University of Oldenburg

Zebrafish Model of Schizophrenia

Author:
Elena Richert
B.Sc. Psychology
Student ID: 4434927

First Examiner:
Prof. Dr. Karl Ægir Karlsson
Department of Biomedical Engineering
School of Technology
Reykjavik University
Menntavegur 1
102 Reykjavik
Iceland

Second Examiner:
Prof. Dr. Christiane Thiel
Department of Psychology
Faculty of Medicine and Health Sciences
University of Oldenburg
Ammerländer Heerstraße 114-118
26129 Oldenburg
Germany

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Abstract

Background and Objective: Schizophrenia is a heterogeneous, severe psychiatric disorder with a poor life outcome, unsatisfactory treatment options, and poorly understood etiology. With its high heritability, genetic models are a suitable tool for studying this disorder. However, already available animal models do not cover the broad range of schizophrenia symptoms and are not disorder-specific.

Recently, a novel high-risk gene (*RBM12*) for schizophrenia had been identified (Steinberg et al., 2017). Using CRISPR/CAS9, three *RBM12* zebrafish mutant strains were created. This thesis aimed to validate a schizophrenia-like phenotype in these *RBM12* mutants.

Methods: The behavior of mutated larval and adult zebrafish was assessed using assays of thigmotaxis, novel tank test, velocity, and sleep. The explored behavioral parameters were based on frequent human symptoms in schizophrenia, including anxiety, positive symptoms, and sleep disturbances. Furthermore, it was attempted to rescue the phenotype with the commonly prescribed antipsychotics haloperidol, chlorpromazine, and the anxiolytic diazepam.

Results: The RBM12 mutants exhibited to some extent anxiety-like behavior and fragmented sleep, which could be rescued with antipsychotics and diazepam. The strains differed from each other in symptom type and severity.

Conclusion: It can be concluded that *RBM12* transgenic zebrafish show at least a partial symptomatology consistent with humans with schizophrenia, providing a novel and promising animal model. However, more research is needed to explore further schizophrenia-like symptoms in this *RBM12* model. A selection of suitable assays for this approach is proposed.

Keywords: zebrafish, schizophrenia, RBM12, anxiety, sleep

Zusammenfassung

Hintergrund und Zielsetzung: Schizophrenie ist eine gravierende und vielgestaltige psychiatrische Erkrankung einhergehend mit einer geringen Lebensqualität, unzufriedenstellenden Behandlungsmöglichkeiten, und kaum erforschter Ätiologie. Durch die hohe Heritabilität von Schizophrenie sind genetische Modelle für die Erforschung dieser Störung geeignet. Allerdings sind die bereits existenten Tiermodelle unbefriedegend, da sie weder die Vielfalt an Symptomten umfassen, noch spezifisch für Schizophrenie sind.

Kürzlich ist ein neues Hochrisikogen (*RBM12*) mit Schizophrenie in Verbindung verbracht worden (Steinberg et al., 2017). Mithilfe von CRISPR/CAS9 wurden drei Linien an *RBM12* Zebrafischmutanten kreiert. Ziel dieser Thesis war es, einen Schizophrenie-ähnlichen Phänotpyen in diesen *RBM12* Mutanten zu validieren.

Methoden: Das Verhalten der mutierten Zebrafischlarven und erwachsenen Fische wurde mithilfe von Assays für Thigmotaxis, Novel Tank Test, Geschwindigkeit, und Schlaf untersucht. Die darin erhobenen behavioralen Parameter wurden in Anlehnung an die häufigen Symptome bei Menschen mit Schizophrenie ausgewählt: Angst, Positivsymptomatik und Schlafstörungen. Darüber hinaus wurden die häufig eingesetzten Antipsychotika Haloperidol, Chlorpromazin, und das Anxiolytikum Diazepam auf ihre Fähigkeit getested den Phänotypen zu retten.

Ergebnisse: Die *RBM12* Mutanten zeigten zu einem gewissen Grad Angst-ähnliches Verhalten und fragmentierten Schlaf mit Unterschieden in Symptomatik und Symptomschweregrad in den jeweiligen Linien. In beiden Fällen konnten die eingesetzten Medikamente den Phänotypen retten.

Fazit: *RBM12* Mutanten weisen zumindest teilweise eine Schizohrenie-ähnliche Symptomatik auf und stellen damit eine neues und vielversprechendes Tiermodell dar. Es darf bedarf allerdings weiterer Forschung, um weitere Schizophrenie-artige Symptome bei *RBM12* Mutanten zu erforschen. Dafür bietet die vorliegende Arbeit eine Auswahl an geeigneten Methoden an.

Schlüsselwörter: Zebrafische, Schizophrenie, RBM12, Angst, Schlaf

1 Introduction

Schizophrenia is a severe psychiatric disorder with a significant economic burden (Jin & Mosweu, 2017; Tyler, Zaldivar-Diez, & Haggarty, 2017) affecting around 1% of the population worldwide (American Psychiatric Association, 2013). The symptoms include a wide range of positive (e.g., hallucinations, delusions, thought disorders), negative (e.g., social withdrawal, catatonia, flattening of affect), and cognitive (e.g., reduced attention, slower processing speed, impaired working memory) symptoms. Additionally, more than 50% of the patients with schizophrenia have comorbid diagnoses such as disturbed sleep patterns, anosognosia (lack of insight or awareness of disorder), substance use, anxiety, panic, and obsessive-compulsive disorders. Patients with schizophrenia also have an increased risk for suicide, with one in five patients attempting suicide at least once in their life and 5-14% completing it (Bachmann, 2018). Moreover, schizophrenia often leads to severe functional consequences such as impaired educational progress, difficulty in maintaining employment, as well as social implications due to limited social contacts, and poor engagement in health maintenance behavior (American Psychiatric Association, 2013).

The cornerstone of schizophrenia treatment are antipsychotics that improve symptoms in many cases and thus, increasing life quality immensely (Freudenreich, 2020). However, while schizophrenia symptoms can often be diminished by medication, antipsychotics have a high potential to cause various side effects, including severe implications such as parkinsonian symptoms, seizures, or cardiovascular symptoms. At the same time, around one-third of the patients remain treatment-resistant (Potkin et al., 2020). These differences in treatment response are not yet fully understood by the scientific community but are hypothesized to be due to underlying neurobiological differences between patient groups (Sawa & Snyder, 2002; Winship et al., 2019).

Several approaches try to explain the psychopathology of schizophrenia. Next to neurodevelopmental and cognitive models, there are hypotheses involving different neurotransmitter systems. However, not one single theory fully elucidates this complex disorder. Nonetheless, even though the exact etiology of schizophrenia is unknown, it was demonstrated that schizophrenia occurs with a high genetic disposition. Schizophrenia shows one of the highest heritability across human mental disorders with 80% (Beidel & Frueh, 2018), meaning that schizophrenia can be attributed to 80% on to genetic factors. Hence, genetic models of schizophrenia are a promising tool for studying the underlying mechanisms of this disorder and finding new treatments.

In the past years, several genetic models of schizophrenia have been introduced. However, manipulation of the same genes produce inconsistent phenotypes (Winship et al., 2019), or often only lead to a few specific symptoms of schizophrenia not covering the full range of the disorder. Recently, a new mutation has been linked to schizophrenia by Steinberg et al. (2017), namely a mutation of the RNA-binding-motif protein 12 gene (*RBM12*). The mutation occurred in a Finnish and Icelandic family with a cumulative occurrence of schizophrenia, schizoaffective disorder, and psychotic bipolar disorder. Additionally, carriers of the mutation who were not diagnosed with psychosis were similar to patients with schizophrenia in their life outcomes and performance in neuropsychological tests. Thus, the further investigation of an *RBM12* model for schizophrenia is especially promising.

So far, except for *RBM12*'s link to schizophrenia, the gene is poorly understood in terms of function, downstream expression, and biology (Steinberg et al., 2017). Therefore, a suitable biomedical model is crucially needed to examine the gene's function and its resulting phenotype. Out of the many options for animal models, there is an especially promising one - the zebrafish. Zebrafish became popular in biomedical research because of their fully sequenced and easy to manipulate genome, high fecundity, rapid development, and neuroanatomical layout, which is similar to the mammalian brain (Kalueff, Stewart, & Gerlai, 2014; Vaz, Hofmeister, & Lindstrand, 2019). Further, unlike nocturnal rodents, zebrafish have diurnal rhythms which are comparable to those of humans, making them notably fitting to study sleep disruptions (Bandmann & Burton, 2010; Shams, Rihel, Ortiz, & Gerlai, 2018). Due to these characteristics, the zebrafish is a suitable model to examine the link between *RBM12* and schizophrenia. However, as there is no available data about whether zebrafish or other animals naturally develop schizophrenia, and there is no satisfying existing animal model so far, it is unclear how this highly complex disorder would be expressed in zebrafish. Therefore, this thesis follows an exploratory approach.

Considering the three main symptom groups of schizophrenia, which are positive, negative, and cognitive symptoms and the most frequently accompanied disturbances, including anxiety and sleep disturbances (Freudenreich, 2020), the following behavioral parameters have been selected as possible indicators for a schizophrenia-like phenotype in zebrafish: Thigmotaxis, also known as "wall-hugging" behavior, describes the tendency to remain close to the borders of an arena and thus, reduced exploratory behavior and is an established indicator of anxiety-like behavior in animals and anxiety humans (Schnörr, Steenbergen, Richardson, & Champagne, 2012). Another measurement is increased locomo-

tion which is a frequently investigated parameter for positive-like symptoms in animals (Winship et al., 2019), as well as anxiety-like symptoms (Jesuthasan, 2012; Kedra et al., 2020). Furthermore, sleep has been selected as a factor for analysis. These behavioral parameters are not only seen frequently in patients with schizophrenia (American Psychiatric Association, 2013) but also highly correlate with other symptoms of the disorder (Ashton & Jagannath, 2020; Korczak & Styła, 2021) making these symptoms suitable candidates for a first phenotype analysis of a new zebrafish model for schizophrenia.

This thesis aims to inspect a novel CRISPR/CAS9 (Jao, Wente, & Chen, 2013) created *RBM12* zebrafish model consisting of three different mutant strains and validate it on the basis of the common schizophrenia symptoms anxiety, sleep disturbances, and positive symptoms. The thesis first presents an overview of schizophrenia, including its history, involved neurotransmitter systems, and treatment. This is followed by covering the background of pharmacological and genetic animal models of schizophrenia and introducing the zebrafish as a biomedical model. The *RBM12* mutant lines and the different setups are depicted, and the findings from these studies are explained. Lastly, the proposed *RBM12* zebrafish model is discussed and evaluated in its suitability as a novel genetic animal model for schizophrenia. Finally, an outlook for further research is presented.

2 Theory

2.1 Schizophrenia

Symptoms of schizophrenia have already been described in ancient ages throughout all cultures (Kyziridis, 2005). Still, it was not identified as a psychiatric disorder until 1887 by Emile Kraepelin as "dementia praecox". Kraepelin believed that schizophrenia was primarily a brain disorder and a particular form of dementia in young adults. Around 20 years later, the Swiss psychiatrist Eugen Bleuler criticized the term "dementia praecox", stating the lack of evidence for a dementing process in this disorder. Instead, Bleuler named this disorder "schizophrenia" (which translates to "split mind") to describe the fragmented thinking in patients and characterized schizophrenia by the "four A's": disturbed Associations, Affect, Ambivalence, and Autistic isolation. Furthermore, Bleuler was the first one to define the symptoms as "positive" (symptoms that are "added" onto usual behavior such as hallucinations), and "negative" (symptoms resulting from "subtraction" of usual behavior such as flattened affect). Since then, schizophrenia has been reclassified several times.

Today, schizophrenia belongs to the chapter "Schizophrenia Spectrum and Other Psychotic Disorders" in the DSM-5 (American Psychiatric Association, 2013) and to "Schizophrenia, schizotypal, delusional, and other non-mood psychotic disorders" in the ICD-10 (World Health Organization, 2004). The diagnosis no requires at least one of the following key features to be present: delusions, hallucinations, disorganized speech, disorganized or catatonic behavior, and negative symptoms. However, schizophrenia is a highly heterogeneous syndrome affecting all life aspects. Next to the diagnosis symptoms, patients often report additional disturbances in self-experience (e.g., experiencing one's thoughts and feelings being controlled externally), sensory processing and inhibition (American Psychiatric Association, 2013), derealization and -personalization, deficits in theory of mind, anosognosia, as well as sleep problems and anxiety.

Altogether, while schizophrenia is defined by positive, negative, and cognitive symptoms, the disorder is highly heterogeneous and frequently accompanied by comorbid diagnoses (American Psychiatric Association, 2013). Two of the most common features are sleep disturbances (Ashton & Jagannath, 2020), and anxiety (Kiran & Chaudhury, 2016). These symptoms do not only have a significant negative impact on the patients' quality of life, but further act as prognostic factors for approaching psychotic episodes (Davies, Haddock, Yung, Mulligan, & Kyle, 2017) and the patients' life outcome (Braga, Reynolds, & Siris, 2013; Ritsner, Kurs, Ponizovsky, & Hadjez, 2004).

Anxiety Schizophrenia shows a comorbidity of around 40-65% with anxiety disorders (Goodwin, Lyons, & McNally, 2002; Kiran & Chaudhury, 2016) making anxiety one of the most frequent symptoms in schizophrenia. Patients often report thoughts of worrying, distress, and social anxiety (Freudenreich, 2020), and in many cases the symptoms are already present throughout the prodromal phase of the disorder (Hall, 2017). Moreover, anxiety is the most important predictor for health-related life quality in patients with schizophrenia according to Kanchanatawan, Sriswasdi, and Maes (2019), and plays a crucial role in suicidial thoughts and behavior (Jian-biao, Lü, Jian, & Zheng-hui, 2020). Further, a recently published meta-analysis revealed a link between anxiety and diminished executive functions in schizophrenia (Korczak & Styła, 2021). While anxiety symptoms are usually seen as a symptom of schizophrenia (American Psychiatric Association, 2013), some researchers hypothesize that it could actually be a risk factor leading to the development of schizophrenia (Hall, 2017).

Sleep Disturbances Sleep disturbances are one of the most common symptoms in individuals with schizophrenia and are reported in around 80% of patients (Ashton & Jagannath, 2020). The presented symptoms are various and include daytime sleeping and nighttime activity (Sharma, Dikshit, Shah, Karia, & De Sousa, 2016), nightmares (M. V. Seeman, 2018), insomnia (Robertson, Cheung, & Fan, 2019), and fragmented sleep (Davies et al., 2017). The underlying mechanisms have not been fully explained, yet. However, research shows a disrupted circadian rhythm in individuals with schizophrenia indicated by physiological circadian parameters, molecular alterations, and mutations in different clock genes (Ashton & Jagannath, 2020; Waite, Sheaves, Isham, Reeve, & Freeman, 2020). It is hypothesised that dopamine is involved in this process due to its central role in the sleep-wake cycle (Kant, Meena, & Pathania, 2021; Monti & Jantos, 2008; Monti & Monti, 2007). Moreover, disruptions in dopaminergic activity have been linked to schizophrenia (Howes & Murray, 2014).

However, sleep disturbances in schizophrenia are not only a life quality diminishing feature by negatively affecting mental health (Ritsner et al., 2004), but are correlated with further symptoms of the disorder, and overall symptom severity (Ashton & Jagannath, 2020). Insomnia for example, which is reported in around 50% of the patients with schizophrenia according to a survey with more than 1809 patients by Freeman, Taylor, Molodynski, and Waite (2019), seems to be especially related to worsening of positive symptoms (Chemerinski et al., 2002) and has been linked to anxiety and depression (Freeman et al., 2019) which in turn are predictors of paranoia. Also nightmares, and fragmented sleep have been

associated with worsening of positive symptoms (Davies et al., 2017). There is also evidence for sleep disruptions affecting negative symptoms in schizophrenia (Blanchard, Andrea, Orth, Savage, & Bennett, 2020), though conducted research on this relation is scarce and ambiguous. Further, the link between sleep disturbances and cognitive symptoms is unclear (Kaskie & Ferrarelli, 2020).

Before the onset of schizophrenia, patients usually undergo a prodromal phase which can last from several days to years (Freudenreich, 2020). During this period, patients often experience poor concentration, depression, and social withdrawal - symptoms frequently reported in relatives without a diagnosis of schizophrenia as well . At the end of the prodromal phase first attenuated psychotic symptoms develop, forecasting the beginning of full schizophrenia. The peak for the onset of schizophrenia is in early adolescence and earlier for men (early-mid-20s) than for women (late-20s) (American Psychiatric Association, 2013). There are also rare cases of childhood-onset / early-onset schizophrenia (Driver, Thomas, Gogtay, & Rapoport, 2020) and (very-)late-onset schizophrenia (Howard, Rabins, Seeman, Jeste, & Late-Onset, 2000).

The life outcome of patients with schizophrenia is usually poor, and an earlier age onset predicts a worse prognosis (Eaton et al., 1992). Over the life course, psychotic symptoms seem to diminish, while negative and cognitive symptoms are more persistent and may worsen (American Psychiatric Association, 2013). The life expectancy of patients with schizophrenia is reduced by around 20 years (Tyler et al., 2017). This is for once due to the increased suicide rate, which remains high throughout life, and also the accompanying medical conditions such as metabolic syndrome, and cardiovascular and pulmonary disease (American Psychiatric Association, 2013). The functional consequences of schizophrenia are associated with impaired educational progress and low employment, social and occupational dysfunction, and limited social contacts. In total, only 20% of the patients experience a favorable course, and a complete recovery is improbable. (Freudenreich, 2020). Most patients require daily living support and stay chronically ill.

2.2 Hypotheses of Schizophrenia

There are various hypotheses about the etiology and pathology of schizophrenia coming from different disciplines. These hypotheses can be grouped into neurochemical and neurodevelopmental, and are briefly described in the following. Furthermore, there are cognitive hypotheses of schizophrenia, but are not further explained within this thesis (Rector, Stolar, & Grant, 2011).

2.2.1 Neurochemical Hypotheses

Different neurotransmitter systems have been implicated in the pathology of schizophrenia. While research points to the involvement of various neurotransmitters such as serotonin (5-HT) (Shah & González-Maeso, 2019), GABA (Sawa & Snyder, 2002), glycine (Javitt, 2012), and others, most pharmacological and functional studies focused on the role of dopamine (DA), followed by glutamate.

Dopamine hypothesis The classic dopamine hypothesis of schizophrenia by Creese, Burt, and Snyder (1976) states that schizophrenia stems from excessive activity of DA. This derives from the discovery that antipsychotics diminish positive symptoms by blocking D_2 receptors (Creese et al., 1976; P. Seeman & Lee, 1975; P. Seeman, Lee, Chau-Wong, & Wong, 1976). Furthermore, patients with schizophrenia show abnormal activity in the four major dopaminergic pathways (Abi-Dargham & Moore, 2003; Howes & Murray, 2014; Patel, Cherian, Gohil, & Atkinson, 2014): the mesolimbic pathway, which is associated with positive symptoms, the mesocortical pathway linked to cognitive and negative symptoms, the nigrostriatal pathway being responsible for motor symptoms when DA levels are low, and the tuberoinfundibular pathway where a decrease in DA levels results in prolactin-mediated effects. The DA hypothesis is further supported by the fact that DA-increasing psychostimulants, such as amphetamine, induce psychosis in individuals without schizophrenia, as well as enhance positive symptoms in individuals with schizophrenia (Sawa & Snyder, 2002). However, the DA hypothesis has been criticized. For example, around 1/3 of the patients do not respond to the DA-blocking antipsychotics (Bartley & Ross, 2020; Ichinose & Park, 2020). Further, these drugs have only a limited effect on negative and cognitive symptoms (Ichinose & Park, 2020). Hence, the DA hypothesis does not fully explain the neurobiological basis of schizophrenia.

Glutamate hypothesis The glutamate hypothesis of schizophrenia stems from the antagonistic effect on glutamatergic receptors of certain drugs (Ichinose & Park, 2020). More specifically, by blocking the N-methyl-D-aspartate (NMDA) receptor (Moghaddam & Javitt, 2012), drugs such as phencyclidine (PCP), dizocilpine (MK-801), or ketamine are known to induce schizophrenia-like positive, negative, and cognitive symptoms in healthy adults (Ichinose & Park, 2020). NMDA is a subtype of the glutamate receptor, with glutamate being the most abundant excitatory amino acid in the brain and central nervous system. Therefore, while the DA hypothesis mainly refers to specific brain areas, the glutamate hypothesis is more global as NMDA receptors are found throughout the cortex. In

schizophrenia, it is believed that NMDA receptors are hypofunctioning, resulting in changes in the glutamatergic-induced excitatory-inhibitory balance of neuronal activity(Moghaddam & Javitt, 2012) which has been confirmed in different genetic, neuroimaging, and post-mortem studies (Ichinose & Park, 2020). Although the glutamate hypothesis can account for positive, negative, and cognitive symptoms, schizophrenia cannot be attributed to NMDA receptor abnormalities alone. The glutamate system interacts highly with other neurotransmitter systems, such as DA. Therefore, different other neurochemical and models have been proposed that intertwine DA and glutamate dysregulation in schizophrenia (Howes, McCutcheon, & Stone, 2015).

