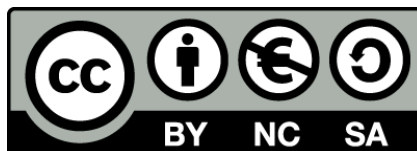




UNIVERSITAT DE
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The role of *RBFox1* and its regulatory network in major depression and comorbid psychiatric phenotypes

Maja Rebecca Adel



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Maja Rebecca Adel

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UNIVERSITAT DE
BARCELONA

Faculty of Biology

Department of Genetics, Microbiology and Statistics

Doctoral program in Genetics

The role of *RBFOX1* and its regulatory network in major depression and comorbid psychiatric phenotypes

El paper de RBFOX1 i la seva xarxa reguladora en el trastorn depressiu major i fenotips psiquiàtrics comòrbids

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to qualify for the

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THESIS SUMMARY

RBFOX1 is a master regulator of large transcriptomic networks with pleiotropic effects on numerous neurodevelopmental and psychiatric disorders, including major depressive disorder (MDD). MDD is a complex disorder characterized by symptoms such as anhedonia, hopelessness, feelings of emptiness and guilt. It frequently co-occurs with other psychiatric disorders, which can be partially explained by an overlapping genetic basis. Its aetiology involves complex interactions of biological predispositions, including a highly polygenic genetic basis, and environmental risk factors.

In this doctoral thesis we aimed to study the role of *RBFOX1* and its regulatory network on MDD and comorbid psychiatric phenotypes and characterize the underlying mechanisms through which this gene exerts its pleiotropic effects by using zebrafish models. To obtain a comprehensive overview, we followed a multi-faceted approach investigating the effects of *RBFOX1* at the levels of genetics, transcriptomics, neurotransmission, brain activity, and behaviour.

Our results show that *RBFOX1* is an essential regulator of a large network of differentially spliced and expressed genes, which substantially contribute to the shared genetic basis of MDD, anxiety, irritability, and neuroticism. The *RBFOX1*-regulated genes associated with MDD and these comorbid traits show a substantial overlap and are mainly related to the neurodevelopment and neurotransmission. We furthermore identified enrichment of *RBFOX1*-regulated genes among genes causal for rare disorders with symptoms of depression, anxiety and irritability, suggesting that *RBFOX1* contributes to these symptoms through shared pathways in psychiatric and rare disorders. Our integrative analyses highlighted 19 genes regulated by *RBFOX1*, including *CADM2*, *CTSB*, *MAPT*, *NSF*, *PAX6*, *SCAI*, *SNCA*, *SP4*, and *TCF4*, with a high pleiotropic effect on MDD and its comorbid traits.

Experiments in two *rbfox1* mutant zebrafish lines showed that the effects of *rbfox1* on behavioural changes mirror various symptoms of different psychiatric disorders. We observed a range of consistent behavioural alterations in both lines, including hyperactivity, thigmotaxis, social impairments, and aggression in only one. The behavioural alterations in *rbfox1* mutant zebrafish could be partially reversed through acute exposure to fluoxetine, a commonly prescribed antidepressant targeting the serotonergic system.

In line with our findings of the involvement of *RBFOX1*-regulated genes in neurotransmission, we observed reduced levels of serotonin, dopamine, GABA, and glutamate in forebrain regions of adult *rbfox1* mutant zebrafish. Interestingly, *in vivo* whole-brain imaging analyses showed

increased neuronal activity in midbrain and hindbrain regions of *rbfox1* mutant zebrafish larvae. Transcriptomic analyses of adult *rbfox1* mutant zebrafish revealed numerous differentially spliced and expressed genes in all brain regions, and were enriched in cell development processes and DNA repair mechanisms. These transcriptomic changes likely explain the alterations in neurotransmission and neural activity.

Keywords

Depression, psychiatric disorders, genetics, zebrafish, behaviour, brain function

RESUM DE LA TESI

RBFOX1 és un regulador mestre de grans xarxes transcriptòmiques amb efectes pleiotròpics en nombrosos trastorns del neurodesenvolupament i psiquiàtrics, incloent el trastorn depressiu major (TDM). El TDM és un trastorn complex caracteritzat per símptomes com l'anhedònia, la desesperança, els sentiments de buit i culpa. Sovint coocorre amb altres trastorns psiquiàtrics, fet que es pot explicar parcialment per una base genètica solapada. La seva etiologia implica interaccions complexes entre predisposicions biològiques, incloent una base genètica altament poligènica, i factors de risc ambientals.

En aquesta tesi doctoral, ens hem proposat estudiar el paper de *RBFOX1* i la seva xarxa reguladora en el TDM i en fenotips psiquiàtrics comòrbids, així com caracteritzar els mecanismes subjacents mitjançant els quals aquest gen exerceix els seus efectes pleiotròpics, utilitzant models de peix zebra. Per obtenir una visió global, hem seguit un enfocament multifacètic, investigant els efectes de *RBFOX1* a nivell genètic, transcriptòmic, de neurotransmissió, activitat cerebral i comportament.

Els nostres resultats mostren que *RBFOX1* és un regulador essencial d'una gran xarxa de gens afectant la seva expressió i *splicing*, els quals contribueixen substancialment a la base genètica compartida del TDM, l'ansietat, la irritabilitat i el neuroticisme. Els gens regulats per *RBFOX1* associats al TDM i a aquests trets comòrbids mostren un gran solapament i estan principalment relacionats amb el neurodesenvolupament i la neurotransmissió. A més, hem identificat un enriquiment de gens regulats per *RBFOX1* entre els gens causals de malalties rares amb símptomes de depressió, ansietat i irritabilitat, el que suggereix que *RBFOX1* contribueix a aquests símptomes mitjançant vies compartides entre els trastorns psiquiàtrics i les malalties rares. Les nostres anàlisis integratives han destacat 19 gens regulats per *RBFOX1*, incloent *CADM2*, *CTSB*, *MAPT*, *NSF*, *PAX6*, *SCAI*, *SNCA*, *SP4* i *TCF4*, amb un gran efecte pleiotròpic al TDM i als seus trets associats.

Els experiments en dues línies mutants de *rbfox1* en peix zebra han mostrat que els efectes de *rbfox1* en els canvis de comportament reflecteixen diversos símptomes de diferents trastorns psiquiàtrics. Hem observat un ampli ventall d'alteracions conductuals consistents en ambdues línies, incloent hiperactivitat, tigmotaxi, alteracions socials, en canvi, agressivitat en només una d'elles. A més, les alteracions conductuals en els peixos mutants per *rbfox1* es van poder revertir parcialment mitjançant l'exposició aguda a fluoxetina, un antidepressiu àmpliament prescrit que actua sobre el sistema serotoninèrgic.

D'acord amb les nostres troballes sobre la implicació dels gens regulats per *RBFOX1* en la neurotransmissió, hem observat una reducció dels nivells de serotonina, dopamina, GABA i glutamat en regions del cervell anterior de peixos zebra adults mutants per *rbfox1*. A més, les anàlisis d'imatge de cervell sencer *in vivo* han revelat una activitat neuronal incrementada en les regions del mesencèfal i el rombencèfal en larves de peix zebra mutants per *rbfox1*. Les anàlisis transcriptòmiques de peixos zebra adults mutants per *rbfox1* han revelat nombrosos gens amb expressió o *splicing* diferencial en totes les regions cerebrals, amb un enriquiment en processos de desenvolupament cel·lular i mecanismes de reparació de l'ADN. Aquests canvis transcriptòmics probablement expliquen les alteracions en la neurotransmissió i l'activitat neuronal.

Paraules clau

Depressió, trastorns psiquiàtrics, peix zebra, comportament, funció cerebral

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ABBREVIATIONS

5-HT_{1A} receptors	presynaptic serotonin autoreceptors
5-HTT	serotonin transporter
A2BP1	<i>Ataxin 2-Binding Protein 1</i>
ACC	anterior cingulate cortex
AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASD	Autism spectrum disorder
BDNF	brain-derived neurotrophic factor
BMI	body mass index
CADD	combined annotation dependent depletion
CBT	cognitive behavioural therapy
CELF4	<i>CUGBP Elav-Like Family Member 4</i>
CNVs	copy number variants
CPP	causal probability
DALYs	disability-adjusted life-years
dIPFC	dorsolateral prefrontal cortex
dpf	days post fertilization
DRD2	<i>Dopamine Receptor 2</i>
ENU	Ethyl-Nitroso Urea
eQTL	expression quantitative trait <i>locus</i>
FDA	Food and Drug Administration
fMRI	functional magnetic resonance imaging
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GWAS	genome-wide association study
HARs	human accelerated regions
HCN	hyperpolarization-activated cyclic nucleotide-gated
HPA	hypothalamic-pituitary
hpf	hours post fertilization
i.p.	intraperitoneal
LSD1	Lysine Specific Demethylase 1
LTP	long-term potentiation
MAO	monoamine oxidase
MAO	monoamine oxidase

MDD	major depressive disorder
mPFC	medial prefrontal cortex
NAc	nucleus accumbens
<i>NEGR1</i>	<i>Neuronal Growth Regulator 1</i>
NMDA	N-methyl-D-aspartate
OCD	obsessive compulsive disorder
OR	odds ratio
<i>PAX6</i>	<i>Paired Box 6</i>
PFC	prefrontal cortex
PGC	Psychiatric Genomics Consortium
PTSD	post-traumatic stress disorder
PVIs	parvalbumin-expressing interneurons
rACC	rostral anterior cingulate cortex
<i>RBFOX1</i>	<i>RNA Binding Fox-1 Homolog 1</i>
SCA2	spinocerebellar ataxia type 2
SERT	serotonin transporter
SMD	standardized mean difference
SNPs	single nucleotide polymorphisms
SNRIs	serotonin-noradrenaline reuptake inhibitors
SNVs	single nucleotide variants
SSRIs	selective serotonin reuptake inhibitors
SSRIs	selective serotonin reuptake inhibitors
SST	somatostatin
TCAs	tricyclic antidepressants
TCAs	tricyclic antidepressants
TRD	treatment-resistant depression
TWAS	transcriptome-wide association study
<i>VAMP1</i>	<i>Vesicle Associated Membrane Protein 1</i>
VGLUT	vesicular glutamate transporter
vmPFC	ventromedial prefrontal cortex
VTA	ventral tegmental area
WES	whole-exome sequencing
WGCNA	weighted gene co-expression network analysis
YLDs	years lived with disability

INTRODUCTION

1 MAJOR DEPRESSIVE DISORDER AND COMORBID TRAITS

Major depressive disorder (MDD), commonly known as major depression, is a multifaceted and complex disorder characterized by symptoms such as anhedonia, excessive feelings of guilt, sleep disturbances, fluctuations in weight, and cognitive impairments¹. It affects over 300 million people worldwide, with depression rates in women being twice as high as in men^{2,3}. The risk factors for MDD include biological components, such as genetic predispositions, and environmental factors, such as early life adverse events³.

1.1 The history of depression

The clinical definition of major depression as we understand it today emerged in the 19th century from what was previously called melancholy⁴. “Melancholia”, characterized mainly by fear and sadness, was first described by Hippocrates (460 – 379 BC), who believed it to result from an excess of “black bile”⁵. Over the centuries, the concept of melancholy evolved. Until the 18th century, the term melancholy was extended to describe not only the affective states of fear and sadness, but also cognitive symptoms such as delusions⁵. Towards the end of the 18th century, the concept of melancholy shifted to emphasise the aspect of partial delusions, i.e., irrational beliefs within certain boundaries, and melancholy was seen more as an intellectual disorder^{4,5}. By the turn of the 20th century, the focus returned to the affective aspects, and irrational beliefs and delusions were classified as “monomania”⁵. Since the end of the 19th century, the hypotheses and theories regarding the origins of psychiatric disorders have evolved several times. Theories ranged from neurological, organic models of the pathophysiology of mood disorders over psychological concepts of mood disorders resulting from actual or perceived losses, to the theory that psychiatric disorders are psychobiological reactions to stress⁴. Our current understanding of depression is that of a clinical syndrome with a distinguishable symptomatology, with its aetiology not included in the definition, as to this date it remains to be fully understood⁴. Depression is now conceptualized as a psychiatric disorder characterized by cognitive, emotional, and neurovegetative symptoms, with genetic and environmental factors interacting to contribute to its complex aetiology³.

1.2 Clinical presentation and prevalence

Core symptoms of MDD comprise sadness, depressed mood, including increased irritability and feelings of emptiness, loss of interest or pleasure, excessive feelings of guilt, feelings of worthlessness, hopelessness, and suicidal ideation, as well as insomnia, hypersomnia, unintentional changes in weight, fatigue, and psychomotor agitation or slowing, as laid out in diagnostic manuals such as the DSM-5 (Table 1)^{1,6,7}.

MDD, or unipolar depression, belongs to a broader category of mood disorders, which also encompasses conditions like persistent depressive disorder, disruptive mood dysregulation disorder, premenstrual dysphoric disorder, and substance-or medication- induced depressive disorder, each with its own distinct diagnostic criteria and characteristics¹.

For a diagnosis with MDD according to the DSM-5, at least five of the listed symptoms in Table 1 must present almost all day for almost every day during at least two weeks, significantly affect the individual’s functioning, and depressed mood or loss of interest must be present¹. The ICD-11 classifies major depression similarly, however, it also includes hopelessness as a symptom⁸.

In MDD, the depressive symptoms typically occur in discrete and recurring episodes¹. MDD has a high phenotypic heterogeneity, as not all patients display all symptoms, and the onset and intensity of the somatic vs cognitive symptoms can also vary⁹. While the symptomatology of MDD partially overlaps with other affective disorders, it can only be diagnosed if there is no other explanation for the symptoms, such as grief, other psychiatric disorders, or side effects of medication^{1,7}. To this day, there are no established biomarkers for a diagnosis with MDD^{10,11}.

Table 1: Diagnostic criteria of major depressive disorder according to the DSM-5¹

A. Five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning: at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure. Note: Do not include symptoms that are clearly attributable to another medical condition.
(1) Depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad, empty, hopeless) or observation made by others (e.g., appears tearful). (Note: In children and adolescents, can be irritable mood.)
(2) Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation).
(3) Significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month) or decrease or increase in appetite nearly every day. (Note: In children, consider failure to make expected weight gain.)
(4) Insomnia or hypersomnia nearly every day.
(5) Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down).
(6) Fatigue or loss of energy nearly every day.
(7) Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick).
(8) Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others).
(9) Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.
B. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

C. The episode is not attributable to the physiological effects of a substance or to another medical condition.

Note: Criteria A-C represent a major depressive episode.

