied with structural abnormalities within certain brain areas, such as a reduction in the volume of the hippocampus or a decreased neuronal density in the prefrontal cortex (for review see Femenia et al., 2012).

The complexity of depression suggests that alterations of several different systems play a role in its pathogenesis.

#### 2.1.3.1 The role of the immune system

One of the hypotheses that have been proposed is the "inflammatory"- or "cytokine hypothesis". The activation of the immune system is thought to provoke a psycho-neuroimmunological dysfunction (Zunszain et al., 2012) that leads to depressive symptoms.

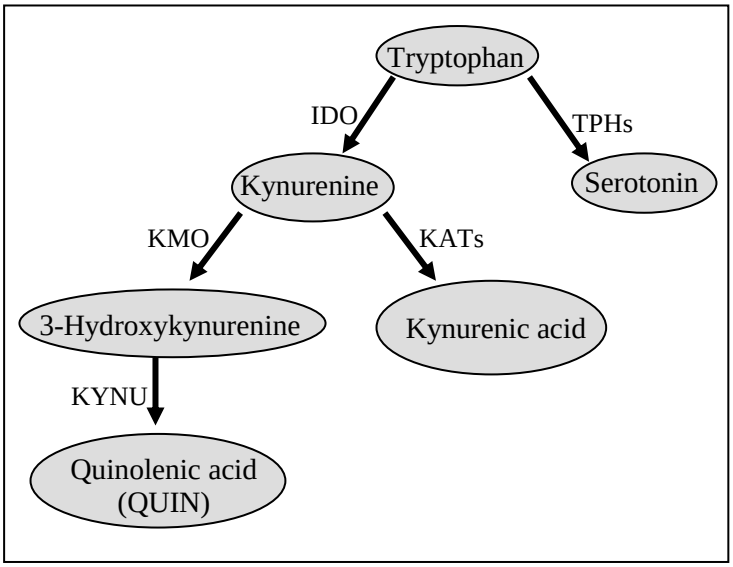
Increased levels of pro-inflammatory cytokines, chemokines, acute phase proteins and cellular adhesion molecules could be found in depressed patients (Raison et al., 2006). Furthermore, it could be observed that patients undergoing an infection, chronic disease or immunotherapy frequently develop symptoms of depression independently of the sickness that is present (Dantzer et al., 2008).

In a study from Capuron and Miller (2004), the initiation of interferon-alpha (IFN-α) immunotherapy was followed by symptoms of influenza-like sickness and later 30% - 50% of the patients suffered from major depression and more than 80% of the patients revealed depressive symptomology, providing a remarkable evidence that pro-inflammatory cytokines are linked to the subsequent depressive disorder. Similar studies have been published by other authors (e.g. Pollak and Yirmiya, 2002, Lotrich et al., 2007). Further support for the role of cytokines in developing depression is provided by the finding from Musselmann et al. (2001), who observed that IFN-α-induced depression in patients undergoing immunotherapy is responsive to treatment with standard antidepressants.

One proposed pathway that may lead to depression is the ability of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), to directly activate the enzyme indoleamine 2,3 – dioxygenase (IDO) (Dantzer et al., 2008). The extrahepatic enzyme IDO can be found in immune cells, including macrophages and dendritic

cells, where it degrades the essential amino acid tryptophan into kynurenine (Wirleitner et al., 2003). Under physiological conditions tryptophan degradation to kynurenine is negligible, but the enzyme is highly inducible by pro-inflammatory cytokines and consequently causes a reduced bioavailability of tryptophan (Dantzer et al., 2008). Kynurenine is a precursor of the bioactive metabolite quinolinic acid (QUIN), which is an N-methyl-D-aspartate (NMDA) receptor agonist, potentially neurotoxic and therefore assumed to contribute to depression (Muller and Schwarz, 2007). But simultaneously kynurenine is also a precursor of kynurenic acid (KYNA) that is generally considered to be neuroprotective because of its characteristic as an NMDA receptor antagonist (Moroni, 1999).

It has been proposed that reduced peripheral availability of tryptophan (the precursor of serotonin) leads to a decreased synthesis of serotonin in the brain and that this constriction as well as imbalances between QUIN and KYNA may result in depression and neurodegeneration (Capuron and Miller, 2004, Dantzer et al., 2008, O'Connor et al., 2009).




IDO indoleamine 2,3–dioxygenase

KAT kynurenine aminotransferase

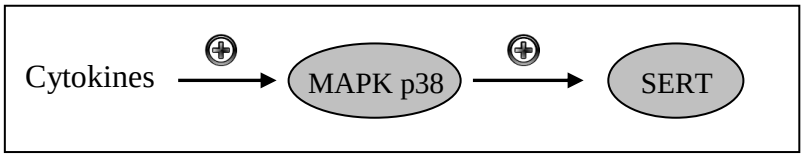
KMO kynurenine 3–monooxygenase

KYNU kynureninase

TPH tryptophan hydroxylase

**Figure 1:** Simplified kynurenine pathway (mod. from Zunszain et al. (2012))

Another way cytokines contribute to the development of depression is their ability to activate the serotonin transporter and cause a reduction of available extracellular serotonin. It has been shown that cytokines such as interleukin-1-beta (IL-1ß) and tumor necrosis factor-alpha (TNF-α) activate the mitogen activated proteinkinase p38 (MAPK p38) increasing serotonin transport activity of the serotonin transporter (Zhu et al., 2006).




MAPK p38 mitogen activated proteinkinase p38

SERT serotonin transporter

**Figure 2:** Simplified effect of cytokines on the serotonin transporter (mod. from text passages - Zhu et al. (2006))

### 2.1.3.2 The role of the HPA axis and stress

The disturbance of the hypothalamic pituitary adrenal (HPA) axis is a main characteristic feature of depression (Palazidou, 2012) and could be demonstrated by Holsboer et al. (1982). In the study, depressed patients had elevated levels of plasma cortisol which could not be suppressed by administration of dexamethasone. Various research has been found to link the HPA axis to the development of depression (for review see Zunszain et al., 2012), but it is still unknown why depressed patients often show these disturbances of the HPA axis (Carvalho and Pariante, 2008).

It is known that cytokines alter the negative feedback mechanism enhancing the release of the corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus (Pace et al., 2007), leading to elevated plasma levels of cortisol and increasing levels of the adrenocorticotropic hormone (ACTH).

Stress is an influencing factor on the HPA axis and thought to be a predominant course in depression development (e.g. Zlotnick et al., 1995, Mullen et al., 1996).

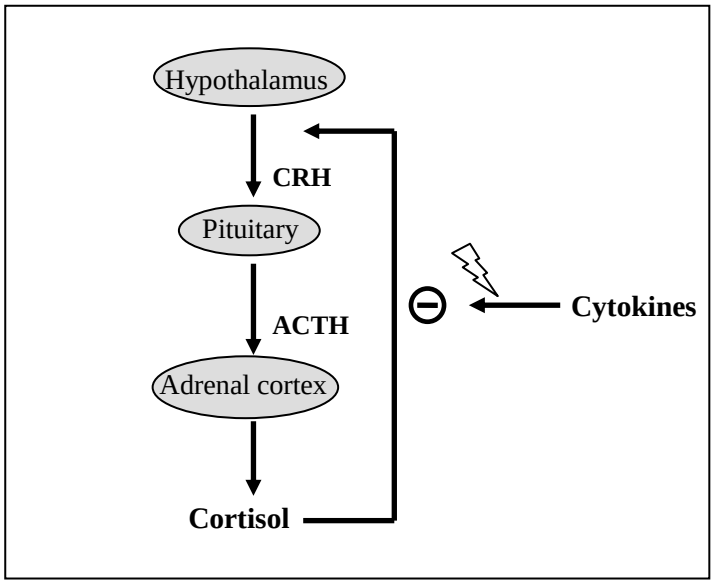



**Figure 3**: Simplified effect of cytokines on the HPA axis
(mod. from text passages - Pace et al. (2007))

### 2.1.3.3 The role of aging

The aging progress is accompanied by various changes in the body: The organism is shifted into a pro-inflammatory state that is characterized by an increased activity of the innate immune system, a disproportional central nervous system (CNS) response when challenged by immune stimuli and a disruption of the periphery-CNS immune communication (for review see Alexopoulos and Morimoto, 2011). An elevated amount of cytokines, especially interleukin-6 (IL-6) (Bremmer et al., 2008) and interleukin-1-beta (IL-1ß) (Thomas et al., 2005), has been found to be linked to depressive symptoms in elderly. These processes lead to elevated numbers of activated microglia cells and an enhanced production of pro-inflammatory cytokines in the brain combined with decreased levels of anti-inflammatory cytokines (Sparkman and Johnson, 2008). The activation of microglia then leads to disturbances in the cell's metabolic mechanisms and results in neuron loss, insufficient clearance of neurotoxic molecules and reduction of neurogenesis (Carpentier and Palmer, 2009).

Based on these observations, Alexopoulos and Morimoto (2011) suggest that aging-related processes may contribute to the etiology of depressive syndromes in older adults. The authors assume that the pro-inflammatory state is likely to induce changes in the emotional and cognitive neural system that predisposes the individuals to geriatric depression.

### 2.1.3.4 Multiple neurotransmitters involved in depression

Although research has shown that various neurotransmitter systems are pathologically involved in the development of depression, no single neurotransmitter system seems to be solely responsible. Much research demonstrates that alterations in the metabolism of various neurotransmitters such as norepinephrine, dopamine and glutamate are linked to the pathophysiology of depression (for review see Saveanu and Nemeroff, 2012).

With this knowledge, this thesis will focus on the serotonergic system, because studying all possible changes would go beyond the scope of the thesis.

### 2.1.3.5 Serotonin alterations in depression

Serotonin (= 5-hydroxytryptamine (5-HT)) is known to be a neurotransmitter of the central and peripheral nervous system and modulates many different physiological processes such as sleep, mood and appetite (e.g. Hipolide et al., 2005, Serretti et al., 2006). In the brain, serotonin is released into the synaptic cleft and is then actively transported back into the presynaptic neurons by the serotonin transporter (SERT) (Fjorback et al., 2009).

Several alterations of the serotonergic system have been postulated to be associated with depression: Evidence of the involvement of the serotonin circuit includes a reduced activity of serotonergic neurons in depressed patients who have made a suicide attempt compared to patients who have not made a suicide attempt (Mann et al., 1996). Further support comes from post-mortem and positron emission tomography (PET) imaging studies that showed a significant reduction in the number of serotonin transporter binding sites in certain brain areas of depressed patients (Drevets et al., 1999). Furthermore, humans that possess the ”s” allele of the promoter region of the serotonin transporter gene, and hence a lower transcriptional efficiency, are more vulnerable to depressogenic effects caused by stressful life events than individuals with the ”l” allele (Caspi et al., 2003).

### 2.1.4 Serotonin receptors and the serotonin agonist DOI

Serotonin targets seven different classes of serotonin receptors (5-$HT_1$ – 5-$HT_7$) that are divided into 14 subfamilies (Nichols + Nichols 2008). The 5-$HT_2$ receptor class contains three subtypes that are G-protein-coupled and classified as 5-$HT_{2A}$, 5-$HT_{2B}$ and 5-$HT_{2C}$ (Halberstadt et al., 2009).

In order to evoke serotonin mimetic effects in the mice described in this thesis, and to investigate potential alterations in the reaction to this substance due to disturbances of the serotonergic system, the serotonin agonist DOI ((±)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane) was used. DOI is relatively selective for activating all $HT_2$ receptor subtypes and has a high affinity for these receptors (Canal and Morgan, 2012).

### 2.1.5 Serotonin transporter

The serotonin promoter region of the serotonin transporter gene (SLC6A4), in humans located on 17q11.2, is modified by sequence elements within the proximal 5´ regulatory region, termed the serotonin transporter gene linked polymorphic region (5-HTTLPR) (Caspi et al., 2003).

The serotonin transporter is exclusively located on the presynaptic cell membrane of serotonergic neurons in various brain areas, such as in the raphe complex, neocortical regions, amygdala, CA3 region of the hippocampus and hypothalamus (Benninghoff et al., 2012).

Although the exact functions of the transporter still need to be investigated, the current state of scientific research assumes that the transporter contains 12 hydrophobic transmembrane domains and binds sodium, chloride and serotonin simultaneously. By performing this procedure the serotonin transporter is a major regulator of the serotonin availability in the synaptic cleft and therefore highly influences the downstream signalling via the pre-and postsynaptic receptors (Fox et al., 2007).

Inhibiting the uptake of serotonin leads to a relative increase of serotonin in the synaptic cleft and enhances the serotonin neurotransmission which might be diminished in a depressed patient (Auerbach and Hjorth, 1995).

### 2.1.6 Glycoprotein M6B

In this thesis mice lacking the glycoprotein M6B (Gpm6b) are described.

M6B belongs to a proteolipid protein family and is expressed in neurons as well as in oligodendrocytes in most brain regions (Werner et al., 2001). Besides that, basal levels of M6B mRNA are also detectable in many non-neuronal tissues, such as testis, lung, heart and spleen (Werner et al., 2001, Isensee et al., 2008).

In humans, the gene for M6B is located at chromosome Xp22.2 (Sebastiani et al., 2008) and an increased expression in females could be demonstrated (Isensee et al., 2008). Interestingly, M6B has been shown to be downregulated in suicide completers which leads to the assumption that elevated levels of this gene may be protective against suicide (Fiori et al., 2011).

M6B is a four-transmembrane protein that interacts with the N-terminal domain of the serotonin transporter in the brain. It is involved in cellular housekeeping functions and mediates a decrease in serotonin transporter surface expression, even though it is still uncertain whether M6B facilitates endocytoses or inhibits exocytosis of the serotonin transporter (Fjorback et al., 2009).

Fjorback et al. (2009) demonstrated that in HEK-MSR-293 cells transfected with the serotonin transporter and either M6B or pcDNA3 (control) the membrane-bound level of the serotonin transporter was reduced about 50% when co-expressed with M6B compared to the cells co-transfected with pcDNA3. The same study demonstrated that co-expression of the serotonin transporter with M6B in HEK-MSR-293 cells also mediated a dose-dependent reduction in the serotonin uptake when compared to cells transfected with pcDNA3.

According to Werner et al. (2001), M6B is additionally involved in myelination and it is upregulated in the brain during terminal neuronal differentiation and myelination.

The glycoprotein M6A, which is expressed only in neurons and has a 55% sequence identity with M6B (Fjorback et al., 2009), is known to interact with the μ-opioid receptor and was demonstrated to facilitate the endocytosis and recycling of this receptor (Wu et al., 2007) which supports the hypothesis that M6B is also involved in alterations of transporter expression (Fjorback et al., 2009).

## 2.2 Depression in animals

### 2.2.1 Animal models of depression

#### 2.2.1.1 Requirements

In order to investigate characteristics and treatment of various diseases, use of animal models are inevitable whenever the study on humans is unethical or impractical.

Animal models are evaluated against different criteria that they should fulfil ideally. Face validity, construct validity and pharmacological validity are key requirements a model should meet to serve as a model for neuropsychiatric syndromes.

The "face validity" of a model of depression describes the symptomatic homology to symptoms as they are found in depressed humans. Many models are able to meet this requirement although the described phenotypes are generally transient and do not occur simultaneously (Krishnan and Nestler, 2010). According to Nestler and Hyman (2010), it cannot be expected that animal models mirror the full extent of a human disorder like depression as it is defined in DSM-IV, as the diagnosis in human patients is often built on judgement of investigators and reviewers and therefore often arbitrary and hazy. Furthermore, the authors remark that specific symptoms observed in animals may not necessarily have a simple, straightforward correspondence to symptoms found in depressed humans. Some symptoms of humans like suicidal thoughts or feelings of guilt are simply not possible to investigate in animals.

"Construct validity" is a model's ability to replicate the etiological background of certain phenotypes by using paradigms of genetic and/or environmental manipulations (Krishnan and Nestler, 2010). Meeting these criteria constitutes a great challenge, as the factors that are implicated in human depression are themselves not entirely understood (Krishnan and Nestler, 2010). For example, different human patients likely have different genetic pathways that contribute to their depressed symptoms (Nestler and Hyman, 2010).

The term "pharmacological" or "predictive validity" refers to the ability of currently available antidepressant modalities to reverse the depressive-like phenotype of the model (Krishnan and Nestler, 2010). Many models already meet this criteria, but considering that generally

available pharmacological drugs are monoamine modulators and only a minority of human patients gain remission after first-line therapies with that type of drug (Li et al., 2012a), it should not be mandatory for a model to fulfil the requirement for pharmacological validity in order to be considered an applicable animal model.

#### 2.2.1.2 Examples for animal models of depression

Various studies could describe depressive-like symptoms in different animal species, suggesting that this disease is particularly suitable to be modelled in animals. Many of the following examples describe depressive-like behavior caused by separation of the animal from the offspring, mother or partner. These observations in animals provide valuable evidence that various species are able to exhibit symptoms that resemble depression in humans.

Monkeys especially aroused researcher's attention in the early studies of depression on animal models. It could be observed that infant rhesus monkeys displayed features of depression after separation from their mother (Harlow and Suomi, 1974). The behavioral changes included a reduction in play, appetite and social interaction and an increase of crying and sleep disturbances. The changes lasted until reunion with the mother. Similar findings were published previously Jensen and Tolman (1962). Other species of monkeys revealed comparable symptoms when separated from the mother or partner (for review see McKinney and Bunney, 1969).

But not only monkeys (a species that is closer related to humans than any other animal) exhibited depressive-like symptoms. In other studies authors describe dog puppies that demonstrated typical symptoms of depression after separation from the investigator (Senay et al., 1966) or a bitch that was "classically depressed" after the loss of her litter (Saul et al., 1962).

Well socialized dogs have been shown to initially develop both agitation as well as acute distress and subsequently retardation, weight loss and finally death when they were isolated for an extended duration (Scott et al., 1973).

A depressive-like phenotype was also described in birds. Species that were characterized include geese, jackdaw birds or the African parrot (for review see McKinney and Bunney, 1969).

More recent studies focus not only on the symptoms of depression in different species but also ask the question how a particular phenotype might be used to serve as a model for human depression. Fureix et al. (2012) investigated the influence of chronic stress on domestic horses and Hymel and Sufka (2012) demonstrated that an isolation stressor provoked depressive-like behavior in chicks.

#### 2.2.1.3 Mouse models of depression

Several characteristics of mice are especially suitable for modelling illnesses that occur in humans: Mice are relatively easy to keep under laboratory conditions, and as mammals they share more similarities with humans than a non-mammal laboratory animal could offer. The majority of the genes in mice have a homolog in the human genome (Pollak et al., 2010). The mouse genome is accessible by gene-targeting and transgenic-techniques and the produced phenotype can be tested in behavioral tests that are standardized, commonly used and accepted (Pollak et al., 2010). Simultaneously, mice have a relatively short generation time which makes it possible to test high numbers and breed mice individualized to particular needs. The high degree of genetic homogeneity in addition to possible variation in genes of interest facilitates the readout of conducted experiments.

On a basis of growing knowledge about the pathogenesis of depression and its treatment, different approaches have been taken to create a rodent model of depression. Although it is not possible to model all the symptoms that can comprise a depressed episode in humans, different attempts show some success. In order to picture particular features of human behavior and taking account of the biological mechanisms, research has addressed the genetic and environmental underlying background of depression in humans and tried to utilize the knowledge to induce depression in rodents.

Depressive-like phenotypes of rodents can be induced by pharmacological or non-pharmacological treatment as well as by genetic manipulations and some authors successfully created a strain which shows a gene and environment interaction (Table 2).

- Models of genetically modified mice

The modulation of a particular protein has been remarkably useful in depression research. Several manipulations across various neurobiological systems that result in depressive-like phenotypes are established.

- Models of pharmacologically induced depression

Various drugs are able to provoke depressive-like behavior in rodents. The most commonly used assay to induce a depressive-like phenotype is the injection of pro-inflammatory drugs, namely lipopolysaccharide (LPS) or cytokines.

LPS that induces the release of pro-inflammatory cytokines, as well as the immediate administration of cytokines, have been shown to interact with the immune system and therefore lead to a depressive-like phenotype.

- Models of non-pharmacological induced depression

Some attempts to create a model without the use of pharmacological substances show some success:

  - Olfactory bulbectomy

    This model is mostly used in rats and comprises the surgical removal of the olfactory bulbs with the consequence of increased open field activity, avoidance-learning deficits and alterations in the endocrine, immune and neurotransmitter system that correlate with many of the changes seen in depressed patients (Pollak et al., 2010).

  - Stress-induced depression

    Stress, as mentioned earlier, is able to cause depression in humans. Several paradigms have been established for rodents using the pathway to create an animal model of depression. The paradigm's main strength is the clinical evidence that stressful life events can trigger depressive episodes in humans (Krishnan and Nestler, 2010).