2.2.2 Neurodevelopmental Hypotheses

Neurodevelopmental hypotheses view the cause for schizophrenia in abnormal brain development (L'upták, Michaličková, Fišar, Kitzlerová, & Hroudová, 2021). Brain imaging (Ren et al., 2013; Van Erp et al., 2018) and post-mortem (Ramaker et al., 2017) studies confirm significant differences in brain anatomy and functioning between individuals with and without schizophrenia especially for areas linked to learning, memory, and emotional functions (L'upták et al., 2021). Pediatric observations further report developmental delay in preschizophrenia children characterized by speech and gross motor difficulties, cognitive deficits, and social anxiety and withdrawal (Jaaro-Peled & Sawa, 2020). Moreover, patient groups seem to differ from each other depending on their predominant symptoms being negative, cognitive, or positive (Ren et al., 2013).

According to the neurodevelopmental hypotheses, the main source for these neurological anomalies is the interaction between genetic and environmental factors (Jaaro-Peled & Sawa, 2020). Next to genetic studies having identified over 145 genetic risk loci associated with schizophrenia (Forsingdal et al., 2019; Thyme et al., 2019), epidemiologic research points out various environmental stressors associated with schizophrenia such as season of birth (C. Wang & Zhang, 2017), urban living (Lederbogen, Haddad, & Meyer-Lindenberg, 2013), and maternal malnutrition and infection (De Matteis, D'Andrea, Lal, Berardi, & Tarricone, 2020), or natal complications (Pugliese et al., 2019).

However, the mechanisms behind the interaction of genetic and environmental factors, and how they initiate abnormal neurodevelopment remain to be elucidated (Rund, 2018).

2.3 Treatment

The first drug for schizophrenia was chlorpromazine which became available in the 1950s (Kyziridis, 2005). Before that, patients with schizophrenia underwent painful procedures including induced fevers, insulin therapy, electroconvulsive treatment, or lobotomies - which were all unsuccessful. Around the 1900s, treatment with chloral hydrate and barbiturates reduced anxiety levels and sleep disturbances but did not help with psychotic symptoms. However, with the discovery of chlorpromazine the treatment of psychotic symptoms became possible and a few years later more antipsychotic drugs were developed. These drugs are also known as the first-generation antipsychotics (FGAs) or "typical" antipsychotics and can be classified as low-potency and high-potency FGAs (Freudenreich, 2020). In short, low-potency FGAs (such as chlorpromazine) are not selective for DA receptors and are prone to cause sedation, metabolic problems, and anticholinergic effects, among others. High-potency FGAs (e.g. haloperidol) on the other hand, are highly selective for the D2 receptor, and side effects are most often related to the motor system.

In the 1960s, the "atypical" or the second-generation antipsychotics (SGAs) were developed, the first one being clozapine. However, it did not become available for treatment until the 1990s because of its association with Agranulocytosis - a life-threatening blood disorder (Kyziridis, 2005). In contrast to FGAs, the SGAs did not only reduce positive but also negative symptoms. Further, SGAs could be used in patients who did not respond to FGAs. Later, in the early 2000s, a new drug named aripiprazole was marketed and became the first third-generation antipsychotic (TGAs) (Norton, 2012). This drug was the first effective partial DA and 5-HT agonist, and the first antipsychotic to be effective on positive, negative, and cognitive symptoms. Since then, several other TGAs have been developed.

It should be noted that patients with schizophrenia also profit from psychotherapy (American Psychiatric Association, 2013). Nonetheless, psychotherapy is more successful in less severe cases of schizophrenia (Bighelli et al., 2020), and usually has as a supporting role for patients (Freudenreich, 2020). The main foundation of schizophrenia treatment is therefore medication. In the following, the commonly prescribed antipsychotics haloperidol, chlorpromazine, and the anxiolytic diazepam will be discussed more extensively.

Haloperidol Haloperidol, an FGA discovered in 1958, is one of the most often prescribed drugs for schizophrenia (Hanafi et al., 2017). It is a high-affinity antagonist for the D_2 receptor, with additional but much less antagonistic activity

at 5-HT2A, and different adrenergic receptors, among others (Tyler et al., 2017). Therefore, it diminishes the dopaminergic activity in the mesolimbic pathway resulting in reduced positive symptoms in schizophrenia. However, haloperidol does not affect negative or cognitive symptoms, and side effects are frequent by decreasing the DA levels in the nigrostriatal, and tuberoinfundibular pathways. Side effects are primarily restricted to the motor system such as extrapyramidal symptoms, for example, acute dystonia or parkinsonism, but can further result in anticholinergic effects such as an elevated temperature, drowsiness, and sedation (Rahman & Marwaha, 2021).

Chlorpromazine Chlorpromazine is another FGA. Since its discovery around 70 years ago, chlorpromazine has remained as the "gold standard" of antipsychotic treatment by which other antipsychotics are evaluated (Boyd-Kimball et al., 2018). In contrast to haloperidol, chlorpromazine is a low-potency D_2 antagonist (Freudenreich, 2020). It successfully reduces positive symptoms through blocking D_2 in the mesolimbic pathway and has a calming effect in terms of anxiety and psychomotor agitation (Sawa & Snyder, 2002). Due to chlorpromazine's strong antihistaminic and anticholinergic effects, it commonly causes side effects such as a dry mouth, blurred vision, anxiety, tremor, weight gain, lowered blood pressure, and dizziness, among other side effects (Boyd-Kimball et al., 2018).

Diazepam Diazepam is an anxiolytic patented in 1963 in the US (Dhaliwal, Rosani, & Saadabadi, 2020). It is GABA allosteric increasing the effect of GABA (Campo-Soria, Chang, & Weiss, 2006), and often prescribed to patients with schizophrenia to reduce stress, anxiety, or prevent a psychotic relapse (Freudenreich, 2020). Further, it can help with sleep problems which are common in schizophrenia. However, this benzodiazepine can have serious side effects such as addiction when used chronically. Another problem is that diazepam can impair cognitive functioning, which is already decreased in patients with schizophrenia.

2.4 Genetics

There are various risk and prognostic factors for schizophrenia, such as environmental, seasonal, and prenatal components. However, a genetic disposition seems to have the most significant influence (American Psychiatric Association, 2013). Still, the inheritance pattern is not fully understood (Ichinose & Park, 2020). Further, even though many different loci have been linked to schizophrenia (Forsingdal et al., 2019), they are also associated with other neuropsychiatric disorders such as ADHD, depression, or autism, and are therefore not schizophrenia-

specific. However, Steinberg et al. (2017) recently linked a previously unknown mutation to psychosis and, as a result, introduced a promising and novel candidate for understanding the genetics of schizophrenia.

RBM12 The recently discovered mutation in the RNA-binding-motif protein 12 gene has been linked to psychosis in a Finnish and Icelandic family (Steinberg et al., 2017). While the mutation is not fully penetrant for psychosis in all variants, carriers are similar to individuals with schizophrenia in their non-psychotic phases. This includes their performance on neuropsychological tests and their life outcome.

RBM12 is expressed in different cell types within the brain (Steinberg et al., 2017). Its highest expression occurs prenatally, and it is believed to have a developmental role. However, besides an indication for its role in the insulin-like growth factor pathway (Krumm et al., 2015), and its recent link to psychosis (Steinberg et al., 2017), the functional biology of the gene is unknown (Coelewij & Curtis, 2018).

2.5 Animal Models of Schizophrenia

Animal models are valuable for studying the etiology of a disorder and the development of new treatments. They allow invasive approaches to understanding structural and molecular changes along with disorder progression and testing of new therapeutic agents (Winship et al., 2019). Further, animal models are useful for the bidirectional translation: Findings from basic research in animal studies can be translated to clinical trials in patients such as testing new drugs which were found to be successful in prior animal studies. Vice versa, if medical treatment is successful in humans, animal models can be used to investigate the underlying mechanisms and develop further therapies. To ensure this translational value, an animal model has to prove construct, face, and predictive (also known as pharmacological) validity (Nestler & Hyman, 2010). Therefore, the model must show a similar etiopathogenesis as the disorder, homologous symptom expression, and comparable treatment response. Table 1 provides an overview of typically measured behavioral and physiological and morphological parameters in rodent models for schizophrenia.

In schizophrenia, neither the etiology of the disease is fully understood nor available treatments are satisfying (Winship et al., 2019). Animal models can help to study the neurobiological bases of this disorder and provide more effective treatments. In the following, different types of already existing animal models for schizophrenia are briefly described.

Table 1

Common Phenotypes Associated with Rodent Models of Schizophrenia

Category	Measure	Description
Behavioural	Locomotor activity (positive symptoms)	Vigor of movement in response to a novel environment, stress, or pharmacological challenge (dopamine enhancers, NMDA receptor antagonists) is quantified.
	Sucrose preference (negative symptoms)	Preference for sweetened water over unsweetened water is measured.
	Social interaction (negative and cognitive symptoms)	Time spent in close proximity or direct contact with an unfamiliar or less familiar animal.
	Latent inhibition (attentional processes, cognitive symptoms)	A classical conditioning procedure whereby previously unreinforced stimuli are slower (less efficient) in generating a conditioned response than novel stimuli.
	Memory (cognitive symptoms)	Many tests have been validated to measure various aspects of working memory, declarative memory, recognition memory (visual, spatial, and olfactory domains), and conditioning.
	Reasoning and problem solving (cognitive symptoms)	Tests of behavioural flexibility in response to dynamic environments include set shifting and reversal learning.
	Prepulse inhibition (sensorimotor deficits)	A reduction in the acoustic startle response is typically observed if the startling stimulus is preceded close in time by a low-intensity prepulse.
	Anxiety-like behaviour	Patterns of exploration in environments such as the elevated plus maze, open field, or light-dark box.
Morphological/ physiological	Enlarged lateral ventricles	Measurements of the volume of lateral ventricles using in vivo imaging techniques such as magnetic resonance imaging or postmortem analysis.
	Morphology of cortical, striatal, thalamic, and limbic areas	Includes measures of size/volume of brain areas, numbers of neurons, dendritic changes, and synaptic proteins.
	Myelination	Assessment of the extent of myelination, particularly of cortical axons.
	Glial cells	Changes in microglia, oligodendrocytes, and astrocytes are commonly studied.
	Integrity of extracellular matrix	Alterations in structures such as perineuronal nets and proteins, including reelin.
	Neurotransmitter-related alterations	Effects on dopamine, glutamate, serotonin (5-HT), GABA (notably parvalbumin-containing interneurons), glycine, D-serine, and neuroactive steroids.
	Patterns of brain activity	Neural activity, dopamine neuron, activity, and patterns of oscillatory activity are measured using in vitro and in vivo electrophysiology techniques.

Note From "An Overview of Animal Models Related to Schizophrenia", by I.R. Winship, S.M. Dursun, G.B. Baker, P.A. Balista, L. Kandratavicius, J.P., Maia-de-Oliveira, ... & J.G. Howland, 2019, *The Canadian Journal of Psychiatry, 64(1)*, p. 7. (doi.org/10.1177/0706743718773728). Copyright SAGE Publications

Pharmacological models In pharmacological models, a neurotransmitter agonist or antagonist is administered. This allows focusing on single neurotransmitter systems and drug-injection into specific brain areas (Nestler & Hyman, 2010). These animal models are a critical source of today's understanding of schizophrenia (Ichinose & Park, 2020) and are based on the psychotic effect of psychoactive drugs in humans. Typically, animals receive DA agonists such as amphetamine or apomorphine, or noncompetitive NMDA antagonists such as phenylcyclohexyl piperidine (PCP), dizocilpine (MK-801), and ketamine (Winship et al., 2019). While amphetamine leads to increased locomotion which is associated with positive symptoms in schizophrenia, it does not reliably produce negative or cognitive symptoms. PCP, on the other hand, creates positive, negative, and cognitive symptoms, and MK-801 induces impaired social behavior and locomotion alterations in zebrafish (Benvenutti et al., 2021) and rodents (Jones, Watson, & Fone, 2011). However, even though pharmacological animal mod-

els can provide translational relevance, their induced symptoms are very limited (Winship et al., 2019). Further, some psychostimulants that cause psychotic-like behavior in animal models have been shown to improve cognitive and negative symptoms in patients with schizophrenia (Zhand et al., 2021).

Developmental models Developmental animal models of schizophrenia focus on peri-/postnatal events and their influence on altered brain development and behavior (Meyer & Feldon, 2010). They are based on human epidemiological studies which have linked the development of schizophrenia to environmental insults such as maternal exposure to stress, infection, or malnutrition. Further, these models are used to study the influence of very early drug exposure or lesions. Developmental models are valuable for understanding the contribution of external factors to the development of schizophrenia and studying the prodromal phase. Eventually, this approach can help to find treatments for preventing the onset of this disorder and its progression (Winship et al., 2019). One example off this type of model is postweaning social isolation in rodents which resulted in altered behavior in adulthood, including locomotor hyperactivity, cognitive impairment, enhanced response to novelty, and increased anxiety and aggression (Winship et al., 2019). Nonetheless, these procedures are not exclusively linked to schizophrenia, but other disorders as well, such as autism.

Genetic models Genetic models allow studying the gene's role in the development of a disorder. For schizophrenia, various risk genes have been identified through genetic studies in patients (Henriksen, Nordgaard, & Jansson, 2017). For many of these genes, animal models have been generated to study the genetic contribution to the disorder and the underlying mechanisms. The most extensively studied gene for schizophrenia is disrupted-in-schizophrenia 1 (*DISC1*) (Millar et al., 2000). *DISC1* is the first gene that has been associated with schizophrenia and has been studied in different animal models. It is involved in neurodevelopment and neurosignaling and produces schizophrenia-associated brain morphology in animals (Winship et al., 2019). However, the results about the behavioral phenotype are inconsistent, and it remains unclear whether *DISC1* produces schizophrenia-like behavior in animals. Moreover, *DISC1* is not schizophrenia-specific and has been linked to other psychiatric disorders such as bipolar disorder and major depression (Daggett, 2016). Other genetic models of schizophrenia include the *22q11.2* deletion syndrome associated with a deletion on chromosome *22q11.2* (Bassett & Chow, 2008), *neuregulin-1 (NRG1)* which is a pleiotropic growth factor (Harrison & Law, 2006), *dystobrevin-binding protein 1 (DTNBP1)*

with its synaptic protein dysbindin (Papaleo et al., 2012), reelin (RELN) being a protein involved in neurodevelopment (Bellon, Le Pen, Matricon, Jay, & Krebs, 2009). Even though these models have contributed to schizophrenia research, so far, no existing genetic model has successfully induced the range of symptoms in schizophrenia, nor been exclusively linked to schizophrenia (Winship et al., 2019).

2.6 Zebrafish as a biomedical model

Zebrafish have been used as biomedical models since the 1950s (Hisaoka & Hopper, 1957). Since then, the possibilities for their use have increased drastically. Due to their small size and their preference to stay in big groups, zebrafish are easily housed (Shams et al., 2018). Further, they reproduce fast, with one female laying up to 200 eggs every other day (Gerlai, 2014). This ex-utero development and their transparency make embryos easily accessible for chemical or mutagenic manipulation (Vaz et al., 2019), and for observing single organs and their rapid development. Their fast life cycle also shows in the development of behavioral repertoire (Orger & de Polavieja, 2017). At only five days post fertilization (dpf), larvae swim freely, allowing studying different locomotor parameters; they display sensorimotor responses such as the startle response to various types of stimuli, and they even show stable sleeping and wake behavior.

Moreover, zebrafish share important characteristics with mammals, including humans. For example, there is a 70 % gene homology between humans and zebrafish (Howe et al., 2013). Due to this high genetic conservation, it is most likely that the zebrafish gene and its human homolog share a similar function (Gerlai, 2014). Further, the neuroanatomical layout of zebrafish is similar to other vertebrates species. Kalueff et al. (2014) showed that the habenula and amygdala of zebrafish share the same function in affective behavior as in humans or rodents, such as a hyperaction leading to depression-like and stress-related behavior. Apart from their similar brain anatomy (see Morris (2009)), zebrafish share the same neurotransmitter system as higher vertebrates, including GABA, glutamate, DA, noradrenaline, 5-HT, histamine, and acetylcholine (Basnet, Zizioli, Taweedet, Finazzi, & Memo, 2019). Further, zebrafish show similar aging trajectories as other vertebrates (Van houcke, De Groef, Dekeyster, & Moons, 2015), and cognitive declines with aging. As diurnal animals, they are even more similar to humans than rodents which are typically used in sleep research (Ruhl et al., 2016), and have been validated as a suitable model for sleep research (Sorribes et al., 2013).

Altogether, the zebrafish "represents an excellent compromise between system complexity and practical simplicity" (Shams et al., 2018). By this, different

zebrafish models have been successfully established over the last few decades including models for Alzheimer's disease (Van houcke et al., 2015), Parkinson's disease, cancer (S. Liu & Leach, 2011), and different neurodevelopmental disorders (Sakai, Ijaz, & Hoffman, 2018). Additionally, zebrafish make it easy to perform high-throughput phenotypic screens - an approach which led to several successful drug discoveries (MacRae & Peterson, 2015).

This thesis aims to validate the novel *RBM12* zebrafish mutant line as a new biomedical model for schizophrenia research regarding the behavioral phenotype. Using CRISPR/CAS9 (Cong et al., 2013; Jao et al., 2013), several different *RBM12* mutant strains have been generated (Maier et al., 2021, in preparation). The selected behavioral parameters are compared for the different mutant lines and genotypes. Therefore, this project focuses on investigating anxiety-like behavior by a thigmotaxis assay, which is measuring wall-hugging behavior, and a novel tank test measuring bottom-dwelling behavior, velocity as an indicator of positive-like and anxiety-like symptoms, and disrupted sleep patterns, along with their drug-induced rescue through the most commonly prescribed antipsychotics haloperidol and chlorpromazine, and the anxiolytic diazepam. Moreover, new approaches for measuring working memory and social behavior in zebrafish are proposed.

2.7 Research questions

The aim is to validate the *RBM12* zebrafish model for schizophrenia based on behavioral parameters (anxiety, velocity, sleep) in three mutant strains. To this end, the following questions were posed:
1. Does the *RBM12* mutation generate a phenotype? As indicated by a:
1.1 thigmotaxis assay
1.2 novel tank test assay
1.3 locomotion assay
1.4 sleep assay
2. Do the *RBM12* mutant lines differ from each other in their phenotype?
3. Can the *RBM12* mutant phenotype be rescued by the antipsychotics haloperidol and chlorpromazine, or the anxiolytic diazepam?

3 Materials & Methods

3.1 Animals

RBM12 mutants and AB Wildtype (AB-WT) zebrafish were used for this thesis. The *RBM12* mutants were generated at 3Z ehf and Reykjavik University in Iceland, whereas the AB-WT were initially obtained from Oregon and have been maintained at Reykjavik University for many generations.

Fish were kept at Reykjavik University at a 14:10 hour light:dark cycle (lights on at 8 am, lights off at 10 pm) in a 3 L or 10 L multi-tank constant flow system (Aquatic Habitats, Apopka, FL, USA) with a constant temperature of 28.5 °C and a daily water replacement of 10 %. Adult fish were fed three times a day with TetraMin flakes (Tetra Holding GmbH, Melle, Germany), Adult Zebrafish Complete Diet (Zeigler Bros, USA), and live Artemia (INVE Aquaculture Nutrition, USA). Fish eggs were collected between 9 and 11 am and kept in 2 L tanks filled with system water mixed with methylene blue (2 ml .1 % methylene blue per 1 L) in an incubator at 28.5 °C. On the following day, dead eggs were removed and the tanks were cleaned. Starting from five days post-fertilization (dpf), larvae were fed with Dry Larval Diet (Zeiler Bros, USA) increasing pellet size according to larval growth and starting from 10 dpf, larvae were fed with live Artemia. After approximately three weeks, larvae were transferred to the multi-tank constant flow system. The data of 1411 larvae and 104 adult fish were analyzed. All procedures were performed according to and approved by the National Bioethics Committee of Iceland (regulation 279/2002); a permit was issued to KÆK on 19th May 2008.