Note: Responses to a significant loss (e.g., bereavement, financial ruin, losses from a natural disaster, a serious medical illness or disability) may include the feelings of intense sadness, rumination about the loss, insomnia, poor appetite, and weight loss noted in Criterion A, which may resemble a depressive episode. Although such symptoms may be understandable or considered appropriate to the loss, the presence of a major depressive episode in addition to the normal response to a significant loss should also be carefully considered. This decision inevitably requires the exercise of clinical judgment based on the individual's history and the cultural norms for the expression of distress in the context of loss.

D. The occurrence of the major depressive episode is not better explained by schizoaffective disorder, schizophrenia, schizophreniform disorder, delusional disorder, or other specified and unspecified schizophrenia spectrum and other psychotic disorders.

E. There has never been a manic episode or a hypomanic episode.

Note: This exclusion does not apply if all of the manic-like or hypomanic-like episodes are substance-induced or are attributable to the physiological effects of another medical condition.

Over 300 million people worldwide were affected by depressive disorders in 2021, with an estimated 2-month prevalence of 4-5% globally^{2,12}. The prevalence varies between countries (**Figure 1**)^{2,12}. However, the reported differences are quite small, and might be influenced by factors such as cultural variations in recognizing and acknowledging mental health issues, stigma, and methodological considerations^{2,12}.

MDD is diagnosed twice as often in women as in men, especially in social emergencies or under significant stress (see chapter 1.3)¹³. This difference in prevalence may be explained by a combination of biological, psychological, and social factors, including genetic and hormonal differences, variations in cognitive processing styles, such as rumination and self-consciousness, as well as societal gender inequalities¹⁴. However, more research is needed to fully understand how these factors affect the aetiology of depression in women and men.

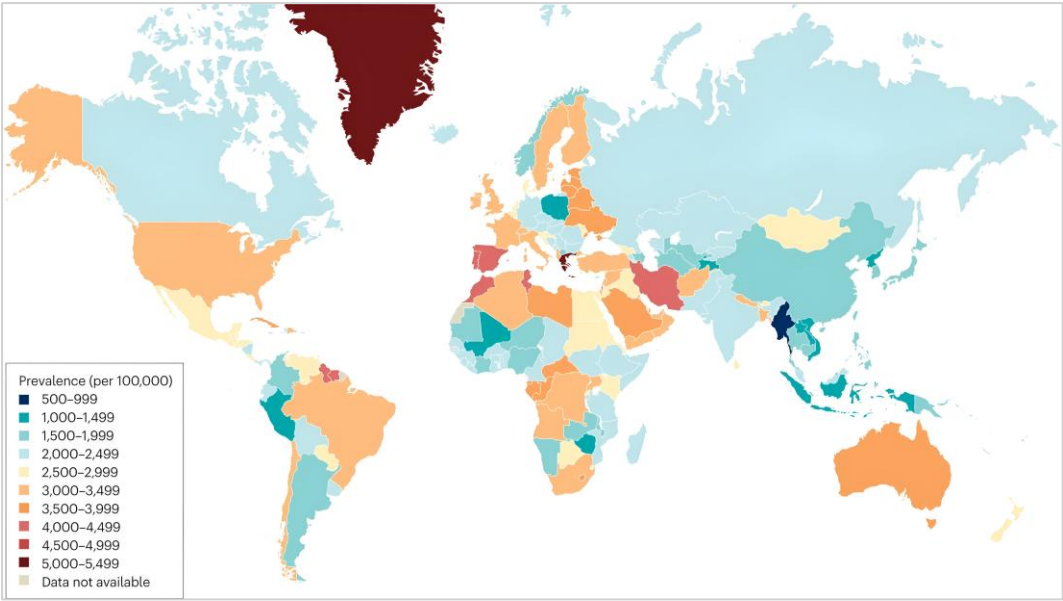


Figure 1: Worldwide prevalence of MDD. Prevalence in 2019 per 100000 individuals by country across all ages and for both sexes. Marx et al. (2023)^{12,15}

In 2019, mental disorders were responsible for 418 million disability-adjusted life years (DALYs), which is 16% of the global total, with depression representing the largest the largest proportion of DALYs among mental disorders (**Figure 2**)^{16,17}. Depressive disorders are furthermore among the leading causes of years lived with disability (YLDs), as the burden of depression increased by 36.5% between 2010 and 2021¹⁶. MDD was responsible for 80% of all depressive disorder YLDs, accounting for 37.2 million YLDs¹⁶.

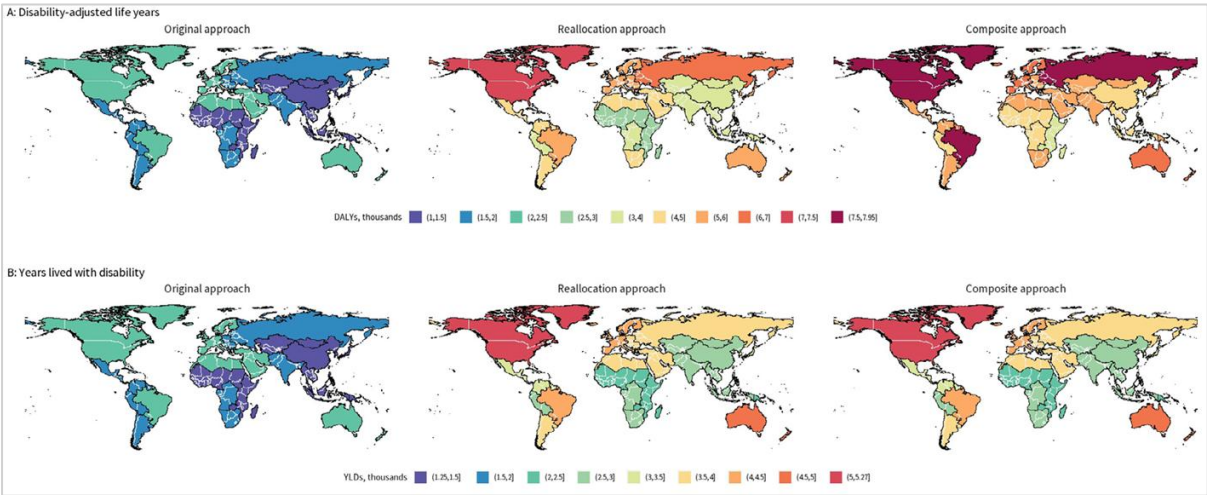


Figure 2: Global burden of mental health disorders in 2019. A DALYs and B YLDs attributable to mental disorders in 2019, by estimation approach, per 100000 population. Values are aggregated by GBD region. Original approach: Conventional GBD method using DALYs, and YLDs. Reallocation approach: Expands mental disorder classification to account for underestimation. Composite approach: Novel method incorporating premature mortality risk and monetary values. DALYs Disability-adjusted life years, YLDs years lived with disability; GBD Global Burden of Disease. Arias et al. (2022)¹⁷

Both education level and family income negatively correlate with depression severity¹⁸. The impact of mental disorders on the quality of life of patients can be severe, especially if they do not receive proper treatment. Additionally, due to medical cost, disability, and premature mortality, mental disorders also impose a significant economic burden¹⁷. The worldwide economic burden of mental disorders were estimated between 3.1 to 6.9 trillion USD, or between 2.7 and 6.1 trillion EUR, in 2019¹⁷. Therefore, it is crucial to further investigate mental disorders to improve patient outcomes, identify effective treatments, and reduce the economic burden.

1.3 Risk factors and comorbidities

MDD is a complex disorder characterized by a multifaceted interaction between genetic predispositions and environmental stressors that increase the susceptibility to the disease and affect its progression. Its heritability ranges from 30-50%, with twin studies suggesting a higher genetic risk in women than in men (40% vs 30%)^{19,20}. The genetic architecture of MDD is highly polygenic, with over 100 associated genetic loci identified in the last genome-wide association study (GWAS) meta-analyses (see chapter 3)¹⁹.

The most prominent environmental risk factors for MDD include early life adverse events, such as childhood sexual abuse and stress²¹. Early life stress is a significant risk factor for the development of MDD and is also linked to an earlier onset²¹. Early life stressful and traumatic events, specifically if they occur during the postnatal or early childhood period and adolescence, are thought to affect the neurodevelopment, especially in brain regions involved in emotional functioning, increasing the susceptibility to later life depression²².

Different risk factors are associated with depression in women and men, likely contributing to the notable difference in the prevalence. In women, personality traits like neuroticism and problems in interpersonal relationships have been found to contribute stronger to depression risk than in men, whereas in men lowered self-worth and different acute stressors, such as financial, occupational, and legal stressors, have a bigger effect²³. Fluctuations in estrogen levels, specifically estrogen withdrawal in combination with psychosocial stress and other pre-existing risk factors, are a potential vulnerability mechanism of depression in women^{24,25}. The gender disparity in MDD prevalence likely includes further hormonal as well as genetic differences, variations in cognitive processing styles, such as rumination and self-consciousness, and societal gender inequalities¹⁴. Other possible explanations for the difference in the prevalence of depression in men and women likely also include other environmental differences and sex-specific differences in the clinical presentation of depression²⁶. For instance, women are more likely to experience early life sexual abuse and gender-based violence throughout their lives¹⁴. This along with other forms of structural gender inequality might partially explain the difference in prevalence¹⁴.

MDD constitutes a risk factor for the development and worse disease progression for a range of comorbidities²⁷. It is a common comorbidity with cancer, cardiovascular disease, diabetes, metabolic syndrome, and obesity, as well as a risk factor for neurological and neurodegenerative diseases like Alzheimer's disease, dementia, and Parkinson's disease^{13,16,28–31}. MDD also frequently co-occurs with other psychiatric disorders, with anxiety disorders constituting some of the most common comorbidities of MDD^{32–34}. Between 45%-85% of MDD patients reported to have at least one comorbid anxiety disorder, which is further associated with increased suicidality and increased antidepressant resistance in MDD patients^{32–37}. Additionally, personality traits such as irritability and neuroticism are correlated with the development of psychiatric disorders such as anxiety and MDD^{38–47}. Irritability is not only a predictor for anxiety and MDD, but also correlates with depression severity, while MDD itself has a causal effect on neuroticism^{38–47}. Given that suicidal ideation is a core symptom of MDD, the disorder constitutes a major risk factor for suicide attempts and completed suicide⁴⁸.

These comorbidities likely contribute to the shorter life expectancy in MDD patients^{3,13}. One study in a Danish cohort found the overall mortality rate ratio (MRR) associated with a unipolar depression diagnosis was 2.07 compared to the general population, with an MRR of 2.32 in men and 1.93 in women, resulting in a decrease in life expectancy of 14.0 10.1 years for men and women, respectively⁴⁹.

1.4 Aetiological aspects of major depressive disorder

Different theories about the biological basis of MDD include alterations in neurotransmitter systems, hyperactivity of the HPA axis and increased inflammation in the brain, reduced neurogenesis and neuroplasticity, and network dysfunctions due to altered anatomical and functional connectivity, with all of these factors influencing and interacting with each other³. While the aetiology of depression has been an important topic of research for decades, the exact working mechanisms remain unclear.

While the involvement of the HPA axis and inflammation are considered important additional aspects in the aetiology of depression, the focus of this thesis lies on the role of key neurotransmitters, i.e., serotonin, dopamine, GABA, and glutamate, as well as alterations in neurocircuitry and connectivity, along with other potential biological processes and pathways involved in depression. Connectivity can be affected at an anatomical, functional, and effective level⁵⁰. The anatomical connectivity is altered if white matter connections are impaired, while functional connectivity usually measures statistical correlations in brain activity⁵⁰. Effective connectivity describes the direct effects of one neural system on another⁵¹. Depression could therefore be conceptualized as a complex network disorder instead of a dysfunction of individual specific brain regions^{50,52}.

1.4.1 Alterations in neurotransmitter systems

Neurotransmitter alterations have been implicated both in the development and treatment of depression, with the strongest connections observed in the monoaminergic systems, consisting of serotonergic, dopaminergic, and norepinephrinergic neurotransmission, as well as the GABAergic and glutamatergic systems^{3,50,52,53}. While the serotonergic system is the most popular target of antidepressants (see chapter 2), there is no consistent evidence for a sole causal role of lowered serotonin levels in depression^{52,54}. The dopaminergic system has been implicated in depression mostly through its involvement in reward processing and anhedonia, and GABA and glutamate have been associated with a reduced signal to noise ratios in depression and stress⁵⁵. The different neurotransmitter systems influence each other, as the dopaminergic and serotonergic systems are closely related to each other and with the norepinephrinergic system³. Dopamine and norepinephrine increase the release of serotonin, while norepinephrine can both inhibit and excite the dopaminergic neurons³. Also, GABA modulates local noradrenergic, dopaminergic, and serotonergic neuronal circuits, and GABA deficiency has been theorised to be implicated in depression since the 1980s^{53,56}.

Serotonin

Lowered levels of serotonin were previously believed to play a causal role in the aetiology of depression. However, the relationship between serotonin and depression is now understood to be more complex, involving mechanisms such as neuroplasticity and neurogenesis. Serotonergic projections in the brain originate primarily from the raphe nuclei and extend to various forebrain and limbic structures (**Figure 3**). Its main functions involve the modulation of aggression, anxiety, learning and memory, mood, sexual behaviour, sleep, and different physiological functions⁵⁰.