- Maternal deprivation

The separation of the mother from her pups within a period critical for physical and mental development has consequences for both individuals: The repeated deprivation of maternal care to the offspring affects the stress and depressive-like behavior later in life, whereas the mother displays inappropriate attention towards the pups and an increase in the level of anxiety and depressive-like behavior (Finamore and Port, 2000, Eklund et al., 2009).

- Learned helplessness

After animals experience an uncontrollable and inescapable stress, for example exposure to inescapable electric shocks, they display an increased escape latency or complete failure to escape the re-exposure to the same event, even when provided with an easy escape route. The state of the animals is referred to as a state of "helplessness" (Seligman et al., 1975).

The paradigm of learned helplessness has been established for research purposes because behavioral correlates of helplessness are seen frequently in depressed humans and due to its exclusive treatment response to antidepressant drugs (Pollak et al., 2010).

However, a very variable amount of animals (10-80%) develop measurable behavioral symptoms of helplessness with great variation of different strains (Krishnan and Nestler, 2011).

### 2.2.1.4 Behavioral tests for depression in rodents

Several tests are thought to be particularly suitable to assess depression-related behavior in rodents, because they contain observations of depression as they are typically found in humans. In this section the most commonly used tests are mentioned. A more detailed description of these tests as well as additional tests that were performed to obtain a more extensive characterization of Gpm6b null mutants can be found in the "Material and Methods" section of this thesis. Despite the settings that are specifically designed to evaluate depressive-like behavior, there are several other tests that are eligible to assess certain features of depression. Although these tests are not exclusively used for depression-related behavior, they are employed for a wide range of behavioral characterizations. The tests include assays for the measurement of altered levels of anxiety, locomotor activity or the assessment of the diurnal pattern, weight and feeding behavior.

The first-generation of behavioral tests for depression were originally designed to detect responses to pharmacological antidepressants. Today, particularly the forced swim test and the tail suspension test are still most widely used because they are not expensive, easy to use and have a potentially high throughput (Pollak et al., 2010).

- Forced Swim Test (FST)

This assay was initially developed by Porsolt et al. (1977) to assess the effect of antidepressants in rodents, but subsequently became the most popular behavioral test to assess depressive-like behavior. The behavior is measured as immobility time which is explained as behavioral despair (Pollak et al., 2010).

- Tail Suspension Test (TST)

Similar to the forced swim test, behavioral despair is assessed as the time spent immobile.

Comparable to the forced swim test, a wide range of pharmacological antidepressants have been shown to reduce the time the mice spent immobile (Pollak et al., 2010).

- Measurements of anhedonia

Anhedonia is one of the core symptoms of depression and is defined as “markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day” (American Psychiatric Association., 2000).

Anhedonic behavior can be reliably reproduced and measured in rodents with two different tests:

- Sucrose preference

  A reduced interest in sucrose solution is assumed to be an indicator for a reduction in the rewarding effectiveness (Jayatissa et al., 2006) in analogy to anhedonic behavior that is described in humans.

- Intracranial self-stimulation (ICSS)

  This paradigm can be reliably applied to mice and rats. The rodents have electrodes implanted into the brain and can self-administer rewarding electrical stimulation. The electrodes are implanted in brain regions that are associated with the rewarding process, such as the medial forebrain bundle at the level of the lateral hypothalamus (Slattery et al., 2007). ICSS is used to evaluate depression-related behavior in various settings and to investigate the properties of the endogenous reward system.

### 2.2.1.5 Table: Rodent models of depression

## INDUCED DEPRESSION

## (pharmacological)

| Model | Author | Species | Relevant behavioral readouts | Remarks |
|---|---|---|---|---|
| Pro-inflammatory agents<br>(LPS, cytokines) | Dunn and Swiergiel (2005)<br>Frenois et al. (2007)<br>Swiergiel and Dunn (2007)<br>Zhu et al. (2010)<br>de Paiva et al. (2010) | Mouse | OFT: reduced activity<br>FST, TST: increased immobility<br>EPM: increased anxiety<br>feeding behavior: reduced food-intake | Performances of different tests highly dependent on dose and testing interval after administration. |
| Isotretinion treatment | O'Reilly et al. (2006) | Mouse | TST, FST: increased immobility<br>OFT: no differences | =13-Cis-retinoic acid, effective treatment for nodular acne |
| Drug-withdrawal-induced | | | | |
| Amphetamine | Cryan et al. (2003) | Rat | ICSS: increased brain reward thresholds<br>FST: increased immobility | |
| | | Mouse | TST: decresed immobility | |
| Morphine | Anraku et al. (2001) | Rat | FST: Increased immobility | |

# INDUCED DEPRESSION
## (non-pharmacological)

| Model | Author | Species | Relevant behavioral readouts | Remarks |
|---|---|---|---|---|
| Olfactory bulbectomy | Review: Song and Leonard (2005) | Rat | Increased exploratory behavior, open field activity, open arms entries (EPM), nocturnal hyperactivity<br>Impaired Morris water maze behavior, food-motivated behavior, sexual activity | Mechanism of action poorly understood, further studies needed, ethical restrictions? |
| Chronic mild stress (CMS) | Review: Willner (2005)<br>Harkin et al. (2002) | Rat<br>Mouse,<br>CD-1 background | Reduced sucrose/saccharin preference, decrease in other hedonic behaviors, potentiation of learned helplessness, decreased male sexual behavior, decreased male aggression, decreased grooming, decreased REM sleep latency, increased immobility in the FST<br>Decreased saccharin preference | |
| Prenatal stress | Miyagawa et al. (2011) | Mouse<br>ICR background | EPM: Increased anxiety-like behavior | |
| Maternal deprivation | Schmidt et al. (2002)<br>Matthews and Robbins (2003) | Mouse<br>Rat | Disturbances of HPA axis<br>Reduced ICSS | |
| Learned helplessness | Review: Yan et al. (2010) | Mouse and rat | Altered REM sleep<br>Reduced body weight, diminished sexual behavior, elevated levels of CRF and corticosterone | |

# GENETIC MODELS

| Model | Author | Species | Relevant behavioral readouts | Remarks |
|---|---|---|---|---|
| **Serotonin** | | | | |
| Serotonin transporter knockout<br>(SERT-KO) | Lira et al. (2003) | Mouse<br>129S6/SvEv line<br>males and females | OFT, EPM: no effect of genotype<br>TST: decreased immobility<br>FST: increased immobility<br>Novelty suppressed feeding paradigm: increased anxiety | |
| | Holmes et al. (2002) | Mouse<br>129S6 background<br>males and females | TST: decreased immobility<br>FST: increased immobility | Reduced neuromuscular strengths may affect performance<br>Analysis does not distinguish between males and females |
| | Perona et al. (2008) | Mouse<br>C57BL/6J-129Sv mixed background | Locomotor activity: no effect of genotype<br>TST: decreased immobility<br>FST: no effect of genotype<br>sucrose preference: no effect of genotype | Mice showed a trend of decreased sucrose consumption (not significant) |
| | Kalueff et al. (2007)<br><br>Zhao et al. (2006)<br><br>Wellman et al. (2007) | Mouse<br>C57BL/6 background | OFT: decreased activity<br>EPM: decreased activity, increased anxiety-like behavior<br><br>OFT: decreased activity, decreased center time<br>TST: increased immobility<br>Marble burying: decreased marble burying<br><br>FST: increased immobility<br>Fear conditioning, fear extinction: no effect of genotype<br>Impaired extinction recall | <br><br>Zhao: trend for increased startle response<br><br>Wellmann: n-numbers quite small<br>FST: repeated exposure necessary |

| | | | | |
|---|---|---|---|---|
| | Holmes et al. (2003) | Mouse<br>C57BL/6 background<br>males and females | OFT: in both genders decreased horizontal/vertical activity and decreased center time<br>EPM: in both genders decreased open arm entries and open arm time<br>Light-dark-exploration test:<br>Decrease of light-dark transitions, increase of time spent in dark compartment (females only)<br>Emergence Test: Decrease of shelter-open field transitions and increase of time spent in the shelter | **General remark to SERT-KO mice:**<br>*Conflicting results might be due to the genetic heterogeneity of different strains*<br>*Increased anxiety can be observed across all strains and tests.* |
| **Noradrenalin** | | | | |
| α-$2_A$ – adrenoreceptor knockout | Schramm et al. (2001)<br><br>Lahdesmaki et al. (2002) | Mouse<br>C57BL/6 background | Open Field Test: no effect of genotype in horizontal activity<br>Decreased rearing after exposure to injection stress<br>Light-dark-box: increased anxiety after exposure to injection stress<br>FST: increased immobility due to decreased climbing (not swimming)<br>24 hour locomotor activity measurement: flattened pattern of activity<br>EPM: increased anxiety | |
| α-2c – adrenoreceptor overexpression | Sallinen et al. (1999) | Mouse<br>FVB/N background | FST: increased immobility | |

| | | | | |
|---|---|---|---|---|
| Dopamine-ß-hydroxylase knockout | Cryan et al. (2001) | Mouse<br>129/SvCPJ-C57BL/6J hybrid | FST: no effect of genotype in baseline immobility<br>Blockade of antidepressant effects | |
| **Miscellaneous** | | | | |
| CRF overexpression | van Gaalen et al. (2002) | Mouse<br>C57BL/6 background | OFT / homecage observation: decreased activity<br>Light-dark-box: decreased activity, increased anxiety<br>FST: increased immobility<br>Fear conditioning: decreased conditioned fear at 1 h but not 24 h after conditioning | Impaired performance on rotarod |
| CRF2 – receptor knockout | Bale et al. (2000)<br><br>Bale and Vale (2003) | Mouse<br>mixed 129 and C57BL/6 background<br><br>males and females | Food intake: Decreased food intake following 24 h of food deprivation<br><br>EPM / OFT: in both genders increased anxiety<br>Light-Dark-Box: No effect of genotype<br>FST: in both genders increased immobility | |
| Overexpression of glucocorticoid receptors in forebrain | Wei et al. (2004) | Mouse | OFT: no effect of genotype<br>FST: increased immobility<br>EPM / Light-dark-box: increased anxiety-like behavior | Increased anxiety and immobility is attenuated by antidepressants |
| CB1 receptor knockout | Review: Valverde and Torrens (2012) | Mouse | EPM, OFT, light-dark-box: increased anxiety<br>Fear conditioning: impairment of extinction<br>Partner recognition test, Operant conditioning test, | |

| | | | | |
|---|---|---|---|---|
| | | | Morris water maze: cognitive impairments<br>Sucrose preference: anhedonia<br>TST: increased immobility | |
| Delta-opioid receptor knockout | Filliol et al.(2000) | Mouse | EPM / Light-dark-box: increased anxiety<br>FST: increased immobility | |
| p11 knockout | Svenningsson et al. (2006) | Mouse | Increased thigmotaxis<br>TST: increased immobility<br>Sucrose preference: anhedonia | p11 increases localization of 5-$HT_{1B}$ receptors |
| BDNF knockout | Chan et al. (2006) | Mouse | TST: increased immobility<br>FST: decreased immobility<br>EPM: no effect of genotype | |

## MIXED MODELS (gene and environment)

| Model | Author | Species | Relevant behavioral readouts | Remarks |
|---|---|---|---|---|
| α-2A – adrenoreceptor knockout<br>+ stressor | Schramm et al. (2001) | Mouse | Decreased rearing after exposure to injection stress<br>Light-dark-box: increased anxiety after exposure to injection stress | |
| CRF2 Receptor knockout<br>+ stressor | Bale et al. (2000) | Mouse | Decreased food intake after 24h lasting food deprivation | No differences in body weight |

5-HT: 5-hydroxytryptamine; BDNF: brain-derived neurotrophic factor; CB1 receptor: cannabinoid receptor type 1; CRF: corticotropin releasing factor; EPM: elevated plus maze; FST: forced swim test; h: hour / hours; HPA: hypothalamus pituitary axis; KO: knockout; OFT: open field test; REM: rapid eye movement; SERT: serotonin transporter; TST: tail suspension test

**Table 2:** Rodent models of depression

# 3 Objectives and hypothesis of the present thesis

Disturbances of the serotonergic system are hypothesized to be a main pathway that may lead to the development of depression in humans.

The use of Gpm6b null mutants in this thesis may indirectly illustrate the impact of M6B on the serotonin transporter of these mice. Since Gpm6b null mutants will not express any M6B, the inhibitory effect of the glycoprotein on the serotonin transporter is no longer present. Mice with a surface overexpression or increased function of the serotonin transporter are expected. This is hypothesized to lead to a more efficient clearing of serotonin from the synaptic cleft.

An induced relative deficiency of serotonin in Gpm6b null mutants should provoke a depressive-like phenotype and provide interesting candidates for modelling depression in mice.

Additionally to a general characterization of this mouse strain, different challenges will be performed. In order to prove the involvement of the serotonergic system, the response to the 5-$HT_{2A/2C}$ receptor agonist (±)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI) of Gpm6b null mutants and wildtype mice will be compared.

Furthermore, the pro-inflammatory substance lipopolysaccharide (LPS) will be applied to investigate a potential enhancement of a depressive-like phenotype.

# 4 Material and Methods

## 4.1 Animals and housing

For the behavioral tests male Gpm6b null mutants and their wildtype littermates were used. Mice were bred by the Department of Neurogenetics (Max Planck Institute of Experimental Medicine, Göttingen). The exact method of gene targeting and mouse genetics was described by Werner et al. (2013). Mice were bred on a C57BL/6J background for more than 10 generations but formally are a C57BL/6J*129SV hybrid (Werner et al., 2013).

At the age of 21 days, mice were weaned and housed in groups of 4-5 animals in standard plastic cages (Tecniplast, type 1284L, 20 x 30 x 12 cm) with wood-chipped bedding (Rettenmeier FN 1-4). Each cage was equipped with a paper tissue, as well as food and water *ad libitum*. The food consisted of pelleted complete feedingstuff (Ssniff V1124-3; dry matter 87,9%, raw protein 22%, raw fat 4,5%, raw fiber 3,9%, raw ash 6,8%, metabolizable energy 13,6 MJ/kg). Tab water was provided in drinking bottles. The temperature in the colony room was maintained at 20-22 °C, with 55 ± 5% air .humidity, and a 12 h light-dark cycle (light on at 8:00 am) was applied. The cages were located in closed shelves that were part of a ventilation system to prevent infections.

Mice got transferred to new cages every 7-10 days and food and water was checked every day and refilled as needed. The responsible person during the testing phase was the person conducting the experiments.

## 4.2 Behavioral testing

All experiments were approved by the governmental Animal Care and Use Committee in accordance with the German Animal Protection Law.

Behavioral experiments were conducted by an investigator, blinded to the genotype, during the light phase of the day (in general between 9:00 am and 6:00 pm). Age of mice at the beginning of testing was 13-14 weeks. Inter-test interval was at least 1 day. Three different cohorts of mice were used.

The order of testing of the first cohort was: elevated plus maze, open field, pre-pulse inhibition, rotarod, visual cliff, hearing, holeboard, hot plate, novel object recognition, social interaction and memory in the 3-compartement chamber, Morris water maze, chimney test, sucrose preference, olfaction / buried food finding, marble burying, tail suspension test, forced swim test, contextual and cued fear conditioning, DOI-project, LPS-project.

The tests conducted with the second cohort were: hearing, prepulse inhibition, DOI-project.

The third cohort was used for the following tests: LABORAS, hearing, prepulse inhibition, fear extinction curve.

All basic behavior tests were performed in accordance to the behavioral protocols of the Department of Neuroscience (Max Planck Institute of Experimental Medicine, Göttingen).

## 4.3 Genotyping

To identify the genotype of the mice, tail biopsies were taken prior to testing and analyzed by the Department of Neurogenetics (Max Planck Institute of Experimental Medicine, Göttingen) using a genotyping protocol based on polymerase chain reaction (PCR). The M6B knockout allele can be discriminated from the wildtype allele with a three primer PCR (for primer sequences see Fig. 4b).

The first primer, M6B.P38.AS, is an antisense primer located in the M6B exon, encoding the first transmembrane domain (exon 4) in a position that is replaced by neo in the knockout. The second primer, M6B.P62, is located in Intron 3. P62 together with P38 produce an amplification product of app. 400 bp for the wildtype allele (Fig. 4a). P62 together with the third primer, neo6, produce an amplification product of app. 700 bp for the knockout allele (Fig 4a).

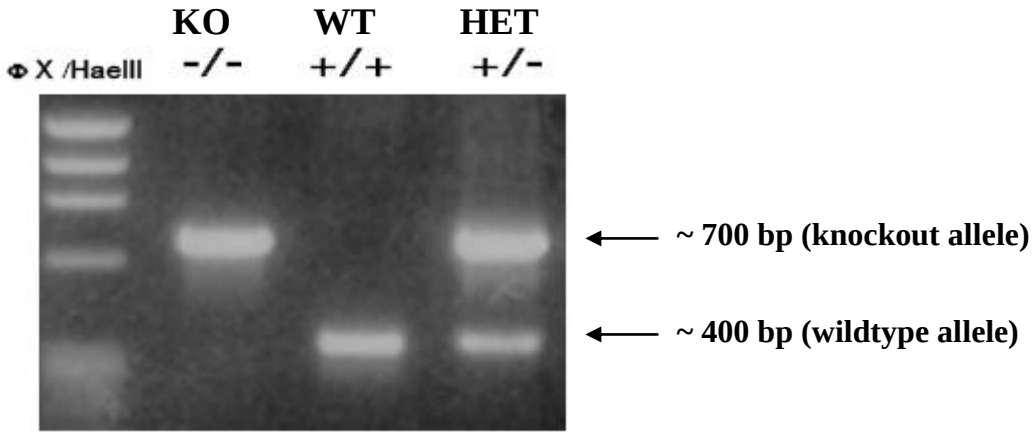
**Protocol:**

1 µl dissolved tail DNA
0.375 µl P38 (10 pmol/µl stock)
0.75 µl P62 (10 pmol/µl stock)
0.75 µl neo6 (10 pmol/µl stock)
0.2 mM dNTPs
1x Red Taq Puffer
0.5 µl Red Taq (Sigma)
ad H2O
total: 20 µl

36 cycles of:
Annealing 30 sec 56 °C
Elongation 60 sec 72 °C
Denaturation 30 sec 95 °C
Run on agarose gel (1-2%)

**Table 3**: Genotyping protocol used for genotyping Gpm6b null mutants

**(a)**




**(b)**

M6B.P38.AS 5‘-CCAGGGAGGCATAGGGAACT-3‘

M6B.P62 5‘-CCCTTTGCCTCCCAGTCAGTTG-3‘

neo6 5‘-GCAATCCATCTTGTTCAATGGC-3‘

**Figure 4:** (a) Agarose gel with amplification products for knockout and wildtype alleles (b) Primer sequences of primers used for genotyping

## 4.4 Behavioral tests

### 4.4.1 Basic behavior testing

#### 4.4.1.1 Elevated plus maze

In this test of anxiety, a maze made of grey plastic with a 5x5 cm central platform, two 30x5 cm open arms and two 30x5x15 cm closed arms was used and an illumination 120 lx was applied. Mice were placed in the central platform, facing an open arm of the maze. Subsequently, behavior was recorded for 5 min by an overhead video camera and a PC equipped with "Viewer 2" software (Biobserve GmbH, Bonn, Germany) to calculate the time spent in open or closed arms, number of arm visits as well as distance traveled and velocity.

#### 4.4.1.2 Open field

Spontaneous activity in the open field was tested in a grey Perspex arena (120 cm in diameter, 25 cm high; illumination 120 lx). Mice were placed in the center and allowed to explore the arena for 7 min. The behavior was recorded by a PC-linked overhead video camera. "Viewer 2" software was used to calculate distance traveled, velocity and time spent in central, intermediate or peripheral zones of the open field.

#### 4.4.1.3 Rotarod

The rotarod test assesses motor function, coordination and balance. The test consisted of a rotating drum (Ugo Basile, Comerio, Varese, Italy), which was accelerated from 4 to 40 rounds per min over the duration of 5 min. Mice were placed individually on the drum and the latency of falling off was recorded using a stop-watch. To evaluate motor learning, the rotarod test was repeated 24 h after the initial trial.

#### 4.4.1.4 Visual cliff

The test was used to examine visual deficiencies of the mice. The test apparatus was comprised of an open-topped box (70 × 35 cm floor, 30 cm high). The base consisted of clear Perspex and the walls of the box were made from white Perspex.

The box was placed on the edge of a laboratory bench with the consequence that half of the base was positioned on the bench ("ground" side), and the other half over the edge of the bench, suspended 1 meter above the floor ("air" side). Mice were then individually placed in the middle of the base at the edge of the cliff. The activity was assessed for 5 min by a computer using video-tracking system Viewer 2 (Biobserve GmbH, Bonn, Germany). The percentage of time mice spent on the "ground" and the "air" side of the box was calculated.