***RBM12* - Mutation** Several different *RBM12* (ENSDART00000170043.2) zebrafish mutant strains were previously generated using CRISPR / Cas9 according to Jao et al. (2013) in the laboratory (Maier et al., in preparation). In short, the single guide RNA (sgRNA) sequences were selected in silico, generated, and microinjected as pre-assembled with Cas9 protein (NEB) into zebrafish eggs at the single-cell stage. Subsequently, a few larvae were sampled and sequenced in the region flanking the CRISPR guide site to determine the success of generating a mutation. Larvae from injections were grown into adulthood resulting in the F0 generation. The F0 generation was then outcrossed to AB-WT fish to generate F1 heterozygous fish.

Three out of initially six sgRNAs designed successfully generated mutations. The position of the mutations generated on the putative *RBM12* protein is shown in Figure 1. Additionally, these strains have been crossed several times with AB-

WT to remove genetic background noise. The resulting fish lines have been selected for further behavioral testing for this thesis.

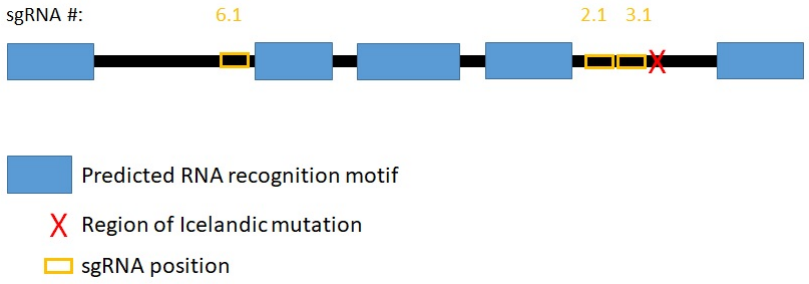

Figure 1. sgRNA positions on the *RBM12* protein

Courtesy of Valerie Maier.)

3.2 Behavioral Tests

3.2.1 Larvae

At five dpf, larvae were individually placed into the wells of microwell plates (Nunc, Roskilde, Denmark) which were filled with system water. The plates were positioned in a custom-built activity monitoring system thermo-regulated at 28.5 °C and blocked from daylight. The system was illuminated from below with white (255 lx in light-phase) and infrared light (0 lx in dark phase) and equipped with 24 infrared cameras (Ikegami, ICD-49E; Ikegami Tsushinki Co, Japan). Recordings were started at 1 pm after larvae acclimated for approximately 24 h and started with a 30 min light phase followed by alternating dark and light phases between 1.30 pm and 6 pm. The lights were again turned off between 10 pm and 8 am to assess sleep behavior.

Thigmotaxis Assay The thigmotaxis assay is an established index of anxiety in zebrafish (Ahmad, Noldus, Tegelenbosch, & Richardson, 2012) and measures thigmotactic behavior in larval zebrafish. This assay requires sufficiently large swimming arenas to allow the larvae to move freely and not restrict their spatial movements. A size of at least 24 microwell-plates is therefore recommended (Ahmad et al., 2012; Schnörr et al., 2012).

Larvae at 5 dpf were placed as described above into 12 microwell-plates. This plate size was chosen to allow for increased exploratory behavior of the larvae (Colwill & Creton, 2011) and thus, an increased possibility to detect differences

between strains and genotypes. The larvae were left to acclimate in the recording system for 24 h followed by a 24 h recording at six dpf. A total of 655 larvae of all three *RBM12* strains as well as AB-WT larvae have been recorded (see Table 2 for specific sample sizes).

For analysis, only the first 30 min (lights on) were used to exclude the effect of changes in lighting conditions which is often used to induce motor responses (e.g., Christensen, Þorsteinsson, Maier, and Karlsson (2020)). Thus, this approach measures particularly spontaneous activity without additional stimuli. The wells were first virtually divided into a center and outer zone. The center zone was defined as 66.6 % of the arena. A total diameter of 22.1 mm of one well equals a center of 14.7 mm diameter, leaving a border of 3.7 mm as the outer zone. Then, the cumulative duration (in percent) for the center zone was calculated. Thigmotactic behavior was indicated by less time spent in the center. As the entire well was divided into two zones, the cumulative duration was not additionally calculated for the outer zone. Furthermore, this assay was repeated with different drugs in the *RBM12* 3.1 strain with 288 larvae (see Table 2).

Locomotion Assay Additionally to thigmotaxis, locomotor activity was analyzed in the same data sets as a supplementary indicator of anxiety (Jesuthasan, 2012) or possibly positive symptoms (Demin et al., 2019; Winship et al., 2019). Therefore, mean velocity (in mm/s) was calculated separately for the center and outer zone and the change in velocity as the absolute value of velocity in the center minus velocity in the outer zone. The analysis of the change in velocity in the two zones is explored as an additional marker of anxiety, and to rule out motor disturbances.

Sleep Assay Patients with schizophrenia often show sleep disturbances, so sleep was assessed in larvae during the lights-off phase between 10 pm and 8 am based on behavioral parameters. The behavior was first categorized as 1 s bins of movement or non-movement based on prior studies defining a speed of 1 mm/s as the threshold for movement and a speed below 1 mm/s as non-movement for larval zebrafish (Christensen et al., 2020). Further, this dichotomous behavior was classified as sleep and wake according to previously established sleep criteria (Sorribes et al., 2013; Yokogawa et al., 2007) where sleep is defined as a minimum of six consecutive 1 s bins of non-movement and wake as the opposite of sleep. Five different sleep parameters were investigated based on sleep and wake bouts: 1) Sleep fragmentation is defined as the number of transitions between sleep and wake bouts per hour. 2) Sleep ratio describing the amount of time a larva was

categorized as asleep during the entire night as a percentage. 3) Wake bout duration (in s) which portrays the average length of a wake period during the night. 4) Sleep bout duration as the average length of a sleeping period (in s). 5) Velocity (in mm/s). The sleep assay was performed in 96 microwell-plates with and without drugs in a total of 384 larvae of the *RBM12* 3.1 strain (see Table 2).

3.2.2 Adult fish

Novel Tank Test Similar to the thigmotaxis assay, the novel tank test (NTT) is a tool to assess exploratory behavior as an indicator for anxiety (Ahmad et al., 2012). When introduced to a new tank, anxious fish tend to explore the novel environment less in comparison to non-anxious fish. This effect is especially prominent in the top part of the tank.

Adult zebrafish were individually netted out of their home tank into a 2 L container for transportation to the recording room. There, the fish was placed on top of the recording box into a 300 ml beaker. After 5 min, the fish was gently placed into the recording tank (24 x 21 x 5 cm; length x width x depth; Figure 2). After 1 min, the fish was recorded for 10 min. The recording tank was placed in front of a white background illuminated from behind with an LED light panel (FLOALT by IKEA). The camera (Basler acA2000-50gmNIR with a PENTAX TV LENS 50MM 1:2.8 C5028-M Mount-C) was positioned in front of the tank to record the fish from the side with 25 fps. The temperature of the beaker was measured, as well as of the recording tank at recording start, after 5 min, and after the recording ended with a commercial infrared thermometer to control for temperature-induced behavioral effects (Toni et al., 2019).

In total, 104 adult fish of all three different *RBM12* strains, and AB-WT were recorded (see Table 2 for specifics). For each strain, fish were tested in a randomized order for genotypes (randomization function in Python). To control for age effects, fish from all strains were roughly the same age. Recordings were performed between 10 am and 2 pm to control for circadian rhythm effects in the animals (J. Wang et al., 2014). All equipment was cleaned between recordings to eliminate odors that could influence the fishes' behavior (Santacà, Dadda, & Bisazza, 2021).

The video files were converted to 10 fps videos in Adobe Premiere to be suitable for analysis in EthoVision where the tank was virtually divided into two same-sized parts (upper and lower part) (Egan et al., 2009). Because of the short recording time and the fast movement of adults, the tracking was less accurate than in the larval assays. Therefore, the cumulative duration was analyzed for both zones as a better estimate.

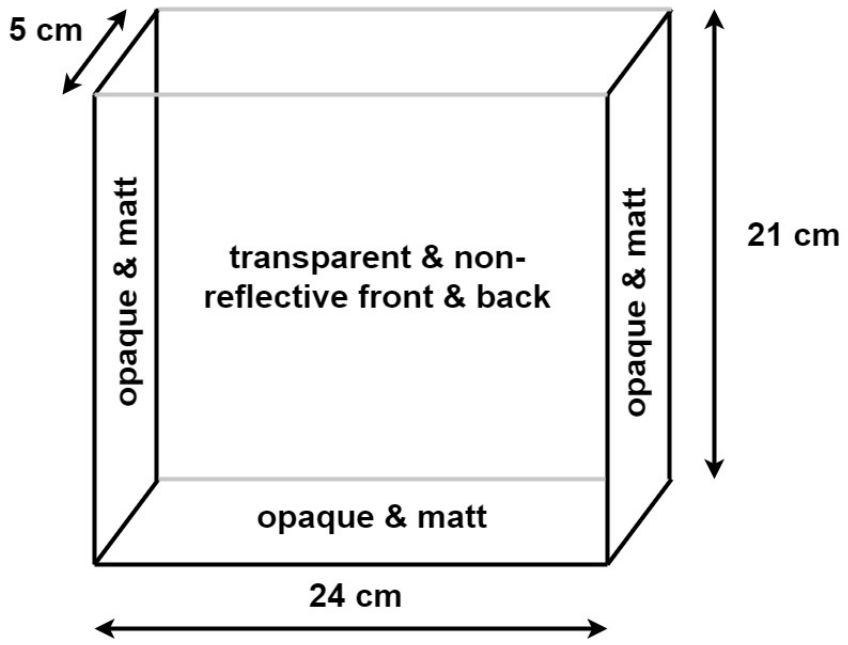

Figure 2. Tank Design for the Novel Tank Test

Table 2

Sample Size

RBM12 strain / -Assay	2.1		3.1		6.1		AB-WT
Thigmotaxis	HOM	89	HOM	103	HOM	58	36
&	HET	19	HET	54	HET	80	
Locomotion	RBM-WT	81	RBM-WT	83	RBM-WT	52	
Thigmotaxis ◇ &			HOM	144			
Locomotion ◇			RBM-WT	144			
Sleep			HOM	48			
			RBM-WT	48			
Sleep ◇			HOM	48			
			RBM-WT	48			
	HOM	10	HOM	12	HOM	10	11
Novel Tank Test	HET	10	HET	12	HET	10	
	RBM-WT	10	RBM-WT	10	RBM-WT	9	

The ⋄ marks assays under the influence of drugs.

Number of individuals used in the different assays according to strain and genotype

3.3 Drug preparation and administration

In the thigmotaxis and sleep assay, some larvae were exposed to different concentrations of the antipsychotics haloperidol (CAS: 52-86-8), and chlorpromazine (CAS: 69-09-0), as well as the anxiolytic diazepam (CAS:439-14-5; all from Sigma–Aldrich, St. Louis, MO, USA). The drugs were solved in dimethyl sulfoxide (DMSO; from Sigma–Aldrich, St. Louis, MO, USA) which further acted as a drug control. The drug solutions were either prepared freshly or one day before the recording and administered 1.5 h before the recording started.

For the 96 microwell-plates with a total volume of 350 µL, 10 µL of the drug solutions were filled into each well. For the 12 microwell-plates with a total volume of 5500 µL, either 10, 20, or 157 µL of the drug solutions were administered dependent on the respective drug stock concentration. Thus, resulting in well-concentrations of 1, 10, and 30 µM for the drugs and .3 % for DMSO (equivalent to 30 µM) as a drug control in both plate types.

3.4 Genotyping

For some recordings of the thigmotaxis assay, offspring of heterozygous fish were used. Therefore, these larvae had to be classified as RBM-WT, HET, or HOM after the behavior recording. First, larvae were transferred from 12 microwell-plates into 96 microwell-plates. All excess water was removed, and larvae were frozen immediately at -80 °C until genotyped. DNA was extracted with Lysis Buffer (10 mM Tris, 1 mM EDTA, .01 % SDS, 100 mM NaCl, 100 µM / ml Proteinase K) followed by a 3 h incubation at 55 °C. KASP genotyping was performed according to the manufacture's recommendation (LGC, UK). For each *RBM12* mutant line, custom-designed Primers for KASP (Table 3) were made (LGC, UK). The primers contained two different dyes (FAM-dye or HEX-dye) to distinguish the DNA samples as WT, HOM, or HET.

The DNA was diluted to 5-10 ng/µL and mixed with the KASP master mix (LGC, UK) and the primers. The plates were placed in a DNA engine tetrad OCR machine (BioRad), and the following cycles were run: 15 min at 94 °C, 10 cycles of 20 sec, denaturation at 94 °C, and 1 min annealing/extension at 61 °C. The second set of 28 cycles was performed with 20 seconds at 94 °C and 1 min annealing/extension at 55 °C. Reading of the plates took place in the Applied Biosystem 7500 Real Time PCR machine. Finally, the samples were grouped into the three genotypes (RBM-WT, HET, HOM) with the SDS 2.0 software.

Table 3

KASP primers for RBM12 mutants.

Strain name	KASP primer sequence
2.1	TGACGTCTCGCTTAGTGTTCCACCACCTATGGTTGGA GGACCTCTGCCTG[GTC/C]CTATTCTAGAGCACCCTG GTTTCAGACCCAGTGTCAATACGGCACCACCT
3.1	CTATTCTAGAGCACCCTGGTTTCAGACCCAGTGTCAAT ACRGCACCACCT[GGTT/TAAAATC]TTGTTGGCGCACC AGAGCCTTTAAGGGTGATGCCACCATTTGATAATGGG
6.1	CCAGGCATTCCTCCACCTGTTGGCCCCGTCCCTGGT GGTTTAGCTGCCTCTG[G/TTTAGC]GTCCACTGTCTC TCGGAAATTCCAATCCAATGTTCCTGAATCCGTTAAA CCCTCTAAACCCCC

The blue amino acids are the WT sequence. The red amino acids are the resulting mutation.

3.5 Data Analysis

The behavior of six dpf larvae and adult fish was tracked with EthoVision XT (Version 11.5.2016, Noldus). Sleep parameters were obtained from a custom-written software called Screeny. Statistical analyses were performed in R Statistics (Version 4.1.0).

The data sets were tested with Shapiro-Wilk-Test for normality and Levene-Test for homoscedasticity. Scheirer-Rey-Hare (SRH) tests with the factors *genotype* and *strain* with Dunn's post hoc analyses were conducted in the thigmotaxis for the naive fish. SRH with the factors *genotype* and *drug group*, or *genotype* and *control* were used for drug influenced fish, followed by Mann-Whitney U-tests. For the NTT data, SRH factors were *genotype* and *strain*. Locomotion analyses were conducted with PERMANOVA (PERmutational Multivariate ANalysis Of Variance - the nonparametric equivalent to MANOVA; adonis function in the vegan package in R Statistics) (Anderson, 2001) with 10000 permutations on the dependent variables velocity in center and velocity in the outer zone (from thigmotaxis assay) or velocity in the top and bottom part (from NTT assay) with the factors *strain* and *genotype*. This was followed by SRH and Dunn's tests. For the change in velocity, one-sample Wilcoxon tests compared to the overall median of velocity change across all individuals within an assay were performed. Simple t-tests or Mann-Whitney U-tests were conducted for the sleep analysis in naive and drug influenced fish. P-values in post-hoc tests were adjusted with the Benjamini-Hochberg (BH) method, and the significance level was set to $\alpha < .05$

for all analyzes.

The results are presented as boxplots (except for velocity in the naive thigmotaxis and the NTT assays). The box ranges from the first to third quartile, with a horizontal line representing the median. The mean is depicted as a black dot. The whiskers range from the minimum (without outliers), to the maximum (without outliers), while the outliers are bordered by squares. Significant difference are marked by a horizontal line between the referring groups. Further, the boxplots are combined with dot plots, where every dot depicts one single individual.

The violin plots show the distribution's density by their width and the mean by a black dot. Significant differences between the center / upper zone are indicated by a light horizontal line, while differences between the outer / bottom zone are marked by a darker line. The dark orange line marks a significant difference in the change of velocity.

3.6 Supplementary assays

When selecting the appropriate assays to use in this project, multiple methods were developed and tested, but later abandoned for various reasons. Some of those, however, may be highly useful in future studies. Some assays, such as for thigmotaxis, have been administered in different ways to develop suitable protocols by exploring different acclimation times. With other assays, data was collected but could not be analyzed due to software problems.

The software (idtracker.ai) (Romero-Ferrero, Bergomi, Hinz, Heras, & de Polavieja, 2019), which allows analysis of group behavior, was intended to be used for the analysis of social behavior by measuring distances between individuals within a group, and group density. The setup was optimized regarding tank shape and size, video quality parameter. However data analysis could not be performed due to the requirement of extensive expertise in Python, which is beyond the scope of a master's thesis. Further, the novel tank test was repeated with groups of adult fish to measure anxiety-like behavior within a group. This data has not been analyzed.

Further, a preliminary setup for a pre-pulse-inhibition (PPI) assay was developed, but could not be used for data collection because of technical limitations, as the necessary high-frequency camera was not available at the time of setup development. The last assay was developed to assess working memory / set-shifting behavior in adult zebrafish. However, data could not be collected as this assay is very time-consuming and did not fit within the time frame of this thesis.

The assays are described in further detail in Appendix A and include setup designs, methodological approaches, and in case of the working memory / set-

shifting assay, a detailed study setup and protocol. Even though these assays have not been applied within this project, they are valuable tools for assessing behavioral parameters in zebrafish associated with schizophrenia symptoms in humans and should be considered for future studies.

4 Results

4.1 Genetics

All three *RBM12* strains in this thesis have been DNA sequenced and show mutations in the anticipated position on the *RBM12* gene (see table 4 for amino acid sequences). In the 3.1 strain, the mutation lies towards the end of the gene between the two last predicted RNA recognition motifs and is most similar to the mutation in the human gene (Steinberg et al., 2017). The 2.1 strain's mutation lies slightly in front of the 3.1 mutation assuming a truncated protein, while the mutation of the 6.1 strain is taking place shortly before the second predicted RNA recognition motif, resulting in a presumed knockout model. Fish of all strains are homozygous viable and able to breed for several generations.

Table 4

Resulting amino acid sequence in RBM12 *mutants.*

Strain name	Guide RNA	Amino acid sequence
2.1	2	...GPLP*ALF*STOP
3.1	3	...NTAPPSTOP
6.1	6	...GLAAS*V*STOP

Blue amino acids are mutated.

4.2 Behavioral Tests

4.2.1 Larvae

Thigmotaxis - Naive One of the assessed indicators of anxiety-like behavior in larval zebrafish was thigmotaxis. The cumulative duration in the center was analyzed in naive AB-WT, and all three *RBM12* strains including all three genotypes. RBM-WT were tested against AB-WT, which showed no significant difference (data not shown). Therefore, AB-WT were excluded from further analyses. Descriptive data can be found in Table 5.