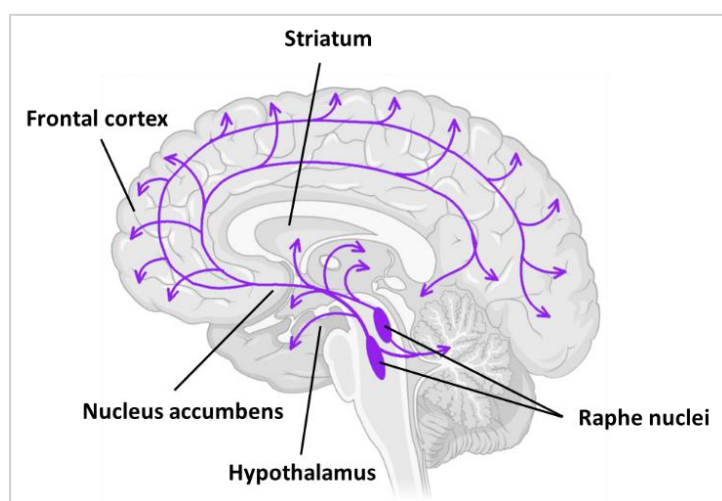


Figure 3: Serotonergic circuitry in the human brain. Serotonergic neurons originate primarily from the raphe nuclei. From there they project to various forebrain and limbic structures, including the hippocampus, nucleus accumbens, frontal cortex, and striatum. Adapted from O'Callaghan et al. (2021)⁵⁷

Serotonin has been thought to be implicated in the aetiology of depression since the 1950s⁵⁰. Catecholamine reuptake inhibitors were shown to induce depressive symptoms in some

experiments which lead to the hypothesis that lowered levels of catecholamines and monoamines were causal for depression⁵⁰. However, these experiments do not provide conclusive evidence⁵⁰. This “monoamine hypothesis” gained further popularity with the discovery of the antidepressant effects of monoamine oxidase (MAO) inhibitors and tricyclic antidepressants (TCAs) (see chapter 2)^{50,54}. It was further reinforced in the 1980s by the observation that selective serotonin reuptake inhibitors (SSRIs) had antidepressant effects in some patients (see chapter 2)^{50,54}. Furthermore, in study participants with a family history of depression, serotonin depletion was associated with a slight decrease in mood^{52,58}. There is some evidence pointing to the involvement of the serotonin precursor tryptophan in depressive mood, as unmedicated patients with depression in remission showed a decrease in mood after tryptophan depletion⁵⁸. Nevertheless, conclusive evidence of a link between tryptophan depletion and depression has yet to be demonstrated⁵⁸. Moreover, during chronic antidepressant treatment, inhibitory presynaptic serotonin autoreceptors (5-HT_{1A} receptors) are downregulated, which increases the probability of serotonin release³. Potential mechanisms through which the serotonergic system contributes to depression include its role in reward processing, which is known to be dysregulated in patients with depression (see chapter 1.4.2)^{52,58}. Additionally, serotonin modulates neuroplasticity, especially during early development, which has been found to be dysfunctional in patients with depression⁵⁹.

However, in their umbrella review about the serotonin depletion hypothesis in depression, Moncrieff et al. (2023) exhaustively analysed numerous different reviews and meta-analyses, and could not find conclusive evidence supporting the involvement of serotonin in depression⁵⁴. The authors highlight that studies examining serotonin activity or availability, tryptophan levels, and genetic factors have not consistently linked altered serotonin levels to depression⁵⁴. However, they emphasize the need for studies with better quality and higher power to conclusively confirm or refute the role of serotonin in depression⁵⁴. They also stress the importance of taking pharmacological treatment into account when studying the effects of neurotransmitters on psychiatric disorders^{54,60}. The serotonergic system is a main target for antidepressant medications, however, the working mechanisms of these drugs are not fully understood⁵⁰. It is crucial to investigate both the role of serotonin in depression and how antidepressant effects are produced by these drugs to develop better treatment outcomes in patients.

Dopamine

The dopaminergic system is thought to contribute to depression most prominently through its involvement in anhedonia, i.e., the loss of interest and pleasure, which is a core symptom of depression and negative prognostic factor for treatment outcome⁵². Disruptions in the reward system play a key role in the development of anhedonia, and dopamine is the main modulator of

reward processes. However, dysfunctions in the dopaminergic system alone do not explain the whole symptomatology or pathophysiology of depression. Dopaminergic neurons primarily originate in the substantia nigra, projecting to dorsolateral regions of the striatum, and from the ventral tegmental area (VTA), projecting towards the ventromedial striatum, including the nucleus accumbens (NAc), to the hippocampus, hypothalamus, and frontal cortex (**Figure 4**). Apart from modulation of reward processing, dopamine is involved in motor control, affective and emotional states, memory, and cognition^{61,62}.

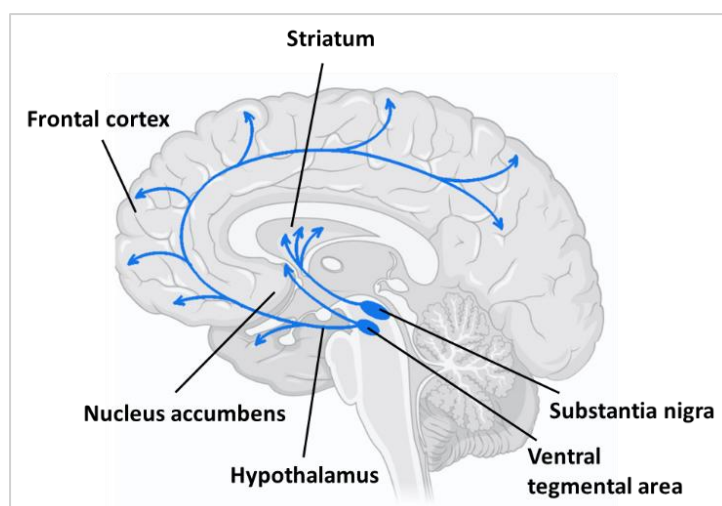


Figure 4: Dopaminergic circuitry in the human brain. Dopaminergic projections originate primarily from the substantia nigra and the ventral tegmental area. From the substantia nigra they project towards dorsolateral regions of the striatum. The ventral tegmental area projects dopaminergic neurons towards the ventromedial striatum, including the nucleus accumbens, the hippocampus, hypothalamus, and frontal cortex. Adapted from O'Callaghan et al. (2021)⁵⁷

Dopamine is the main neurotransmitter modulating reward processing, which has been modelled in animals, and shown to be dysfunctional in patients with depression^{3,52,61,63}. In patients with depression, anhedonia is closely related to a lack of afferent control of medial frontal cortical regions over the dopaminergic system (see chapter 1.4.2)⁶². Dopamine release in response to reward has been shown in the striatum, specifically the NAc, and dopamine release has been correlated with a hemodynamic response in the substantia nigra, VTA, and ventral striatum during reward anticipation^{52,64}. Moreover, dopamine release was associated with an increase of activity in the limbic system and hippocampus in response to high reward⁶⁴. Findings of reduced dopamine transporter binding and increased D₂ receptor binding in striatal regions of MDD patients suggest compensatory mechanisms resulting from the downregulation of the dopaminergic system⁶⁵. However, the afferent circuits that regulate these mechanisms remain unclear. While dopamine is the most prominent modulator of reward processing circuits in the brain, it is also regulated by the serotonergic, GABAergic and glutamatergic systems^{52,66}.

In animals, depression is often modelled through chronic and/or unpredictable stress, which correlates with a decrease in dopamine in the VTA⁶². After acute activation of the dopaminergic system, a prolonged state of decreased responsivity follows⁶². As a result of prolonged stress, tonic dopamine population activity first increases, sensitizing the system to stimuli, and then significantly decreases after a longer time⁶². This state of decreased dopaminergic neuronal

activity, reflected in depressive-like behaviour, could be reversed through the activation of dopaminergic neurons⁶².

GABA and glutamate

GABA and glutamate are thought to contribute to the pathophysiology of depression mainly through imbalances in neuronal inhibition and excitation, which can then lead to alterations in brain connectivity and reduced neuroplasticity⁶⁷. GABA and glutamate are the main inhibitory and excitatory neurotransmitters in the human brain, respectively. Glutamate is transformed into GABA in GABAergic terminals by the glutamic acid decarboxylase⁵³. In MDD, lower levels of both neurotransmitters were found in cortical and limbic brain regions, and animal studies showed a causal relationship with depressive-like behaviours⁶⁸. GABA deficits result in impaired cortical inhibition, which was also modelled in animals^{68,69}. Interestingly, these inhibitory deficits have also been found in other psychiatric disorders such as obsessive compulsive disorder (OCD) and schizophrenia⁶⁹. The implication of glutamate in depression has been further highlighted by the discovery of the rapid antidepressant effects of ketamine, a N-methyl-D-aspartate (NMDA) receptor antagonist^{3,70}. Ketamine is thought to target NMDA receptors on GABAergic interneurons, reducing the inhibition of glutamate release in glutamatergic neurons³. The effects of Ketamine are furthermore linked to increased neuroplasticity and synaptogenesis⁶⁷. Structural and functional alterations in GABAergic and glutamatergic neurons could lead to signalling dysfunctions in cortical and hippocampal regions in patients with depression. Alterations of GABAergic neurotransmission reduce the efficacy and precision of neural circuits by missing inhibitory control of excitatory systems in these brain regions⁶⁸.

Another link between the disruption of the GABAergic and glutamatergic systems, which in turn could be related to alterations in brain connectivity in patients with depression, is stress⁶⁸. In animal models, acute stress increased extracellular glutamate in the medial prefrontal cortex (mPFC) and hippocampus, possibly leading to neuronal atrophy in these regions, while chronic stress lowered glutamate cycling in the PFC⁶⁸.

1.4.2 Neurocircuitry and connectivity in the depressed brain

Depressive symptoms are associated with dysfunctional emotional processing, driven by bottom-up hyperactivity in subcortical regions and impaired top-down inhibition from cortical areas. This includes upregulation and increased connectivity of the affective and default mode networks, and downregulation and decreased connectivity of the cognitive control and reward networks.

These disruptions in emotional processing are described in Beck's cognitive model of depression, in which negative thought patterns and cognitive distortions play a central role in the onset and

persistence of depressive symptoms. According to this model, patients with depression are biased towards negatively experiencing and processing stimuli, excessively recalling depressive episodes, ruminating on negative thoughts, and building negative internal representations^{63,71}. These negative representations of the self, the external world, and future perspectives result in depressive schemas, and are thought to stem from early adverse events, as well as genetic and personality factors (**Figure 5**)⁶³. The depressive schemas in turn influence how external and internal stimuli are processed and remembered, further increasing the negative perception bias, and leading to certain cognitive impairments associated with depressive symptoms (**Figure 5**)⁶³.

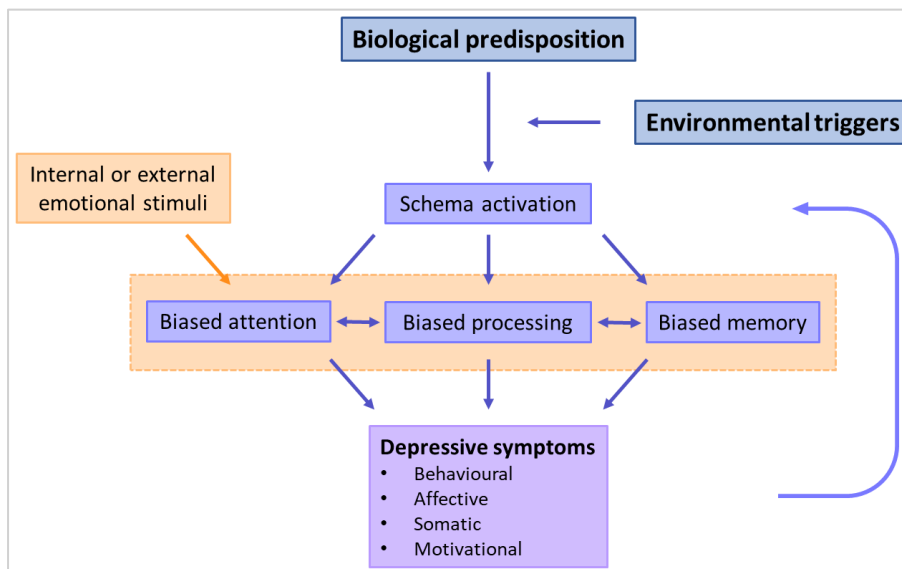


Figure 5: Information processing in the cognitive model of depression. Biased attention, processing, and memory are triggered by external or internal stimuli through activation of depressive self-referential schema, leading to depressive ideation and depressive symptoms. Adapted from Disner et al. (2011)⁶³

This cognitive model of depression has been demonstrated to have a robust biological basis. Negative cognitive biases are believed to stem from dysfunctional emotional processing, caused by hyperactivity in subcortical regions and impaired top-down inhibition from the PFC^{3,63,72,73}. The main networks involved in these processes are the affective network, the default mode network, the cognitive control network, and the reward networks⁵⁰. Studies show upregulation and increased connectivity of the affective and the default mode networks, and downregulation and decreased connectivity of the cognitive control and reward networks in patients with depression (**Figure 6**)⁵⁰. The affective network processes emotional stimuli and regulates emotional responses, and spans the orbitofrontal cortex, anterior cingulate cortex (ACC), and limbic structures like the amygdala and hippocampus (**Figure 6**)⁵⁰. The default mode network, which is associated with resting-state introspection and rumination, includes cortical regions such as the precuneus, mPFC, and inferior parietal cortex, and the posterior cingulate cortex, which is part of the limbic system (**Figure 6**)⁵⁰. The cognitive control network, which is responsible for impulse control, emotion regulation, and other higher cognitive functions, includes the ACC, dorsolateral PFC (dlPFC), and parietal cortex (**Figure 6**)⁵⁰. The reward network, which is involved in processing rewarding stimuli, motivation, and pleasure, includes the frontal cortex and striatum (**Figure 6**)⁵⁰.

These disruptions in the affective and cognitive control networks suggest connectivity disruptions between limbic regions and higher cognitive centres. Limbic areas process and relay information through the subgenual cingulate cortex, ACC, caudate, putamen, NAc, and hippocampus to the PFC and frontal cortex, while areas of the PFC direct the cognitive processing of emotions and inhibit the limbic system from the top down^{3,63,72}. In MDD patients, misregulation of this processing circuit, due to lack of top-down inhibition from the PFC and rostral ACC (rACC) and/or chronic bottom-up hyperactivity in limbic areas, is thought to impair proper emotional processing (**Figure 6**)^{3,63,72,73}. This is supported by functional magnetic resonance imaging (fMRI) studies showing distinct rACC activation patterns in patients with depression and healthy controls^{63,74–76}. In patients with depression, rACC activity was increased when attention to negative stimuli was inhibited, whereas healthy controls showed increased rACC activity when inhibiting attention to positive stimuli^{63,74–76}.