#### 4.4.1.5 Holeboard

The holeboard test, to measure exploratory activity, consisted of a 51×51×33 cm transparent Perspex chamber with a non-transparent floor that was equipped with 16 equally spaced holes, each 2 cm in diameter and 2 cm deep. Mice were allowed to freely explore the apparatus for 5 min and the number of explored holes (measured as nose pokes) was registered by a computer software (TSE GmbH, Bad Homburg, Germany). The illumination in the testing room was 120 lx.

#### 4.4.1.6 Hot plate

The hot plate test was used as a measure of pain sensitivity. Mice were placed on a metal plate (Ugo Basile) that was preheated up to 55 °C. The latency till the mice showed either hind paw licking or jumping was recorded. Immediately after showing the response mice were removed from the platform. 40 sec cut-off time was supposed to prevent wounds, although none of the tested mice reached it.

#### 4.4.1.7 Marble burying

The marble burying and digging test measures impulsive and stereotyped behavior in rodents. The test box (34.5x56.5x18.5 cm) is filled with relatively deep bedding (about 5 cm) and the surface is smoothened. Marbles are set in a grid-like pattern using a cardboard template, which is removed prior to testing. Mice are put in the chamber for 30 min and allowed to freely explore. The number of buried marbles in both the periphery (16 edge marbles) and the center (8 middle marbles) is quantified. Marbles are considered buried if at least two-thirds of the marble is obscured from view.

#### 4.4.1.8 Olfaction / Buried food finding

Starting 4 days prior to testing, mice received several pieces of chocolate cookies of 1.6 g daily with water ad libitum. All mice consumed all cookies within 24 h. Then, mice were deprived of food for 12 h before testing, with water ad libitum. For testing, individual mice were placed into clear cages (29.5x18.5x13 cm), in which a piece of a chocolate cookie was positioned at the end of the cage. The mouse was put 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 arrived at the cookie, was recorded. In a second trial the cookie was hidden under 1.5 cm standard bedding instead of being visible and the time the mouse needed to find the cookie, e.g. started digging, was recorded. As soon as the cookie was uncovered, the mouse was removed from the cage. A fresh cage and bedding was used for each trial, and all mice were subjected to identical testing procedures.

#### 4.4.1.9 Hearing

The hearing test used the same apparatus as for assessment of the prepulse inhibition. The startle reaction to an acoustic stimulus (pulse), 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 100 ms and stored for further evaluation. The recording window was defined from the onset of the acoustic stimulus. An experimental session consisted of 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, stimuli of different intensity and fixed 40 ms duration were presented. Stimulus intensity was varied between 65 dB and 120 dB, such that 19 intensities from this range were used with 3 dB steps. Stimuli of each intensity were presented 10 times in a pseudorandom order with an interval ranging from 8 to 22 sec. The amplitude of the startle response (expressed in arbitrary units) was defined as the difference between the maximum force detected during a recording window and the force measured immediately before the stimulus onset. Amplitudes of responses for each stimulus intensity were averaged for individual animals.

#### 4.4.1.10 Prepulse inhibition

For this test of sensorimotor gating, individual mice were placed in small metal cages (90x40x40 mm) to restrict major movements and exploratory behavior. The movable platform floor of each cage was attached to a sensor that records vertical movements of the floor. The cages were placed in sound-attenuating isolation cabinets (TSE GmbH, Bad Homburg, Germany). Startle responses were evoked by acoustic stimuli delivered from a loudspeaker suspended above the cage and connected to an acoustic generator.

The startle reflex to an acoustic stimulus evokes a movement of the platform and a transient force resulting from the movement. The reaction was recorded with a computer during a time window of 260 ms (beginning with the onset of prepulse) and stored for further evaluation. The recording window was defined from the onset of the acoustic stimulus.

An experimental session consisted of a habituation period of 2 min 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 in order to decrease influence of within-session habituation. These data were not included in the analysis of the prepulse inhibition. For tests of prepulse inhibition, the 120 dB / 40 ms startle pulse was applied either alone or preceded by a prepulse stimulus of 70 db, 75 db, or 80 dB sound pressure level and 20 ms duration. An interval of 100 ms with background white noise was applied between each pre-pulse and pulse stimulus. The trials were presented in a pseudorandom order with an interval ranging from 8 to 22 sec. The amplitude of the startle response (expressed in arbitrary units) was defined as the 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 prepulse). Pre-pulse inhibition was calculated as the percentage of the startle response using the following formula: *% pre-pulse inhibition = 100 – [(startle amplitude after pre-pulse and pulse) / (startle amplitude after pulse only) x 100]*.

## 4.4.2 Social behavior testing

### 4.4.2.1 Social interaction and memory in the 3-compartement chamber

The social testing arena was a rectangular, 3-chambered box. Each chamber was 20x40x22 cm in size. Dividing walls were made from clear Plexiglas, with rectangular openings (35x35 mm) allowing access into each chamber. The chambers of the arena were cleaned, and fresh paper 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 two-side chambers were obstructed by plastic boxes during this habituation phase.

After the habituation period, an unfamiliar C57BL/6 male mouse without prior contact to the subject mouse was placed in one of the side chambers. The location of the stranger mouse in the left versus right side chamber was systematically alternated between trials. The stranger mouse was enclosed in a small (60x60x100 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 heavy cup was placed on top of each 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 was recorded by the video tracking system “Viewer 2” (Biobserve GmbH).

A second, unfamiliar mouse was then placed into the previously empty wire cage. The test mouse had a choice between the first, already explored mouse (familiar), and the novel unfamiliar mouse (stranger). As described above, measures were taken of the amount of time spent in each chamber during the second 10-min-session.

### 4.4.3 Learning and memory testing

#### 4.4.3.1 Novel object recognition

The object recognition test is based on the natural tendency of mice to investigate a novel object instead of a familiar one. The choice to explore the novel object reflects the use of learning memory.

In the object recognition task, a mouse was placed in a box and was exposed for 10 min to two objects (object1 and object2) that were similar in shape and colour and were located a specified distance from each other. The mouse was then removed from the environment and immediately after the exploration period testing takes place. The mouse was retested in the same environment for 10 min except that one of the two previously used (familiar) objects was replaced with a novel one that differs from the familiar object in shape and appearance.

A week later the mice were again exposed to two similar objects (object1 and object2) and allowed to explore freely. After removing the mice from the environment a delay of 30 min was awaited before the mice were returned to the box with one familiar and one novel object.

The behavior was recorded by a video/computer controlled system which measures the number of visits and the duration mice spent on exploring the objects. Subsequently, preference for each object was calculated.

#### 4.4.3.2 Morris water maze

Spatial learning and memory was assessed in a water maze. A large circular tank (diameter 1.2 m, depth 0.4 m) was filled with opaque water (25±1 ˚C, depth 0.3 m) and the escape platform (10x10 cm) was submerged 1 cm below the surface. The swim patterns were monitored by a computer and the video-tracking system “Viewer 2”. The escape latency, swim speed, path length, and trajectory of swimming were recorded for each mouse. Every day, mice went through 4 trials with an inter-trial interval of 5 min.

During the first 2 days, mice were trained to swim to a clearly visible platform (visible platform task) that was marked with a 15 cm high black flag and placed pseudo-randomly in different locations across trials (non-spatial training). The extra-maze cues were hidden during these trials.

After 2 days of visible platform training, hidden platform training (spatial training) was performed. For 8 days, mice were trained to find a hidden platform (i.e. the flag was removed) that was located in the center of one of the four quadrants of the pool. The location of the platform was fixed throughout testing. Mice had to navigate using extra-maze cues that were placed on the walls of the testing room. The mice were placed into the pool facing the side wall randomly at one of four start locations and allowed to swim until they found the platform, or for a maximum of 90 sec. Any mouse that failed to find the platform within 90 sec was guided to the platform. The animal then remained on the platform for 20 sec before being removed from the pool.

The day after completion of the hidden platform training, a probe trial was conducted in order to determine whether mice used a spatial strategy to find the platform or not. The platform was removed from the pool and the mice were allowed to swim freely for 90 sec. The percentage of time spent in each quadrant of the pool was recorded.

To investigate the flexibility of cognitive processes in mice, the reversal water maze test was performed. The experimental procedure was identical to the one used for the hidden platform training with the exception that the escape platform was moved from the original position to the neighboring quadrant. The reversal task was performed on 4 days and subsequently a reversal probe trial was conducted as described earlier.

#### 4.4.3.3 Contextual and cued fear conditioning

For fear conditioning mice were trained within the same session for context and cue. Training consisted of exposing mice for 120 sec to the context to assess the baseline level of activity. This period was followed by a 10 sec, 5 kHz, 85 dB tone (conditioned stimulus, CS). Immediately after the tone, a 2 sec, 0.4 mA foot shock (unconditioned stimulus, US) was applied. This CS-US pairing was repeated 13 sec later. All mice remained in the conditioning chambers (interior: 305x241x210 mm) for an additional 23 sec following the second CS–US pairing. The contextual memory test was performed 48 h after this training. Mice were monitored over 2 min for freezing in the same context as used for training. The cued memory test was performed 52 h after training in a new chamber. First, mice were monitored for freezing over a 2 min pre-cue period with no tone to assess freezing in the new context. Next, a 2 min

lasting cue period followed in which the tone was presented. Duration of freezing behavior, defined as the absolute lack of movement (excluding respiratory movements), was recorded by a video camera and a PC equipped with 'Video freeze' software (MED Associates, St. Albans, Vermont, USA).

#### 4.4.3.4 Fear conditioning – extinction curve

Mice underwent the fear conditioning training as described above. 24 h later mice were exposed to the same context they experienced during the training and freezing levels were assessed. Subsequently, the animals were tested in the same context repeatedly in order to obtain an extinction curve.

### 4.4.4 Tests for depressive-like behavior

#### 4.4.4.1 Sucrose preference

The sucrose preference test was performed using a two-bottle procedure, during which mice had free access to both water and a sucrose solution. Animals were first habituated for 48 h to consume water from the 2 small (100 ml) bottles. After habituation, mice were deprived of water and the sucrose preference was measured during the next 4 days. The first 2 days served as a habituation to sucrose solution. The average consumption of sucrose solution in comparison to the amount of water consumed in the 4 days was used for the evaluation of sucrose preference. Each day, group-housed mice were placed individually into small plastic cages and 2 bottles were presented to them for 60 min - one with tap water and one with a 2% sucrose solution. Consumption of water or sucrose solution was measured by weighing the bottles before and after the session. Bottles were counterbalanced across the left and the right sides of the cage, and their position was alternated from test to test. Sucrose preference (percent) was calculated as follows:

Preference [%] = [sucrose solution intake (ml) / total fluid intake (ml)] × 100

#### 4.4.4.2 Chimney test

The chimney test measures whether a mouse can escape an inverted tube by climbing backwards. This model serves as a metric for neurological impairment and motoric functions, but also for the investigation of a lack of motivation to escape.

No training is required for the task. Mice crawl into a horizontal tube, which is inverted. Normally, mice will attempt to escape by climbing backwards out of the space. The escape latency of the mice was recorded.

#### 4.4.4.3 Tail suspension test

In the tail suspension test a tape gets attached to the mice's tail (about 1 cm from the tip), and the tape is hung up on a bar so that mice are midair. Then the movements are recorded for 6 min by a video camera and analyzed by trained observers. Typically, mice will initially struggle in an attempt to escape and subsequently begin to remain motionless after having "given up" their efforts.

#### 4.4.4.4 Forced swim test

The forced swim test assays for depression in a similar manner to the tail suspension test.

Mice are placed in a clear container with ~25 ºC water and video-recorded over the duration of the trial (6 min). The time the mouse remains immobile is scored by trained observers.

Immobility is defined as floating or small movements that allow the mouse to keep the head above the water level.

### 4.2.4.5 LABORAS

"Laboratory Animal Behavior Observation Registration and Analysis System"

The LABORAS system (Metris b.v., Hoofddorp, The Netherlands) characterizes and records rodent behavior through analysis of movement-induced forces generated by the animals. The system consists of a triangular shaped sensor platform (Carbon Fiber Plate 1000x700x700x30 mm, Metris b.v.). The sensors on the measurement plate amplify and match vibrations to stereotyped profiles of known actions. Recognized actions include climbing, grooming, eating, drinking, locomotion, scratching and seizure.

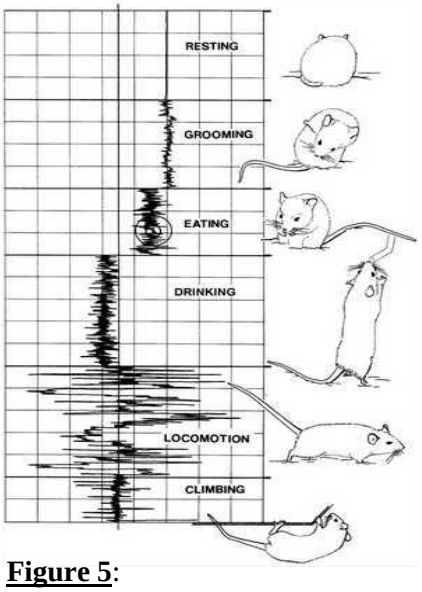



**Figure 5**:
Specific vibration patterns of rodent behavior analyzed with the LABORAS system

LABORAS also tracks the positional information and speed of test animals. During LABORAS recording, mice are housed in clear polycarbonate cages (Makrolon type II cage, 22x16x14 cm) that are directly placed onto the platform and have a wood-chip bedding covered floor as well as food and water provided.

For the assessment of home cage behavior, mice were habituated to the room under single housed conditions on 2 consecutive days prior to testing. On day 3 mice were placed in the LABORAS cages 2 h before the dark phase. They remained undisturbed for 15 h and were removed from the cages 1 h after beginning of the light phase.

## 4.4.5 DOI – Project

### 4.4.5.1 Pilot experiment

The 5-$HT_{2A/2C}$ receptor agonist (±)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI) was purchased from Sigma-Aldrich (Germany) and dissolved in 0.9% sodium chloride solution.

10 wildtype mice were used in the pilot project to assess the optimal dose of DOI provoking changes in distinct behavioral patterns, such as grooming and locomotion. One mouse at a time was removed from its cage, gently weighted and placed in a cage that was part of the LABORAS system and located in an undisturbed and lit room. Subsequently, the mouse was left alone for 30 min while its behavior was recorded by a video camera and LABORAS in order to assess its behavior without any manipulations.

After 30 min the mouse was removed from the observation cage and either injected with a dose of 0.5 mg/kg DOI i.p. or 0.75 mg/kg DOI i.p.. Immediately after the injection, the mouse was returned to the observation cage and remained undisturbed for another 30 min.

Grooming behavior was scored from video recordings and data for locomotor activity was obtained from LABORAS. The sessions were divided into 3 parts (each 10 min) that were rated separately and scored how much time the mouse spent revealing a particular behavior.

### 4.4.5.2 Final protocol

After identifying a suitable dose of DOI, wildtype mice and Gpm6b null mutants were used to assess potential variations in the grooming duration and locomotor activity in dependence of the genotype. The hypothesis was that Gpm6b null mutants show a different reaction to DOI than wildtype mice due to altered extracellular serotonin levels.

Each mouse underwent the same procedure as described previously, but this time all mice received the same dose of DOI (0.5 mg/kg) i.p.. Video recordings and LABORAS data were analyzed as described previously.

## 4.4.6 LPS – Project

### 4.4.6.1 Pilot experiment

In order to challenge the mice pharmacologically, lipopolysaccharide (LPS) extracted from *E. coli*, was injected to elderly Gpm6b null mutants and wildtype mice. The aim was to find a threshold dose of LPS that increases the slightly depressive-like phenotype observed in Gpm6b null mutants during previous testing. Lipopolysaccharide was purchased from Sigma-Aldrich and dissolved for injection in 0.9% sodium chloride solution.

The protocol used for this project is illustrated in Figure 6. For the first approach, wildtype mice and Gpm6b null mutants (at the age of about 15.5 months) received a single i.p.-injection of lipopolysaccharide at a dosage of 100 µg/kg body weight. 24 h later, mice were tested in the open field for a period of 6 min to detect potential sickness behavior, revealed as reduced locomotor activity. If no reduced locomotor activity was observable and it could therefore be assumed that mice had overcome the induced sickness, they were given a 1-h rest before performing the tests for depressive-like behavior. 50% of the mice performed the forced swim test and the other 50% underwent the tail suspension test. The allocation of the genotypes was evenly distributed. After a break of 6 h the mice were tested in the opposing test.

### 4.4.6.2 Final protocol

A dose of 100 µg/kg LPS did not prove to be adequate, since it did not have any effect on neither wildtype nor Gpm6b null mutants. Therefore, wildtype mice and Gpm6b null mutants (at the age of about 17 months) were injected i.p. with a dose of 250 µg/kg LPS on two consecutive days. After the injection the same protocol as used in the pilot project was employed (Fig. 6). The locomotor activity was assessed repeatedly (24 h, 48 h and 96 h after the second injection) for 6 min in the open field until no reduction of activity was observable. After the open field test mice were given a rest of 1 h before proceeding with the forced swim test and the tail suspension test as described previously.

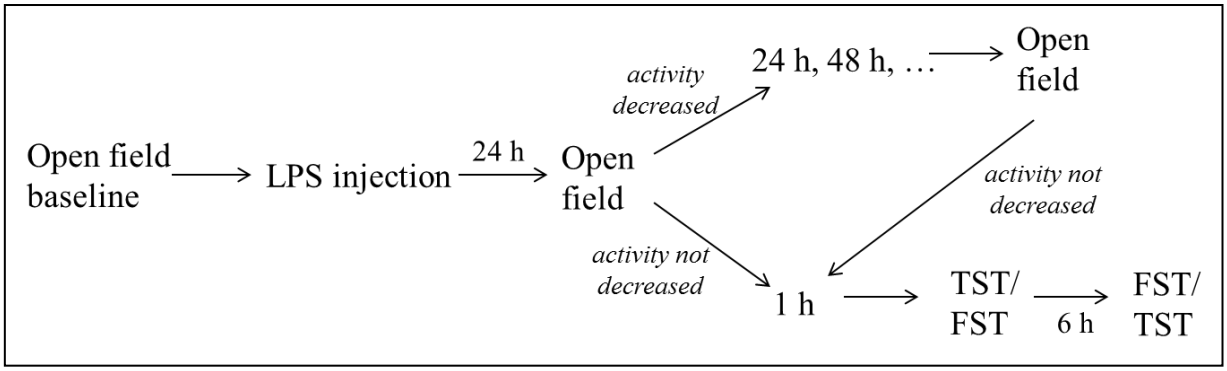



**Figure 6:** Abstracted schedule for LPS experiment

## 4.3 Statistical analysis

Unless stated otherwise, the results are expressed as mean±SEM and a p-value below 0.05 was considered to be significant.

The data were analyzed mainly using Prism4 (GraphPad Software, San Diego, CA, USA) and SPSS Software to compare the data by 2- or 3-way analysis of variance (ANOVA) with post-hoc planned comparisons, or by ANOVA for repeated measurements, respectively Mann-Whitney U and student t-test where appropriate.

In the forced swim test, a mouse was excluded from the analysis when it was not able to swim properly (e.g. repeatedly sank to the ground).

In the graphs shown in the result section, wildtype mice are referred to as “WT” and Gpm6b null mutants are referred to as “KO”.

# 5 Results

## 5.1 Basic behavior testing

**– Differences only in the elevated plus maze and prepulse inhibition test**

The basic behavior testing shows that Gpm6b null mutants only differed from the wildtype littermates in the elevated plus maze and prepulse inhibition test.

In the elevated plus maze (EPM) test (Fig. 7a - 7e) all mice spent significantly less time in the open arms than in the closed arms (Fig. 7a; $p < 0.001$) and an equal amount of time in the center of the EPM (Fig. 7b; $p > 0.05$), independent of the genotype.

Assessment of the total number of visits revealed that Gpm6b null mutants visited the open arms as well as the closed arms more often than wildtype mice (Fig. 7d; $p = 0.028$) which was also reflected by a significantly higher amount of total arm entries (Fig. 7e; $p = 0.0204$). Both genotypes preferred to visit the closed arms rather than the open arms, which could be seen as a higher number of closed arm entries compared to open arm entries (Fig. 7d; $p < 0.001$).

The velocity that was measured could show that Gpm6b null mutants traveled a significantly higher distance per second than wildtype mice (Fig. 7c; $p = 0.0297$).

In the open field test (OFT) (Fig. 7f – 7i) both genotypes spent most of the time in the periphery and less time in the intermediate or the central section (Fig. 7f).

There was no significant difference in the number of center visits during the testing period between the genotypes (Fig. 7g); $p > 0.05$). It is interesting to note that Gpm6b null mutants had the tendency of a higher velocity in comparison to wildtype mice (Fig. 7j; $p = 0.1577$). Also, the total distance traveled tended to be greater in Gpm6b null mutants (Fig. 7h; $p = 0.1611$). These results point in the same direction as those of the EPM, i.e. underscore a mild hyperactivity phenotype of Gpm6b null mutants.