Differences between genotypes within each strain were calculated with separate Dunn-tests for *RBM12* 2.1 3, *RBM12* 3.1 4, and *RBM12* 6.1 5. There was no significant difference between genotypes in *RBM12* 2.1. In *RBM12* 3.1, HOM spent less time in the center than HET ($p < .001$) and RBM-WT ($p < .001$), while there was no difference between HET and RBM-WT. The same pattern was found in *RBM12* 6.1, where HOM spent less time in the center than HET ($p =$

.028) and RBM-WT (p = .017), with no difference between HET and RBM-WT (Table

5 for descriptive data, Table B.1 for results from Dunn-tests)

Table 5

Cumulative Duration in Center in Percent

RBM12 Strain	Genotype	*M*	*SD*	Min	Max
	HOM	26.602	16.910	0.734	98.802
2.1	HET	26.696	19.711	3.559	80.339
	RBM-WT	26.889	15.719	0.565	65.062
	HOM	14.445	12.812	1.458	66.407
3.1	HET	20.741	13.932	4.588	65.254
	RBM-WT	21.025	15.555	2.147	95.164
	HOM	13.158	13.256	1.695	59.299
6.1	HET	19.409	16.740	0	73.186
	RBM-WT	19.633	16.503	0.723	65.175

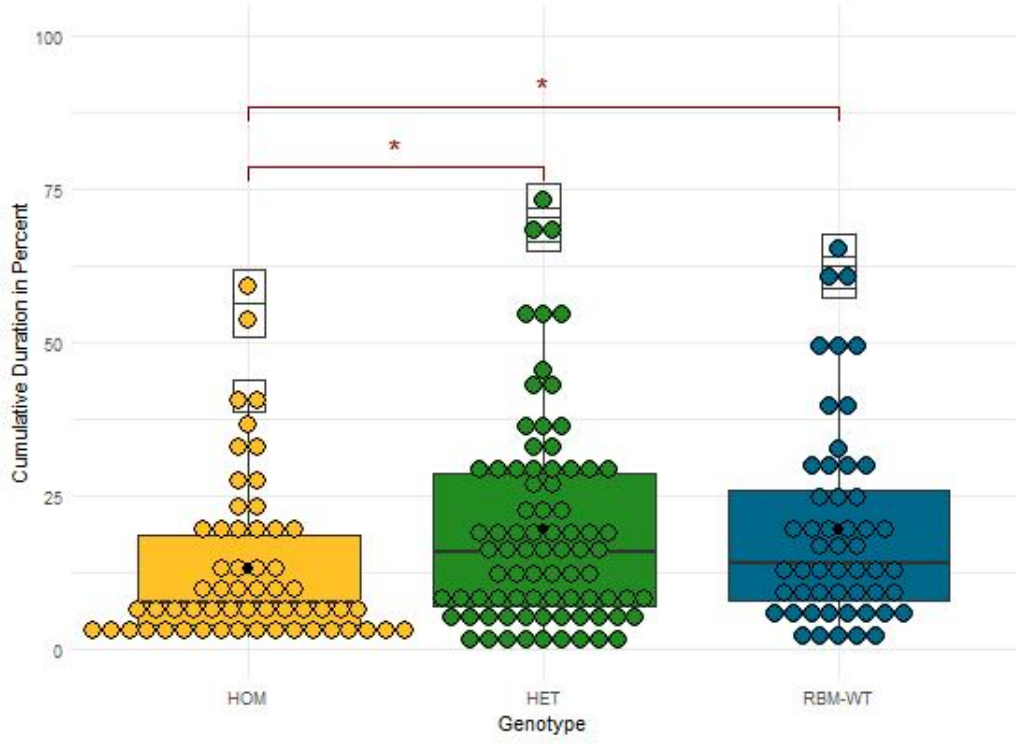

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Figure 5. Cumulative Duration in Center in *RBM12* 6.1 larvae

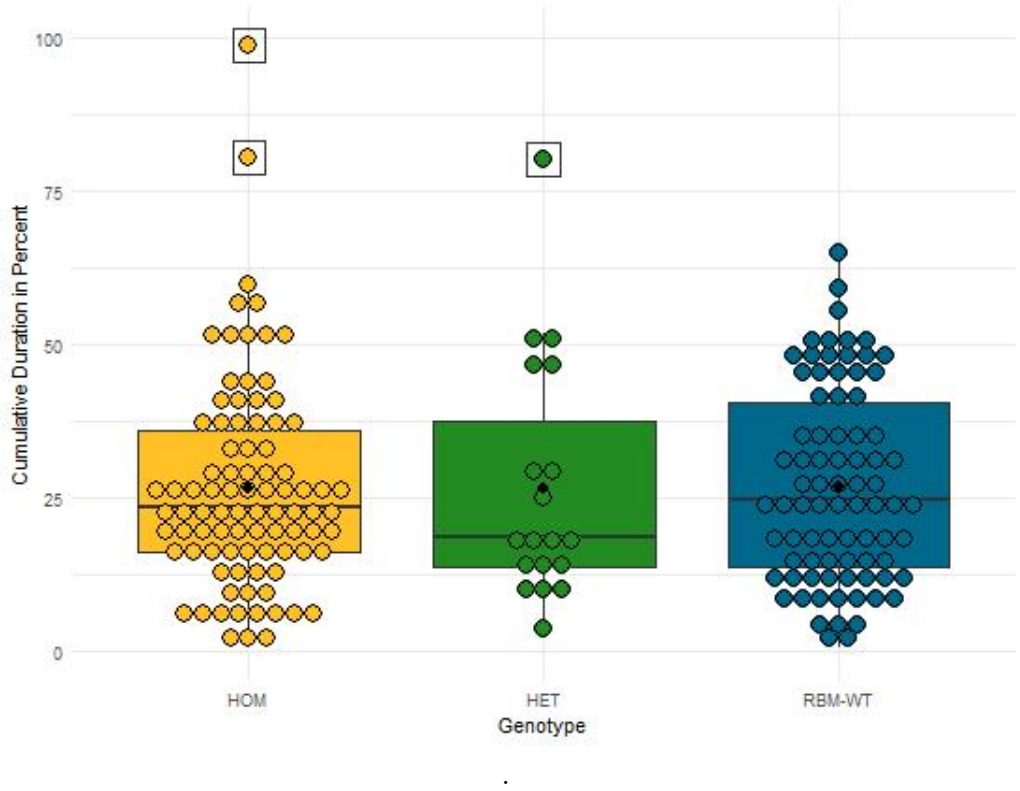

Figure 3. Cumulative Duration in Center in *RBM12* 2.1 larvae

For deeper-focused analysis in order to assess overall differences between genotypes and strains, a SRH was conducted with factors *strain* and *genotype*. This analysis was significant for main effects (strain: $H(2) = 50.980$, $p < .001$; genotype $H(2) = 16.520$, $p < .001$), but not for their interaction ($H(4) = 7.856$, $p = .097$). Next, Dunn-tests with BH adjustment were conducted separately for the factors *genotype* and *strains* for further analysis.

To explore overall differences between strains, all three genotypes within each strain have been pooled together. A Dunn-test showed that *RBM12* 2.1 larvae spent significantly less time in the center than the other two strains (compared to *RBM12* 3.1: $p < .001$; compared to *RBM12* 6.1: $p < .001$), while there was no difference between *RBM12* 3.1 and *RBM12* 6.1.

A separate Dunn-test where all strains have been pooled together to analyze overall genotype effects showed a lower cumulative duration in the center for HOM compared to RBM-WT ($p < .001$), whereas no difference was found between the other genotypes.

Furthermore, Dunn-tests were conducted to compare the individual strains separately within each genotype. In HOM (Figure B.2), *RBM12* 2.1 showed a higher cumulative duration in the center than *RBM12* 3.1 ($p < .001$) and RBM6.1 ($p < .001$), with no difference between *RBM12* 3.1 and 6.1. The same pattern

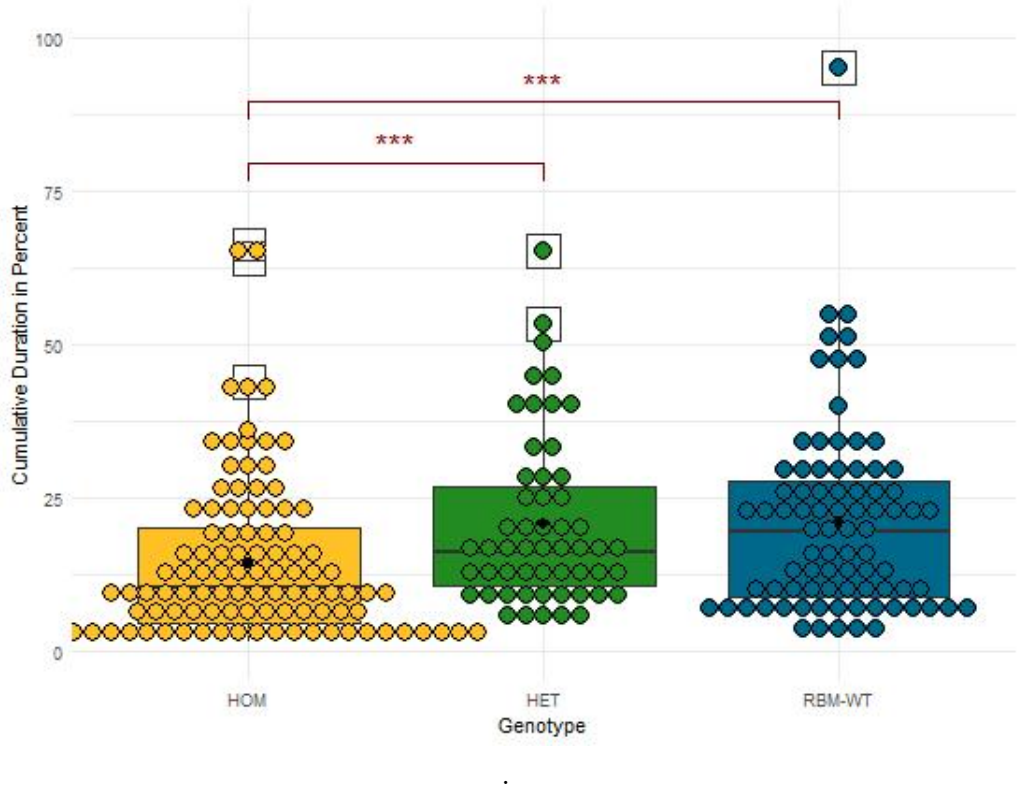

Figure 4. Cumulative Duration in Center in *RBM12* 3.1 larvae

was seen in RBM-WT (Figure B.4) where *RBM12* 2.1 spent more time in the center zone than *RBM12* 3.1 ($p = .014$) and *RBM12* 6.1 ($p = .007$), while *RBM12* 3.1 and *RBM12* 6.1 did not differ. No significant difference was found in HET (Figure B.3) for the different strains.

Exact p- and Z-values are depicted in Table B.1 for each comparison. Boxplots for the comparisons within each genotype are found in Appendix B.

Velocity - Naive Velocity was analyzed as an indicator for anxiety-like behavior, while it is often additionally seen as a marker for positive-like symptoms in schizophrenia. The same recordings as used in the thigmotaxis analysis have been analyzed for locomotion, i.e., velocity in the center and velocity in the outer zone. The change in velocity between the two zones was explored as an additional variable. First, RBM-WT were compared to AB-WT, which did not differ (data not shown). AB-WT have been therefore discarded from further analyses. Descriptive data are shown in Table 6.

Separate Dunn-tests conducted for each of the three strains showed that HOM in *RBM12* 2.1 larvae (Figure 6) moved slower in the center zone than RBM-WT ($p = .026$). In *RBM12* 3.1 (Figure 7), HOM moved faster in the center zone compared to HET ($p = .044$) and RBM-WT ($p = .009$). No significant difference

in velocity in the center zone was found for *RBM12* 6.1 (Figure 8).

Comparisons of the velocity in the outer zone with Dunn-tests within each strain, indicated that *RBM12* 2.1 RBM-WT moved faster than HET ($p = .013$) and HOM ($p < .001$). Dunn-tests for *RBM12* 3.1 and 6.1 did not show significant effects.

Table 6

Descriptive Data for Velocity in larval zebrafish

Velocity (mm/s) in	Strain	Genotype	*M*	*SD*	Min	Max
		HOM	3.950	3.052	0.676	20.680
	2.1	HET	4.505	2.121	1.113	9.111
		RBM-WT	4.551	2.755	1.287	19.887
		HOM	5.950	3.297	0.969	17.104
center zone	3.1	HET	4.738	2.437	1.099	10.819
		RBM-WT	4.650	2.543	1.174	13.915
		HOM	5.707	3.051	1.877	14.679
	6.1	HET	5.116	2.730	1.029	13.565
		RBM-WT	4.711	2.274	1.601	12.882
		HOM	2.182	1.560	0.346	14.484
	2.1	HET	1.996	0.757	0.715	3.885
		RBM-WT	2.680	1.027	0.739	6.300
		HOM	2.573	1.169	0.622	6.296
outer zone	3.1	HET	2.565	1.157	0.771	6.211
		RBM-WT	2.600	1.586	0.625	14.458
		HOM	2.279	0.909	0.449	4.971
	6.1	HET	2.500	1.101	0.286	4.933
		RBM-WT	2.375	0.943	0.685	4.394
		HOM	2.112	3.075	0.012	19.218
	2.1	HET	2.578	1.757	0.335	7.263
		RBM-WT	1.971	2.604	0.036	18.037
		HOM	3.409	2.920	0.019	13.900
change in velocity	3.1	HET	2.224	2.018	0.049	8.133
		RBM-WT	2.337	2.594	0.010	11.916
		HOM	3.430	2.766	0.002	11.298
	6.1	HET	2.620	2.379	0.111	10.549
		RBM-WT	2.336	2.170	0.170	11.103

Additionally, the change in velocity was calculated for each genotype within each strain, and compared against the overall median of all larvae ($M = 1.684$, $SE = 0.107$). The separate Wilcoxon signed-rank tests revealed a significant change in velocity for *RBM12* 2.1 HET ($p = .027$), *RBM12* 3.1 HOM ($p < .001$), *RBM12* 6.1 HOM ($p < .001$) and HET ($p < .007$).

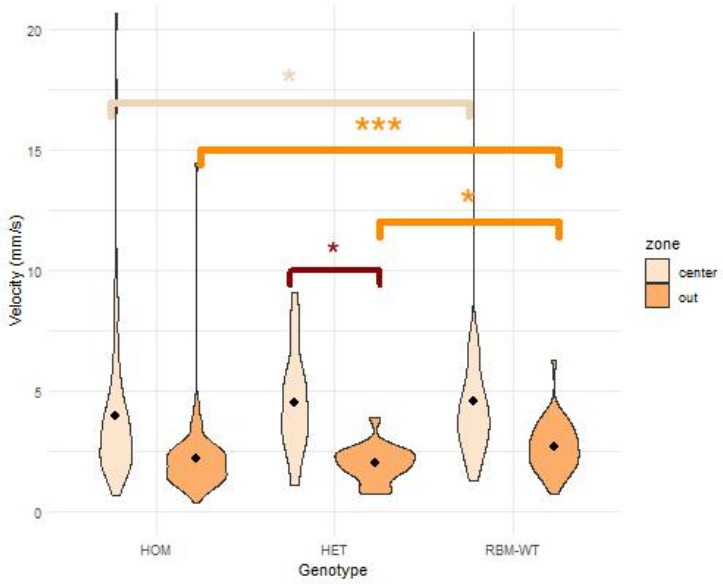

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Figure 6. Velocity in *RBM12* 2.1 larvae

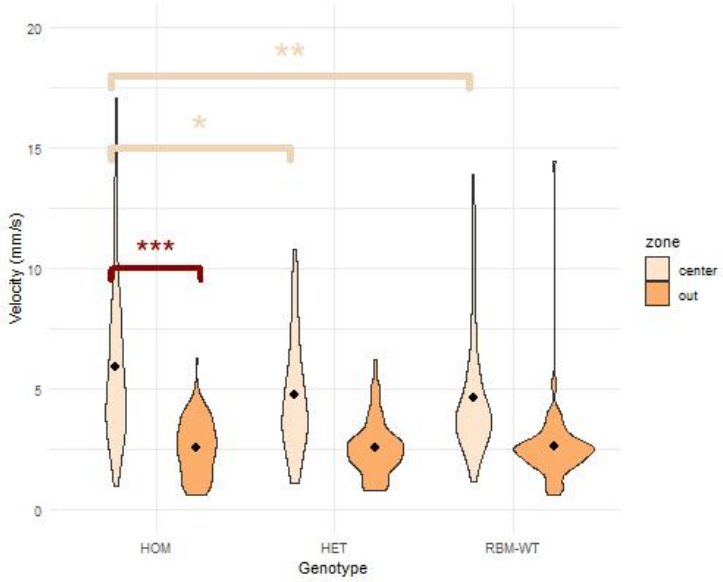

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Figure 7. Velocity in *RBM12* 3.1 larvae

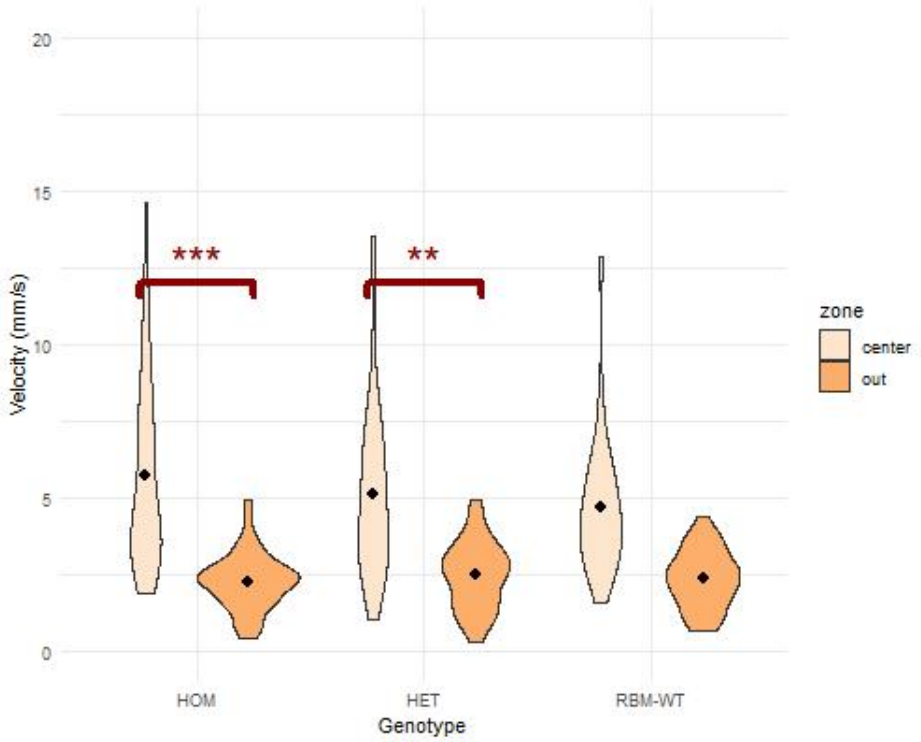

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Figure 8. Velocity in *RBM12* 6.1 larvae

For detailed analyses between the different strains, a three-factor PERMANOVA was conducted with 10,000 permutations with the factors *strain*, *genotype*, and their interaction term, on the dependent variables velocity in center, and velocity in outer zone. The PERMANOVA was significant for *strain* [$F(2, 617) = .385$, $p < .001$], and the *genotype:strain* interaction [$4(2, 617) = .0487$, $p = .001$], but not for *genotype* [$F(2, 617) = .085$, $p = .135$]. As post-hoc analyses, SRH with BH adjustments for p-values were calculated separately for the two zones.

For velocity in the center, a SRH test with the factors *strain* and *genotype* showed a significant effect of strain ($H(2) = 22.384$, $p < .001$), and of the interaction of strain and genotype ($H(4) = 16.927$, $p = .002$), but not for genotype ($H(2) = 1.212$, $p = .546$).

Additional Dunn-Tests with BH corrections showed that overall, *RBM12* 2.1 moved slower than *RBM12* 3.1 ($p <.$ 001) and *RBM12* 6.1 ($p < .001$) in the center. There was no difference between *RBM12* 3.1 and *RBM12* 6.1.

The inspection of overall differences between genotypes with Dunn-Tests revealed that in the center zone, HOM larvae of *RBM12* 2.1 demonstrated significantly lower velocity compared to *RBM12* 3.1 ($p <.$ 001) and *RBM12* 6.1 ($p <.$ 001).

For the outer zone, a SRH test with the factors *strain* and *genotype* was

significant for the interaction [$H(4) = 14.460$, $p = .0189$], but not for main effects [strain: $H(2) = 5.227$, $p = .073$; genotype: $H(2) = 5.662$, $p = .073$]. Separate Dunn-Tests for overall genotype effects in the outer zone, showed that *RBM12* 2.1 HOM larvae moved slower than *RBM12* 3.1 ($p = .003$). No other differences were noted (Figures B.5, B.6, B.7).

Sleep - Naive Sleep was analyzed in *RBM12* 3.1 HOM and RBM-WT (descriptive data in Table 7). After removing one extreme outlier from RBM-WT, the two genotypes were compared with independent t-tests or Mann-Whitney U-tests. Out of the five analyzed sleep parameters, these analyses showed significantly fragmented sleep in HOM [($t(90.95) = 2.079$, $p = .040$], as well shorter sleep duration ($U = 774$, $p = .038$). No difference was found for sleep ratio ($U = 914$, $p = .202$), velocity ($U = 1069$, $p = .930$), or wake duration ($U = 1088$, $p = .96$) (see Figure 9).