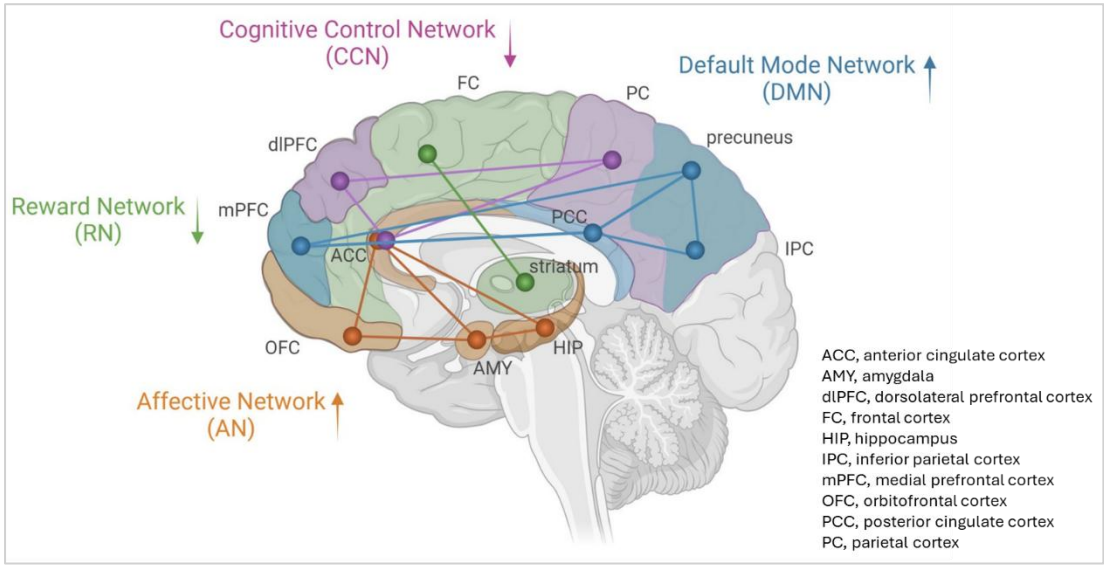


Figure 6: Four core networks involved in depression. Activity and connectivity were increased in the affective and default mode network in depression, whereas the cognitive control network and reward network, they were decreased Bremshey et al. (2024)⁵⁰

Patients with depression have been shown to have an attention bias and a heightened neural response to negative stimuli, and require greater cognitive effort to shift their focus, whereas healthy individuals show a natural bias towards positive stimuli^{63,77}. In healthy controls, suppression of unattended fear stimuli activated the right dIPFC, whereas patients with depression showed increased amygdala activation, which indicates that the suppression of emotional responses to fear stimuli is impaired⁷⁸. Additionally, during emotional processing trials, the amygdala exhibited increased and prolonged activity in patients with depression compared to healthy controls, indicating biased stimulus processing (**Figure 7**)⁷⁹. Interestingly, when directly attending to fear stimuli, the dIPFC showed increased activation in patients with depression, while in healthy controls, the amygdala showed increased activation⁷⁸. This indicates

higher cognitive control and affect regulation in patients with depression when directly attending to fear stimuli⁷⁸.

This bias towards negative stimuli in patients with depression is further increased by alterations in the thalamocortical pathway (**Figure 7**)⁶³. This pathway includes the thalamus, the dorsal ACC, and the subgenual cingulate cortex⁶³. It is essential for organizing and processing environmental stimuli⁶³. During depressive episodes, the thalamus is more active in dysphoric individuals, potentially compensating for decreased connectivity and inhibition of the dorsal ACC over the medial thalamus (**Figure 7**)⁶³. With reduced dorsal ACC inhibition of the limbic system, emotional information relaying from the limbic system is thought to be disproportionately led by the subgenual cingulate cortex (**Figure 7**)^{63,80}. This could lead to an increase in the perceived emotionality of the processed stimuli⁶³. This theory is supported by findings of increased functional connectivity of the subgenual cingulate cortex and thalamus with the default-mode network in patients with depression, with stronger subgenual connectivity correlating to the duration of the depressive episode⁸⁰.

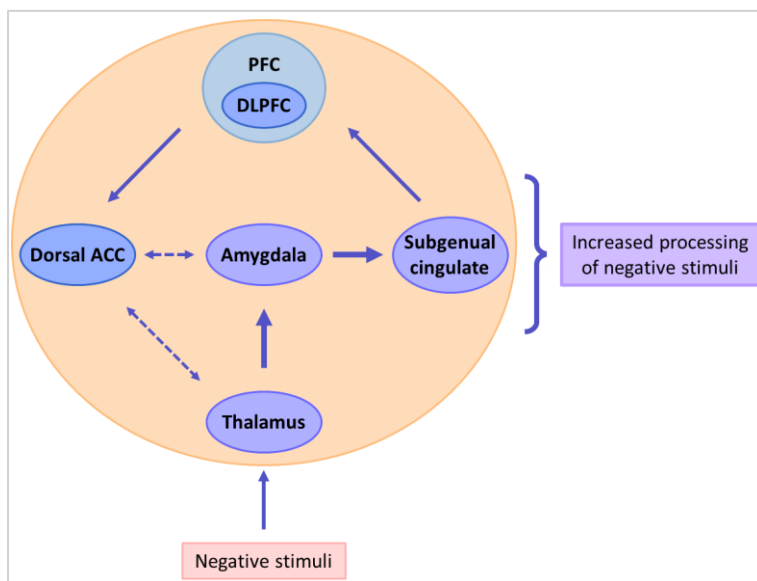


Figure 7: Cognitive model of negative processing bias in depression. In depression, negative stimuli cause hyperactivity in the thalamus, amygdala, and subgenual cingulate cortex, shifting focus toward negativity. Hypoactivity in the dorsolateral prefrontal cortex (dlPFC) and dorsal anterior cingulate cortex (ACC) impairs cognitive control over these regions, reinforcing the negative bias. Solid arrows indicate intact connections, dashed arrows show weakened connections, and thicker arrows represent increased signal. Adapted from Disner et al. (2011)⁶³

In addition to a bias perception of negative stimuli, patients with depression also show a bias in remembering and internalizing them^{63,81}. This bias towards negative memories is correlated with increased activation of the amygdala and greater functional connectivity with the hippocampus and caudate-putamen⁸¹. In patients with depression, increased amygdala activity was associated with better encoding of negative stimuli, which was in turn correlated with increased hippocampal, caudate, and putamen activity, facilitating the recollection of negative information^{63,81}. Negative biases in memory recall may also be facilitated by hypoactivation and hyperactivation of the ventromedial PFC (vmPFC) when recalling sad and happy life events, respectively⁸². The vmPFC is implicated in the contextualisation and abstract representation of

rewarding stimuli⁸². These findings indicate that depressed patients need higher top-down efforts to recall happy memories, but not for the recollection of sad memories⁶³.

In addition to negative attention and processing biases, the experience of reduced pleasure, or anhedonia, is a core feature of depression, and stems from disruptions in the reward processing system. Anhedonia is characterized by reduced positive affect in response to positive stimuli or decreased pleasure, and can manifest differently across patients^{52,63,83}. Additionally, reward networks may influence the treatment response in depression, with treatment-resistant depression linked to reduced connectivity between frontal cortical regions and subcortical areas like the striatum⁵². However, this connection is not yet fully understood.

Reward processing and positive emotion regulation are largely mediated by dopaminergic mesolimbic pathways, including the VTA, NAc, bed nucleus of the stria terminalis, amygdala, hippocampus, and the fronto-striatal network (**Figure 6**)^{52,63,83,84}. This pathway extends to the mPFC, forming the mesocorticolimbic reward circuit, where overexcitability of the mPFC and reduced striatal-mPFC connectivity are thought to disrupt reward processing⁵². Different disruptions in specific regions can have distinct effects on reward anticipation, sensitivity, processing, and response⁵². For instance, in depression, cortical areas such as the cuneus, middle and superior frontal gyrus, fusiform gyrus, and lingual gyrus are more active in response to positive emotional stimuli, while subcortical and limbic areas show reduced activity⁸⁵. This suggests that more cognitive effort is needed for a positive response⁸⁵. The insula, precuneus, PFC, and inferior parietal lobule are selectively responsive to monetary rewards, whereas the PFC is primarily involved in the cognitive evaluation and regulation of reward processing⁸⁵. Lowered sensitivity to rewards, which is another aspect of anhedonia, is likely due to reduced top-down PFC control over the NAc^{63,83}. This was shown in fMRI studies, where decreased activity in the NAc and PFC in response to positive stimuli was observed, even when individuals attempted to sustain a positive mood^{63,83}. This suggests a disrupted positive emotion regulation due to difficulties maintaining NAc engagement and reduced fronto-striatal connectivity in the context of positive affect^{63,83}. Furthermore, the left dorsomedial frontal gyrus and the ventral striatum, regions are associated with cognition and self-reflection, and processing of reward and stimuli, respectively. Both regions show reduced activation in patients with depression in response to positive stimuli⁸⁶.

In summary, a growing body of evidence supports the theory that dysregulation in emotional processing and memory plays a central role in developing negative cognitive biases and anhedonia in depression. These biases are driven by hyperactivity in subcortical regions and impaired top-down control from the PFC. Together, these findings highlight the complex interplay of different brain networks underlying major depression.

1.4.3 A unified theory of depression

The complex aetiology of major depression has been investigated in numerous studies using various approaches that provide different insights into the involvement of various mechanisms, including psychological and biological predispositions and environmental risk factors. In their 2017 review about a “Unified Theory of Depression”, Dean and Keshavan present a comprehensive framework of the aetiology, pathophysiology, and symptomatology of MDD, integrating how the genetic, neurobiological, psychological, and other physiological mechanisms interact with each other (**Figure 8**)³. They suggest that genetic predispositions and environmental factors activate reciprocally interacting pathophysiological mechanisms, which then manifest in a depressive phenotype³.

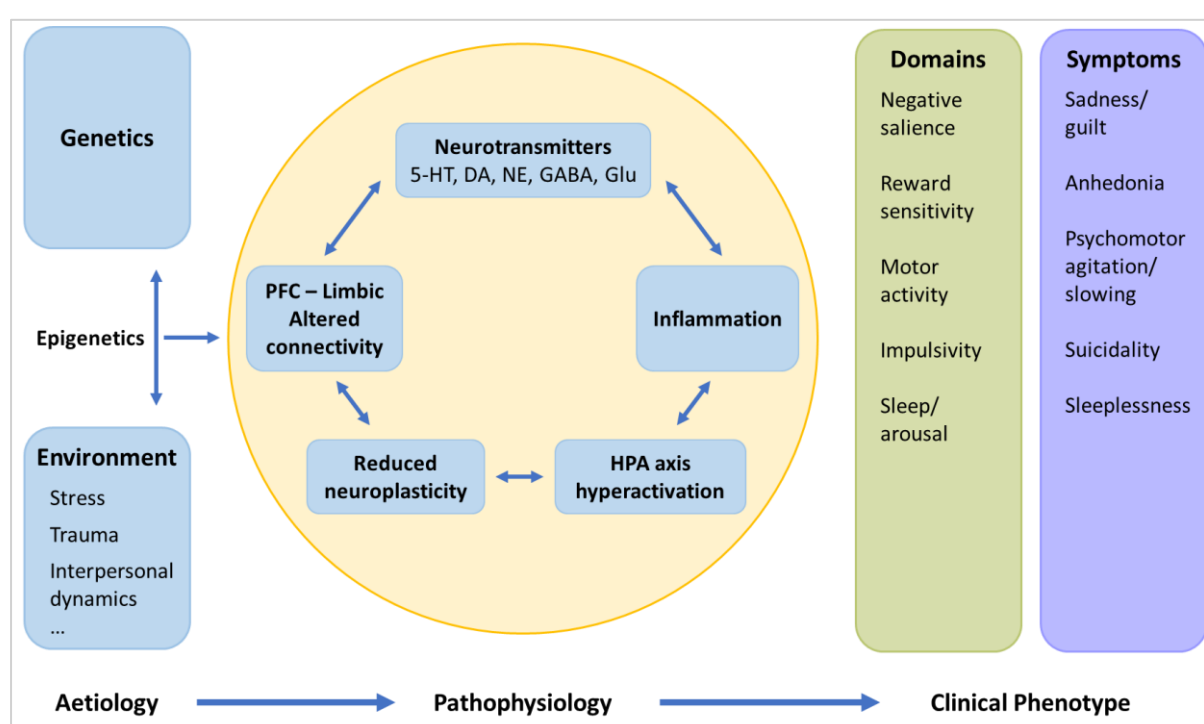


Figure 8: A unified theory of depression. Certain biological components, including genetic contributions, as well as environmental risks, can facilitate the development of depression. However, each of these factors in isolation is likely not sufficient to cause this disorder. Rather, it is their interaction that leads to the phenotype. For instance, environmental stressors, especially early life adverse events, may lead to epigenetic modifications facilitating the activation of certain pathophysiological mechanisms. These mechanisms include alterations in neurotransmitter levels, neuroinflammation and HPA axis activation, and reduced neuroplasticity. These factors are all connected between each other, and alterations in one aspect can trigger the development of others. For instance, if neuroinflammation is caused by excessive stress, it can lead to the activation of the HPA axis, which can increase the inflammation. This feedback loop of pathophysiological mechanisms can then result in the clinical phenotype observed in depression. Some of the depressive symptoms can also reciprocally increase the described feedback loop. For instance, lack of sleep and anxiety may increase stress, which can increase inflammation and HPA axis activation. The complexity of the different factors could also partially explain the heterogeneity of the symptoms observed in patients with depression. Adapted from Dean and Keshavan (2017)³

It is well established that genetic and epigenetic risk factors as well as environmental stressors like early life adverse events contribute to the aetiology of depression and the development of depressive schemas³. These schemas bias the processing of stimuli, triggering an overly strong response from the limbic system, which is insufficiently inhibited by cortical areas, leading to a negative emotional response^{3,63}. Additionally, alterations in neurotransmitter systems and hyperactivity of the HPA axis, due to genetic factors, increased stress responses and inflammation³. This affects different brain circuitries, potentially reducing neuroplasticity and altering connectivity in the depressed brain³. Reduced neuroplasticity can hinder the recovery from the depressive state of the brain³. As the proposed mechanisms are interconnected, different pathways can trigger the depressive pathophysiology, and can lead to depressive symptoms in different patients³. Due to the great heterogeneity of the symptoms, specific findings are often found in only a fraction of MDD patients³. The authors therefore mention that “depression” could also be an umbrella term for different underlying networks, biological mechanisms, or even distinct disorders, resulting in the different symptoms³.