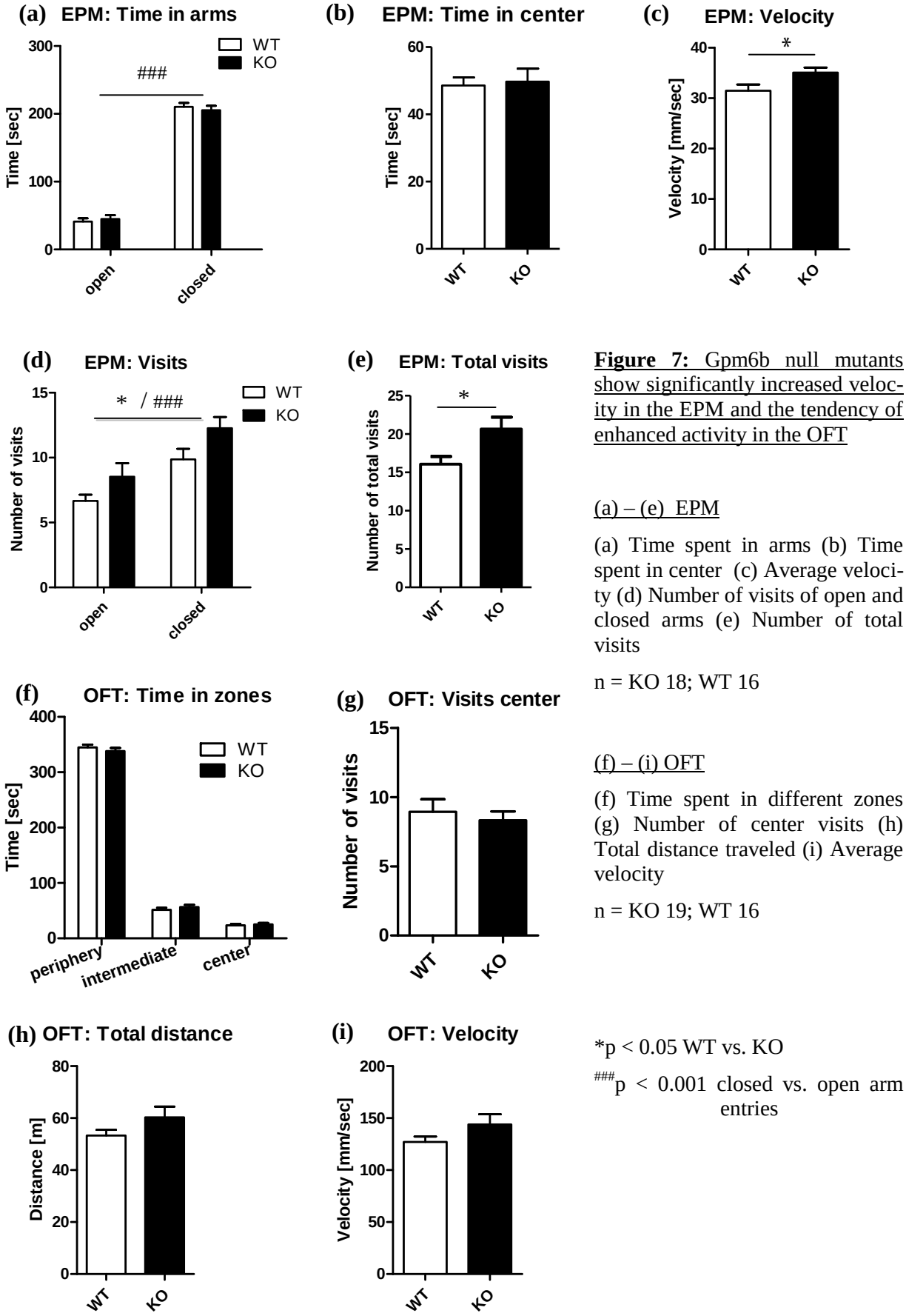



**Figure 7:** Gpm6b null mutants show significantly increased velocity in the EPM and the tendency of enhanced activity in the OFT

(a) – (e) EPM

(a) Time spent in arms (b) Time spent in center (c) Average velocity (d) Number of visits of open and closed arms (e) Number of total visits

n = KO 18; WT 16

(f) – (i) OFT

(f) Time spent in different zones (g) Number of center visits (h) Total distance traveled (i) Average velocity

n = KO 19; WT 16

*$p < 0.05$ WT vs. KO

###$p < 0.001$ closed vs. open arm entries

The results of the rotarod (Fig. 8a) show that there was no initial difference in the genotype with regard to motor functions (genotype: $p > 0.05$) and that both genotypes were able to improve their performance when tested again 24 h later (day: $p < 0.004$). Therefore, it can be concluded that Gpm6b null mutants do not have impairments in motor functions or motor learning.

When analyzing the data from the visual cliff test (Fig. 8b), there was no significant difference between the genotypes observable ($p > 0.05$). Both genotypes preferred staying on the ground side of the box which indicates an unimpaired vision of Gpm6b null mutants.

The exploratory activity, measured by the number of nose pokes that the mice showed in the holeboard test (Fig. 8c), did not yield any differences between genotypes ($p > 0.05$).

The data obtained from the hot plate test (Fig. 8d) for testing reaction to pain revealed that there were no differences between Gpm6b null mutants and wildtype mice ($p > 0.05$). Both genotypes showed signs of pain after a similar duration spent on the hot plate.

Assessing the motor impulsivity and stereotypy with the marble burying test (Fig. 8e) revealed that there were no differences in Gpm6b null mutants as compared to wildtype littermates in the number of buried marbles ($p > 0.05$).

In the buried food finding test (Fig. 8f) both genotypes were faster finding the visible cookie than discovering the hidden one. Independent of the differences between the visible and the hidden cookie, the performance of Gpm6b null mutants was comparable to the performance of wildtype mice ($p > 0.05$), excluding an applicable olfactory deficit.

The hearing ability (Fig. 8g) of the mice did not show any significant difference ($p > 0.05$). Also, the body weight of the different genotypes (Figure 8h) was comparable ($p > 0.05$) and therefore did not influence the outcome of the test.

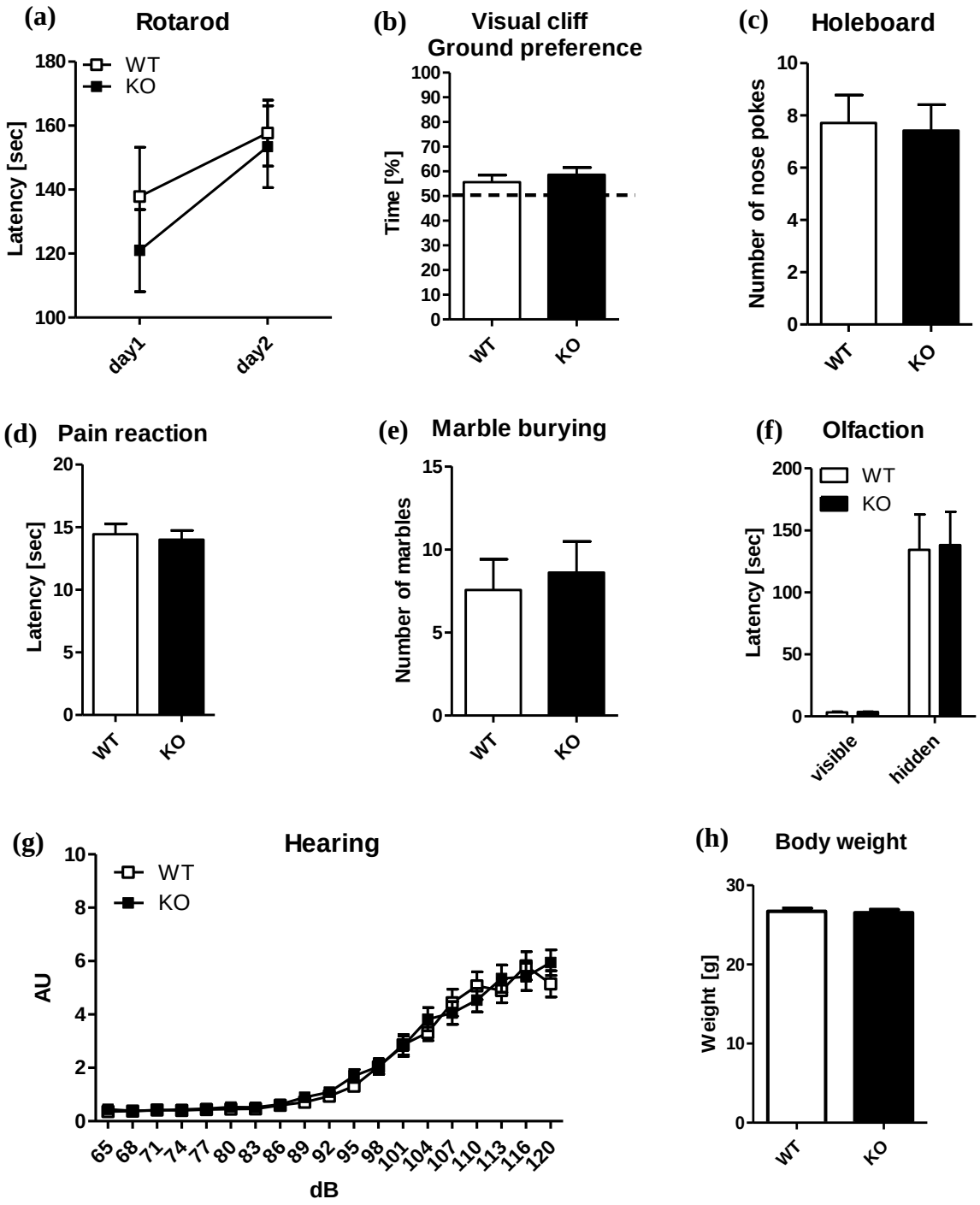



**Figure 8**: No abnormalities of Gpm6b null mutants in various basic tests

(a) Latency time on rotarod (b) Percentage of time spent on the ground side of the visual cliff (c) Number of nose pokes in the holeboard test (d) Latency until observable pain reaction on the hot plate (e) Number of buried marbles (f) Time needed to find the cookie (g) Hearing capabilities (h) Body weight at the time of hearing assessment

(a) n = KO 19; WT 15 (b)-(e) n = KO 19; WT 16 (f)+(h) n = KO 36; WT 39

In the prepulse inhibition test (Fig. 9b) Gpm6b null mutants showed a reduction compared to wildtype mice ($p = 0.013$). All mice showed a significantly different inhibition of their response after the prepulse dependent on the sound intensity ($p < 0.001$). There was, however, no interaction of genotype and sound intensity present ($p > 0.05$).

Neither the startle response (Fig. 9a) nor the body weight at the time of the prepulse inhibition test (data not shown) differed between Gpm6b null mutants and wildtype mice ($p > 0.05$) which is essential because both factors could otherwise affect the outcome of the prepulse inhibition test.

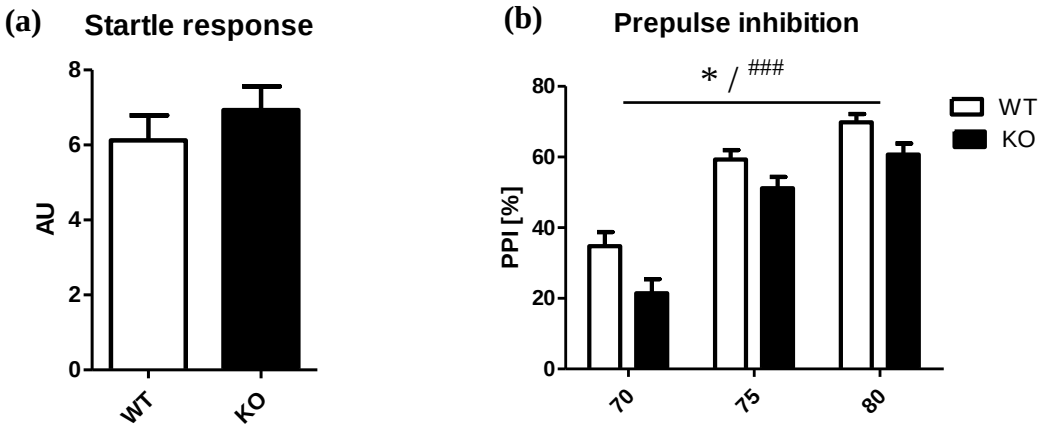



**Figure 9:** Unaltered startle response but reduced prepulse inhibition of Gpm6b null mutants

(a) Startle response (b) Percentage of prepulse inhibition

n = KO 34: WT 33 *$p < 0.05$ WT vs. KO ###$p < 0.001$ sound intensity

## 5.2 Social behavior testing

**– No differences in social interaction and social memory in Gpm6b null mutants**

The results of the social interaction and memory test revealed that both genotypes preferred to interact with an unfamiliar mouse instead of spending time in the compartment with the empty cage (Fig. 10a). The amount of time mice spent in the compartment equipped with the stranger was significantly higher than the duration mice spent in the empty compartment ($p < 0.001$). When mice had the choice to interact with either a familiar mouse or with an unfamiliar mouse (Fig. 10b), both genotypes preferred to explore the new mouse, reflected in a greater time amount they spent in the chamber with that mouse ($p = 0.0017$).

It can be summarized that Gpm6b null mutants do not have any differences with regard to social interaction and memory in the 3-compartment chamber as compared to wildtype mice.

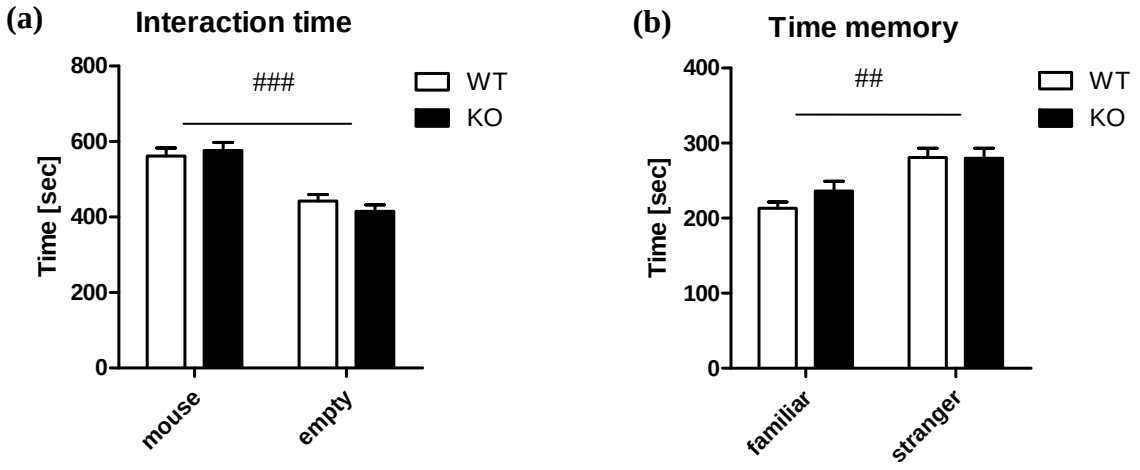



**Figure 10:** Normal social behavior of Gpm6b null mutants

(a) Interaction time with stranger mouse as compared to empty compartment (b) Interaction time with familiar mouse as compared to stranger mouse

n = KO 19; WT 16

$^{###}p < 0.001$ mouse vs. empty compartment $^{##}p < 0.01$ familiar vs. stranger mouse

## 5.3 Learning and memory testing

- **Gpm6b null mutants show hints of an enhanced fear memory but no further alterations in learning and memory tasks**

During the first trial of the novel object recognition test, in which two identical objects could be explored by the mice, both genotypes spent about 50% exploring either of the objects (Fig. 11a).

In the following session with no delay (Fig. 11b + 11c) mice spent significantly more time exploring the novel object, which resulted in an increased number of visits (Fig. 11c; $p = 0.002$) and a longer duration of time exploring the novel object (Fig. 11b; $p = 0.002$) without a genotype effect being observable ($p > 0.05$).

During the object exploration trial that was performed about 1 week later (Fig. 10d) wildtype mice preferred exploring object1 ($p = 0.0047$), whereas Gpm6b null mutants explored both objects equally. However, after a delay of 30 min all mice seemed to prefer spending time with the unfamiliar object which is reflected in a recognition index of about 60% for both genotypes although the p-value is only borderline significant (Fig. 11e; $p = 0.052$).

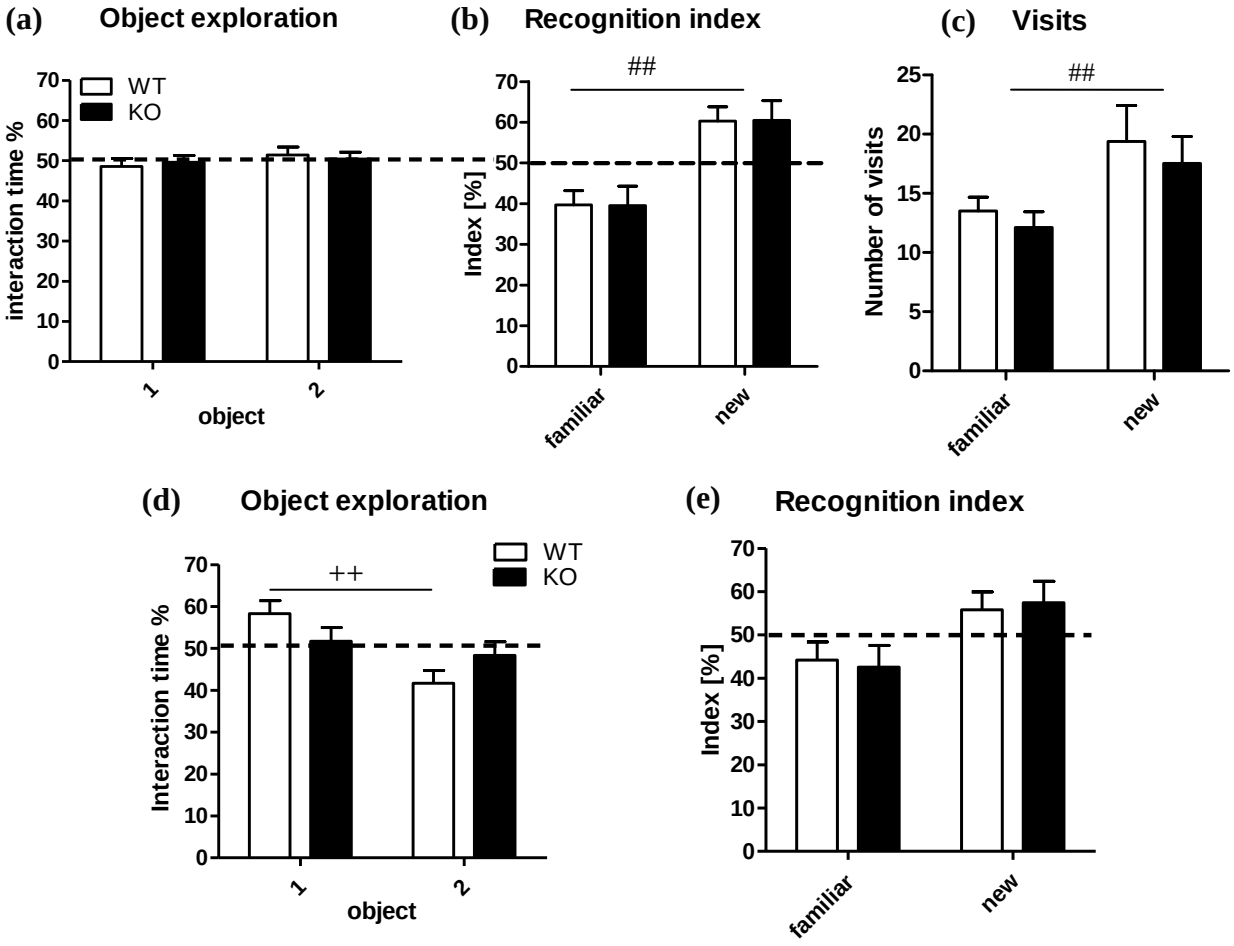



**Figure 11**: The recognition of a familiar object did not show any variances of Gpm6b null mutants compared to wildtype mice

(a) Percentage of interaction time with object1 and object2 (b) Percentage of time spent with new and familiar object when no delay was applied after the exploration trial (c) Number of visits of new and familiar object when no delay was applied after the exploration trial (d) Percentage of interaction time with object1 and object2 (e) Percentage of time spent with new and familiar object when a 30 min delay was applied after the exploration trial

##$p < 0.01$ familiar object vs. new object

++$p < 0.01$ exploration time of object1 vs. object2 in wildtype mice

n = KO 17; WT 16

The Morris water maze (Fig. 12a – 12g) as a test for spatial learning and memory did not show any significant differences between Gpm6b null mutants and wildtype mice.