Table 7

Descriptive Data for Sleep Assay in Naive Fish

Sleep Parameter	Genotype	*M*	*SD*	Min	Max
Sleep Fragmentation	HOM	130.36	12.56	94.37	159.25
	RBM-WT	125.06	12.00	100.84	153.92
Sleep Ratio	HOM	0.54	0.08	0.38	0.74
	RBM-WT	0.55	0.10	0.10	0.72
Velocity	HOM	0.21	0.02	0.15	0.26
	RBM-WT	0.21	0.04	0.14	0.36
Wake Bout Duration	HOM	12.59	1.42	9.77	16.41
	RBM-WT	13.00	2.91	9.96	27.63
Sleep Bout Duration	HOM	15.01	3.28	9.59	22.31
	RBM-WT	16.33	3.48	8.05	25.74

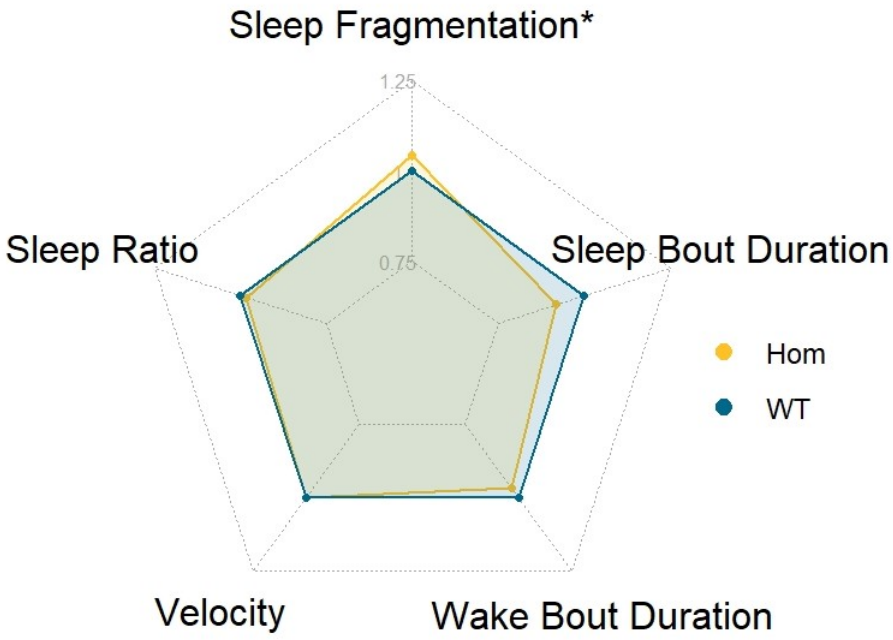

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Figure 9. Comparisons of Sleep Parameters in *RBM12* 3.1 HOM compared to RBM-WT

All sleep parameters are depicted in ratios with data from RBM-WT set to 1 as a control.

Thigmotaxis - Drug Assay As the thigmotaxis assay with naive larvae revealed an anxiety-like phenotype in *RBM12* 3.1 and 6.1, this assay has been repeated with the antipsychotics haloperidol and chlorpromazine, and the anxiolytic diazepam in order to rescue the phenotype. *RBM12* 3.1 HOM and RBM-WT larvae have been selected as the first strain for this drug administered thigmotaxis assay. Descriptive data are shown in Table (Table 8).

First, the three DMSO control groups from the three different recordings have been compared to each other for cumulative duration in the center. A SRH test with the factors *genotype* and *recording* was significant for *genotype* ($H(1) = 8.070$, $p = .005$), but showed no effect for *recording* or the interaction. Therefore, it was assumed that there is no difference between the three DMSO recordings, and the DMSO groups were pooled for each genotype.

To evaluate the effect of haloperidol, chlorpromazine, and diazepam, each in the concentration of 1 µM, 10 µM, and 30 µM, as well as DMSO in the corresponding concentration of 30 µM, drug influenced HOM were compared to naive RBM-WT to evaluate potential phenotype-rescuing drug effects. This was calcu-

Table 8

Drug Assay - Cumulative Duration in Center in Percent

Drug	Concentration	Genotype	*M*	*SD*	Min	Max
DMSO	30 µM	HOM	20.274	16.427	2.249	63.977
		RBM-WT	34.543	21.455	0.655	83.446
haloperidol	1 µM	HOM	19.359	18.08	4.678	70.102
		RBM-WT	24.172	12.646	6.893	49.955
	10 µM	HOM	15.219	10.993	1.638	39.164
		RBM-WT	25.19	10.057	10.06	36.80
	30 µM	HOM	12.616	7.961	4.136	27.684
		RBM-WT	12.283	11.098	1.661	40.915
chlorpromazine	1 µM	HOM	32.935	24.773	6.136	93.989
		RBM-WT	31.946	24,440	6.576	96.723
	10 µM	HOM	45.459	37.689	0	99.525
		RBM-WT	40.714	37.817	0.01	100
	30 µM	HOM	21.290	34.302	0	99.605
		RBM-WT	37.444	34.348	0.079	83.808
diazepam	1 µM	HOM	13.990	13.159	2.475	49.887
		RBM-WT	29.695	25.211	4.599	92.452
	10 µM	HOM	23.751	34.869	4.237	75.774
		RBM-WT	54.271	29.271	6.486	100
	30 µM	HOM	54.377	30.953	2.746	99.627
		RBM-WT	31.746	27.790	0.045	80.463

lated with individual Mann-Whitney U-tests. Altogether, DMSO, haloperidol in all three concentrations, chlorpromazine in the concentration of 1 µM and 10 µM, and diazepam in the concentration of 1 µM elevated the cumulative duration in the center zone towards a naive RBM-WT level. Comparisons are summarized in Figure 10.

Velocity - Drug Assay The same recordings as analyzed in the drug influenced thigmotaxis assay have been inspected for a phenotype-rescuing effect of velocity with haloperidol, chlorpromazine, and diazepam, where each drug was administered in three concentrations. First, the three DMSO groups have been compared to each other. A 3-factor PERMANOVA with 10,000 permutations was not significant for the factors *genotype* [$F(1, 70) = .925$, $p = .385$], *drug* [$F(2, 70) = .031$, $p = .636$], nor their interaction-term [$F(2, 70) = .306$, $p = .883$] on the variables velocity in center and velocity in outer zone. A SRH with the factors *genotype* and *drug* on change in velocity did not show significant effects, neither

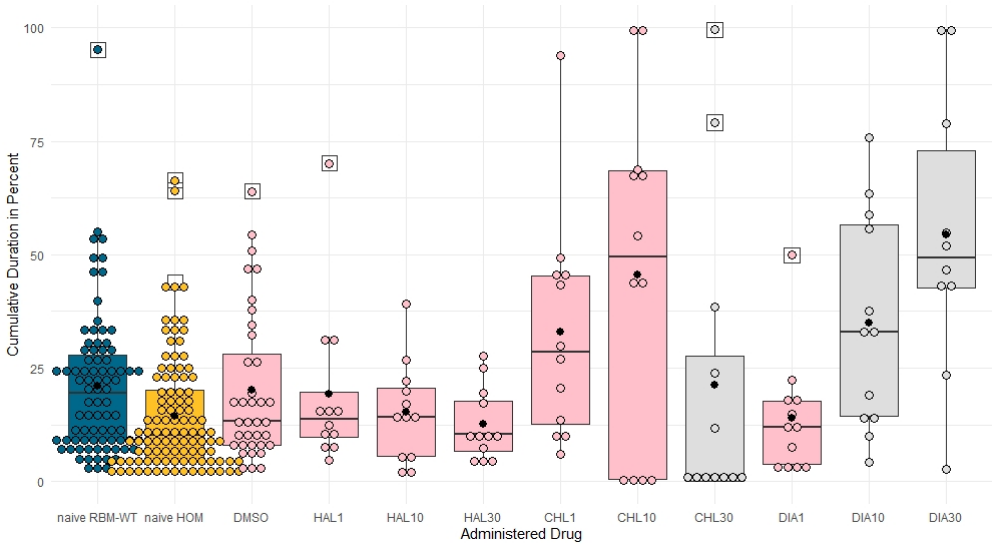

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Figure 10. Drug Effects on Cumulative Duration in *RBM12* 3.1 HOM

Depicted are *RBM12* 3.1 naive RBM-WT as control, naive HOM, and the drug effects on HOM. A rescuing effect is marked with pink boxplots. HAL = haloperidol, CHL = chlorpromazine, DIA = diazepam. The numbers 1, 10, and 30 are indication the concentrations of 1, 10, and 30 µM.

[*genotype:* H*(1)* = *41.1,* p = *.756*; drug: $H(2) = 1248.5$, $p = .231$; interaction: $H(2) = 350.9$, $p = .663$]. Therefore, DMSO groups have been pooled together.

The effect of haloperidol, chlorpromazine, diazepam, and the DMSO control has been first evaluated with a Kruskal-Wallis test [$(H(11) = 61.064$, $P < .001$] followed by individual Mann-Whitney U-tests for each drug in each concentration. Comparisons of drug influenced HOM and naive RBM-WT showed that haloperidol in the concentrations of 1 µM, 10 µM, 30 µM, 10 µM chlorpromazine, diazepam in 1 µM, 10 µM, and 30 µM, as well as DMSO, reduced the velocity in the center of *RBM12* 3.1 HOM towards a naive RBM-WT level (Figure 11).

Sleep - Drug Assay The sleep assay was repeated in order to investigate a rescuing effect of haloperidol and chlorpromazine on sleep fragmentation in *RBM12* 3.1 HOM. The antipsychotics were administered in concentrations of 1 µM, 10 µM, and 30 µM, with one DMSO control per drug. Analysis of 30 µM chlorpromazine was discarded due to total lethality. Altogether, 10 µM haloperidol, and 1 and 10 µM chlorpromazine had a rescuing effect on HOM (Figure 12).

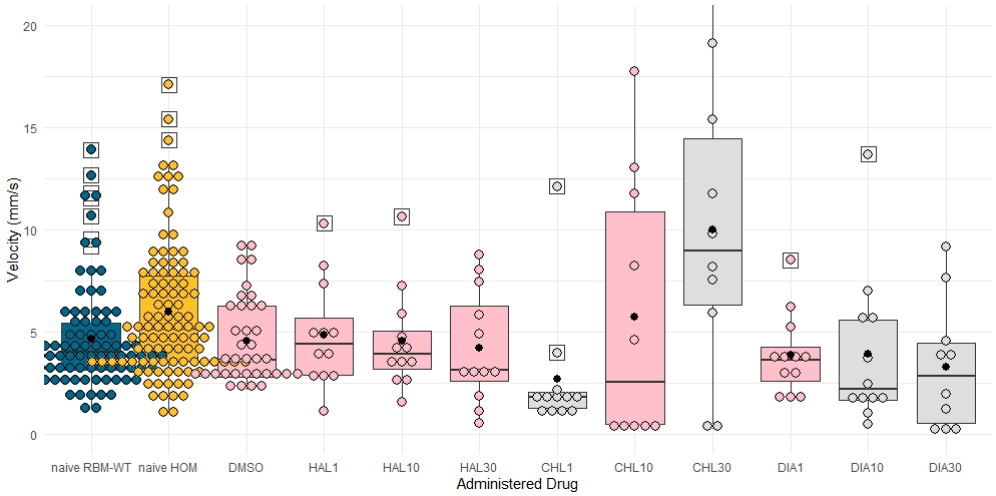

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Figure 11. Drug Effects on Velocity in Center Zone in *RBM12* 3.1 HOM

Depicted are *RBM12* 3.1 naive RBM-WT as control, naive HOM, and the drug effects on HOM. A rescuing effect is marked with pink boxplots. HAL = haloperidol, CHL = chlorpromazine, DIA = diazepam. The numbers 1, 10, and 30 are indication the concentrations of 1, 10, and 30 µM.

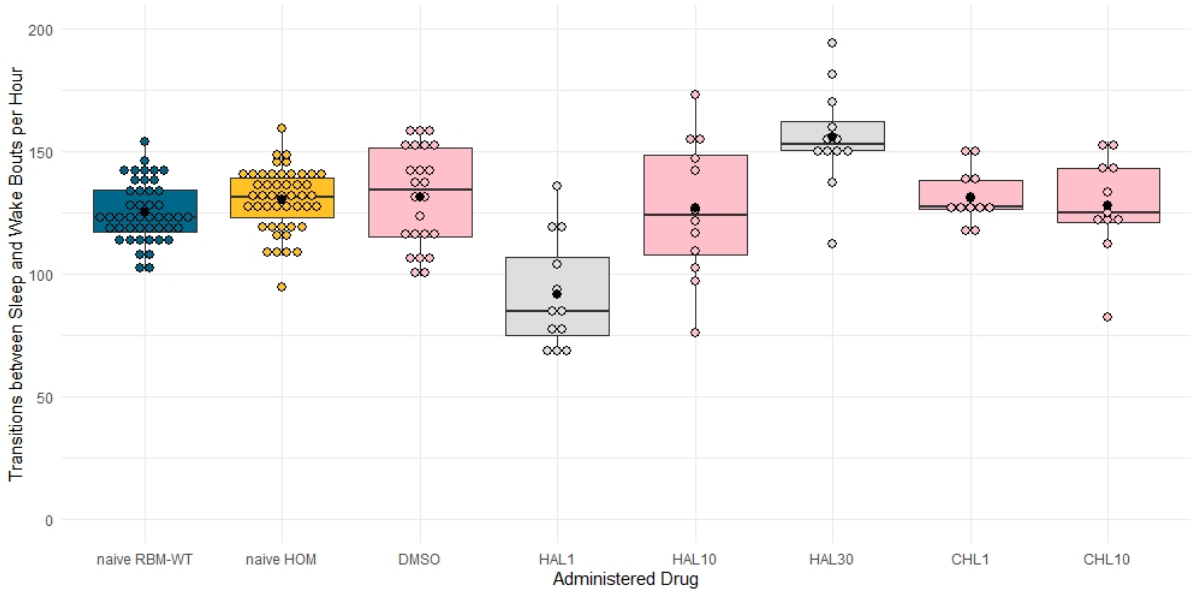

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Figure 12. Drug Effects on Sleep Fragmentation in *RBM12* 3.1 HOM

Depicted are *RBM12* 3.1 naive RBM-WT as control, naive HOM, and the drug effects on HOM. A rescuing effect is marked with pink boxplots. HAL = haloperidol, CHL = chlorpromazine. The numbers 1, 10, and 30 are indication the concentrations of 1, 10, and 30 µM.

4.2.2 Adult fish

Novel Tank Test As an indicator of anxiety-like behavior in adult zebrafish, the NTT was performed in AB-WT and all three *RBM12* strains including the three genotypes. Due to inconsistencies in the tracking process of the recordings, the cumulative duration was analyzed for both zones. However, the comparison of the two zones showed mirrored effects and trends. Therefore, only data for the bottom zone is presented in this thesis.

First, AB-WT were compared against *RBM12* RBM-WT. This comparison showed that AB-WT spent less time in the bottom zone ($Mdn = 72.854$) compared to RBM-WT of the 6.1 strain ($Mdn = 90.274$, $p = .031$). However, as 6.1 RBM-WT differed from all other RBM-WT, AB-WT were excluded from further analyses. Descriptive data for cumulative duration in the bottom zone for all strains and genotypes are depicted in Table 9.

Differences between genotypes within each strain were calculated with separate Dunn-tests with BH adjustment for *RBM12* 2.1 (Figure 13), *RBM12* 3.1 (Figure 14), and *RBM12* 6.1 (Figure 15). In *RBM12* 2.1, HOM spent more time at the bottom in comparison to HET ($p = .004$), and RBM-WT ($p < .003$). There was no difference in the bottom-dwelling behavior between genotypes in *RBM12* 3.1 fish. *RBM12* 6.1 HET showed higher cumulative duration at the bottom compared to HOM ($p = .028$), and RBM-WT ($p = .04$).

For further analyses and in order to assess differences between genotypes and strains, a SRH was performed with the factors *strain* and *genotype*. The SRH showed a significant effect for *strain* [$H(2) = 32476$, $p < .001$], the interaction between strain and genotype [$H(4) = 8722$, $p = .0176$], but not for *genotype* [$H(2) = 181$, $p = .883$]. Next, Dunn-Tests with BH adjustment were conducted separately for the factors *genotype* and *strains* for further analysis.

For investigation of overall differences between strains, all three genotypes within each strains have been pooled together, and the strains were compared. The Dunn-Test showed that *RBM12* 2.1 displayed increased bottom-dwelling behavior in comparison to *RBM12* 3.1 ($M = 73.919$, $SD = 12.805$ vs. $M = 58.071$, $SD = 19.362$, $p = .006$), and decreased bottom-dwelling behavior compared to *RBM12* 6.1 ($M = 73.919$, $SD = 12.805$ vs. $M = 89.352$, $SD = 11.361$ $p < .001$). Further, *RBM12* 6.1 spent more time in the bottom part than *RBM12* 3.1 ($p < .001$).

A separate Dunn-test to explore overall genotype differences showed significant differences between the three genotypes when data is pooled from the three strains.

Moreover, Dunn-tests were conducted to explore differences between strains

Table 9

Descriptive Data NTT

Velocity (mm/s) in	Strain	Genotype	*M*	*SD*	Min	Max
		HOM	1.254	0.414	0.7444	2.004
	2.1	HET	1.665	0.674	0.572	2.719
		RBM-WT	2.313	0.750	1.067	3.444
		HOM	6.279	1.813	4.129	10.751
upper zone	3.1	HET	6.055	1.032	4.082	7.758
		RBM-WT	5.610	1.249	4.056	7.745
		HOM	4.978	1.301	3.324	8.078
	6.1	HET	5.729	0.717	4.711	6.242
		RBM-WT	4.529	1.295	3.514	7.518
		HOM	4.858	3.516	2.022	14.458
	2.1	HET	3.419	1.296	1.401	5.857
		RBM-WT	4.479	1.592	2.868	7.661
		HOM	4.572	1.088	3.021	6.812
bottom zone	3.1	HET	5.002	1.231	3.201	7.802
		RBM-WT	4.649	0.706	3.614	6.021
		HOM	3.593	1.163	2.179	5.6344
	6.1	HET	2.128	1.234	0.735	3.929
		RBM-WT	2.937	1.241	1.434	4.910
		HOM	2.537	0.859	1.182	3.861
	2.1	HET	1.748	1.032	0.804	3.373
		RBM-WT	2.167	1.216	0.635	4.217
		HOM	1.706	1.216	0.041	5.827
change in velocity	3.1	HET	1.159	1.449	0.091	3.361
		RBM-WT	1.123	1.100	0.060	2.555
		HOM	1.385	0.827	0.327	3.030
	6.1	HET	2.903	0.832	1.815	3.564
		RBM-WT	1.434	0.992	0.120	3.369

within each genotype. In HOM (Figure B.8), *RBM12* 3.1 spent less time at the bottom than *RBM12* 2.1 ($p < .001$), and ($p < .001$), while there was no difference between *RBM12* 2.1 and *RBM12* 6.1. In HET fish (Figure B.9), *RBM12* 6.1 differed from the other two strains with increased bottom-dwelling behavior [compared to*RBM12* 2.1 ($p = .002$); compared to *RBM12* 3.1 ($p < .001$)]. This is also seen in RBM-WT (Figure B.10, where *RBM12* 6.1 presented higher cumulative duration in the bottom than *RBM12* 2.1 ($p < .001$) and *RBM12* 3.1 ($p = .001$).