2 PHARMACOLOGICAL TREATMENTS OF MAJOR DEPRESSIVE DISORDER AND COMORBID TRAITS

Antidepressant medication typically targets monoaminergic systems, mostly the serotonergic system⁸⁷. These medications are usually combined with psychotherapy to achieve the best treatment outcomes, however, the focus of this thesis lies on pharmacological interventions^{3,6}. Additionally, medications targeting different neurotransmitter systems are often used in preclinical studies to investigate their role in the aetiology of MDD.

2.1 The history of antidepressants

Antidepressant treatments have evolved significantly since the 1950s and have mainly targeted serotonin and norepinephrine neurotransmission. The first antidepressants were TCAs and MAO inhibitors (see chapter 1.4.1), followed by SSRIs and serotonin-noradrenaline reuptake inhibitors (SNRIs), which inhibit the reuptake of these neurotransmitters more selectively. More recently, ketamine, which targets glutamate neurotransmission, has emerged as a rapid-onset antidepressant and a promising option for treatment-resistant depression.

The first antidepressants were TCAs and MAO inhibitors, both of which were developed in the 1950s⁸⁸. TCAs inhibit the reuptake of serotonin and norepinephrine, and are originally derived from antihistaminic compounds^{50,88}. Despite response rates of 43-70%, TCAs are less commonly prescribed due to their higher risk of side effects caused by their lack of specificity compared to newer treatments^{50,89}. MAO inhibitors block the action of MAO enzymes, which break down neurotransmitters, including serotonin, but were discontinued due to severe side effects⁸⁸.

In the 1980s, SSRIs were subsequently introduced⁵⁰. SSRIs primarily act by inhibiting the reuptake of serotonin into the presynaptic neuron, prolonging its action on postsynaptic receptors (see chapter 2.3.1)⁵⁰. Following SSRIs, SNRIs were developed, which inhibit both serotonin and norepinephrine reuptake in a more specific manner than TCAs. As of 2021, SSRIs made up over 60% of all prescribed antidepressants, making them the most commonly prescribed antidepressants worldwide, with TCAs accounting for over 25%, and newer antidepressants like SNRIs making up over 14%^{50,87,89}.

Most recently, ketamine, and specifically esketamine, has become a point of interest as an antidepressant, and was approved as such by the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) and in 2019, after previously being used as an anaesthetic. Ketamine is a non-competitive, high affinity NMDA receptor antagonist, with additional effects on α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, opioid receptors, as well as monoaminergic

receptors⁷⁰. Its antidepressant effects are noted considerably faster in MDD patients than those of SSRIs^{50,90}.

Antidepressants targeting the GABAergic system are less common, although benzodiazepines, which act on GABA_A receptors and are widely used as anxiolytics, might also offer some long-term benefits as antidepressants, and can be prescribed for comorbid anxiety⁶⁸.

Despite the wide range of different medication available, up to 30% of patients can be considered to have treatment-resistant depression (TRD)^{91–93}. TRD is defined as a lack of response to at least two different antidepressants after an adequate trial period, typically 6–8 weeks, although definitions vary⁹¹. So far, there are no reliable genetic biomarkers to identify patients at risk for TRD⁹³. Although the high percentage of treatment resistance could also be influenced by other factors like non-adherence to treatment or inadequate treatment trials, it is still an important focus of current research^{91–93}. Notably, Ketamine's rapid onset of action has been shown to be particularly effective for TRD, with a reported response rate of 64%, which was significantly higher than placebo (odds ratio (OR) = 2.18; 95% CI, 1.21 to 4.14), making it an interesting target for future research^{50,90}.

2.2 Effects of selective serotonin reuptake inhibitors

SSRIs inhibit the reabsorption of serotonin into the presynaptic membrane, increasing serotonin levels in the synaptic cleft and enhancing serotonergic neurotransmission⁵⁰. SSRIs such as Citalopram or Fluoxetine were consistently found more effective than placebo (OR = 1.62, 95% CI 1.51–1.72) using symptom severity change from baseline the outcome measure (standardized mean difference, SMD –0.27, 95% CI –0.31 to –0.23), especially in alleviating depressed mood and psychic anxiety⁹⁴. They were also more prone to side effects than placebo (OR = 1.73, 95% CI 1.58 to 1.89)⁹⁴. Despite these side effects, SSRIs are the most commonly prescribed antidepressants globally, with response rates ranging from 53–64%^{50,87,89}. Their effects are generally seen within weeks, though individual responses can vary^{95,96}.

In treatment responders, SSRIs alleviate different symptoms of MDD through both direct and indirect effects (**Figure 9**)⁹⁶. The primary impact of SSRIs is the rapid improvement of depressed mood and psychic anxiety (**Figure 9**)⁹⁶. These initial changes lead to secondary effects. For instance, reduced depressed mood results in fewer cognitive symptoms, such as suicidal thoughts and loss of interest (**Figure 9**)⁹⁶. Similarly, decreased psychic anxiety further reduces depressed mood and improves arousal and somatic symptoms, as well as cognitive symptoms⁹⁶. However, SSRIs may also increase somatic anxiety and agitation compared to cognitive behavioural therapy (CBT), indicating a complex therapeutic profile, and highlighting the importance of combining treatment approaches⁹⁶.

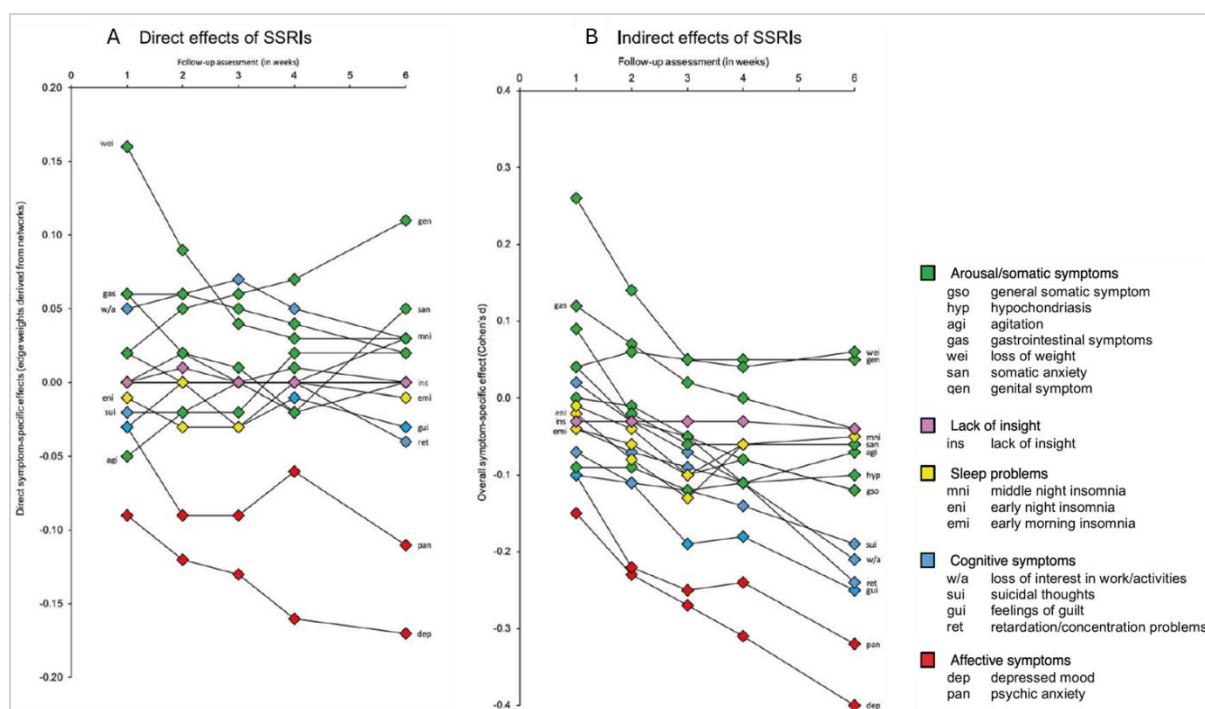


Figure 9: Direct and indirect effects of SSRIs within the first six weeks of treatment. A Direct effects of SSRIs. The strongest direct effects of SSRIs were consistently found to be the improvement of affective symptoms. These symptoms include depressed mood and psychic anxiety. B Overall effect of SSRIs. The overall effects include direct and indirect improvements in symptoms. Reduced depressed mood results in fewer cognitive symptoms, such as suicidal thoughts, loss of interest, and feelings of guilt. Decreased psychic anxiety further reduced depressed mood, and improved arousal and somatic symptoms such as agitation and somatic anxiety. It furthermore improved cognitive symptoms, including feelings of guilt and loss of interest. Adapted from Boschloo et al. (2023)⁹⁶

Microdialysis studies in rats showed substantial changes in brain serotonin levels within the first week of SSRI treatment in a region-dependent manner⁹⁵. Interestingly, extracellular serotonin levels in the frontal cortex first decreased, but over the next two weeks, they increased by up to 350% of baseline levels⁹⁵. This pattern is consistent with the hypothesis that desensitization of 5-HT_{1A} autoreceptors over time leads to increased serotonin release⁹⁵. In contrast, in other brain regions, including the hippocampus, PFC, NAc, and VTA, serotonin levels increased within the first three days of SSRI administration, suggesting that additional mechanisms may also contribute to SSRI effects⁹⁵. However, as discussed in chapter 1.4.1, changes in serotonin alone cannot fully explain depressive symptoms. The antidepressant effects of SSRIs are now thought to result not only from serotonin modulation but also from secondary changes in neural plasticity at structural and functional levels (see chapter 2.3.1)⁹⁷.

2.3 Fluoxetine, quinpirole, and diazepam in treatment and research

SSRIs, along with other medications, are also commonly used in preclinical studies to investigate the effects of various neurotransmitter systems on MDD. For example, three popular medications

used in the treatment and research of MDD and other psychiatric disorders are fluoxetine, quinpirole, and diazepam (**Figure 10**). Fluoxetine is an SSRI that primarily targets the serotonergic system (chapter 2.3.1), quinpirole is a dopamine receptor agonist commonly used for studying the role of the dopaminergic system in psychiatric disorders (chapter 0), and diazepam is a benzodiazepine that modulates the GABAergic system and is commonly used to treat comorbid anxiety in depression (chapter 2.3.3). These neurotransmitter systems have all been implicated in the pathophysiology of depression and other psychiatric disorders (see chapter 1.4.1). Fluoxetine, quinpirole, and diazepam were used in this thesis to assess the effects of the different neurotransmitter systems on depressive-like behaviours in a zebrafish model (see chapter).

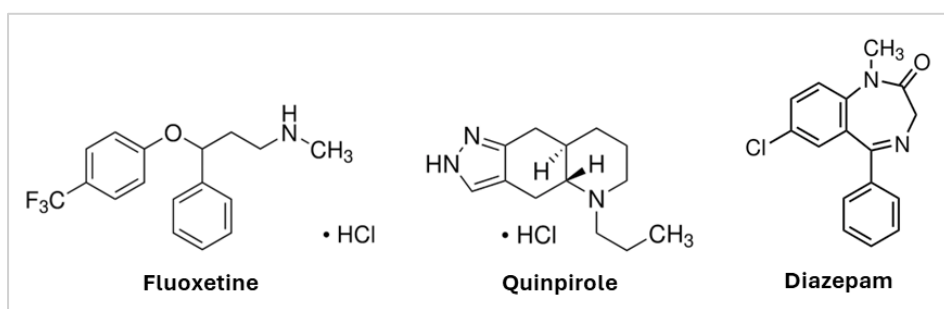


Figure 10: Molecular forms of fluoxetine, quinpirole, and diazepam. Pictures from sigma-aldrich, fluoxetine hydrochloride ref F132, quinpirole hydrochloride ref Q102, diazepam ref D0899.

2.3.1 Fluoxetine

Fluoxetine was the first SSRI to be approved by the FDA for the treatment of depression in 1987 (and not be withdrawn), and it remains one of the most commonly prescribed antidepressants today, with around 27 million prescriptions in the US alone in 2018^{50,98}. As an SSRI, its primary working mechanism is by increasing serotonin availability in the synaptic cleft (**Figure 11**)^{99,100}. It is commonly used in preclinical studies of the role of serotonin in anxiety and depression models, where it has been shown to affect neuroplasticity and behaviour.

Fluoxetine increases the availability of serotonin in the synapse, as well as to some extent dopamine and noradrenaline^{99,100}. It inhibits the serotonin transporter (SERT), preventing the reuptake of serotonin into the presynapse, potentiating serotonergic signalling (**Figure 11**).

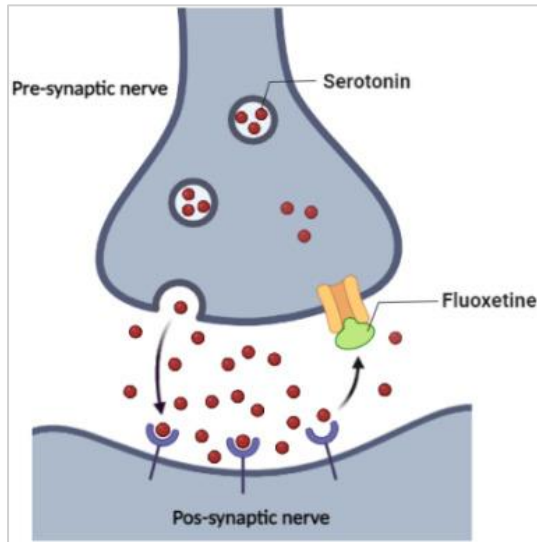


Figure 11: Mode of action of fluoxetine on the central nervous system. Fluoxetine binds to presynaptic serotonin transporters, preventing serotonin reuptake, resulting in an increase of serotonin levels in the synaptic cleft, which potentiates serotonergic signalling. Correia et al. (2023)⁹⁸

Discontinuation rates in fluoxetine are slightly lower than in other antidepressants, however, it was not found to be more effective (OR = 1.52 in fluoxetine vs OR = 1.62 in SSRIs in general) (Figure 12)¹⁰¹. While its main use is as an antidepressant, fluoxetine is also used for treating other psychiatric disorders, including anxiety, eating disorders, OCD, and panic disorder^{98,99,102,103}.