In the visible platform task performed on two consecutive days (Fig. 12a), the duration Gpm6b null mutants needed to reach the platform was similar to the time wildtype mice required ($p > 0.05$).

The performance of Gpm6b null mutants in all other trials that measured spatial learning and memory was similar to the ones wildtype mice displayed. In the hidden platform training, where spatial training was performed, both genotypes learned where to find the new location of the platform equally so that the resulting duration curve needed of reaching the platform is almost identical (Fig. 12b). The distance traveled before finding the platform (Fig. 12c) and the average velocity during the search for the platform (Fig. 12d) did not show any differences between genotypes ($p > 0.05$). Subsequently, both genotypes spent the same amount of time searching in the quadrant where the platform used to be during the spatial training (Fig. 12e; $p > 0.05$).

When the platform was positioned in a different quadrant during the reversal water maze test (Fig. 12f), the duration Gpm6b null mutants required to reach the platform did not show any differences as compared to the performance of wildtype mice ($p > 0.05$).

Also, there was no significant difference between Gpm6b null mutants and wildtype mice when the platform was again removed from the tank (Fig. 12g): Both genotypes spent the same amount of time in the quadrant where the platform had been previously during reversal training ($p > 0.05$).

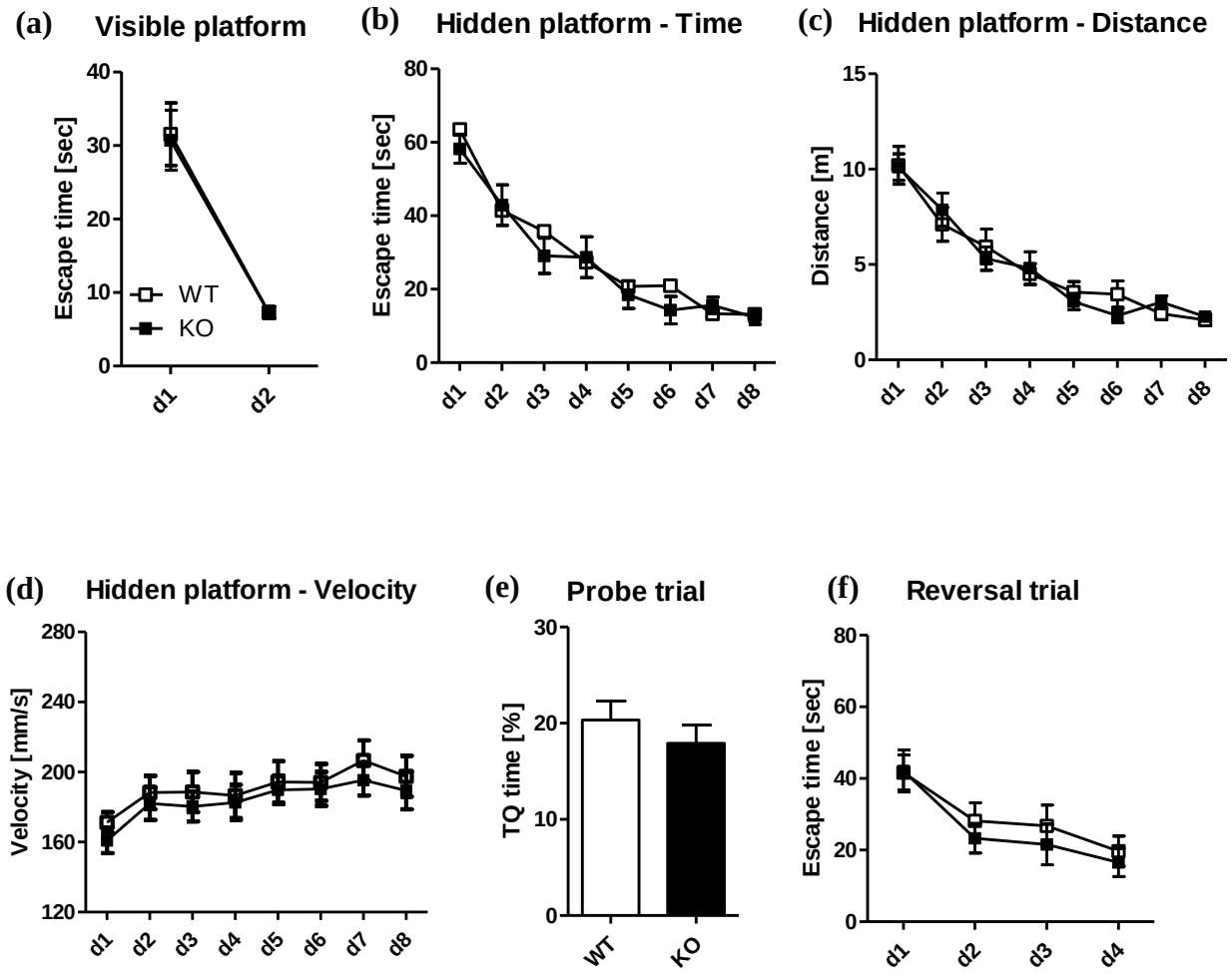



**Figure 12**: No differences in the Morris water maze task

(a) Time needed to reach the visible platform (b) Time needed to reach the hidden platform (c) Distance traveled until finding the hidden platform (d) Average velocity when searching for the hidden platform (e) Percentage of time spent in quadrant (f) Time needed to reach the platform when moved to another quadrant (g) Percentage of time spent in quadrant

n = KO 16; WT 15

In the fear conditioning test (Fig. 13a) all mice showed a comparable baseline freezing level ($p > 0.05$). When exposed to the same context 48 h later, Gpm6b null mutants showed a significantly higher amount of time freezing as compared to wildtype mice ($p = 0.04$).

The freezing level that was obtained in another context before and after the mice could hear the tone (basecue and cue, respectively) did not show any differences between Gpm6b null mutants and wildtype mice ($p > 0.05$).

In contrast to the initially obtained fear conditioning results, the fear extinction curve (Fig. 13c) that was assessed with another cohort of mice starting 24 h after conditioning, did not show impairments in fear extinction of Gpm6b null mutants. Gpm6b null mutants had a similar course of reduction in freezing behavior as wildtype mice when exposed to the same context repeatedly without experiencing the foot shock ($p > 0.05$). The baseline freezing was again not altered (Fig. 13b; $p > 0.05$).

Comparing the freezing levels of the first exposure to the same context after the training session (Fig. 13d), it could be shown that Gpm6b null mutants spent about 60% of the time freezing in both protocols (24 h and 48 h after conditioning, respectively) ($p > 0.05$), whereas wildtype mice revealed a reduced freezing when the interval between the training and the contextual protocol was greater ($p = 0.026$).

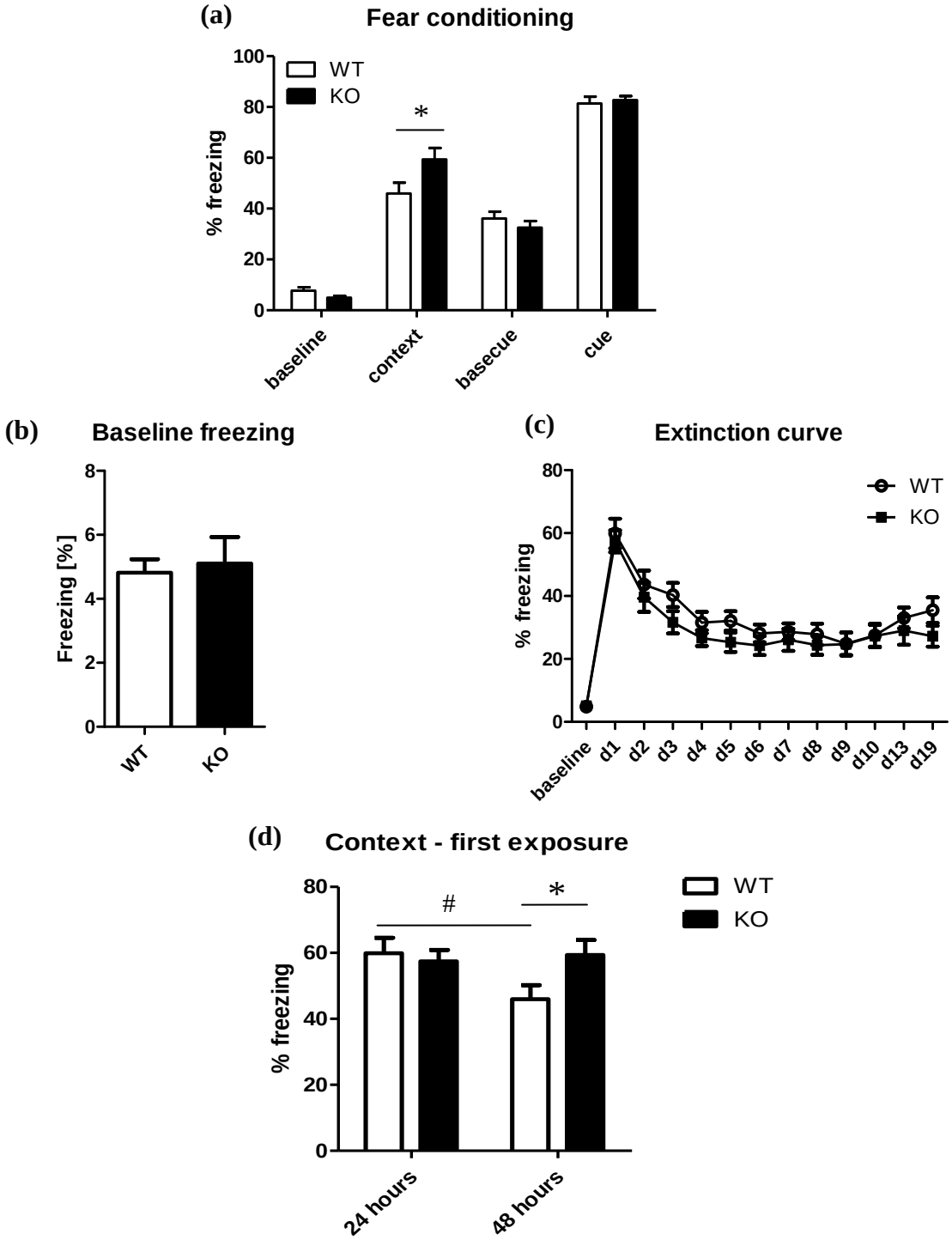



**Figure 13**: Hints for a prolonged contextual fear memory

(a) Freezing levels of initial fear conditioning (b) Baseline freezing level for extinction curve (c) Fear extinction curve (d) Freezing level at time of first exposure to context

(a) n = KO 17; WT 16 (b) – (c) n = KO 16; WT 18

*p < 0.05 WT vs. KO; #p < 0.05 WT 24 h vs. 48 h

## 5.4 Tests for depressive-like behavior

**– Gpm6b null mutants exhibited an increased immobility time in the FST**

Gpm6b null mutants displayed a strong sucrose preference (about 80%) and therefore did not show any significant differences to wildtype mice (Fig. 14a; $p > 0.05$).

In the chimney test (Fig. 14b) the performance of Gpm6b null mutants did not show any differences as compared to the performance of wildtype mice ($p > 0.05$). Both genotypes escaped the tube in less than 30 sec.

The tail suspension test (TST) (Fig. 14c) did not reveal any significant differences between Gpm6b null mutants and wildtype mice. Both genotypes spent the same time immobile during the trial ($p > 0.05$).

In the forced swim test (FST) (Fig. 14d) Gpm6b null mutants spent significantly more time immobile than wildtype mice ($p = 0.033$).

The LABORAS analysis (Fig. 15a - 15e) revealed that there is no difference in the assessed parameters between genotypes. None of the assessed parameters (locomotion, immobility, climbing, eating, grooming) did show any significant difference between wildtype mice and Gpm6b null mutants over the duration of the trial ($p > 0.05$).

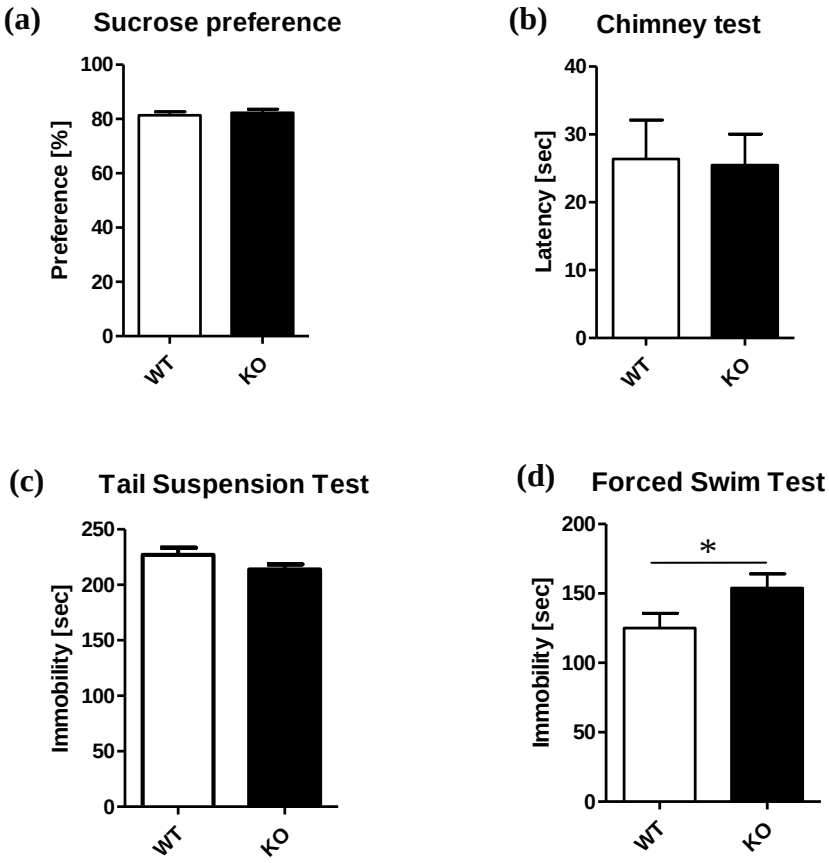



**Figure 14**: The forced swim test revealed a significant difference between genotypes

(a) Preference for sucrose solution (b) Time needed to escape the inverted tube (c) Immobility time in the tail suspension test (d) Immobility time in the forced swim test

(a) n = KO 18; WT 16 (b) n = KO 19; WT 15 (c)+(d) n = KO 16; WT 16

*$p < 0.05$ WT vs. KO

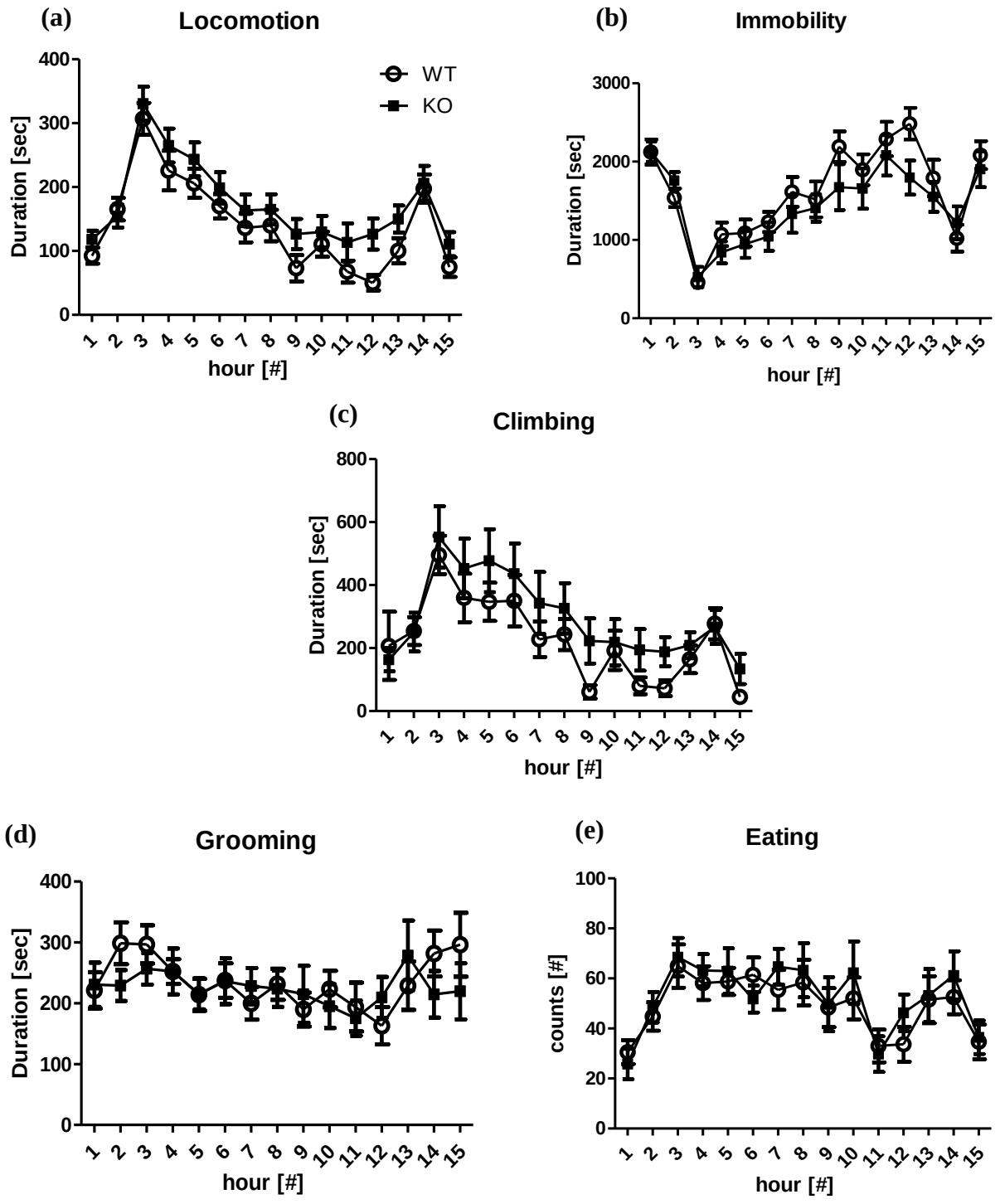



**Figure 15**: LABORAS data assessed over 15 h did not show a genotype difference

Duration of (a) locomotion (b) immobility (c) climbing (d) grooming and (e) eating

KO 17; WT 19

## 5.5 DOI – Project

**- DOI had different effects on locomotion in Gpm6b null mutants and wildtype mice**

### 5.5.1 Pilot experiment

The pilot project in wildtype mice performed in order to identify the appropriate dosage ("threshold dose") of DOI revealed no significant difference between the two used dosages of 0.5 mg/kg and 0.75 mg/kg ($p > 0.05$; Fig. 16a + 16b) with regard to locomotor activity and grooming.

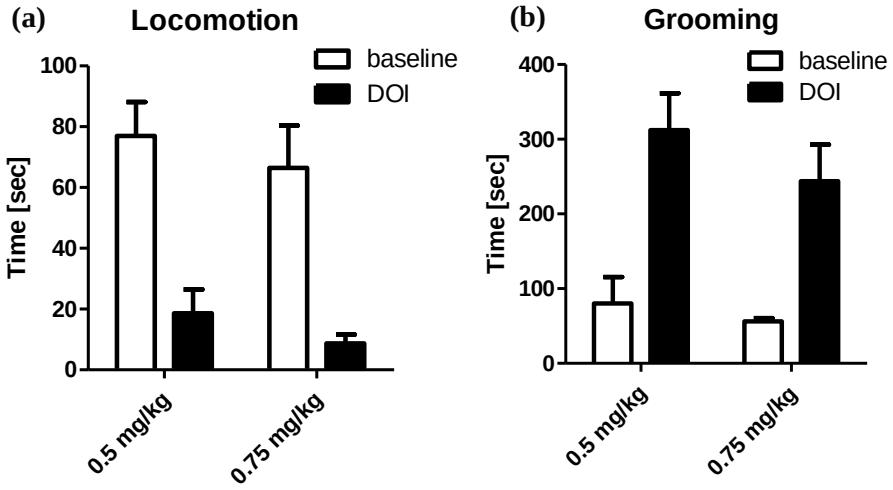



**Figure 16**: Pilot project: Comparison of different doses of DOI in wildtype mice

(a) Locomotor activity and (b) grooming behavior at 0.5mg/kg and 0.75 mg/kg DOI

n = 5 per group

### 5.5.2 Final protocol

In consequence of the results of the pilot project, it was decided to proceed with a dose of 0.5 mg/kg DOI.

The initially assessed baseline of locomotor activity (Fig. 17a) as well as the grooming duration (Fig. 17c) was the same in Gpm6b null mutants compared to wildtype mice.