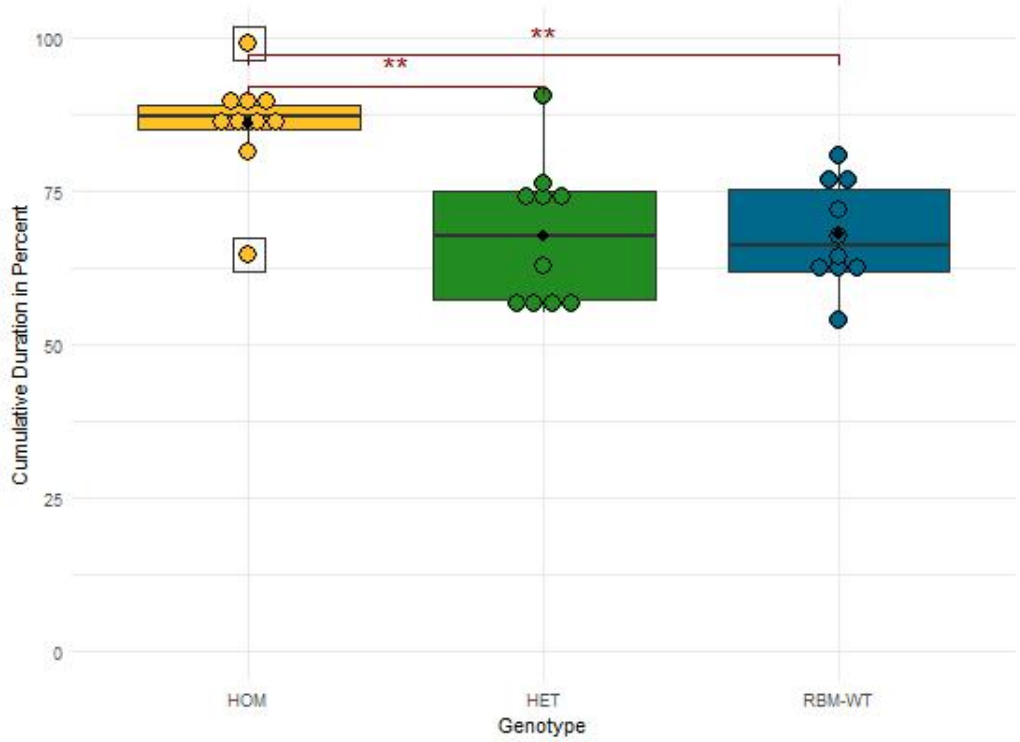

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Figure 13. Cumulative Duration in Bottom Zone in *RBM12* 2.1

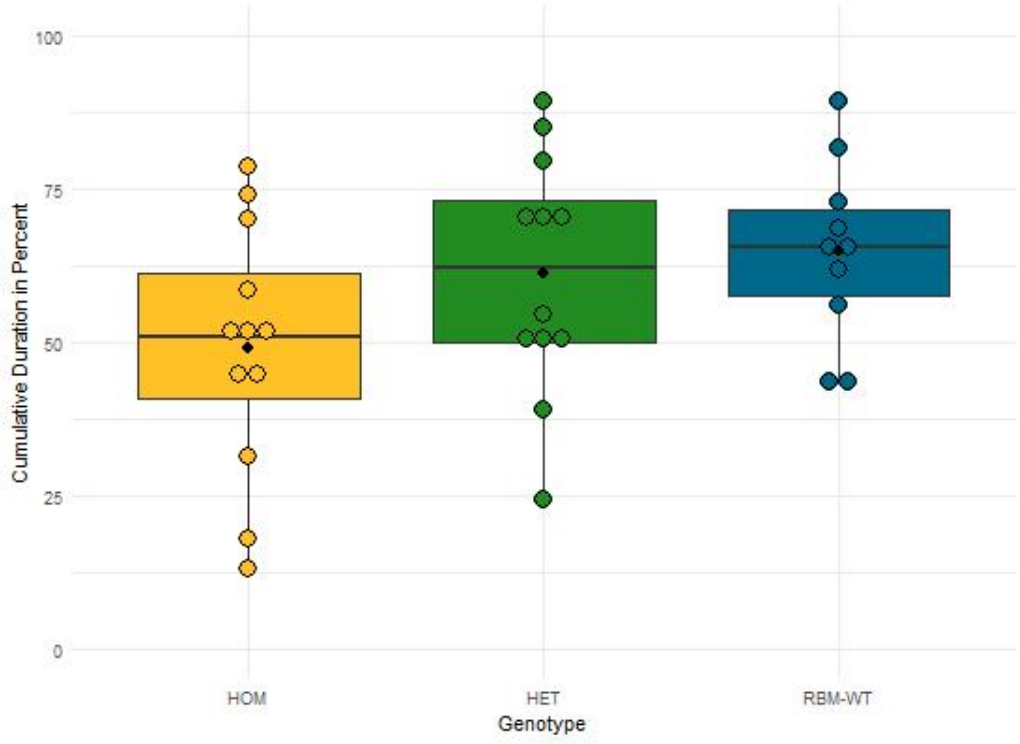

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Figure 14. Cumulative Duration in Bottom Zone in *RBM12* 3.1

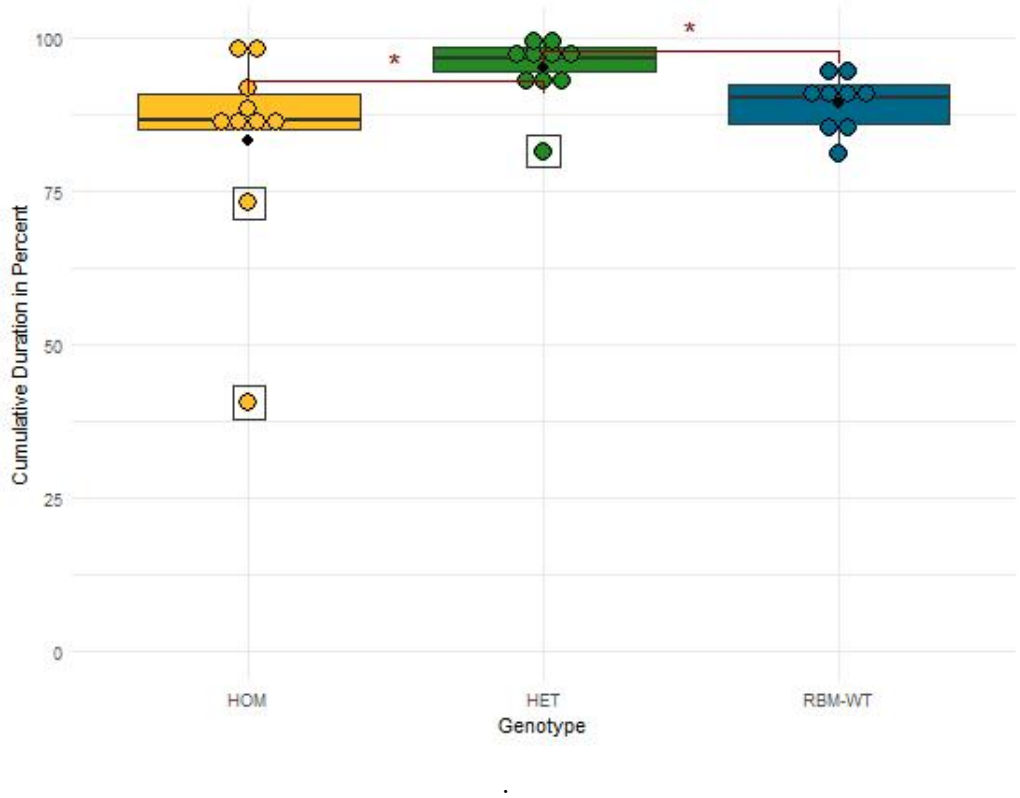

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Figure 15. Cumulative Duration in Bottom Zone in *RBM12* 6.1

Velocity Additionally to the NTT assay, velocity was analyzed in the recordings as an indicator for anxiety-like, and positive-like symptoms in adult zebrafish. Therefore, velocity in the top zone, velocity in the bottom part, and the change in velocity have been inspected. Descriptive data are presented in Table 9.

Dunn-tests were conducted separately for each of the three *RBM12* strains and the two zones. For velocity in the upper part, *RBM12* 2.1, HOM moved slower than RBM-WT ($p = .007$). No other comparison was significant.

Additionally, the change in velocity was calculated for each genotype within each strain, and compared against the overall median ($Mdn = 1.446$, $SE = 0.124$) of all fish with Wilcoxon signed-rank tests (Figures 16, 17, 18). Of all comparisons, only *RBM12* 2.1 HOM displayed a significant change in velocity ($p = .006$).

A detailed analysis to explore differences strains was performed with a three-factor PERMANOVA with 10,000 permutations with the factors *strain*, *genotype*, and their interaction term, on the dependent variables velocity in top part, and velocity in bottom zone. The PERMANOVA was significant for all three factors [*strain*: $F(2, 92) = 41.126$, $p < .001$; *genotype*: $F(2, 92) = 3.433$, $p = .012$; *interaction*: $F(4, 92) = 2.849$, $p = .005$]. This was followed by SRH with BH

adjustments for p-values for analyses of velocity in the two zones.

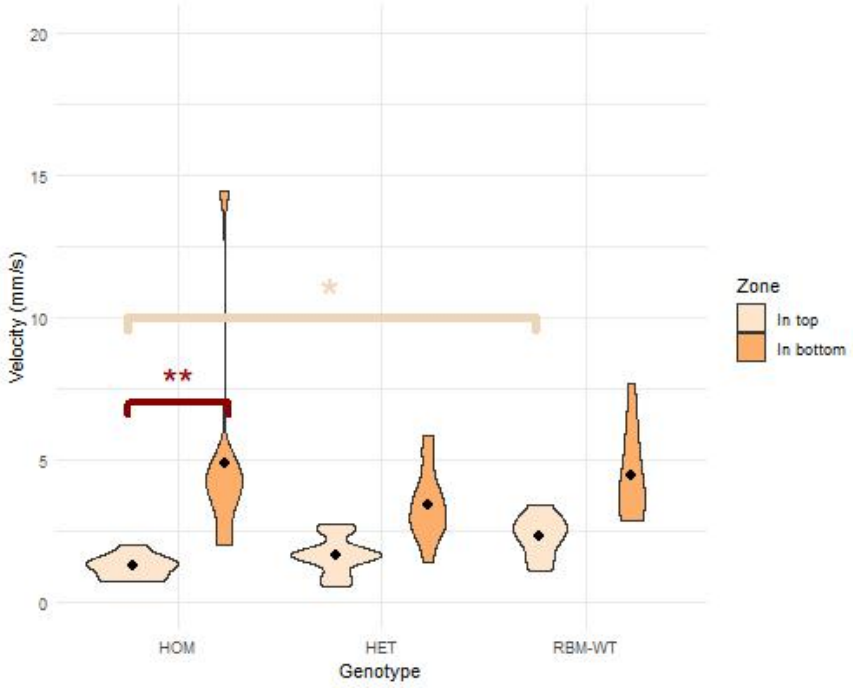

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Figure 16. Velocity in *RBM12* 2.1 adults

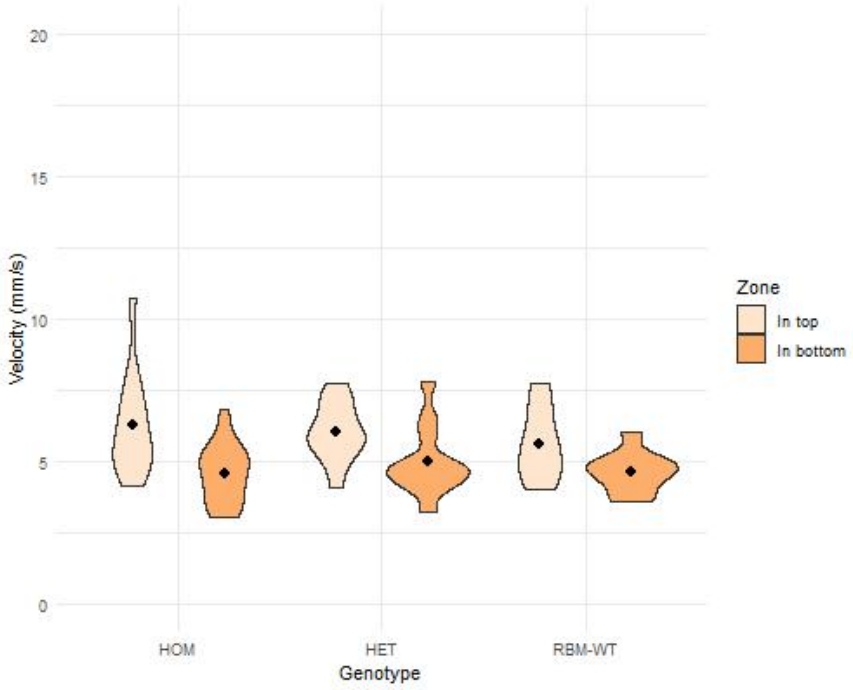

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Figure 17. Velocity in *RBM12* 3.1 adults

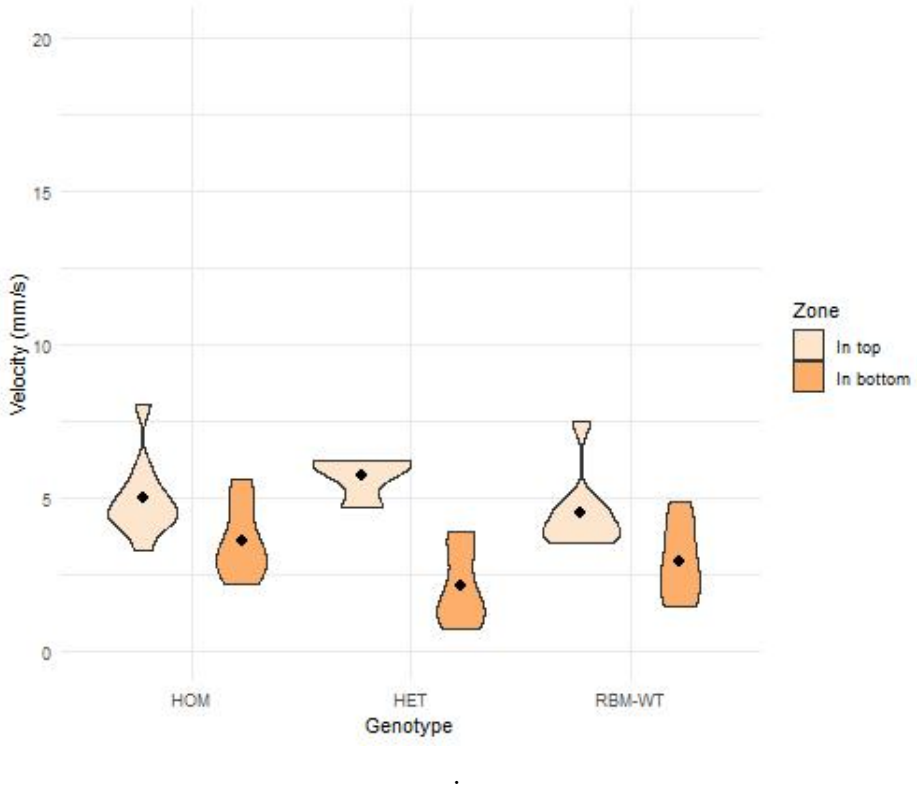

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Figure 18. Velocity in *RBM12* 6.1 adults

The SRH test for velocity in the center with the factors *strain* and *genotype* was significant for *strain* [$(H(2) = 58.777,\ p < .001)$], but neither for *genotype* [$(H(2) = 0.611,\ p = .737)$], nor factor interaction *strain* [$(H(4) = 1622,\ p = .737)$].

A Dunn-test with BH adjustments for the factor *strain* showed that *RBM12* 2.1 moved significantly slower in the upper part of the tank compared to *RBM12* 3.1 ($p <.\ 001$) and *RBM12* 6.1 ($p <.\ 001$).

For exploration of differences in velocity in the bottom part, a SRH with the factors *strain* and *genotype* was significant for *strain* [$(H(2) = 25.768,\ p < .001)$], but neither for *genotype* [$(H(2) = 2.321,\ p = .313)$], nor factor interaction *strain* [$(H(4) = 3.837,\ p = .428)$].

A following Dunn-test BH adjustments for the factor *strain* revealed that *RBM12* 2.1 moved slower in the bottom of the tank than *RBM12* 3.1 ($p = .01$), but faster than *RBM12* 6.1 ($p = .022$). Further, *RBM12* 3.1 are overall faster in the bottom zone than *RBM12* 6.1 ($p < .001$).

5 Discussion

This thesis aimed to validate a novel *RBM12* zebrafish model for schizophrenia. This approach was based on behavioral parameters which were selected according to common symptoms seen in humans with schizophrenia including anxiety, and sleep disturbances - in zebrafish analyzed with the thigmotaxis and NTT assays (anxiety-like behavior), velocity analysis (anxiety-like and positive-like symptoms), and sleep parameters. Three previously created *RBM12* mutant strains and their three genotypes were analyzed. Further, the effect of the two most common antipsychotics, haloperidol and chlorpromazine, and the frequently prescribed anxiolytic diazepam have been used in an attempt to rescue the *RBM12* 3.1 HOM phenotype in the larval assays.

5.1 Genetics

The project confirms that *RBM12* mutations alter zebrafish behavior, depending on the sgRNA position. Furthermore, in terms of *RBM12* functions, it can be assumed that the gene is not essential for survival as even the HOM of the presumed knockout strain 6.1 are viable into adulthood and breeding successfully. However, in-detail genetic analyses, for example with antibodies, should be performed in order to verify that the mutation results in the lack of protein (Zhang et al., 2021) and to rule out additional, accidental mutations due to CRISPR / Cas9 (H. Lee & Kim, 2018).

5.2 Anxiety-like symptoms

Anxiety-like symptoms were assessed in larvae in the thigmotaxis assay through the cumulative duration in the center zone (Schnörr et al., 2012), and in the NTT assay (Ahmad et al., 2012) by bottom-dwelling behavior in adult fish. Overall, there were differences between strains, genotypes, and larval and adult zebrafish.

While genotypes of *RBM12* 2.1 larvae did not differ in their thigmotactic behavior, HOM adult fish demonstrated significant bottom-dwelling behavior indicating anxiety-like behavior in adult zebrafish (Maximino et al., 2010). The reversed effect was seen for zebrafish of the *RBM12* 3.1 strain, where HOM larvae showed anxiety-like behavior in the thigmotaxis assay compared to HET and RBM-WT, but no phenotype was visible in adult fish. Mutants of the *RBM12* 6.1 strain demonstrated an anxiety-like phenotype in HOM larvae compared to HET and RBM-WT. *RBM12* 6.1 adult zebrafish did not differ regardless of genotype but showed overall increased bottom-dwelling behavior in all three genotypes.

The additional investigated indicator of anxiety-like behavior in zebrafish was velocity (Jesuthasan, 2012; Kedra et al., 2020). In comparison to the usually analyzed velocity within an entire arena (Cachat et al., 2011), this project investigated velocity separately for the two zones of the thigmotaxis and NTT assays (center and outer zone; bottom and upper part respectively), as well as the change in velocity between the two zones as an exploratory variable of measurement of anxiety-like behavior. The idea behind this approach was that if avoidance of the center zone, and bottom-dwelling behavior, as well as increased velocity, indicate anxiety-like behavior in zebrafish, then animals should move faster in the center zone and top zone of the thigmotaxis assay and NTT respectively.

Overall, *RBM12* 2.1 HOM larvae were slightly slower in the center zone than RBM-WT which was also seen in adult fish in the NTT for the upper and bottom zones indicating less anxiety-like behavior in HOM than RBM-WT. In change of velocity, *RBM12* 2.1 HOM adults moved significantly faster in the bottom zone of the NTT than in the upper zone of the NTT which leads to the conclusion of less anxiety-like behavior in HOM adults compared to the other genotypes. In larvae, on the other hand, there was only a significant change in *RBM12* 2.1 HET moving faster in the center compared to the outer zone which can be interpreted as more anxiety-like behavior in HET larvae.

In the *RBM12* 3.1 strain, HOM larvae moved faster in the center zone in the thigmotaxis assay compared to HET and RBM-WT indicating an anxiety-like behavior, but no difference was found between the adult genotypes. Further, *RBM12* 3.1 HOM larvae moved faster in the center than the outer zone leading to an interpretation of anxiety-like behavior, but no significant difference was found for the adult HOM fish, nor other larval or adult genotypes.

In the *RBM12* 6.1 strain, there was a descriptive trend for HOM and HET larvae exhibiting an anxiety-like behavior shown by increased velocity in the center zone, but the differences are not significantly relevant. Nevertheless, *RBM12* 6.1 HOM and HET larvae moved faster in the center zone than the outer zone, but no difference was found in adult fish.

Altogether, based on the two parameters of cumulative duration and locomotion, *RBM12* 2.1 zebrafish differ from the other two *RBM12* strains. *RBM12* 2.1 mutants seem to show only slight indications of anxiety-like behavior, restricted to behavior in adults. On the other hand, *RBM12* 3.1 and *RBM12* 6.1 are more alike with HOM *RBM12* 3.1 larvae and *RBM12* 6.1 HOM and HET larvae displaying anxiety-like behavior. In *RBM12* 3.1 and *RBM12* 6.1 adults, however,

the phenotype becomes less visible, and for *RBM12* 6.1 all adults seem to express anxiety-like behavior.

While the change in phenotype in the *RBM12* 2.1 strain could be explained by neurodevelopmental effects (Owen, O'Donovan, Thapar, & Craddock, 2011), as schizophrenia is a progressive disorder (American Psychiatric Association, 2013) with its onset usually during early adulthood, the disappearing phenotype of the *RBM12* 3.1 strain in adult fish is unexpected and requires further research. Nevertheless, this change in the *RBM12* 3.1 phenotype could be due to various factors. For example, fish have not been tested in a longitudinal, but a cross-sectional study design, it has to be considered that the animals in the two assays are not easily comparable. The phenomenon that the same mutation does not produce a consistent phenotype has been observed in other animal models (Winship et al., 2019). The reason behind this remains to be elucidated but could be due to different environmental experiences or previous social interactions. Also, during data collection, the fish' sex has not been documented and could have led to a bias within the data. These aspects are further discussed in the section Methodological Considerations and Limitations. These aspects could also explain the anxiety-like behavior in adults within the entire *RBM12* 6.1 strain.

5.3 Sleep

Sleep disruptions are one of the most common symptoms in schizophrenia and are related to positive symptoms (Davies et al., 2017) and possibly to negative (Blanchard et al., 2020) and cognitive symptoms (Kaskie & Ferrarelli, 2020). Therefore, sleep was analyzed as a crucial part of the validation of the *RBM12* mutants. However, only *RBM12* 3.1 HOM and RBM-WT have been used in this assay.