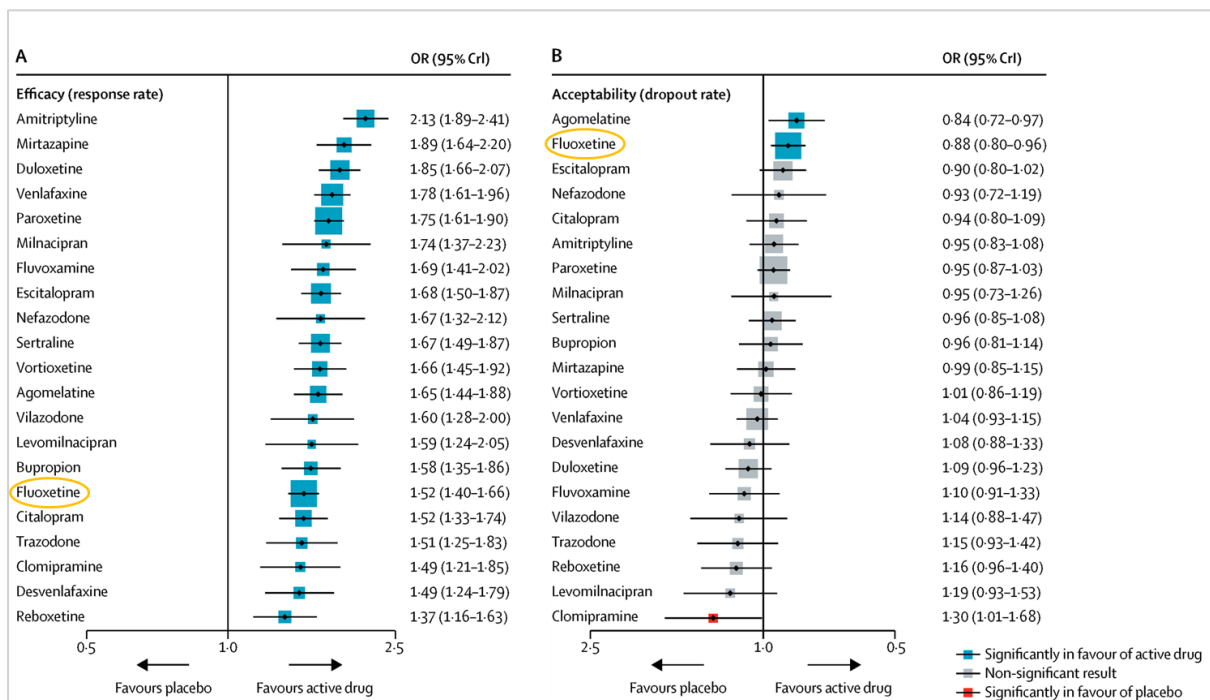


Figure 12: Efficacy and acceptability of fluoxetine. A The response rate of fluoxetine is was not higher than in most antidepressants (OR = 1.52). B The dropout rate for fluoxetine use was lower in comparison to most of the other antidepressants (OR = 0.88). Antidepressants compared to placebo, which was the reference compound. OR, odds ratio. CrI, credible interval. Cipriani et al. (2018)¹⁰¹

Effects on neuroplasticity

The antidepressant effect of fluoxetine is typically delayed, suggesting that the antidepressant properties are not entirely a direct result of increased serotonin availability in the brain^{99,100}. Nowadays the antidepressant effects of fluoxetine are thought to stem to some extent from complex changes in neuronal plasticity, which in turn is thought to facilitate the overcoming of negative schemas^{97,99,104}.

Fluoxetine was found to be involved in several mechanisms of plasticity in the adult CNS, including increased hippocampal neurogenesis, structural remodelling of pyramidal neurons, increasing dendritic spine density in interneurons in the mPFC and hippocampus of mice, and altering the expression of plasticity-related molecules^{105–107}. Its neuroplastic effects can occur independently of the serotonin transporter (5-HTT), as it was found to promote neuroplasticity by activating signalling pathways of the brain-derived neurotrophic factor (BDNF) and its receptor TrkB both in wild-type and 5-HTT knockout mice¹⁰⁸. Additional evidence for the effects of fluoxetine on neuroplasticity comes from studies in reserpine-treated mice, a common depression model¹⁰⁴. Reserpine inhibits catecholamine reuptake and induces depressive-like symptoms in human patients and preclinical models⁵⁰. In reserpine-treated mice, fluoxetine was found to enhance neuroplasticity by increasing the expression of the synaptic protein markers SYP and PSD95, and inhibited reserpine-induced synapse loss in the hippocampus¹⁰⁴. In contrast, while fluoxetine promotes several forms of plasticity, its effects on synaptic plasticity can be pathway- and dose-dependent, and it has also been shown to impair neuronal plasticity or have no effect in some cases^{104,109}. For example, it was found to impair long-term potentiation (LTP) at specific synapses in the CA1 subfield in the hippocampus of rats¹¹⁰. This contrast in findings suggests that the effects of fluoxetine on plasticity may depend on the specific type of plasticity being studied, the brain region, and the duration of treatment.

Effects on behaviour

In preclinical studies fluoxetine has been shown to produce behavioural changes in locomotion and anxiety-like behaviour, among others, in mice and zebrafish^{98,104,111–114}. Importantly, preclinical studies show varying effects of fluoxetine depending on the dosage, exposure time, and other factors^{98,112}. While acute fluoxetine acts ‘neurochemically’ by inhibiting SERT and enhancing serotonergic neurotransmission, its long-term effects are seen only after chronic administration and are mainly associated with altered expression of serotonin receptors and changes in neuroplasticity¹¹². Acute treatment is therefore typically used to investigate the immediate effects of raised serotonin levels on the investigated behaviour, whereas chronic treatment can resemble treatments in patients more closely.

Like its effects on neuroplasticity, the behavioural effects of fluoxetine in mice are complex and depend on factors such as dosage, treatment duration, sex, strain, and other experimental conditions¹¹⁵. The most commonly observed effects are the reduction of anxiety-like and depressive-like behaviours, but findings are not entirely consistent. Chronic treatment of fluoxetine that lasted for more than 12 days was found to reduce depressive-like behaviours at different doses. For instance, 18 mg/kg/day fluoxetine reduced depressive-like behaviours between 12 and 28 days of treatment¹¹⁶. Sub-chronic treatment of fluoxetine at the same dose for four days had no antidepressant effects¹¹⁷. Administration of 10 mg/kg/day fluoxetine for 14 days, but not 7 days, also reduced depressive-like and anxiety-like behaviours¹¹⁸. Treatment with fluoxetine doses as low as 0.5 mg/kg fluoxetine for 14 days also reduced behavioural despair, anhedonia, and anxiety in reserpine-treated mice¹⁰⁴. These studies demonstrate that chronic treatment of fluoxetine can produce robust antidepressant effects in mice at doses from 0.5 mg/kg to 18 mg/kg.

In zebrafish, different studies testing different exposure times and doses of fluoxetine at different ages show various behavioural alterations, including stress response, locomotion, and social behaviour. Correia et al. (2023) summarize behavioural alterations in zebrafish after exposure to fluoxetine in concentrations ranging from 0.00000345 µg/L – 10 mg/L with different exposure times from 3 minutes to 43 days, and across different life stages, from embryonic stages into adulthood (Table 2)⁹⁸. Several studies reported a tendency for stress reduction as well as alterations in exploratory behaviour (Table 2), but not entirely consistent findings were observed⁹⁸. Zebrafish larvae were found to exhibit both reduced and increased swimming activity in response to different fluoxetine concentrations and exposure times (Table 2)⁹⁸. In adult zebrafish, changes in locomotion were also found, with some studies reporting reductions at higher concentrations and others showing increased activity at lower concentrations⁹⁸. Changes in aggressive behaviour were also found to vary depending on the dose, with aggression being generally reduced at higher doses (Table 2)⁹⁸. Exposure to fluoxetine was also shown to reduce social interactions in adult zebrafish⁹⁸. Importantly, serotonergic neurotransmission likely regulates zebrafish behaviour differently in various tasks, and the time of exposure to fluoxetine is an important factor in modulating the behaviour as well¹¹². Therefore, the same treatment can lead to different responses based on the underlying motivation for the behaviour, individual differences, as well as other factors.

Table 2: Behavioural alterations in zebrafish after fluoxetine exposure. Adapted from Correia et al. (2023)⁹⁸

Life stage	Concentration tested	Exposure duration	Behaviour effects observed	Reference
Larvae	2000 µg/L	2h prior to behavioural testing	Decreased swimming speed.	Richendrfer et al. (2012) ¹¹⁹
Embryos	0.00345, 0.345, 4.84 and 50 µg/L	96h	Concentration-dependent reduction in stress-related swimming activity in the dark period (in all concentrations tested).	Zindler et al. (2020) ¹²⁰
Larvae	0.05 µg/L	96h	Under light-dark conditions, zebrafish larvae exhibited a peak swimming activity during the light phase and sleep-like behaviour during the dark phase; After light condition, the locomotor activity of zebrafish larvae increased rapidly and markedly, followed by a slow decline; Exposed larvae displayed reduced locomotor activity, especially at 7 and 8 days post fertilisation. This decrease in locomotor activity was mainly reflected in the daytime.	Wei et al. (2022) ¹²¹
Larvae	0.05 and 0.5 µg/L	96h	Reduced swimming activity, covering a shorter distance (30 % decrease in 0.05 µg/L and more than half in 0.5 µg/L of exposure, after receiving an external stimulus).	Parolini et al. (2019) ¹²²
Larvae	0.1, 1, 10, 100 and 1000 µg/L	120h	Increase in swimming distance and swimming speed (100 µg/L).	Pan et al. (2018) ¹²³
Larvae	0.88, 15.8, 281.2 and 500 µg/L	168 hours post fertilization	Decreased swimming activity of zebrafish larvae at concentrations of 15.8, 281.2, and 500 µg/L.	Farias et al. (2019) ¹²⁴
Juvenile	100 µg/L	1h per day for 14 days	No changes in basal cortisol levels; No changes in anxiety behaviour.	Petrunch-Rutherford (2019) ¹²⁵
Juvenile	0.1, 1, and 10 µg/L	12 and 21 days	After 12 days of exposure, zebrafish juveniles altered swimming time during the dark period at the highest concentrations (1 and 10 µg/L); After 21 days of exposure, fish exposed to all fluoxetine concentrations showed significant decreases in swimming activity during the light period.	Correia et al. (2022) ¹²⁶
Adult	10000 µg/L	3 min	Decreased stress levels (higher positioning in the water column, decreased latency time to enter the top layer of the aquarium, and greater exploitation of the same).	Singer et al. (2016) ¹²⁷

Adult	1, 25 and 50 µg/L	15 min	Lower stress reaction (cortisol levels) to chase with a net for 2 min of animals exposed to the highest concentration.	Abreu et al. (2014) ¹¹³
Adult	50 µg/L	15 min	Decreased stress levels, social interaction, and aggression; No effect on memory acquisition.	Giacomini et al. (2016) ¹¹⁴
Adult	100 µg/L	30 min	No effects were observed in the novel tank test; No effects were observed in animals' scototaxis.	Fontana et al. (2022) ¹²⁸
Adult males	5000 µg/L	2h	Decreased aggressive behaviour.	Theodoridi et al. (2017) ¹²⁹
Adult	0.54 and 54 µg/L	6 days	Increased locomotor activity and exploratory behaviour (novel tank test) (0.54 µg/L).	Vera-Chang et al. (2018) ¹³⁰
Adult	Fluoxetine: 200 µg/L Fluoxetine: 200 µg/L + 2000 µg/L TPH2 irreversible inhibitor, p-chlorophenylalanine (pCPA)	72h	Fish exposed decreased the time spent in the bottom layer and increased the time spent in the upper layer and the distance from the tank's bottom; The combination of pCPA with fluoxetine significantly attenuated the effects of the drug on the endpoints mentioned above.	Evsiukova et al. (2021) ¹³¹
Adult	0.005, 0.016 and 0.04 µg/L	96h	Decreased exploratory behaviour; Decrease in the number of top-bottom and bottom-up transitions; Increase in freezing behaviour.	Orozco-Hernández et al. (2022b) ¹³²
Adult	10 µg/L	1 week	Decreased number of transitions to the upper zone of the tank.	Marcon et al. (2016) ¹³³
Adult	33 and 100 µg/L	2 weeks	Decreased stress levels (more time in the upper area and lower latency to enter it) (100 µg/L).	Wong et al. (2013) ¹³⁴
Adult	100 µg/L	2 weeks	Decreased stress levels (increased time spent in the upper half of the tank).	Pittman and Hylton (2015) ¹³⁵
Adult	100 µg/L	2 weeks	More time spent in the light area (scototaxis).	Pittman and Ichikawa (2013) ¹³⁶

Adult	0.54 and 54 µg/L	0–15 and 15–42 days post-fertilization	Adults exposed between 0 and 15 days post-fertilization exhibited a reduction in exploratory behaviour while adults exposed between 15 and 42 days of exposure exhibited an increase.	Vera-Chang et al. (2019) ¹³⁷
Adult	0.005, 0.01, 0.05, 0.1, 0.5, 1 and 5 µg/L	7, 14, and 28 days	Increased swimming behaviour (0.01, 0.05, 0.1 and 5 µg/L); After 7 day-exposure, the swimming speed decreased at the highest concentrations tested (0.5, 1, and 5 µg/L); After 7 day-exposure, the time spent motionless (%still) increased (0.005, 0.01, 0.5 and 1 µg/L); At 14 day-exposure, %still was significantly higher (0.005, 0.5, 1 and 5 µg/L); At the end of exposure, fish exposed to 0.01, 0.05 and 5 µg/L spent significantly more time motionless.	Al Shuraiqi et al. (2021) ¹³⁸
Adult	0.01, 0.1, 1, 10 and 100 µg/L	15 and 30 days	Trend to swim mainly in the upper layer of the tanks (10 and 100 µg/L); No effect on feeding behaviour.	Farias et al. (2020) ¹³⁹
Adult	50 µg/L	43 days	Increased freezing behaviour. Decreased time at the top of the tank.	Fior et al. (2018) ¹⁴⁰
Adult	1000, 2000, 4000, and 6000 µg/L	(Not reported)	Increase in dark permanence (4000 µg/L) and a decrease in the number of crossings (2000 and 4000 µg/L).	Magno et al. (2015) ¹⁴¹

In summary, fluoxetine can produce behavioural changes mirroring antidepressant effects in mice and zebrafish. These effects depend on the dosage and exposure time, and might differ depending on the behavioural paradigms in which they are assessed.