After the injection of 0.5 mg/kg DOI, the locomotor activity (Fig. 17b) during the first 10 min of the recording was almost identical between genotypes. However, over the course of the following 10 min the locomotor activity of wildtype mice decreased considerably, whereas Gpm6b null mutants remained at a higher level. Only during the last third of the trial the locomotor activity of Gpm6b null mutants decreased slightly.

The observed patterns of locomotor activity result in a significant interaction effect of time and genotype ($p = 0.01$).

Compared to baseline level, the grooming duration after injection of DOI (Fig. 17d) increased during the first 10 min and decreased in both genotypes during the following 10 min, and then remained at this level. There was no difference between genotypes observable ($p > 0.05$).

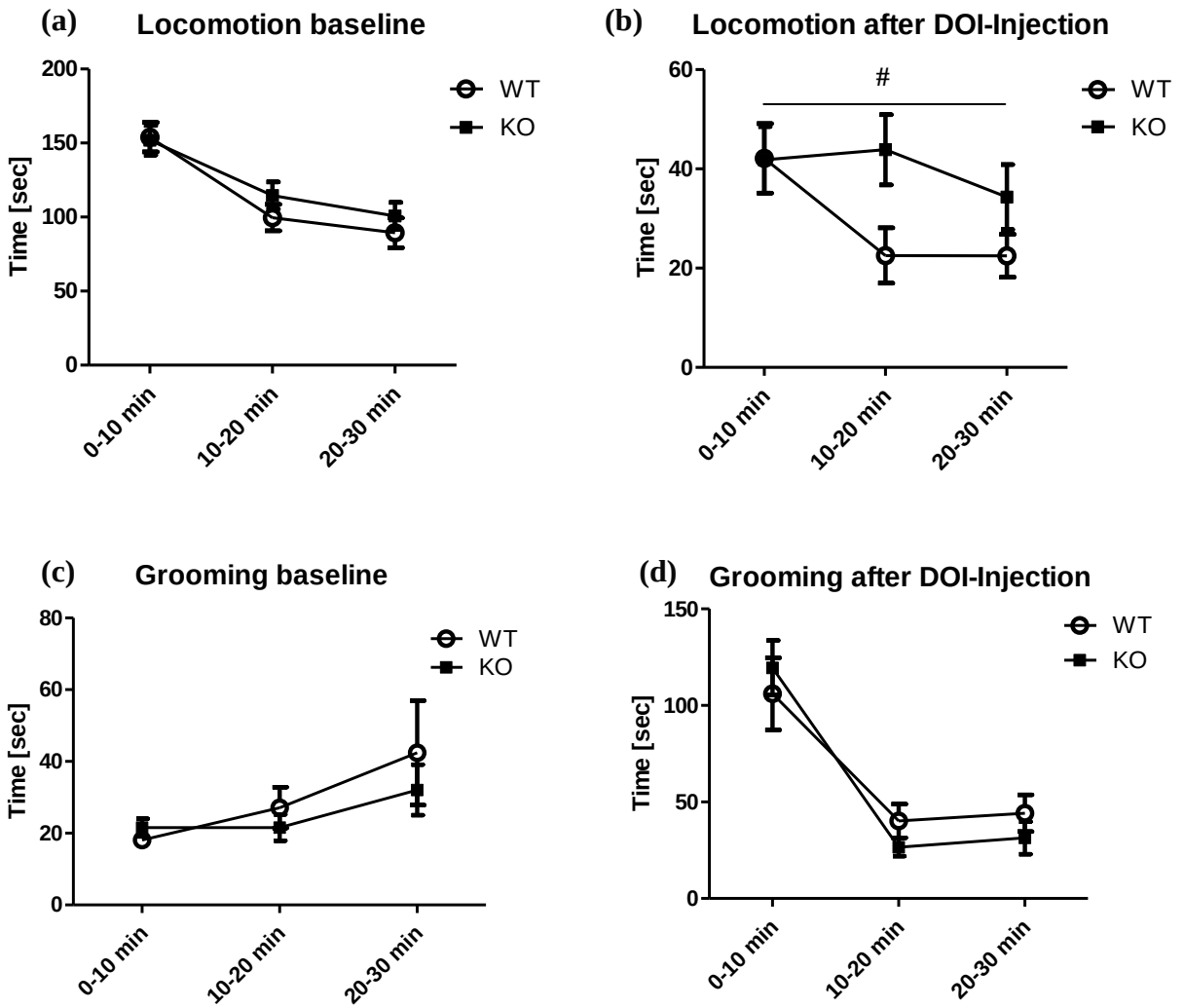



**Figure 17**: Genotype-dependent effects of DOI on locomotion but not on grooming behavior

(a) Time mice spent with locomotion without treatment (b) Time mice spent with locomotion following DOI-administration (c) Time mice spent grooming without treatment (d) Time mice spent grooming following DOI-administration

n = KO 21; WT 17 #$p < 0.05$ interaction effect of time and genotype

## 5.6 LPS - Project

**- LPS reduced locomotor activity in wildtype mice but not in Gpm6b null mutants**

At a dose of 100 µg/kg LPS, neither genotype exhibited reduction in activity 24 h after administration. Additionally, the attempt to enhance the depressive-like phenotype with that dose failed (data not shown). Therefore, it was decided to increase the dose of LPS to 250 µg/kg injected on two consecutive days.

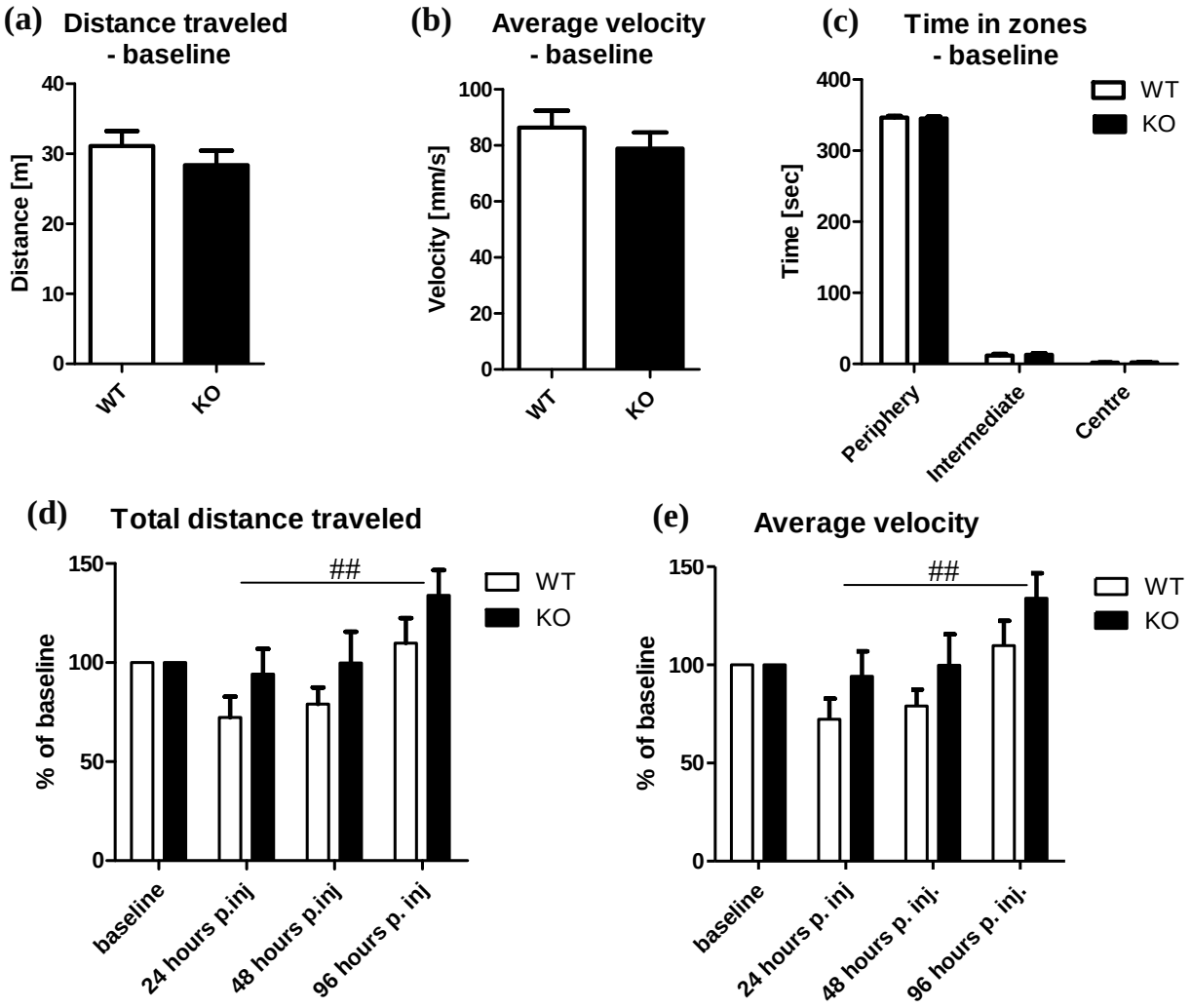
The baseline of the open field activity (Fig. 18a - 18c) before the injection of 250 µg/kg LPS did not show any differences between genotypes ($p > 0.05$).

Over the course of 4 days following LPS treatment (Fig. 18d + 18e), velocity and distance traveled in the open field revealed a significant effect over time ($p = 0.001$) and a genotype effect that shows a trend towards significance ($p = 0.095$).

Posthoc comparisons between activity at baseline level and activity after LPS injection showed differences in the effect of LPS treatment between genotypes (for p-values see table of Fig. 18f): 24 h as well as 48 h after injection, wildtype mice showed a significant reduction in locomotor activity and velocity as compared to baseline level. In contrast, Gpm6b null mutants did not show a decreased activity compared to their baseline level. 96 h after treatment, wildtype mice were back to baseline level, whereas Gpm6b null mutants even showed an elevated activity compared to baseline.

The assessment of the body weight over the whole trial revealed that both genotypes exhibited a similar curve of temporary weight loss (Fig. 19a).

The tests for depression performed after the last assessment of the open field activity at 96 h post LPS treatment, could support the previously observed genotype effect in the FST (Fig. 19c) showing that Gpm6b null mutants spent significantly more time immobile ($p = 0.048$). However, the effect was not enhanced by the treatment of LPS compared to the assessment with untreated mice (Fig. 14d), nor did Gpm6b null mutants develop a depressive-like phenotype in the TST after LPS administration (Fig. 19b; $p > 0.05$).




**(f)**

| | Total distance traveled | | Average velocity | |
|---|---|---|---|---|
| genotype | **WT** | **KO** | **WT** | **KO** |
| baseline vs. 24 h p. inj. | 0.0086 | 0.3605 | 0.0086 | 0.3605 |
| baseline vs. 48 h p. inj. | 0.0083 | 0.6504 | 0.0083 | 0.6504 |
| baseline vs. 96 h p. inj. | 0.966 | 0.0498 | 0.966 | 0.0498 |

**Figure 18**: In the OFT only wildtype mice develop a reduced activity following LPS treatment

(a) – (c): Baseline assessments of (a) Total distance traveled (b) Average velocity (c) Time spent in different zones (d) Total distance traveled and (e) Average velocity at baseline and different time points after LPS injection with baseline equals 100% (f) Comparisons between baseline level and different time points after LPS administration in wildtype mice and Gpm6b knockout mice

n = KO 13; WT 14 ##p < 0.01 time effect (24 h until 96 h after LPS)

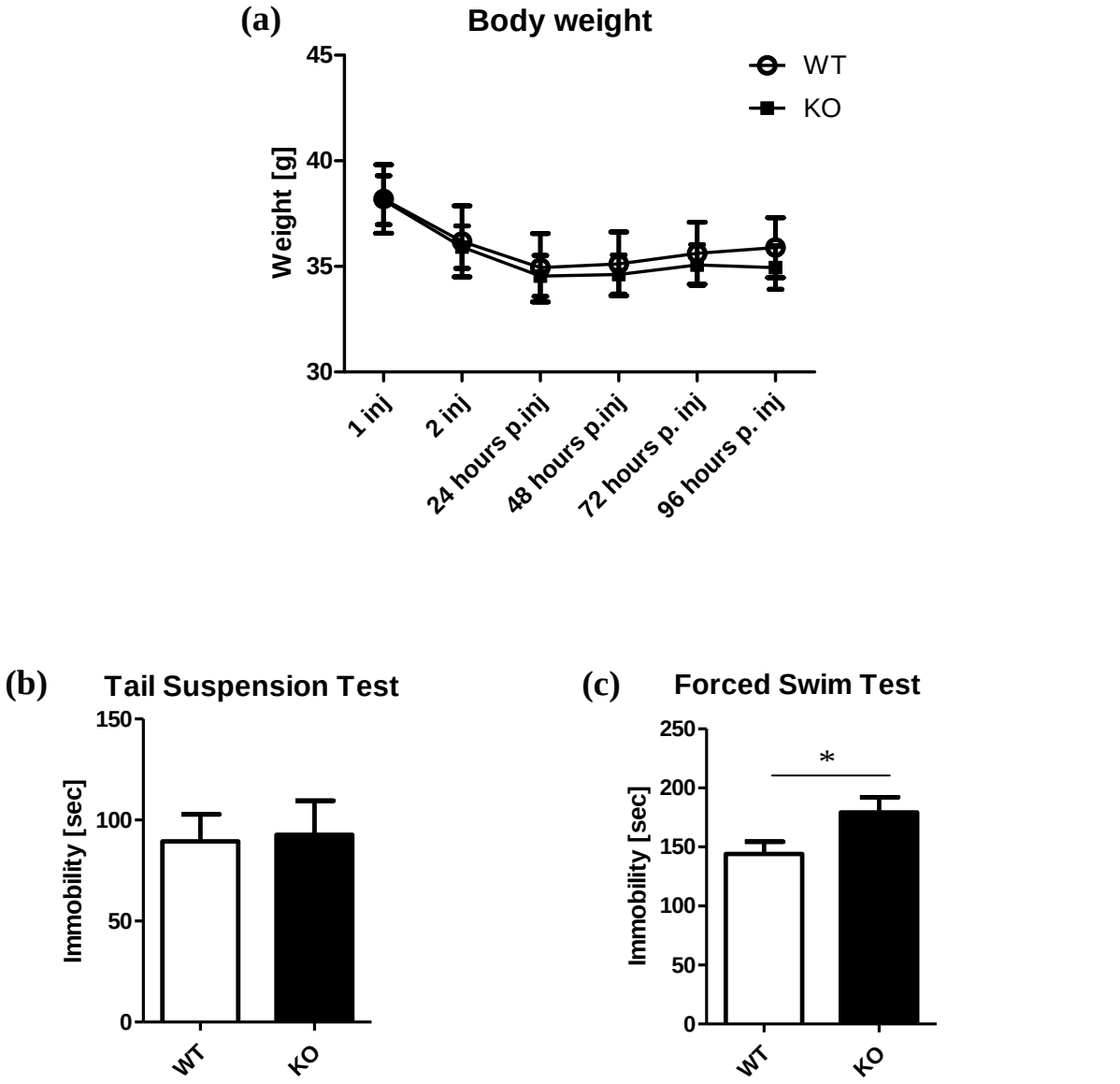



**Figure 19**: Confirmation of the increased immobility in the FST, but no further differences between genotypes

(a) Body weight during LPS experiment (b) Immobility time in the tail suspension test (c) Immobility time in the forced swim test

(a) + (b) n = KO 13; WT 14 (c) n = KO 12; WT 12

*p < 0.05 WT vs. KO

# 6 Discussion

The results of the behavioral testing exhibit distinct differences between Gpm6b null mutants and their wildtype littermates. Gpm6b null mutants showed an increased activity in the elevated plus maze, an impaired prepulse inhibition and a greater immobility time in the forced swim test. There are hints for an impaired extinction of contextual fear memory. Challenge with the serotonin agonist DOI revealed variations in the effect of this drug dependent on the genotype – whereas wildtype mice developed a reduced locomotor activity after injection, Gpm6b null mutants were less susceptible. Also, the injection of LPS caused a reduction of locomotor activity in wildtype mice, but not in Gpm6b null mutants. Altogether, this data underscores a depressive-like phenotype in Gpm6b null mutants, associated with altered serotonergic mechanisms.

## 6.1 Basic functions - Gpm6b null mutants do not have fundamental impairments

It is important to emphasize that Gpm6b null mutants do not suffer from fundamental impairments that could affect the outcome of other tests. Gpm6b null mutants did not show disabilities in terms of vision, olfaction, motor function, hearing or pain sensation compared to wildtype mice and therefore a bias of the testing results can be excluded.

## 6.2 Elevated plus maze - Gpm6b null mutants are prone to enhanced agitation

Even though the results of the EPM might point to a hyperactive phenotype, it is important to note that the EPM test is not a typical test for activity, but for anxiety. Therefore, the increase in velocity and the elevated number of arm entries cannot simply be interpreted as hyperactivity. Instead, it is important to compare the results obtained from the EPM to other tests that assess differences in the activity level such as the OFT, the holeboard test and the Morris water maze test.

Looking at the velocity that the mice displayed when performing the OFT, it is notable that the activity of Gpm6b null mutants also tended to be slightly increased. However, the exploratory activity in Gpm6b null mutants when performing the holeboard test and the

velocity in the Morris water maze test were unchanged, disproving that just a general hyperactive phenotype is present. In fact, the increased activity of Gpm6b null mutants in the EPM may be evoked by the environment of the experimental setting. It is likely that the mice felt irritated by the novelty and complex structure of the EPM and therefore showed an increased agitation. This hypothesis is supported by the fact that the EPM was the first test that was performed and the procedure of being tested was still unfamiliar to the mice. The observed hyperactivity may be due to the higher likelihood of Gpm6b null mutants to become insecure and hence agitated by novel surroundings and is expressed as enhanced activity.

This phenotype can be transferred to human studies, where patients show a higher prevalence of psychomotor and verbal agitation than the healthy control group during depressive episodes (Chung et al., 2011, Judd et al., 2012, Majic et al., 2012).

6.3 Forced swim test - Gpm6b null mutants exhibit depressive-like features

Interestingly, Gpm6b null mutants showed a depressive-like phenotype only in the FST, but not in any other test that is designed to measure depression specifically. It is especially remarkable that the immobility time in the TST did not show any differences, since this test also measures the time mice spent in an unpleasant situation without struggling for escape.

It is preferable to have a model which depressive-like phenotype extents to more than one of the tests, but it is not uncommon to find divergent results in the FST compared to other tests and it can be assumed that the FST and TST do not measure the same processes. Lira et al. (2003) as well as Holmes et al. (2003) found that serotonin transporter knockout mice with a 129S6/SvEv background had an increased immobility in the FST but a decreased immobility in the TST. In contrast, Perona et al. (2008) observed that serotonin transporter knockout mice on a C57BL/6J-129Sv mixed background exhibited a reduced immobility in the TST whereas the FST did not show any differences between genotypes.

These findings point to two conclusions: Firstly, as already described in earlier studies, the mouse strain seems to play an important role and results are not comparable between strains (Bai et al., 2001, Petit-Demouliere et al., 2005). And secondly, the FST and TST cannot be considered as interchangeable but must be seen as two individual readouts (Petit-Demouliere et al., 2005), although research still needs to investigate the specific differences of these paradigms.

Anhedonia, measured as lowered preference for sucrose, was not observed in Gpm6b null mutants, whereas some other studies could link anhedonia to the depressive phenotype of certain mouse strains or treatments. For example, chronic mild stress as well as prenatal stress could be proven to evoke anhedonia in rodents (Harkin et al., 2002, Willner, 2005, Miyagawa et al., 2011) and p11 knockout mice exhibited anhedonia as compared to wildtype mice (Svenningsson et al., 2006).

Taken these studies into account, it can be concluded that mice showing a depressive-like phenotype in one of the tests can be considered as a sufficient model of depression.

## 6.4 Prepulse inhibition - Changes in the serotonergic system may lead to an impaired PPI

Startle, a reaction to a sudden stimulus that can be of tactile, visual or acoustic nature, is characterized by a fast twitch of body and facial muscles, an arrest of ongoing behavior and an acceleration of the heart beat (Koch, 1999). If the startle stimulus is preceded by a low-intensity, non-startling prepulse, a reduction of the startle amplitude is observable. This phenomenon is referred to as prepulse inhibition (PPI) (Hammond et al., 1972). The startle response can be reliably quantified, and by using almost identical stimulus parameters in humans and rodents, it is possible to compare the readouts across species (Swerdlow et al., 1992).

Impairments in PPI, a test for sensorimotor gating, are thought to reflect the loss of the physiologic ability to properly suppress or gate certain irrelevant motor, sensory or cognitive information and are associated with diseases such as schizophrenia, obsessive-compulsive disorder, Tourette´s syndrome or Huntington disease. The patients may experience the impairment in gating as intrusive thoughts, adventitious movements or sensory information (Koch, 1999). According to MacLeod and Mathews (1991), the failure of patients with depression to inhibit intrusive negative thoughts can also be characterized by clinical gating deficits.