Results show a significantly fragmented sleep pattern for HOM larvae which is commonly seen in patients with schizophrenia (Ashton & Jagannath, 2020). In humans with schizophrenia, fragmented sleep characterizes insomnia which is shown in approximately 50% of the patients (Freeman et al., 2019). The remaining sleep parameters (sleep ratio, wake bout duration, sleep bout duration, velocity) did not differ significantly from each other, though there seems to be an indication for shorter sleep bout duration on a descriptive level.

Even though not all sleep parameters were abnormal in the *RBM12* 3.1 HOM larvae, the fragmented sleep indicates sleep disturbances. Considering the larvae's young age of 6dpf at the time of data collection, it should be noted that disrupted sleep is not only a symptom (American Psychiatric Association, 2013),

or predictor of symptom severity (Ashton & Jagannath, 2020). Recent research points out sleep disturbances as an additional risk factor for the development of schizophrenia in humans at ultra-high-risk for psychosis, namely individuals in the prodromal phase (Bradley et al., 2018; Lunsford-Avery, LeBourgeois, Gupta, & Mittal, 2015; Nuzum, Hammoud, Spencer, Akande, & Tognin, 2021; Zaks et al., 2021). Thus, individuals with subthreshold positive symptoms during the last year, brief psychotic symptoms not lasting longer than a week and dissolve spontaneously, or a genetic vulnerability for schizophrenia, are more likely to develop full schizophrenia resulting from sleep disturbances (McHugh et al., 2018).

On the other hand, this could make sleep a protective factor if sleep disturbances are detected and treated early in ultra-high-risk individuals and have already proven as favorable in some cases (Bradley et al., 2018). As zebrafish cannot be treated in the same way as humans in this regard, such as with psychotherapy, the successful rescue of sleep fragmentation in *RBM12* 3.1 HOM by dose-dependent haloperidol and chlorpromazine should be considered one possibility of prevention of schizophrenia. Future studies should investigate a possible protective effect of antipsychotics in young zebrafish larvae and their development. Although, it should be noted that these drugs have various side effects, and could even cause further sleep disturbances (Freudenreich, 2020).

5.4 Rescue of Phenotype

RBM12 3.1 larvae were selected in order to rescue the presented anxiety-like behavior with haloperidol, chlorpromazine, and diazepam. Sleep disturbances have been attempted to be rescued with the respectively mentioned antipsychotics. All drugs have been administered in three different concentrations with DMSO as a control and tested for their effect on HOM larvae. Altogether, the phenotype was successfully rescued.

Regarding the anxiety-like phenotype, it was attempted to enhance the cumulative duration in the center zone of the thigmotaxis assay, while reducing the velocity in the center zone, bringing both parameters towards the RBM-WT level. In the sleep assay, the goal was to reduce sleep fragmentation towards the RBM-WT level. While some drug applications were only effective on single parameters, others successfully rescued the phenotype in all three conditions.

As the effects of diazepam were not analyzed in the sleep assay, only its anxiolytic effect was evaluated. Here, 1 μM diazepam significantly enhanced the cumulative duration in the center in the thigmotaxis assay towards, and in higher concentrations above the RBM-WT level, while it reduced velocity towards the RBM-WT level in all three concentrations. Altogether, it can be stated that

diazepam successfully rescued the anxiety-like behavior in larval zebrafish. This is consistent with studies showing an anxiolytic effect of diazepam on zebrafish larvae (Richendrfer, Pelkowski, Colwill, & Creton, 2012; Schnörr et al., 2012), and patients with schizophrenia (Freudenreich, 2020)

The effect of haloperidol and chlorpromazine was tested in all three assays and showed an overall rescuing effect of 10 μM haloperidol, and 1 μM and 10 μM chlorpromazine on all three assessed behavioral parameters. Thus, these antipsychotics enhanced cumulative duration in the center, inhibited velocity in the center, and reduced sleep fragmentation. These effects are aligned with findings from rodent schizophrenia models regarding anxiety (Canetta & Kellendonk, 2018; Jones et al., 2011; Varga et al., 2021), but no data appears to be available about the effects of these antipsychotics on sleep in animal models for schizophrenia at the time of writing. In humans, "[a]ntipsychotics have been shown to reduce anxiety, increase anxiety, or have no effect" (Howells, Kingdon, & Baldwin, 2017). For example, while chlorpromazine can act as an anxiolytic (Meng, Li, Hou, & Zhang, 2018), it can also have an anxiogenic effect (Boyd-Kimball et al., 2018) like haloperidol (Rahman & Marwaha, 2021). Regarding sleep disturbances, studies show that chlorpromazine (Meng et al., 2018) seems to improve sleep, while results for haloperidol are inconclusive (Stummer, Markovic, & Maroney, 2018).

Nevertheless, the commonly prescribed drugs for schizophrenia were able to rescue the phenotype in the mutants, which contributes to the predictive validity of the *RBM12* model. However, DMSO, which was used as a solvent and control, improved the symptoms as well. It is therefore unclear if the rescuing effect is due to the actual drug effects or DMSO. Future studies could, instead of including only one DMSO control group in the highest dose like it was performed in this project, expand the control groups and find a more suitable control group and rectify the DMSO effect.

5.5 Positive-like symptoms

Increased velocity is often used as an indicator for positive-like symptoms in animal models, especially in drug-induced models (Winship et al., 2019). In this project, velocity was only analyzed separately for the two zones of the thigmotaxis and NTT assays, but not globally. However, based on the interpretation of increased velocity as an indication of positive-like symptoms, velocity should be also increased in the outer zone in the thigmotaxis assay and the bottom part in the NTT which was not found in the presented data of this project.

Handling increased velocity as a marker for positive-like symptoms in animals has already been highly criticized by the scientific community as the locomotion

and psychosis result from different neuronal pathways (Demin et al., 2019). Nevertheless, increased velocity correlates with anxiety, which again, is connected to positive symptoms in humans, and could therefore be an indirect predictor of these symptomatic.

5.6 Methodological Considerations and Limitations

Data collection During the time of data collection, there were frequent earthquakes that could have affected the fish' behavior and the data recordings. Therefore, it is advised to collect and analyze data from several recordings over different plates and days. Even though this was considered in most assays, the naive and drug induced sleep analyses, and the drug induced thigmotaxis and locomotion analyses are based on one single recording each. Another problem is the low sample size in the drug induced assays in larvae and the NTT for adults which should be increased in future studies. While this is easily applicable in larvae with the option for high-throughput screenings, data collection in adult zebrafish, such as for the NTT, is more laborious. Here, it is advised to perform a power-analysis beforehand (Charan & Kantharia, 2013).

Further, in the drug influenced assays, the drugs were prepared with different approaches causing different concentrations of the stock solutions. Even though this resulted in the same well-concentrations for the different drugs, the process of solving and distribution of the drug solutions may have had influenced zebrafish. Also, assays should be performed as standardized as possible, including the drug application with the same volumes.

Controlling for sexual dimorphism One aspect which has been omitted throughout data collection was controlling for sex. In humans, there are sexually dimorphic differences in incidence, disorder onset, symptom type and severity, comorbidity, life outcome, mortality, and drug response (Sommer, Tiihonen, van Mourik, Tanskanen, & Taipale, 2020). For example, women typically show a later disorder onset than men (American Psychiatric Association, 2013), and experience rather affective symptoms while men present more negative symptoms (Li, Ma, Wang, Yang, & Wang, 2016; Sommer et al., 2020). These sex-dependent disparities can also be seen in some rodent models for schizophrenia (Hill, 2016; Winship et al., 2019). However, it remains unclear whether a sexually dimorphic effect can also be seen in zebrafish as the few existing zebrafish models of schizophrenia at the time of writing this thesis did not control for this aspect. Generally, most animal studies either exclude females from their research or do not control for sex (Kekesi, Petrovszki, Benedek, & Horvath, 2015). Nevertheless,

this is a factor that should be controlled for in future studies.

Social Interaction In terms of the effect of social interaction, studies have shown that interaction with other individuals has behavioral consequences in zebrafish (Oliveira, 2013). As highly social animals, they are influenced by various factors such as sex and number of their shoal members, body size, or stripe patterns (Shams et al., 2018) which not only affect the fish' behavior towards others, e.g. through aggression (Shams et al., 2018), but also cognitive functions (Yu, Tucci, Kishi, & Zhdanova, 2006), and anxiety-like behavior (Shams, Chatterjee, & Gerlai, 2015).

Here, it has to be noted that during fish husbandry, the offspring of HET fish, which produces all three genotypes, was growing up in the same tank until genotyped as (young) adults. Thus, fish of different genotypes were interacting with each other until growing into young adulthood, which could have affected the individual fish. It should therefore be considered to note down if fish are offspring of a clean (parent fish are HOM or RBM-WT) or mixed line (parent fish are HET). Furthermore, fish are housed in tanks with transparent walls enabling them to see and possibly interact with individuals from neighbor tanks. Studies show that zebrafish do not only pay attention to conspecifics within their shoal (Shams et al., 2018), but also observe interactions between other individuals as a bystander (Abril-de Abreu, Cruz, & Oliveira, 2015b). This is also known as "social eavesdropping" and does not require physical interaction between the bystander and the observed conspecifics (Abril-de Abreu, Cruz, & Oliveira, 2015a) as fish can be separated by a physical barrier, or the observed conspecifics can be replaced by a video. This social eavesdropping does not only influence the zebrafish' social behavior towards others (Abril-de Abreu et al., 2015a, 2015b; Shams et al., 2018) and social learning (Nunes, Ruhl, Winberg, & Oliveira, 2017), but can also result in anxiety-like behavior in the bystander (Meshalkina et al., 2018). This is especially interesting in the context of schizophrenia as both, impairment in social behavior (Pinkham et al., 2020), and anxiety (Buonocore et al., 2017), are not only frequent symptoms in this disorder but are often comorbid (Pallanti, Quercioli, & Hollander, 2004).

5.7 Considerations for Future Studies

In order to validate the *RBM12* zebrafish mutants as a new model for schizophrenia, further research is needed beginning with all *RBM12* strains to undergo the same assays. This allows comparisons between the different strains and there-

with concludes about the behavioral effect of the different mutations. Further, the same assays should be used in larval and adult zebrafish. Following the neurodevelopmental hypothesis of schizophrenia (L'upták et al., 2021), some behavioral parameters might be not expressed by larval, but only adult fish. Thus, even if larval zebrafish do not show a schizophrenia-like phenotype or just a very mild form of it, it may develop in adulthood.

Furthermore, as schizophrenia results from the interaction of genetic and environmental factors (Jaaro-Peled & Sawa, 2020), and not all individuals with a genetic predisposition for schizophrenia develop the disorder (Ashton & Jagannath, 2020), future studies could use the *RBM12* strains for so called multi-hit models of schizophrenia (Bouet et al., 2021; Monte et al., 2017). In these models, several "hits" are used to combine several risk factors into one model. For example, Bouet et al. (2021) combined a genetic predisposition for schizophrenia through genetic mutations (first hit) with an early maternal separation (second hit), and a cannabinoid exposure during adolescence (third hit). This model resulted in a more severe phenotype the more risk factors got involved. For zebrafish, a possible multi-hit model could look like in a genetic predisposition due to a genetic mutation in the *RBM12* gene combined with early social isolation, which is linked to behavioral consequences in zebrafish (Shams et al., 2018), and chronic exposure to drugs, for example, cannabidiol (Hasumi, Maeda, & Yoshida, 2020).

Another consideration for future studies is the application of different antipsychotics to assess their rescuing effects on the phenotype and evaluate the predictive value of the model. For example, drugs of the second and third generation such as clozapine or aripiprazole could be administered. In contrast to haloperidol, and chlorpromazine, which belong to the first generation antipsychotics and have been used within this project, these drugs target other receptors causing different effects and side effects (Freudenreich, 2020).

While this project focused on the behavioral phenotype resulting from the *RBM12* mutation, future studies should include neurohistological and neuroimagig techniques. Considering the involvement of prominently DA (Patel et al., 2014) and glutamate (Ichinose & Park, 2020) in schizophrenia, these analyses would contribute to the validation of the *RBM12* model for schizophrenia by finding possible disruptions in the schizophrenia-linked pathways. Furthermore, these analyses will provide information on the *RBM12* gene, and help to discover its biological functionality.

5.8 Conclusion and Outlook

Altogether, this project demonstrates that *RBM12* mutations produce a strain-dependent phenotype with anxiety-like behavior indicated by thigmotactic or bottom-dwelling behavior and increased velocity during exploration, as well as sleep disturbances. Thus, showing frequent symptoms seen in humans with schizophrenia. However, the model requires further research to confirm its translational value as a model for schizophrenia as the explored behavioral parameters in this thesis are not schizophrenia-specific. Therefore, further indicators such as PPI, which is probably the easiest translatable symptom between humans and zebrafish, social behavior, and cognitive functions (proposed assays in detail in Appendix A), as well as their drug-induced rescue should be investigated in the future to meet the criteria of construct, face, and predictive validity of animal models.

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Appendix A Additional Assays

A.0.1 Larval Zebrafish

Thigmotaxis As there is no standardized protocol about the length of acclimation and recording time, one approach was to record the larvae without acclimation to save time in data collection. Larvae at 6 dpf were individually placed in 12 microwell-plates as described above. Larvae were recorded for 30 min immediately after placement in the wells. To avoid plate order effects, larvae were placed in parallel (first into well A1 of all plates, then well A2 of all plates, etc.). A total of 60 HOM and 60 RBM-WT per RBM12 2.1 and RBM12 6.1 were recorded in two runs each. Due to immense variances and scattered data, this approach was ceased (data not shown).

Pre-Pulse-Inhibition While a sudden sensory impulse (acoustic, visual, or cutaneous) triggers a startle reflex, this startle can be reduced whenever a weaker impulse is presented 30 - 500 ms before the actual impulse (Braff, Geyer, & Swerdlow, 2001). This phenomenon is known as pre-pulse-inhibition (PPI) and is robust across species. However, in patients with scz the PPI is deficient (see Swerdlow et al. (2018) for overview). Therefore, the application of a PPI assay in the RBM12 lines is a suitable approach to validate these mutants as a new model for scz.

Several studies successfully performed PPI assays in larval zebrafish (Bhandiwad, Zeddies, Raible, Rubel, & Sisneros, 2013; Burgess & Granato, 2007). However, the methodology is difficult to reproduce as all studies, published at the time of this thesis, seemed to have used custom-designed setups, except for Banono and Esguerra (2020). They presented a protocol for assembling a setup with commercially available elements which inspired the following design: After building a recording chamber and setting up the required equipment, the difficulty was to find the perfect volume and interval of pulse and pre-pulse since every setup has its own room acoustics. However, the main problem was the tracking of the video and identification of the startle movement. In larval zebrafish, the startle response is measured by a specific movement - ie, the C-start. This movement describes an escape behavior and happens within a few milliseconds (Y.-C. Liu, Bailey, & Hale, 2012). Eventually, the setup required a camera with a higher frame rate - according to the paper of Banono and Esguerra (2020) of at least 1000 fps - to be able to capture this behavior and to allow working on the remaining parameters. Due to the COVID-19 pandemic, it was not possible to purchase such a camera within the time of this thesis and the PPI assay was therefore

excluded.

A.0.2 Adult Zebrafish

Novel Tank Test The same NTT assay as described prior for individual fish has been repeated for two groups of six fish of each genotype of the RBM12 3.1 strain, as well as two groups of AB-WT. The aim was to analyze the influence of conspecifics on the anxiety response. Additionally, the groups were recorded a second time after spending 1 h in the tank to investigate habituation effects in groups in response to a new environment. However, due to software problems, this data could not be analyzed.

Social Behavior To assess social behavior, groups of fish were recorded in a commercially available bowl filled with approximately 1.5 L of system water. Two groups of six fish of each genotype of the strains RBM12 3.1 and 6.1, and AB-WT have been recorded. After netting the animals out of their home tank, they were placed in a 2 L tank and transported to the recording room. There, they were placed in the recording bowl which was illuminated from below in a custom-designed recording box and surrounded by a black curtain to eliminate external light. The camera (Basler acA2000-50gmNIR with a PENTAX TV LENS 50 mm 1:2.8 C5028-M Mount-C) was secured above the bowl and recorded 5 min long videos at 25 fps. The water temperature was measured immediately after the fish were placed into the bowl. For the RBM12 3.1 and AB-WT strain, fish were left to acclimate for 60 min before the water temperature was measured again, followed by a 5 min recording. For the RBM12 6.1 strain, fish were recorded after 5, 30, and 60 min acclimation for 5 minutes. After the recordings, fish were taken back to their home tank, and all the experimental equipment was cleaned.

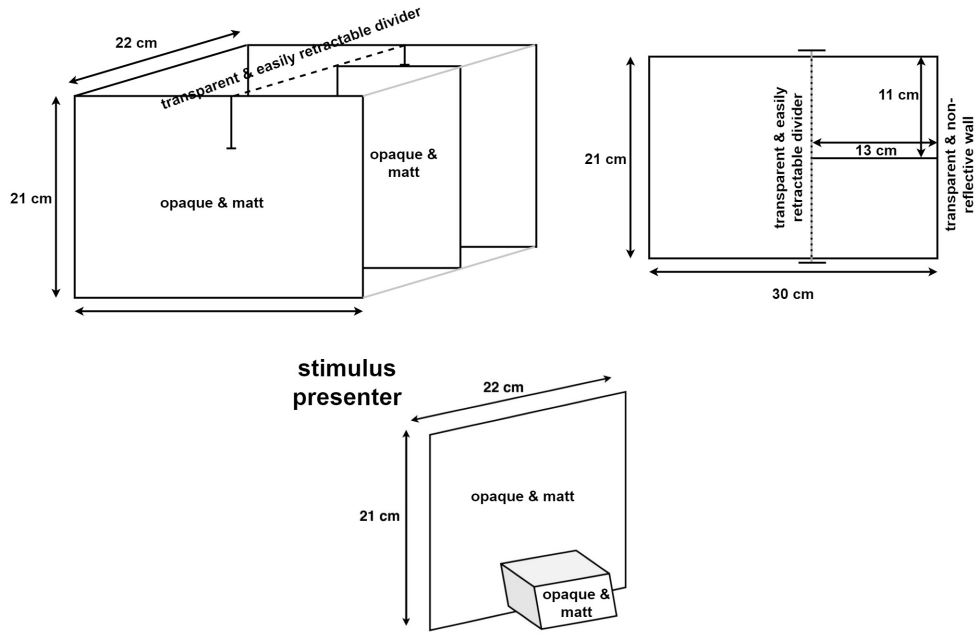
The idea was to use idtracker.ai - a recently developed software from the Champalimaud Centre in Portugal Romero-Ferrero et al. (2019). It was intended to track the trajectories and analyze the individual distances between fish, as well as the density of the group in Python. Social behavior was supposed to be indicated by smaller individual distances and higher group density. However, due to initial difficulties in the software and setup-adjustment (different bowls, lenses, etc.), the data analysis of this assay was ceased.

Working Memory / Set-Shifting One of the most impaired cognitive functions in patients with scz is working memory (Ichinose & Park, 2020). Working memory is described as a storing system with limited capacity which processes information simultaneously (Baddeley, 1992). It is involved in complex cognitive

tasks such as reasoning, comprehension, and learning. Because of consistent findings of the dysfunction of working memory in patients with scz (J. Lee & Park, 2005), its assessment in zebrafish is a promising approach for the validation of scz mutants. Within the scope of this thesis, a detailed working memory protocol has been developed but could not be applied due to limited time. The proposed approach is similar to the Wisconsin Card Sorting Test (WCST) - a neuropsychological test to assess different executive functions such as working memory and set-shifting behavior (Berg, 1948).

The WCST gives a detailed overview of cognitive abilities (Everett, Lavoie, Gagnon, & Gosselin, 2001). Especially its advantage to measure abstraction and cognitive flexibility makes this test a popular tool in scz research (Polgár et al., 2010). In the WCST, cards with four different figures in four different colors are presented. The number of figures can vary on each card. In the beginning, four different stimulus cards are presented to the participant. The participant then has to sort the remaining cards into the four groups according to color, form, or number. However, the participant is not informed about the sorting rule. After the participant puts down a card, the instructor is only allowed to say whether the participant sorted the card correctly or not. After the sorting of five successive correct cards, the sorting category is shifted to another category without warning. This is repeated until all categories are completed or all cards have been used. The number of cards and categories vary in different test versions. Several different measurements and scores of executive functions can be obtained from this test. Patients with scz typically make more perseverative errors (tendency to stick to a rule even though a new rule has been applied) and need more trials to succeed in a category (Everett et al., 2001).