2.3.2 Quinpirole

Quinpirole is a selective D₂ receptor agonist, and has been used in the treatment and research of psychiatric disorders like anxiety, MDD, OCD, and schizophrenia^{142,143}. Quinpirole is a high-affinity agonist of dopamine D₂ and potentially D₃ autoreceptors¹⁴⁴. These receptors modulate dopaminergic neurotransmission by decreasing the excitability of dopamine neurons and the release of dopamine¹⁴⁴. Interestingly, acute exposure to quinpirole may alter dopamine levels in a region-dependent manner. It was shown to increase dopamine levels in the NAc and right PFC, and decrease dopamine metabolites in the amygdala in rats¹⁴⁵. It additionally increased dopamine stores in the NAc, caudate, and right PFC¹⁴⁶. These findings support the theory that acute exposure to quinpirole could stimulate dopaminergic autoreceptors, inhibiting the release

of dopamine in certain brain regions¹⁴⁵. Chronic exposure to quinpirole might have distinct effects. Quinpirole treatment over several days is associated with a downregulation of D₂ receptors and upregulation of dopamine in the striatum¹⁴⁵.

Exposure to quinpirole has produced various behavioural alterations in animal models, including changes in locomotion, stereotypy, and cognition, however, these findings are not always consistent across studies^{143,147,148}. For example, one study found that in mice, chronic quinpirole administration in form of intraperitoneal (i.p.) injection of 1 mg/kg every three days or nights altered locomotion depending on the diurnal rhythm¹⁴⁹. When quinpirole was administered during the day, mice became hyperactive, whereas quinpirole administration by night led to hypomotility and tolerance¹⁴⁹. However, another study monitoring activity in home cages after quinpirole administration (i.p. injection of 0.5 mg/kg) found increased activity during the night and decreased activity during the day¹⁵⁰. Diurnal mechanisms may therefore be important for the development of dopaminergic behaviours and should be taken into account when designing experiments¹⁴⁹. Acute effects of quinpirole were measured in a 2013 study by Luque-Rojas et al., after i.p. injection of 0.05, 0.1, and a 1 mg/kg quinpirole¹⁵¹. They observed a biphasic temporal response, in which for the first 50 min, mice were immobile, followed by increased locomotion for the next 70 min, including stereotyped behaviours¹⁵¹. In pubertal mice, quinpirole-exposure (i.p. injection of 0.5 mg/kg) had sex-specific effects: in female mice, exploration was lowered, whereas in male mice, depressive-like behaviour was observed¹⁵². Given these findings, the effects of quinpirole on behaviours such as locomotion, stereotypy, exploration, and depressive-like behaviours can vary based on factors such as dosage, age, sex of the animals, and the time of administration. These differences, observed in various studies, highlight the complexity of its impact and the need to carefully consider these factors when planning experiments.

Quinpirole has been studied in zebrafish to explore the effects of dopamine on various behaviours, although the results have not always been consistent and depend on exposure time, treatment dose, and age of testing, as well as other factors. For example, acute exposure to 5.5 and 16.7 μ M quinpirole was found to increase larval activity¹⁴⁷. However, zebrafish larvae that were exposed to 0.01, 0.1, and 1 μ M quinpirole for three hours before the experiments, displayed slightly decreased locomotor activity in response to 0.01 and 1 μ M, with no anxiolytic effects¹⁵³. Additionally, the effects of quinpirole differ across life stages. In 2021, Nabinger et al. investigated the effects of early quinpirole exposure (5.5, 16.7, and 50 μ M) on behavioural alterations later in life¹⁴². They found that exposure to quinpirole for 24h at 5 days post fertilization (dpf) increased anxiety-like behaviours, stereotypic swimming, and memory impairment, but did not alter social and aggressive behaviour, at the testing age of three months¹⁴². These findings are supported by another study, in which zebrafish received i.p. injections of 0.5, 1.0, or 2.0 mg/kg quinpirole twice with an inter-injection interval of 48h and were tested 24h after the second injection¹⁴³. These

injections resulted in hypolocomotion, anxiety-like behaviour, and memory impairment, as well as stereotypic swimming¹⁴³.

Overall, these studies show that the effects of quinpirole vary across different experimental designs, and suggest that the effects on the dopaminergic system influences locomotion, stereotypy, and anxiety, may evolve throughout the lifespan.

2.3.3 Diazepam

The non-selective benzodiazepine diazepam is a GABA_A receptor modulator commonly used for the treatment of anxiety since the early 1960s, and it has been shown to be beneficial in the treatment of epilepsy, muscle spasms, and alcohol withdrawal^{154–156}. Diazepam interacts with all GABA_A receptor subunits, has a high bioavailability, and produces sedative and anaesthetic effects in higher doses^{155,156}. It has been shown to potentiate GABA in the limbic system, thalamus, hypothalamus, and cerebral cortex^{154,157}.

In preclinical studies, diazepam has been shown to produce anxiolytic and locomotion changes in mice and zebrafish, however, depending on the testing paradigm, dosage, genetic background, sex, and age, the reported effects varied^{113,114}.

The anxiolytic effects of diazepam on mice are complex and depend on a range of experimental factors. For example, i.p. injections of 2 mg/kg diazepam had anxiolytic effects in mice that were not previously stressed, but not on stressed mice¹⁵⁸. Notably, two reviews focussing on the open field and elevated plus maze test found that the anxiolytic effects of diazepam are not consistent^{159,160}. In the open field test, only 55.8% of the studies found anxiolytic effects^{159,160}. Similarly, diazepam had no consistent anxiolytic effects in the elevated plus maze test¹⁶⁰. The authors suggest that observed alterations in locomotion likely stem from sedation effects of diazepam¹⁶⁰. They recommend caution when using diazepam as a positive control for an anxiolytic in preclinical studies¹⁶⁰.

In zebrafish, diazepam has been shown to produce anxiolytic effects in various studies, however, as with fluoxetine and quinpirole, these effects are influenced by different experimental factors. For instance, acute exposure to diazepam for 3 min was found to produce anxiolytic effects at doses of 1.25 and 5 mg/L in adult zebrafish¹⁶¹. It also alleviated some stress-related behaviours and reduced cortisol levels after exposure to 16 µg/L for 15 min, though it decreased social interaction as well^{113,114}. Interestingly, a study by Generio et al. (2020) found sex differences in the anxiolytic responses to diazepam¹⁶². After 24-hour exposure to 16 µg/L diazepam, only male zebrafish exhibited an anxiolytic response, while females did not¹⁶². In addition to its potential dosage and sex specific effects, diazepam might also have time-dependent effects. In adult

zebrafish, diazepam that was directly added to the testing water at 0.1, 0.5, 2.5, 5, 10, and 20 μM reduced locomotion and increased exploration in a time-dependent manner¹⁶³.

These findings demonstrate that the effects of diazepam in zebrafish are influenced by factors such as dosage, sex, and exposure time, emphasizing the need to consider these variables when designing experiments.

3 GENETICS OF MAJOR DEPRESSIVE DISORDER AND COMORBID TRAITS

The genetic basis of depression is complex and highly polygenic, with twin studies suggesting a moderate heritability of 30-50% for MDD²⁶. Interestingly, differences in the heritability of MDD between the sexes have been reported, with MDD in women having a significantly higher heritability than men (40% vs 30%)^{19,20,26,164,165}. More research into the sex-specific contributions of the genetic basis of MDD is needed to fully understand the differences in the prevalence and presentation of MDD in men and women. Here, the focus lies on findings of studies including both men and women, without explicitly testing for sex differences. Additionally, while there is growing evidence linking epigenetic alterations with depression risk and severity¹⁶⁶, this thesis focuses on genetic studies rather than epigenetic mechanisms.

3.1 Candidate genes studies

Until the emergence of GWASs, the genetic basis of MDD was investigated by focussing on candidate genes based on their involvement in drug mechanisms or the pathophysiology, as well as their interactions with the environment²⁶. However, so far little support for the involvement of the most historical candidate genes could be found in GWASs, potentially due to lack of power or correction for population stratification^{20,26,167}. Another potential issue is the heterogeneity of the displayed symptoms in depression, as well as different assessment strategies, which can hide genetic effects¹⁶⁸. The main effects of polymorphisms, polymorphism-by-environment interactions, and gene-level effects for 18 candidate genes associated with depression, each of which had been studied at least ten times previously, found no clear evidence linking these genes to depression¹⁶⁹. The only exception was *DRD2* (*Dopamine Receptor 2*), which showed a significant gene-level effect on lifetime depression diagnosis in one analysis, and was subsequently identified in GWASs^{169,170}.

3.2 Whole exome sequencing studies

In the past years, whole-exome sequencing (WES) analyses found several rare variants in MDD-related genes. In a study on five families with affected individuals, 67 genes with novel deleterious variants shared among affected relatives were identified¹⁷¹. Interestingly, 18 of the identified genes encode transcription regulators, including the gene *ZNF34*, which is associated with bipolar disorder¹⁷¹. Additionally, an analysing of the UK Biobank 200643 exome dataset found four genes that were significantly associated with MDD, *OR8B4*, *TRAPPC11*, *SBK3*, and *TNRC6B*, including a novel rare risk variant within the *OR8B4* gene¹⁷². notably, some of these genes are related to neurological, cognitive, psychiatric, and behavioural phenotypes¹⁷². *TNRC6B* is related to micro-RNA gene silencing and has been associated with ADHD, ASD, and developmental

delay, among others¹⁷². *TRAPPC11* has also been associated with intellectual disability, and *SBK3* was associated with cognitive, psychiatric, and behavioural traits¹⁷². In 2024, a large-scale exome-wide association study on 296199 participants from the UK Biobank identified 22 genes associated with depressive symptoms, including six novel genes¹⁷³. Interestingly, the identified genes were associated with immune responses¹⁷³. Two of the identified genes, *TRIM27* and *UBD*, were also associated with psychiatric and cognitive traits, among others¹⁷³.

Although these studies provide valuable insights into the involvement of genes associated with psychiatric and cognitive phenotypes in depression, larger sample sizes are needed to uncover additional MDD-associated genes through WES.

3.3 Copy number variants

Copy number variants (CNVs) have been shown to contribute to neurodevelopmental and psychiatric disorders such as ADHD, ASD, and schizophrenia, and might also contribute to the genetic risk of MDD¹⁷⁴.

MDD patients were shown to have an increased burden of short deletions (<100 kb)¹⁷⁵. Most of these deletions lied in intergenic regions and mapped to enhancers, suggesting that rare CNVs contribute to MDD by disrupting regulatory regions¹⁷⁵. Interestingly, an overlap of 53 CNVs contributing to neurodevelopmental disorders and self-reported MDD was identified¹⁷⁴. The three CNVs showing the strongest associations were the 1q21.1 duplication, Prader-Willi syndrome duplication, and 16p11.2 duplication¹⁷⁴. Additionally, CNVs were identified in 17 regions in Korean patients with MDD¹⁷⁶. The most frequently detected regions containing MDD-related genes were 16q22.1, 7q11.21, and 22q11.23¹⁷⁶. Interestingly, copy number gains on 16q22.1–q22.2 are associated with headache and anxiety disorders, and copy number loss on 7q11.21 was associated with schizophrenia^{177,178}.

These studies provide interesting insights into the contribution of CNVs to MDD through disruptions in regulatory regions. They also highlight how the genetic risk of CNVs could be shared between psychiatric phenotypes. However, largest sample sizes might be needed to fully understand the role of CNVs in depression.

3.4 Genome-wide association studies

During the past decade, numerous GWAS studies as well as GWAS meta-analyses have been conducted on MDD, including mostly individuals with European ancestry²⁶. The first GWAS studies on MDD had limited power due to small sample sizes. A critical inflection point occurred at around 75000 cases (**Figure 13**)²⁶. Before this point, larger sample sizes were needed to uncover new loci, but after reaching 75000 cases, they were discovered roughly every 1500 cases.

In other psychiatric disorders like schizophrenia, a critical inflection point was observed at 15000, with new loci being discovered around every 230 cases (**Figure 13**)²⁶.

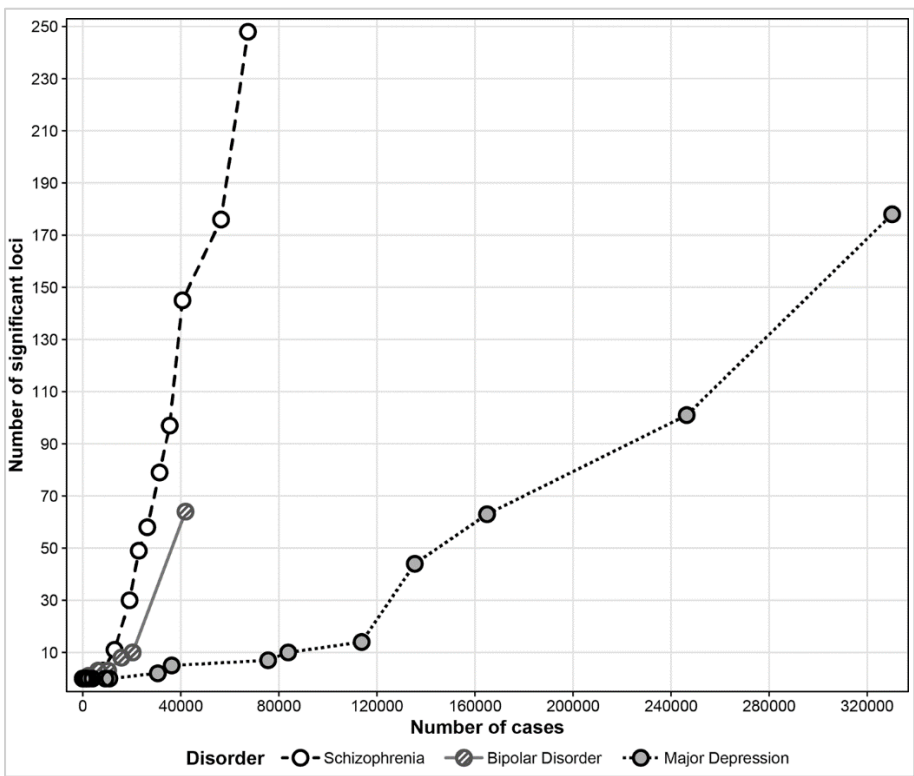


Figure 13: Identified GWAS loci in relation to case numbers in schizophrenia, bipolar disorder and major depression. Schizophrenia GWAS shows an inflection at ~15000 cases, discovering a new locus every 230 new cases. Bipolar disorder currently discovers a new locus for every 400 new cases. Major depression reaches its inflection at ~75000 cases, with liberal phenotype definitions identifying a locus every 1500 cases. Slopes starting at the inflection point were estimated using linear regression. Kendall et al. (2021)²⁶

Since then, a steadily increasing number of GWAS continues to improve our understanding of the genetic basis of MDD and other psychiatric conditions. The NHGRI-EBI GWAS catalogue alone includes 352 MDD-related studies, with 122 providing full summary statistics, covering 3894 associations^{179,180}. Many of the studies focus exclusively on MDD, while others also identify pleiotropic loci associated with multiple psychiatric disorders.