Assessing startle response and PPI of Gpm6b null mutants revealed an unaltered startle response but a reduction of PPI compared to wildtype mice.

Reduced PPI is observed under a variety of experimental conditions that influence different neurotransmitter systems in animals (Koch, 1999). In particular, manipulations of the dopaminergic system (Swerdlow et al., 1994), the noradrenergic system (Sallinen et al., 1998) and the glutamatergic system (Mansbach and Geyer, 1989) have been associated with disruptions of PPI. Also, numeral studies focussing on the serotonergic system could demonstrate that this system is linked to the mechanisms of sensorimotor gating. In a study by Fletcher et al. (2001) the tryptophan hydroxylase inhibitor p-chlorophenylalanine (PCPA) was able to decrease PPI without altering basal startle response. In the same study, the authors could show that the 5-$HT_{1A}$ receptor agonist 8-OH-DPAT disrupted PPI in rats and this effect was potentiated when the animals were depleted of serotonin. The 5-$HT_{1A}$ receptor functions as an autoreceptor: Stimulation of the receptor leads to an inhibition of the activity of raphe seroto-

nin neurons and a reduced serotonin release (Hjorth and Sharp, 1991). Therefore, it may be concluded that the effect of 8-OH-DPAT to decrease PPI could be due to reduced serotonin functions (Fletcher et al., 2001).

In contrast, various drugs have been shown to disrupt PPI in rodents via stimulation of serotonin function including the serotonin releasers fenfluramine, p-chloroamphetamine and 3,4-methylenedioxy-methamphetamine (MDMA) (Kehne et al., 1996). Manipulation of multiple serotonin receptor subtypes can influence PPI. Selective blockage of 5-$HT_{2A}$ receptors (Sipes and Geyer, 1995), non-selective blockage of 5-$HT_2$ receptors as well as deletion of 5-$HT_{1B}$ receptors (Dulawa et al., 1997) prevented the disruptive effects of receptor agonists without altering the PPI itself.

Considering these observations it can be postulated that both increases and decreases in serotonin activity disrupt PPI in rodents and that various receptor subtypes play a role in PPI (Fletcher et al., 2001).

Interestingly, it was shown that certain serotonin uptake inhibitors (such as fluoxetine or fluvoxamine) fail to have an effect on PPI in rats (Martinez and Geyer, 1997) or humans (Phillips et al., 2000). This observation indicates that an unphysiological function of the serotonin transporter may not be the only cause of an impaired PPI, but that also other pathways, such as disturbances of the hypothalamic pituitary adrenal (HPA) axis should be considered. As already described previously, disturbances of the HPA axis are thought to be a main characteristic feature of depression (Palazidou, 2012). Dirks and colleagues were able to show that the corticotropin-releasing factor (CRF) may also be involved in alterations of PPI in rodents (Dirks et al., 2002). In their study mice overexpressing CRF showed reduced PPI compared to wildtype controls. Recently, Conti (2012) could additionally reveal the link between CRF levels and the serotonergic system. In Wistar-Kyoto rats, neither CRF infusion nor the 5-$HT_{1A}$ receptor agonist 8-OH-DPAT alone decreased PPI, but the combination of both treatments led to a significant reduction. Similar observations were made when performing the same experiment with Brown Norway rats (Conti, 2012). These findings are supported by the fact that the two CRF receptors (CRF1 and CRF2) are expressed in brain regions known to modulate startle response and prepulse inhibition, such as the basolateral amygdala, frontal cortex and hippocampus (Swerdlow et al., 2001) and there is a projection from

CRF-containing neurons located in the central nucleus of the amygdala to the major dopaminergic, noradrenergic and serotonergic nuclei (for review see Gray, 1993).

Not only animal experiments were performed addressing this subject, but also studies with depressed human patients were conducted. Ludewig and Ludewig (2003) did not find any differences in PPI between depressed patients and healthy controls. In contrast, a study by Perry et al. (2004) assessed PPI in patients suffering from depression or schizophrenia and compared the results to healthy controls. Depressed patients showed PPI levels that were less reduced than the levels of schizophrenic patients, but showed a non-significant tendency towards lower PPI as compared to the control group. The finding by Perry and colleagues is in line with the previously described reduced PPI found in Gpm6b null mutants.

Means and standard deviations for percent prepulse inhibition for schizophrenia (SCZ ) patients ($n = 14$), non-psychotic patients with major depressive disorder (MDD) ($n = 19$), and non-patient comparison (NC) subjects ($n = 13$)

| | PPI at | | |
|---|---|---|---|
| | 30 ms ISI | 60 ms ISI | 120 ms ISI |
| SCZ patients | 14.6 (21.4) | 30.5†* (24.4) | 42.1†* (19.5) |
| MDD patients | 28.7 (19.6) | 54.5 (20.2) | 69.6 (17.6) |
| NC subjects | 20.9 (20.6) | 66.1 (15.8) | 78.6 (12.3) |

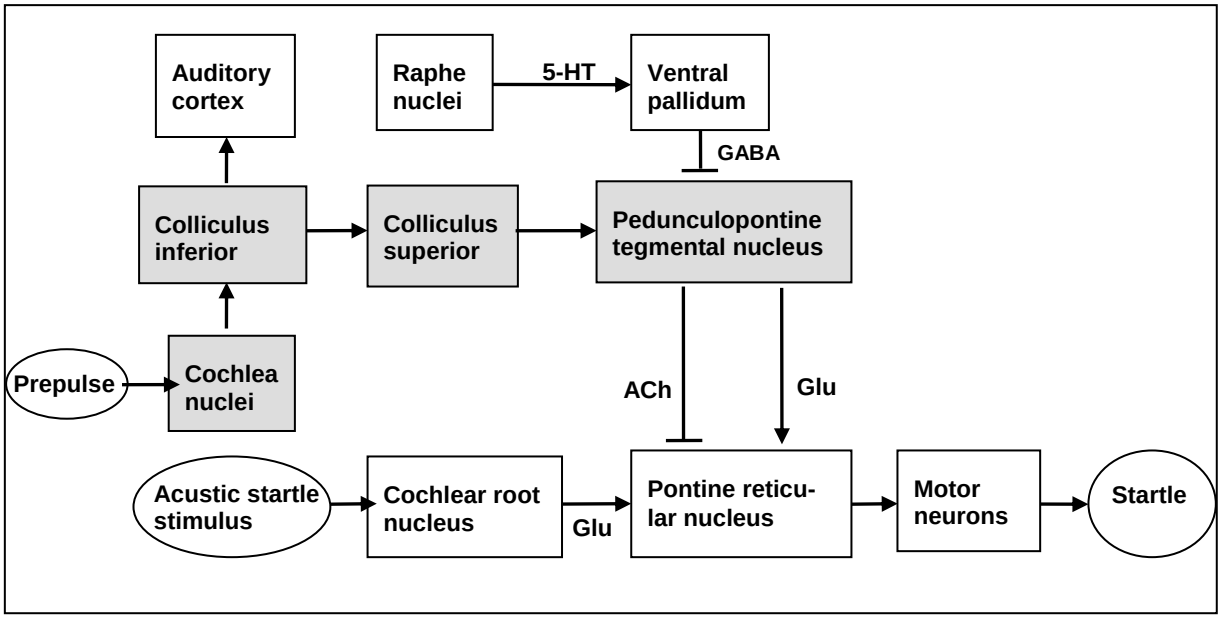
† $P < 0.01$, significantly different from MDD patients. * $P < 0.01$, significantly different from normal comparison subjects.

**Figure 20:** Comparison of PPI results from healthy controls, patients suffering from schizophrenia or depression (Perry et al. (2004))

The underlying mechanisms leading to PPI are still not fully understood (for review see Koch, 1999). The PPI circuit for auditory prepulses is assumingly composed of the lower parts of the ascending auditory system (including cochlea nuclei, superior olivary complex and nuclei of

the lateral lemniscus), the inferior and superior colliculus, and the pedunculopontine tegmental nucleus (PPTg).

The attenuating influence of the prepulse is thought to act at the level of the pontine reticular nucleus (PnC) by inhibitory cholinergic (muscarinergic) input to the PnC (for review see Koch, 1999).




Ach: Acetylcholine; GABA: γ-aminobutyric acid; Glu: glutamate; 5-HT: serotonin

**Figure 21:** Simplified hypothetical circuit of startle response and PPI (mod. from Koch (1999)):

PPI, as a test of sensorimotor gating, may also be reduced when the involved axons and the associated myelin are impaired and M6B has been shown to be associated with the myelination process (Werner et. al., 2001).

M6B constitutes only 0.05% of the total myelin protein and is therefore much less abundant than the most frequently found protein of CNS myelin, the proteolipid protein (PLP) with 15.43% (Werner et al., 2013). The authors could show that M6B, which shares 57% sequence identity with PLP, is associated with the same cholesterol-rich oligodendroglial membrane-microdomains. Even though both proteolipids can bind membrane-cholesterol, whose accumulation in the myelin membrane is critical for normal myelination, it could be

demonstrated that only PLP is efficiently incorporated into mature myelin and therefore eligible to contribute to the high cholesterol content of myelin. Mice lacking M6B or PLP were fully myelinated and myelin assembly was comparable to wildtype mice, whereas mice lacking PLP and M6B revealed severe impairments including hypomyelination and enhanced neurodegeneration, reflected in motor defects such as ataxia, tremor, and spasticity and resulted in premature death at 4-5 months (Werner et al., 2013).

Analyzing myelination with electron microscopy, it was found that the adaxonal glial membrane in mice lacking only M6B appeared occasionally “fused” with the axonal membrane and axons were sometimes double-myelinated and the oligodendroglial plasma were occasionally invaginated into the axon (Werner et al., 2013).

It has to be further investigated whether the interaction of Gpm6b with the serotonin transporter or the involvement of this protein in myelination and hence a possible delay in conduction leads to the observed reduction in PPI.

## 6.5 Fear conditioning and extinction – Hints for a longer lasting memory of aversive events in Gpm6b null mutants

The results of the contextual fear conditioning test with elevated freezing levels of Gpm6b null mutants 48 h after the training indicated a potential impairment of the reduction of aversive memories. It has been shown that patients suffering from depression often have a better memory, especially for negative stimuli, compared to neutral or positive ones (Davidson et al., 2002), although cognitive dysfunction is a common feature of depression (Thomas and O'Brien, 2008). Enhanced fear conditioning or impaired fear extinction are also linked to the development of anxiety disorders, which often co-occur with depressive symptoms and both conditions exhibit a more severe course when expressed simultaneously (Holtzheimer et al., 2005).

Animal studies could support the observations of human samples especially with regard to manipulations of the serotonin transporter: Genetic knockout of the serotonin transporter has been shown to increase anxiety as well as depressive-like behavior in mice (Zhao et al., 2006, Kalueff et al., 2007). Furthermore, Wellmann and colleagues could show that serotonin transporter knockout mice revealed impaired fear extinction and corticolimbic alterations (Wellman et al., 2007). In another study conducted by Yu et al. (2011), mice with depressive-like behavior due to social defeat exhibited an enhanced fear memory when tested 24 h after the training session.

Contextual fear conditioning is mainly a process of hippocampal learning (Tronson et al., 2012) and an impairment of the cognitive capabilities of the hippocampus may also have an effect on the performance in the Morris water maze test as it is used to assess hippocampal learning deficits. However, Gpm6b null mutants did not show any differences in the results of the Morris water maze as compared to wildtype mice. The study mentioned previously by Yu et al. (2011) observed that the enhanced fear memory of socially defeated mice was not associated with impairments in the performance of the Morris water maze. This finding may indicate that the two readouts are not necessarily related.

Fear conditioning is thought to be processed mainly by the hippocampus and the amygdala with the hippocampus being attributed to contextual memory, whereas the amygdala is associated with the processing of the cue and cued memory. During the extinction process,

freezing behavior decreases reflecting new learning about a negative relationship between the context and the foot shock (Tronson et al., 2012). The reduction in freezing levels displays the learning curve of the mice that a previously threatening stimulus is now regarded as safe (Bouton, 2004). During extinction, the infralimbic medial prefrontal cortex (PFC) is involved in this circuit by inhibiting the amygdala (Tronson et al., 2012).

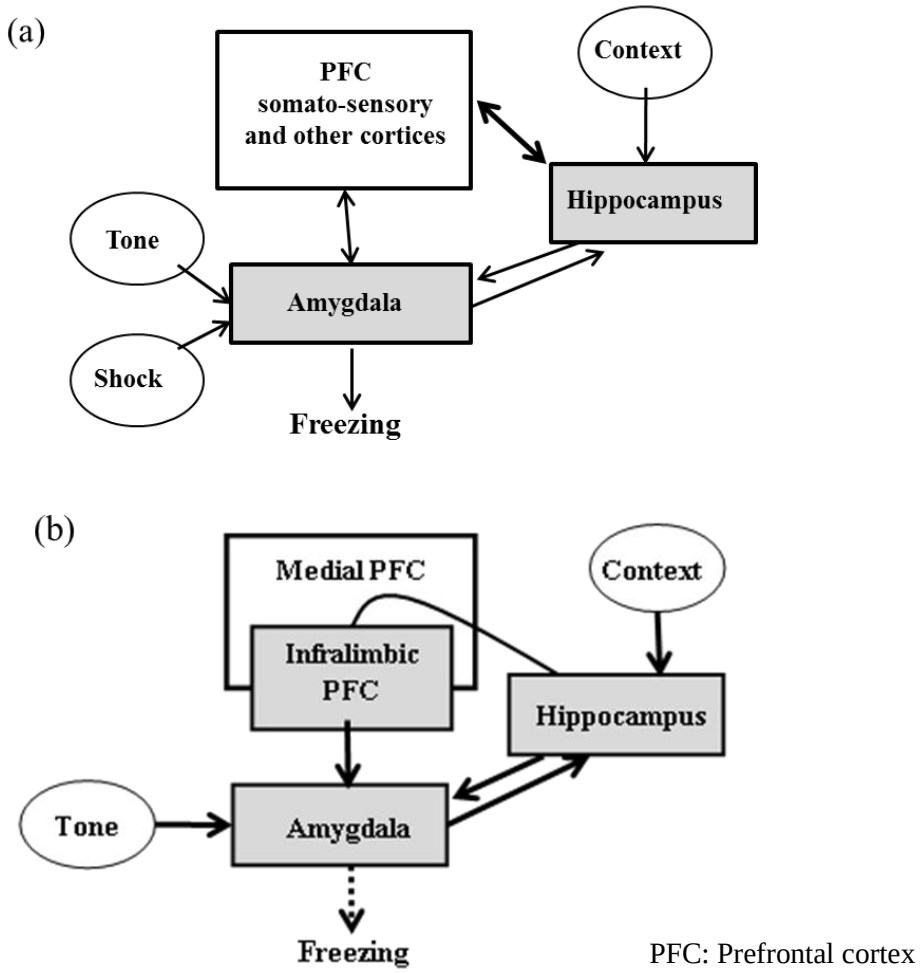



**Figure 22:** (a) Fear condition circuit (b) Extinction process
(mod. from Tronson et al. (2012))

The extinction curve assessed with Gpm6b null mutants and wildtype mice did not mirror the results of the previously obtained fear conditioning paradigm. Both genotypes displayed the same pattern of fear extinction and further discussion is required to investigate the differences: It is possible that the time period between the training session and the first exposure to the same context makes a difference regarding fear extinction. For the initial fear conditioning test, the time interval had been 48 h, whereas the first exposure for the extinction protocol was conducted 24 h after training. Interestingly, Gpm6b null mutants displayed a similar amount of freezing time independently of the time passed, whereas the wildtype littermates revealed a reduction of freezing behavior when the training-testing interval is greater. Therefore, it may be postulated that Gpm6b null mutants have a longer lasting memory of the aversive event when not exposed to the same context in the intervening time.

Unfortunately, all accessible extinction protocols started testing the mice at a 24 h interval after the training session and therefore it is not possible to compare the findings. It would be interesting to investigate if a new cohort tested initially at 48 h after training can support the previous finding and possibly reveal additional alterations in the extinction curve.

The discrepancies in the results between fear conditioning testing and fear extinction may be the history of the different cohorts used influencing the outcome. Even though the mice were about the same age when tested (6-7 months), the first cohort had already absolved more experiments before performing the fear conditioning test. It should be considered that the previous testing associated with cognitive challenges as well as stress and other factors has caused changes in the brain that subsequently affected fear conditioning and extinction. To eliminate this bias in future research, mice should not have been tested in other tests prior to the assessment of the fear conditioning and extinction data.

6.6 DOI – Project - DOI effects are dependent on the serotonin levels

DOI, a potent 5-$HT_2$ agonist, evokes different responses in mice (for review see Canal and Morgan, 2012). The head-twitch response (HTR) as a fast retraction of the neck muscles is thought to be mainly mediated by activation of $5HT_{2A}$ receptors, whereas the ear-scratch response (ESR) that is described as "rapid scratching movement of the head, neck, or lateral area by either hind limb" (Darmani et al., 1996) is assumed to be provoked mainly by 5-$HT_{2C}$ receptors (Darmani and Gerdes, 1995). There are several other behavioral responses to serotonergic compounds classified as the "serotonin syndrome" and contain behavioral readouts such as flat body posture, forepaw treading, hind limb abduction, lower lip retraction or backwards walking (Canal and Morgan, 2012). However, these observations are primarily induced by agonists at 5-$HT_1$ receptor subtypes (Tricklebank et al., 1984).

DOI also has an effect on locomotor activity, although the changes in the activity level do not seem to be consistent among species. Several studies could show the inhibitory effect of small doses of DOI (e.g. 0.27 mg/kg) in rats on locomotion; an effect that could be antagonized by 5-$HT_{2A}$ receptor antagonists (Wing et al., 1990). A study with the least shrew could reveal that DOI at relatively higher doses (1.25 – 2.5 mg/kg) significantly reduced locomotor activity (Darmani et al., 1994), whereas the same author observed that similar doses of DOI (1 – 2.5 mg/kg) were able to significantly enhance locomotor activity in mice (Darmani et al., 1996).

The relatively low dose (0.5 mg/kg) that was used to compare the effects between Gpm6b null mutants and wildtype mice led to a significant interaction effect of time and genotype with a reduction in locomotor activity that was more pronounced in wildtype mice. This result seems to be contrary to the findings of Halberstadt et al. (2009). In the study, the authors describe that DOI produced an inverted U-shaped dose-response function in male C57BL/6J mice. Lower doses of 0.625 – 5 mg/kg DOI increased the locomotor activity in these mice and only doses higher than 10 mg/kg caused a reduction in locomotion (Halberstadt et al., 2009).

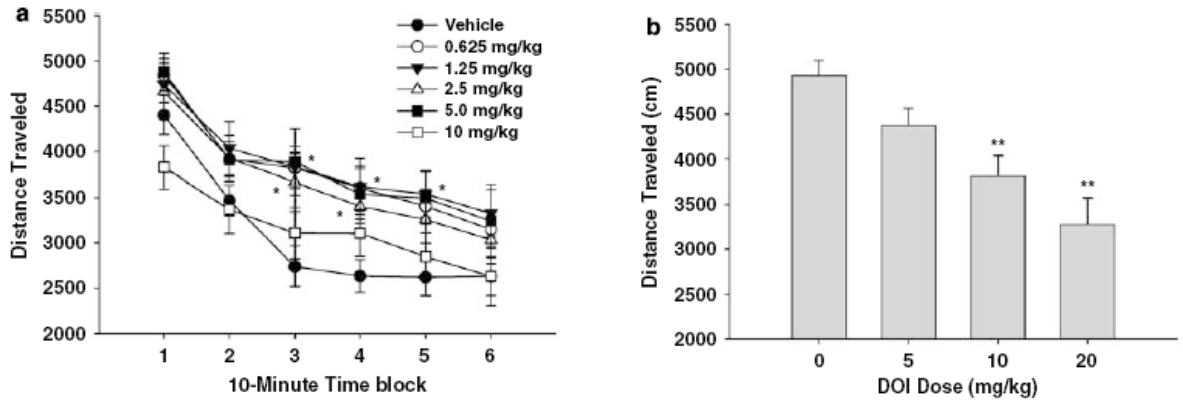



**Figure 23**: Effects of DOI on locomotor activity (a) Dose response of DOI effects on distance traveled (in cm). Mice used were male C57BL/6J. Data are mean ±SEM. *p < 0.05, Dunnett's test vs. vehicle control. (b) The effects of high doses of DOI on locomotor activity were evaluated in a second dose-response experiment. Data shown are distance traveled (cm) during the first 10 min of testing. Data are mean ±SEM. *p < 0.05, Dunnett's test vs. vehicle control (Figures from Halberstadt et al. (2009))

Halberstadt et al. (2009) could additionally demonstrate that the enhancement of locomotor activity provoked by a dose of 1 mg/kg DOI was absent in 5-$HT_{2A}$ receptor knockout mice and that the reduction of activity induced by 10 mg/kg was potentiated in 5-$HT_{2A}$ knockout mice and attenuated when the mice were pretreated with the selective 5-$HT_{2C/2B}$ antagonist SER-082. This finding indicates that the 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors provoke opposing effects with 5-$HT_{2A}$ receptors (assumed to be primarily targeted by lower doses of DOI) leading to an increase in locomotor activity and 5-$HT_{2C}$ receptors (assumed to be primarily targeted by higher doses of DOI) leading to a reduction of locomotor activity (Halberstadt et al., 2009). The authors could support this result by proving that the selective 5-$HT_{2C}$ receptor agonist WAY 161.503 reduces locomotion levels in these mice and that this effect is potentiated in 5-$HT_{2A}$ knockout mice (Halberstadt et al., 2009).