At the time this thesis was written, there were two studies conducted by Parker et al. (2012) and Daggett (2016) that used a set-shifting assay to examine executive functions in zebrafish. The proposed approach was highly inspired by their designs. The test tank (30 x 21 x 22; length x height x depth) consisted of a holding area and two stimulus chambers (13 x 11 cm). The area and the chambers were divided by a retractable, transparent divider. Except for the side of the chambers, all walls were opaque and matt to avoid reflection. A stimulus presenter was designed to allow an easy presentation of color cards at the walls of the stimulus chambers (see Figure A.1 for a setup plan and picture). The chosen color pairs were red and green, and blue and orange.



Supplementary Figure A.1. Setup design for assessing set-shifting behavior

Fish were supposed to be tested individually with the following approach: On the first day, the fish is habituated to the tank without the divider for 2.5 h. Food is given as a reward whenever a stimulus chamber is entered. After habituation (phase I from day 1-7) the fish is restricted to the holding area for 10 s with the divider. Then, the divider is lifted and the fish is rewarded for entering a stimulus chamber where it is restricted for 10 s. After seven days, phase II begins and it is randomly assigned which of the two colors is rated as the correct one. In this phase, the fish is confronted with 30 discrete choice trials. First, the fish is allowed to swim freely for 30 s before it is restricted to the holding area for 30 s. Then, the divider is lifted and the fish has to enter a stimulus chamber within 90 s. By entering a chamber, the fish is restricted for 10 s and only rewarded with food if it has entered the chamber with the correct color. Next, the divider is lifted and the next trial starts as soon as the fish enters the holding area. This phase is repeated until the fish reaches six consecutive correct trials. In phase III, the procedure from phase II is repeated but the rule is reserved so that the previously incorrect color becomes the correct one and vice versa. Finally, phases IV and V are a repetition of phases II and III with a new color pair.

During the experiment, the number of correct and incorrect trials, as well as the latency to enter a chamber should be noted down. Fish should be pair-housed during the time of the experiment to allow individual identification while avoiding social deprivation which is not only stressful for the animals but also

leads to alteration in hormonal levels (Shams et al., 2018) and in behavioral responses (Cleal et al., 2020). Preferably, a pair of a female and male should be housed together to prevent aggressive behavior. Individual fish should always be tested at the same time of day to ensure consistency in between experiments and control for performance fluctuation due to circadian rhythm (Zhdanova et al., 2008). Food should only be provided during the experiments as motivation. However, if a fish does not reach enough correct trials including food rewards, food should be substituted after the experiment. Further, the fish should be acclimated for at least 10 min prior to testing to decrease stress from netting and the environmental change. According to the studies by (Daggett, 2016; Parker et al., 2012), this procedure will probably take at least three to four weeks to complete.

Appendix B Additional Results

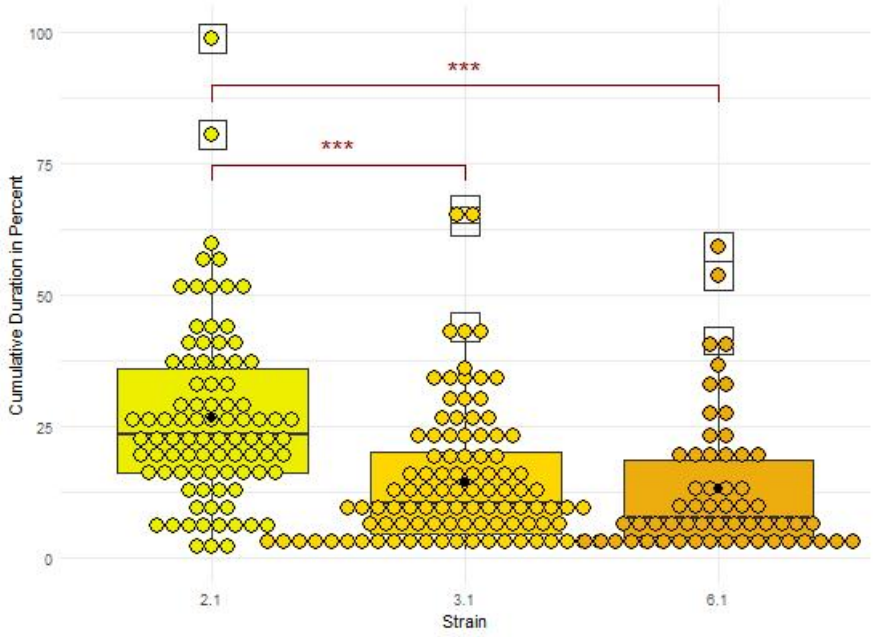
larvae

Supplementary Table B.1

Results from Comparisons for Cumulative Duration in Center of the Larval Thigmotaxis Assay

		2.1			3.1			6.1	
		HOM	HET	RBM-WT	HOM	HET	RBM-WT	HOM	HET	RBM-WT
2.1	HOM	—	$Z = 0.398$, $p = 1$	$Z = -0.219$, $p = .837$	$\boldsymbol{Z = 5.709}$, $\boldsymbol{p < .001}$					
	HET		—	$Z = -0.527$, $p = 1$		$Z = 1.057$, $p = .291$				
	RBM-WT			—			$\boldsymbol{Z = 2.589}$, $\boldsymbol{p = .014}$			
3.1	HOM				—	$\boldsymbol{Z = -3.43}$, $\boldsymbol{p < .001}$	$\boldsymbol{Z = -3.618}$, $\boldsymbol{p < .001}$	$Z = 0.935$, $p = .350$		
	HET					—	$Z = 0.245$, $p = .807$		$Z = 1.111$, $p = .400$	
	RBM-WT						—			$Z = 0.789$, $p = .430$
6.1	HOM							—	$\boldsymbol{Z = -2.598}$, $\boldsymbol{p = .0281}$	$\boldsymbol{Z = -2.540}$, $\boldsymbol{p = .0166}$
	HET								—	$Z = -0.208$, $p = .835$
	RBM-WT									—

Depicted results derive from Dunn tests with Benjamini-Hochberg adjustments. Separate tests were formed within each strain and each genotype. Significant comparisons are marked as bold.



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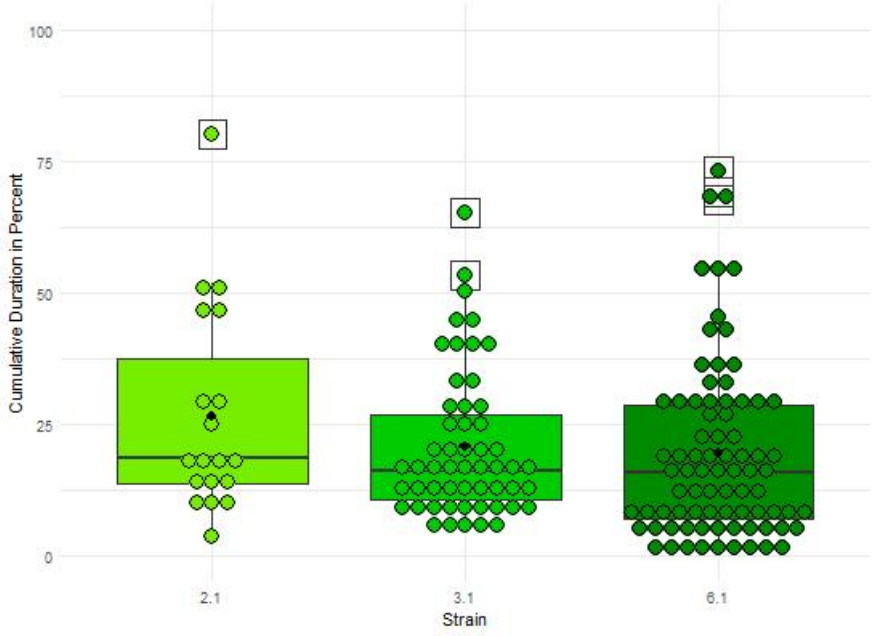
Supplementary Figure B.2. Cumulative Duration in Center in *RBM12* HOM larvae



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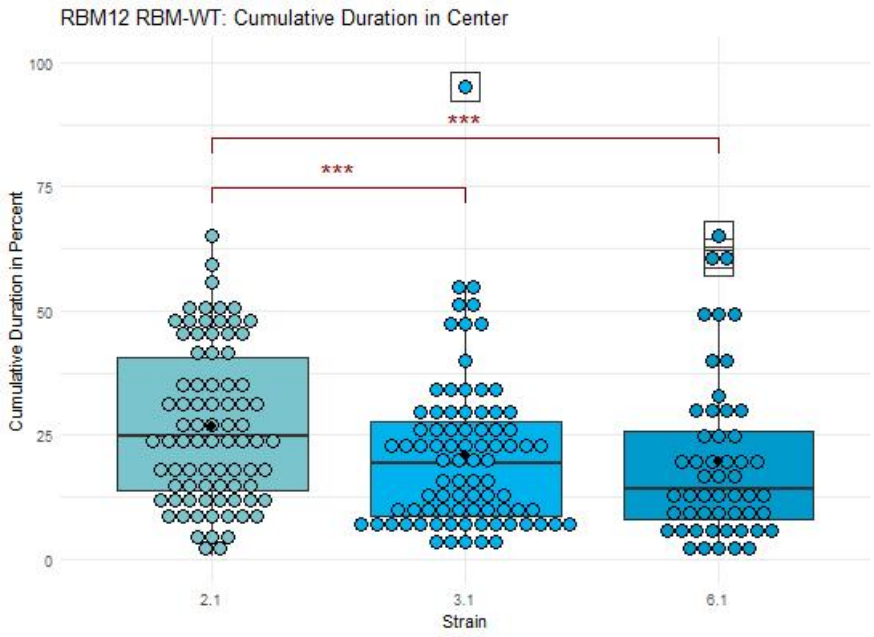
Supplementary Figure B.3. Cumulative Duration in Center in *RBM12* HET larvae



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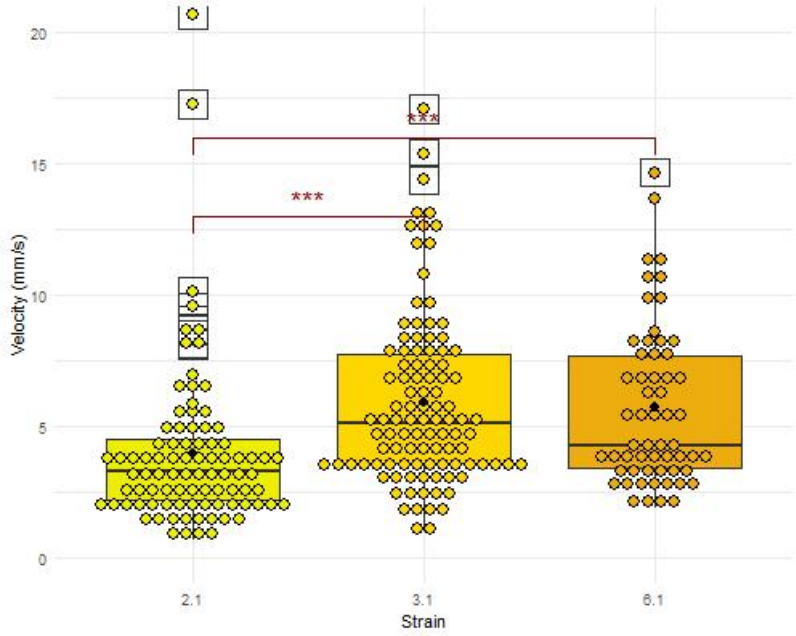
Supplementary Figure B.4. Cumulative Duration in Center in *RBM12* RBM-WT larvae



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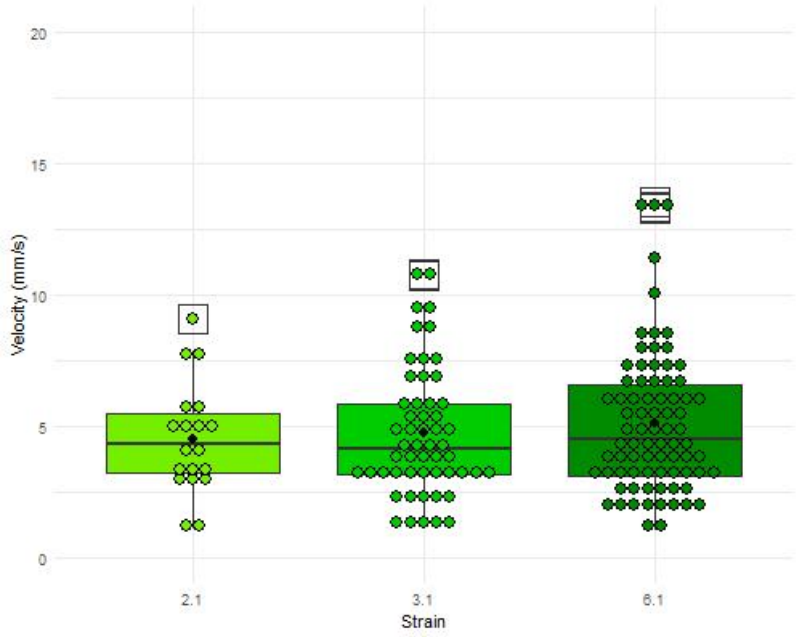
Supplementary Figure B.5. Velocity in Center for *RBM12* HOM larvae



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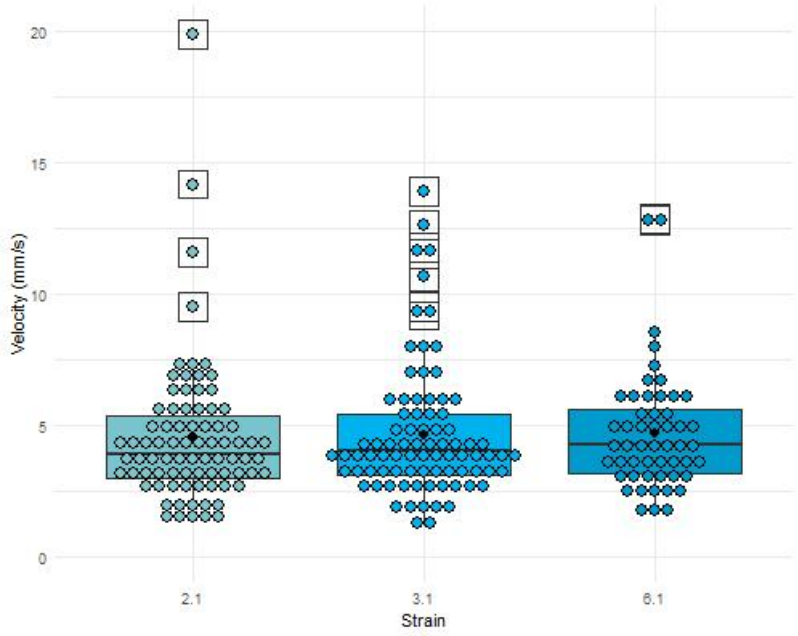
Supplementary Figure B.6. Velocity in Center for *RBM12* HET larvae



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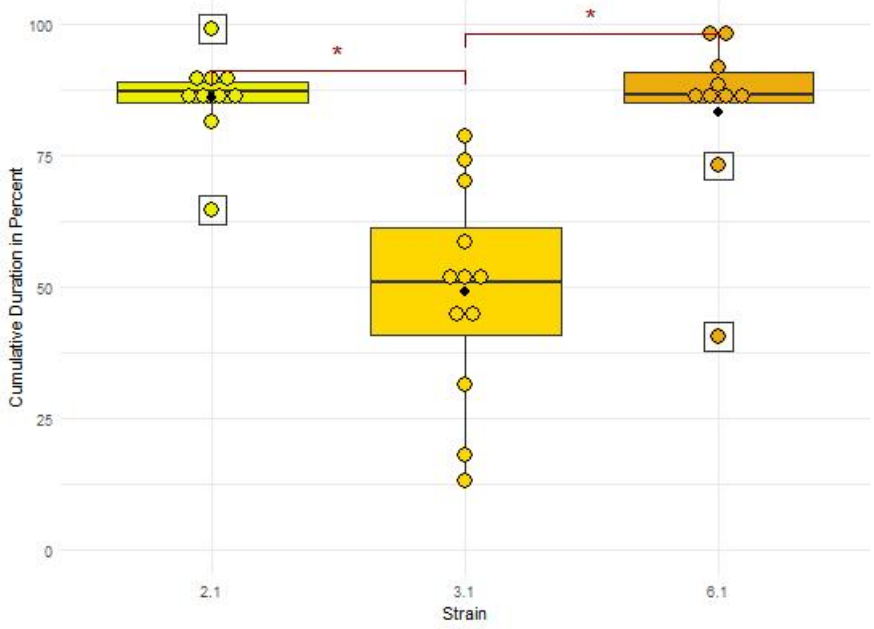
Supplementary Figure B.7. Velocity in Center for *RBM12* RBM-WT larvae

adults .



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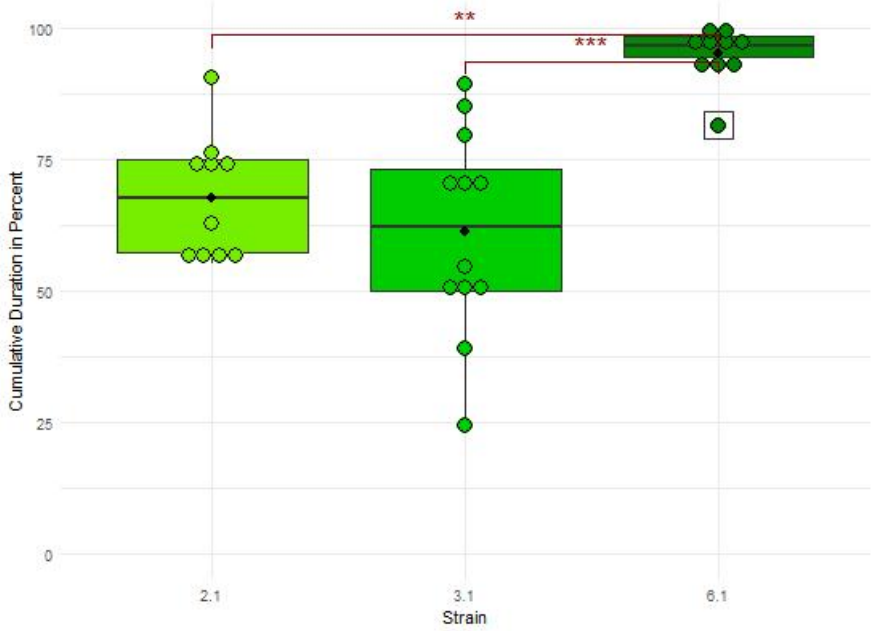
Supplementary Figure B.8. Cumulative Duration in Bottom Zone in *RBM12* HOM adults



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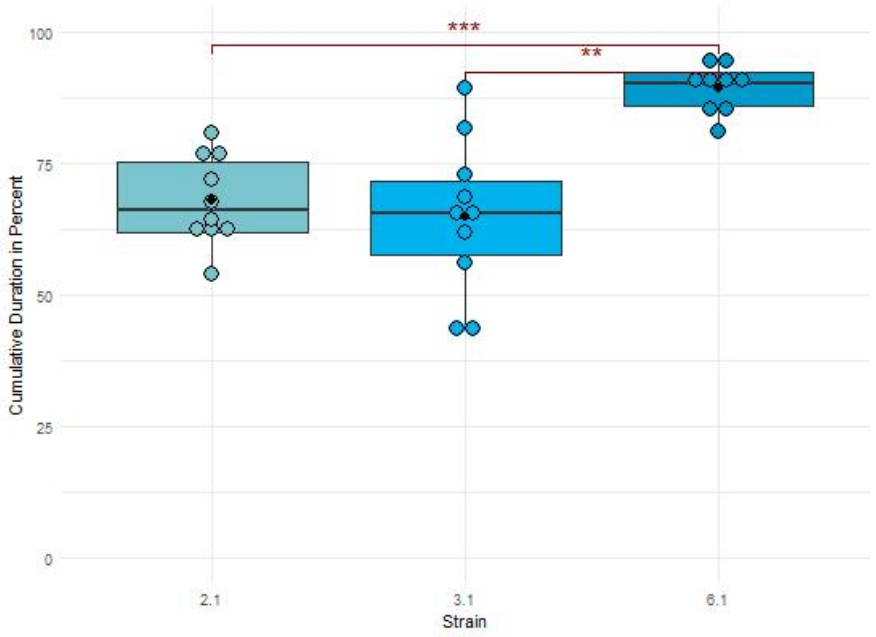
Supplementary Figure B.9. Cumulative Duration in Bottom Zone in *RBM12* HET adults



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Supplementary Figure B.10. Cumulative Duration in Bottom Zone in *RBM12* RBM-WT adults

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Datum, Ort

E. Richert

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