Unfortunately Halberstadt and colleagues did not mention the exact age of the tested mice in their publication. This would have been important information since it has been assumed that the effects of DOI are also age dependent.

Another study tested male and female ICR mice across a wide age range investigating the effects of DOI on HTR, ESR and locomotor activity (Darmani et al., 1996). The authors could show that DOI (1 mg/kg and 2.5 mg/kg) was able to increase the locomotor activity compared

to vehicle control mice with the maximal locomotor activity seen between the age of 28-35 days. Following that peak, the locomotor activity subsided in a pattern that was similar to the one observed in vehicle control mice (Darmani et al., 1996). The age of onset of HTR as well as ESR occurred about 2-3 weeks postpartum with maximal HTR on 28 days of age and maximal ESR frequency between the age of 22-35 days. Following that peak the number of HTR tended to decrease with increasing age without reaching significance, whereas ESR frequency decreased significantly till day 180 where no difference to vehicle control animals was observable anymore (Darmani et al., 1996).

Interestingly, it has been shown that 5-$HT_{2A}$ receptor density levels decrease with increasing age in both rodents and humans (for review see Canal and Morgan, 2012). This finding could explain the reduction of locomotor activity in the mice described in this thesis (which were about 7 months old at the time of testing), whereas most other authors describe an increase of locomotor activity at similar low doses. One possible explanation for the discrepancy could be that the reduction of 5-$HT_{2A}$ receptors in older mice causes DOI to evoke mainly 5-$HT_{2C}$ related effects and therefore a reduction of locomotion levels even though relative low doses (such as the used dose of 0.5 mg/kg) are generally associated with an increased locomotor activity (e.g. Darmani et al., 1996, Halberstadt et al., 2009). This explanation is supported by the study from Halberstadt et al. (2009), in which the locomotor-decreasing effect of 10 mg/kg DOI is potentiated in 5-$HT_{2A}$ knockout mice, indicating that this receptor serves to mask the locomotor-reducing effects that the 5-$HT_{2C}$ receptor mediates when targeted by high doses of DOI.

No differences were found in grooming activity, which is consistent with the finding from Darmani et al. (1996) that ESR was completely absent at the age of 180 days and in general a dose of at least 1 mg/kg DOI was needed to induce robust ESR (Darmani and Gerdes, 1995).

Based on video recordings, it was not possible to reliably rate HTR neither in Gpm6b null mutants nor in wildtype mice after injection of 0.5 mg/kg DOI. This may be due to insufficient screen resolution that lacks details which are necessary to detect HTR or to the lack of experience of the observer. However, it is more likely that the mice did not exhibit any HTR. As mentioned earlier, HTR are mediated by 5-$HT_{2A}$ receptors (Darmani et al., 1996) and taking into account that with increasing age the number of this particular receptor is reduced,

it can be assumed that DOI failed to provoke HTR due to a lack of mediating receptors. Additionally, the dose of 0.5 mg/kg is quite low compared to the dose used by other authors.

Darmani et al. (1996) tested mice that were injected with a dose of 1 mg/kg respectively 2.5 mg/kg DOI. Canal et al. (2010) observed a maximal HTR in 8-week-old mice when injected with 0.8 mg/kg DOI and Weiss et al. (2003) described substantial but submaximal HTR in 3-months-old-mice at dosages of 0.75-1 mg/kg.

For testing Gpm6b null mutants, a relatively low dose was used with the intention to find a threshold dose of DOI that has an effect on wildtype mice but not on Gpm6b null mutants. This difference was successfully proven by the interaction effect in terms of the locomotor activity. Gpm6b null mutants were less susceptible to the reducing effects of DOI on locomotor activity compared to wildtype mice. This finding is in line with the postulation that Gpm6b null mutants may have a lack of serotonin in the synaptic cleft and therefore more DOI is tolerated before behavioral effects are observable.

It is possible that alterations of the behavioral pattern in Gpm6b null mutants after DOI injection are not due to the serotonin levels directly but to adaptations of the serotonergic system, e.g. changes in the receptor density. It has not been investigated yet if Gpm6b null mutants have alterations regarding the receptor numbers. According to Fox et al. (2007), serotonin transporter knockout mice that also serve as a model of depression due to altered serotonin levels (Zhao et al., 2006, Kalueff et al., 2007, Wellman et al., 2007), exhibit only modest changes in 5-$HT_{2A}$ and 5-$HT_{2C}$ binding sites, whereas another study with serotonin transporter knockout mice revealed alterations of density of both receptor subtypes in a brain region-specific manner (Li et al., 2003).

In summary, Gpm6b null mutants exhibit a different reaction to DOI administration compared to wildtype littermates. It can be postulated that a higher dose of DOI may have been able to provoke HTR, but this gain would be potentially associated with a loss of the readout for locomotor activity. Also, a higher dose may veil the differences between genotypes.

## 6.7 LPS – Project – Gpm6b null mutants did not develop a reduced locomotor activity

When assessing the effects of LPS on mouse behavior, it is mandatory to distinguish between the terms sickness behavior and depressive-like behavior.

Lipopolysaccharide (LPS) presents a typical molecular pattern that is eligible to provoke sickness behavior due to a systemic inflammation in the body. LPS is a fragment of gram negative bacteria and binds to toll-like receptor-4 on monocytes and macrophages which activates a complex intracellular signalling cascade (Dantzer, 2004). Systemic administration of LPS induces the expression of pro-inflammatory cytokine mRNAs and proteins in the brain and leads to typical behavioral changes associated with sickness such as social withdrawal, reduced motor activity, altered cognition, decreased food and water intake as well as a hunched posture (Dantzer et al., 2008).

The severity of the effects caused by LPS is highly associated with the age of the animals. It could be demonstrated that the lethal dose (LD) where 50% of the 6-7 weeks old mice died equalled 25.6 mg/kg, whereas the LD50 for mice that ranged from 98-102 weeks of age was only 1.6 mg/kg (Tateda et al., 1996).

Depressive-like behavior is thought to occur subsequently to sickness behavior, but since depressive-like behavior shares several features with sickness behavior, such as anorexia, cognitive changes, and mood changes, it can be a confounding factor (Moreau et al., 2008). It cannot be generalized at which time point sickness ends and depression starts. This is mainly due to the different protocols, strains, and age ranges various authors used, which make the results barely comparable. For example, Frenois et al. (2007) postulates that sickness behavior after a single i.p. injection of 830 µg/kg LPS in 8 week old mice is already negligible at 24 h post injection, whereas Godbout et al. (2008) found that at a dose of 330 µg/kg that was injected to 3-6 months old mice provoked sickness behavior that lasted up to 48 h. Various studies measured the immobility time in the FST or TST shortly after LPS-injection and therefore assumingly assessed the sickness behavior rather than the depressive-like behavior as claimed (e.g. Dunn and Swiergiel, 2005, de Paiva et al., 2010).

The pathways through which LPS is thought to evoke depressive-like behavior have already been discussed in the introductory section. In summary, the increased release of cytokines is

assumed to activate the enzyme IDO which causes a degradation of tryptophan down the kynurenine pathway instead of synthesizing serotonin (Dantzer et al., 2008). Additionally, cytokines alter the negative feedback mechanism of cortisol (Zunszain et al., 2012), which leads to elevated plasma levels of cortisol and ACTH and cytokines are able to activate the serotonin transporter over MAPK p38 (Zhu et al., 2010).

At a dose of 250 µg/kg injected on two consecutive days, sickness behavior was observable shortly after injection as hunched posture and extremely reduced locomotion.

In order to evaluate if sickness behavior, measured as reduced locomotor activity, is still present at various time points after LPS injection, mice were tested in the open field test at 24 h, 48 h and 96 h after treatment. This way it was hoped to circumvent the bias of confounding sickness – and depressive-like behavior. With this higher dosage, wildtype mice showed reduced motor activity when tested in the open field 24 h and 48 h after the second injection and the activity level was back to baseline at 96 h post injection. In contrast, Gpm6b null mutants did not reveal a reduced locomotor activity in either of the trials. This leads to the thought that Gpm6b null mutants have a mechanism that prevents them from exhibiting a decreased activity.

One hypothesis for this finding is that reduced serotonin levels are the determining factor. In both genotypes the induction of inflammatory processes possibly caused a reduction of extracellular serotonin. The remaining serotonin could bind on 5-$HT_{2C}$ receptors and lead to a decrease in the activity level similar to the observations made after the injection of the serotonin receptor agonist DOI. Hypothetically, wildtype mice had still more serotonin available compared to Gpm6b null mutants and therefore the effects of the LPS injection were more pronounced in wildtype mice. In contrast, Gpm6b null mutants did not show a significant reduction of locomotor activity.

The reduction of the body weight displayed a very similar curve in the genotypes suggesting that the LPS indeed affected both genotypes in a similar manner and that the differences are restricted to locomotor activity.

Another possible explanation (purely hypothetical at this point) is that different corticosterone levels contribute to the altered behavioral readouts of Gpm6b null mutants and wildtype mice. Taking the cytokine hypothesis of depression into consideration it may be postulated that

Gpm6b null mutants have increased levels of corticosterone (which functions are comparable to human cortisol) that are multiplied by LPS injection, whereas wildtype mice do not have increased basal corticosterone levels. It is known that glucocorticoids are potent inhibitors of many inflammation-associated processes and thought to upregulate anti-inflammatory mediators (van der Velden, 1998). Consequently, the inflammatory response to LPS injection is less distinct in Gpm6b null mutants than in wildtype mice due to the inflammatory suppressing effects of elevated corticosterone levels. But postulating this, Gpm6b null mutants could be expected to have a less severe loss of body weight compared to wildtype mice, which was not observable in this study.

Gpm6b null mutants revealed an increased activity in the open field test 96 h after LPS injection compared to baseline assessment. An increased activity in the open field test after injection of LPS could also be demonstrated by Zhu et al. (2010). At a dose of 200 µg/kg LPS, mice showed an increased activity in the open field test 24 h after injection compared to saline-injected mice. Why the activity level increases at that time point must be studied. It is possible that over-compensatory mechanisms cause that appearance, but the exact pathways still have to be investigated.

In summary, the pharmacological challenge with LPS did not provoke an enhancement of the depressive-like phenotype in Gpm6b null mutants, but LPS had different effects depending on the genotype.

# 7 Conclusion and perspective

Testing of Gpm6b null mutants provided valuable new insights into this mouse strain and its relation to the serotonergic system, with the possibility to use the strain as a mouse model of depression. Behavioral differences in Gpm6b null mutants compared to their wildtype littermates were found in the elevated plus maze, prepulse inhibition, fear conditioning, and forced swim test. The responses to DOI and LPS differed between genotypes supporting an involvement of serotonergic pathways. The majority of the conducted tests did not reveal differences in the behavior, which makes the results of significant outcomes even more distinct as confounding factors are negligible.

By testing a large number of animals in various tests it was possible to characterize this mouse strain in a very comprehensive manner and demonstrate its qualification to serve as a mouse model of depression. However, research should not cease at this point, but further pursue that path and extent the knowledge about this interesting mouse strain.

Pharmacological validity of this model should be evaluated by investigating whether the depressive-like phenotype of Gpm6b null mutants is reversible by administration of serotonin re-uptake inhibitors. Furthermore, data from female Gpm6b null mutants should be obtained and compared to the tested males. Another interesting approach would be to investigate whether it is possible to enhance the depressive-like phenotype of these mice by using paradigms such as learned helplessness, chronic stress or maternal deprivation models.

Additional molecular studies should be conducted in order to investigate the amount of serotonin in the synaptic cleft, serotonin transporter density, and detect potential alterations of serotonin receptors. Finally, human data should be added to the behavioral results of these mice to support the association of the glycoprotein M6B with depression in human patients.

# 8 Summary

Caroline Ritter: **Comprehensive behavioral characterization of Gpm6b null mutants as a mouse model of depression**

In this thesis male Gpm6b null mutants on a C57BL/6J background were characterized in various behavioral test settings. Gpm6b null mutants, as yet an entirely uncharacterized mouse strain, are lacking the glycoprotein M6B that inhibits the serotonin transporter surface expression. Knockout of the Gpm6b gene may lead to a depressive-like phenotype in these mice due to a surface overexpression of serotonin transporters and hence a more efficient clearing of serotonin from the synaptic cleft.

The performed tests could show distinct changes in the behavior of Gpm6b null mutants making this strain a promising mouse model of depression: Gpm6b null mutants demonstrated an increased velocity in the elevated plus maze test interpreted as mild hyperactivity similar to agitation seen in human patients suffering from depression. Gpm6b null mutants revealed an increase in immobility time in the forced swim test - a test that is typically used to assess depressive-like behavior in rodents. Hints for reduced contextual fear extinction were observable in these mice suggesting an impaired extinction of aversive memories. Additionally, an impaired PPI would be in line with the involvement of the serotonergic system.

The i.p.-injection of the 5-$HT_{2A/2C}$ receptor agonist (±)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI) at a dose of 0.5 mg/kg demonstrated the divergent effects of the drug depending on the genotype: Whereas the administration of DOI caused a reduction of locomotor activity in wildtype mice after a short delay, Gpm6b null mutants were less susceptible, consistent with less serotonin in the synaptic cleft. Furthermore, the injection of the pro-inflammatory substance lipopolysaccharide (250 µg/kg on two consecutive days, i.p) to wildtype mice and Gpm6b null mutants revealed that Gpm6b null mutants did not exhibit the temporarily reduced locomotor activity found in wildtype mice.

To conclude, behavioral tests revealed a mild depressive-like phenotype in Gpm6b null mutants. Pharmacological challenges (DOI, LPS) support an involvement of the serotonergic system in the observed phenotype.

# 9 Zusammenfassung

Caroline Ritter: **Umfangreiche Verhaltenscharakterisierung von Gpm6b-Nullmutanten als Mausmodell für Depression**

Im Rahmen der Dissertation wurden männliche Gpm6b-Nullmutanten vom Typ C57BL/6J in zahlreichen Verhaltenstests charakterisiert. Bei den beschriebenen Tieren handelt es sich um eine genetisch entwickelte Mauslinie, der das Glykoprotein M6B (Gpm6b) fehlt. M6B reduziert unter physiologischen Bedingungen die Oberflächenexpression des Serotonintransporters. Hypothetisch kommt es somit bei Gpm6b-Nullmutanten zu einer Überexprimierung des Serotonintransporters und daher zu einer effizienteren Wiederaufnahme von Serotonin aus dem synaptischen Spalt. Es wird postuliert, dass *knockout* des Gpm6b-Genes auf diese Weise zu der Ausprägung eines depressions-ähnlichen Phänotyps der Mäuse führt.

Bei den durchgeführten Tests konnten deutliche Unterschiede im Verhalten der Gpm6b-Nullmutanten im Vergleich zu Wildtyp-Mäusen nachgewiesen werden, was diese Mauslinie zu einem vielversprechenen Model für Depression macht.

Gpm6b-Nullmutanten zeigten eine erhöhte Aktivität im *elevated plus maze*, welche als ein Zustand der Agitation interpretiert werden kann, wie dieser auch teilweise bei depressiven Patienten beobachtet wird. Zusätzlich zeigten Gpm6b-Nullmutanten eine erhöhte Immobilitätszeit im *forced swim test* – einem Test, welcher üblicherweise genutzt wird um depressions-ähnliches Verhalten bei Mäusen und Ratten zu beurteilen. Im *fear conditioning test* konnten Hinweise auf eine reduzierte Extinktion aversiver Erlebnisse aufgezeigt werden und die beobachtete gestörte *prepulse*-Inhibition der Gpm6b-Nullmutanten spricht für eine Beteiligung des serotonergen Systems.

Um die zu Grunde liegenden Abweichungen im serotonergen System zu bestätigen, wurde den Mäusen der 5-$HT_{2A/2C}$ Rezeptoragonist (±)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI) intraperitoneal (i.p.) in einer Dosis von 0.5 mg/kg injiziert. Dies führte bei den Wildtyp-Mäusen zu einer deutlichen Abnahme der Lokomotion nach einer kurzen Latenzzeit, während die Gpm6b-Nullmutanten weniger ausgeprägt reagierten, übereinstimmend mit einem reduzierten Serotoningehalt im synaptischen Spalt.

Auch die i.p.-Injektion der pro-inflammatorischen Substanz Lipopolysaccharid (LPS), injiziert in einer Dosis von 250 µg/kg an zwei aufeinander folgenden Tagen, führte bei den Gpm6b-Nullmutanten nicht zu einer zeitweise reduzierten Aktivität wie sie bei den Wildtyp-Mäusen beobachtet werden konnte.

Zusammenfassend lässt sich festhalten, dass die durchgeführten Verhaltenstests einen milden depressiven Phänotyp der Gpm6b-Nullmutanten aufzeigen konnten. Pharmakologische Herausforderungen (DOI, LPS) unterstützen eine Beteiligung des serotonergen Systems an dem beobachteten Phänotyp.

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# 12 Appendix

## 12.1 List of abbreviations

| | |
|---|---|
| % | Percentage |
| 5-HT | 5-hydroxytryptamine = serotonin |
| 5-HTTLPR | Serotonin transporter gene linked polymorphic region |
| Ach | Acetylcholine |
| ACTH | Adrenocorticotropic hormone |
| ANOVA | Analysis of variance |
| bp | Base pair |
| CNS | Central nervous system |
| CRH | Corticotropin-releasing hormone |
| dB | Decibel |
| DOI | (±)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane |
| DSM-IV-TR | Diagnostic and Statistical Manual of Mental Disorders, 4$^{th}$ edition, textrevision |
| *E. coli* | Escherichia coli |
| et al. | Et alii |
| EPM | Elevated plus maze |
| ESR | Ear-scratch response |
| Fig. | Figure |
| FST | Forced swim test |
| g | Gram |
| GABA | γ-aminobutyrid acid |
| Glu | Glutamate |

| | |
|---|---|
| Gpm6b | Glycoprotein M6B |
| h | Hour; hours |
| HEK-MSR-293 | Human embryonic kidney-293 |
| HPA axis | Hypothalamo-pituitary-adrenal axis |
| HTR | Head-twitch response |
| ICSS | Intracranial self-stimulation |
| IDO | Indoleamine 2,3 – dioxygenase |
| i.e. | Id est |
| i.p. | Intraperitoneal; intraperitoneally |
| IFN-α | Interferon-alpha |
| IL-1ß | Interleukin-1-beta |
| IL-6 | Interleukin-6 |
| KAT | Kynurenine aminotransferase |
| kHz | Kilohertz |
| kg | Kilogram |
| KMO | Kynurenine 3–monooxygenase |
| KO | Knockout mice |
| KYNA | Kynurenic acid |
| KYNU | Kynureninase |
| LPS | Lipopolysaccharide |
| m | Meter |
| mA | Millampere |
| MAPK p 38 | Mitogen activated proteinkinase p 38 |
| MDMA | 3,4-methylenedioxy-methamphetamine |
| min | Minute; minutes |

| | |
|---|---|
| ml | Milliliter |
| mod. | Modified |
| mm | Millimeter |
| ms | Millisecond; milliseconds |
| NMDA | N-methyl-D-aspartate |
| OFT | Open field test |
| pcDNA3 | Mammalian expression vector |
| PCR | Polymerase chain reaction |
| PET | Positron emission tomography |
| PFC | Prefrontal cortex |
| PLP | Proteolipid protein |
| PnC | Pontine reticular nucleus |
| PPI | Prepulse inhibiton |
| PPTg | Pedunculopontine tegmental nucleus |
| QUIN | Quinolinic acid |
| sec | Second; seconds |
| SERT | Serotonin transporter |
| SSRIs | Selective serotonin re-uptake inhibitors |
| TNF-α | Tumor necrosis factor-alpha |
| TPH | Tryptophan hydroxylase |
| TST | Tail suspension test |
| µg | Mikrogram |
| µl | Mikroliter |
| vs. | Versus |
| WT | Wildtype mice |