In a bi-ancestral GWAS on depression by Levey et al. (2021), 223 independently significant single nucleotide polymorphisms (SNPs) at 178 genomic risk loci were identified for individuals of European ancestry (**Figure 14**)¹⁷⁰. In this study, a combination of causal probability (CPP) and functional annotations was used to identify the most likely causal variants¹⁷⁰. To fine-map the identified loci, they filtered and annotated SNPs using combined annotation dependent depletion (CADD) scores to assess their functional impact, selecting most likely to be relevant¹⁷⁰. These SNPs were then mapped to 107 genes within 100 kb, with a focus on genes that overlapped with multi-tissue transcriptome-wide association study (TWAS) results (see chapter 3.5), further

refining their findings to highlight the most promising candidates¹⁷⁰. They highlighted the 15 most relevant genes associated with depression risk (**Figure 14**)¹⁷⁰. One of the highlighted genes is *CELF4* gene, which had been previously associated with depression³⁸. It encodes an RNA-binding protein implicated in the regulation of pre-mRNA alternative splicing, and has been associated with ASD and epilepsy¹⁸¹.

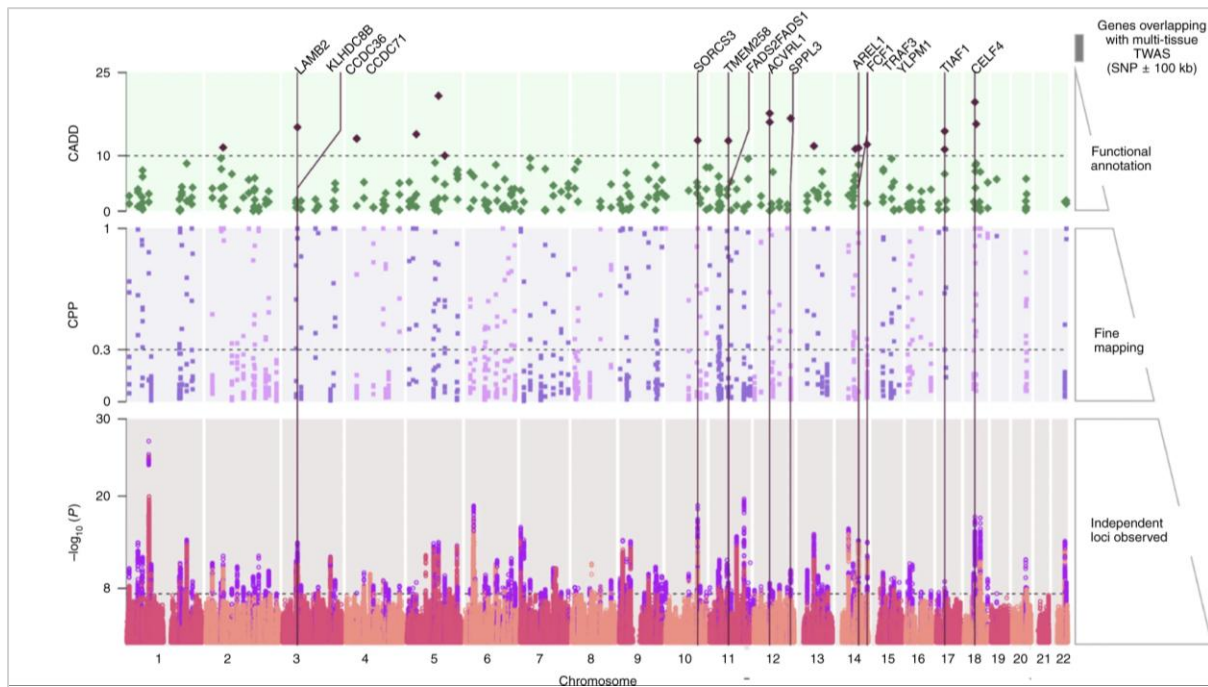


Figure 14: SNP prioritization using fine-mapping and functional scoring. Bottom row, Manhattan plot showing each genomic risk locus in violet. Middle row: Each locus was fine-mapped, and the CPP on the y axis is shown for SNPs from the causal set. The SNPs that had $CPP \geq 0.3$ (30%) were annotated using CADD scores. Top row: The SNPs with $CADD \geq 10$ are highlighted in purple; these SNPs were positionally mapped to 107 genes within 100 kb. Only positional genes overlapping with multi-tissue TWAS results (**Figure 16**) are annotated with vertical lines. CPP causal probability, combined annotation dependent depletion CADD, SNP single nucleotide polymorphism. Levey et al. (2021)¹⁷⁰

Interestingly, the *NEGR1* (*Neuronal Growth Regulator 1*) and *DRD2* genes were highlighted in multiple MDD GWAS^{170,182}. Notably, *DRD2* was the only candidate gene later identified in GWAS studies (see chapter 3.1). It was also highlighted in a study of our group which investigated the genetic contribution of the dopaminergic and serotonergic pathways to eight psychiatric disorders, including MDD, as well as a cross-disorder meta-analysis of these disorders¹⁸³. *DRD2* was associated with both MDD and schizophrenia at a gene-wide level, and was the only gene that overcame the Bonferroni correction for multiple testing in the gene-based results of the cross-disorder meta-analysis¹⁸³. It was also highlighted in a trans-ancestry GWAS, as it had the strongest evidence of association with MDD¹⁸⁴.

Another gene that has been highlighted in multiple GWAS on depression is *RBFOX1* (*RNA Binding Fox-1 Homolog 1*). One of the first GWAS studies identifying a substantial number of associated

loci with MDD, 44 risk loci, discovered *RBFOX1*, *RBFOX2*, *RBFOX3* and *CELF4* regulatory networks (Figure 15)³⁸.

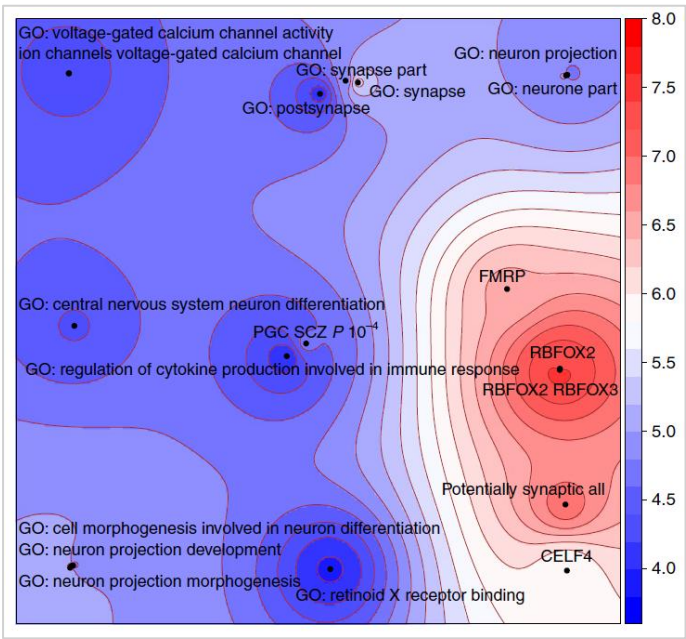


Figure 15: Networks regulated by *RBFOX1*, *RBFOX2*, *RBFOX3* and *CELF4* genes were highly enriched in MDD. The average position of each pathway on the map is represented by a point. Nearby points are more similar in gene overlap than more distant points. The colour surrounding each point indicates significance per the scale on the right. Wray et al. (2018)³⁸

RBFOX1 was also highlighted in a meta-analysis of the three largest GWAS on depression at that time GWAS by Howard et al. (2019)¹⁸². They identified 102 independent variants mapping to 269 putative genes and 15 gene sets associated with depression¹⁸². These genes and gene pathways were mainly involved in synaptic structure and neurotransmission¹⁸². *RBFOX1* was also found as one of the associated loci in a recent GWAS of MDD from 29 countries across diverse and admixed ancestries¹⁸⁴. It was also found highlighted in a cross-disorder study of eight psychiatric disorders, including MDD¹⁸⁵.

3.5 Transcriptome-wide association studies

To gain a deeper insight into the biological implications of the identified genetic associations, these associations can be integrated with gene expression data across different tissues in TWAS studies. This allows for the prioritization of genes that are differentially expressed in relevant tissues like brain tissues. A notable example of this approach is the aforementioned study by Levey et al. (2021)¹⁷⁰. They integrated GWAS findings and eQTL data from brain and whole-blood tissues in the GTEx v8 database, identifying 153 genes predicted to be differentially expressed across 13 brain tissues and whole blood (Figure 16)¹⁷⁰. Key genes identified for each tissue included *CELF4* and *PAX6* (*Paired Box 6*) in the caudate, *NEGR1* in the hypothalamus, *DRD2* in the NAc among others (Figure 16)¹⁷⁰.

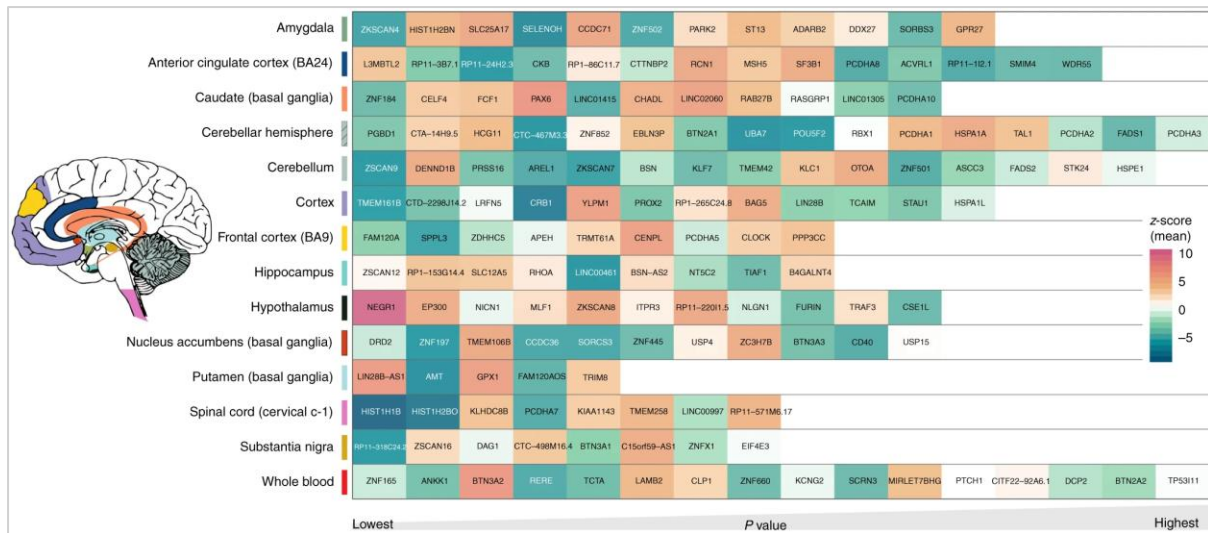


Figure 16: Tissue-based gene association study. Genes were tested using MetaXcan for 13 brain tissues and whole blood from GTEx v8. Genes are ordered from left to right by respective tissue-specific P value, with the lowest value on the left. The colour scale for the gene matrix is based on mean z-score. Levey et al. (2021)¹⁷⁰

3.6 Associated biological processes and pathways

To explore the underlying biological processes and pathways that are affected by genetic mutations, gene-based, gene-set and gene pathway analyses can be performed. By aggregating data in a biologically meaningful way, these analyses allow for a more comprehensive understanding of complex traits, making them a valuable addition to single-marker analyses¹⁸⁶. While GWAS use individual genetic markers to identify associations with traits, gene-based analyses consider the collective effect of all genetic markers within a gene. This can enhance statistical power, reduce the burden of multiple testing, and provide more biologically interpretable results at the gene level. To identify the biological processes to which these genes belong, gene-set, gene ontology, gene pathway, and weighted gene co-expression network analyses can be conducted. These analyses can be useful for understanding how the genetic basis of psychiatric disorders translates into the clinical findings, and can be useful for discovering new medication targets as well as repurposing other drugs¹⁸⁷.

Gene-based analyses of MDD have identified genes relating to neurotransmitter systems, as well as RNA binding proteins²⁶. Additionally, genes involved in neurite outgrowth, synaptic function and plasticity, as well as inflammation, among other functions, were found to be associated with MDD²⁶. Enriched biological processes in MDD include nervous system development, synapse assembly and organization, as well as cell adhesion and neuron differentiation¹⁷⁰. Gene sets related to synaptic processes as well as neuron projection were also found to be enriched^{182,184}. These findings align with previous studies which identified gene sets and pathways related to excitatory synapse and neuron spine and dendrite, as well as cell junction and cell-cell junction organization, and regulation of histone modification^{188,189}. A weighted gene co-expression

network analysis by Gandal et al. (2018) revealed a module enriched for G-protein coupled receptors, cytokine-cytokine interactions, and hormone activity pathways to be upregulated specifically in MDD¹⁹⁰. These findings emphasize the role of inflammation and dysregulation of the hypothalamic-pituitary (HPA) axis in the pathophysiology of MDD¹⁹⁰. Interestingly, the same study revealed that gene expression overlapped between different psychiatric disorders, namely ASD, schizophrenia, bipolar disorder, MDD, and alcoholism (AAD), in correlation with the degree of shared genetic risk¹⁹⁰. Gene expression patterns were mainly shared between ASD, schizophrenia, and bipolar disorder¹⁹⁰.

A detailed insight into enriched cell types was provided in the aforementioned trans-ancestry study of MDD¹⁸⁴. MDD SNP-heritability was enriched in neural tissues, most prominently excitatory and inhibitory neurons in the diencephalon, mesencephalon, and telencephalon (**Figure 17**)¹⁸⁴.

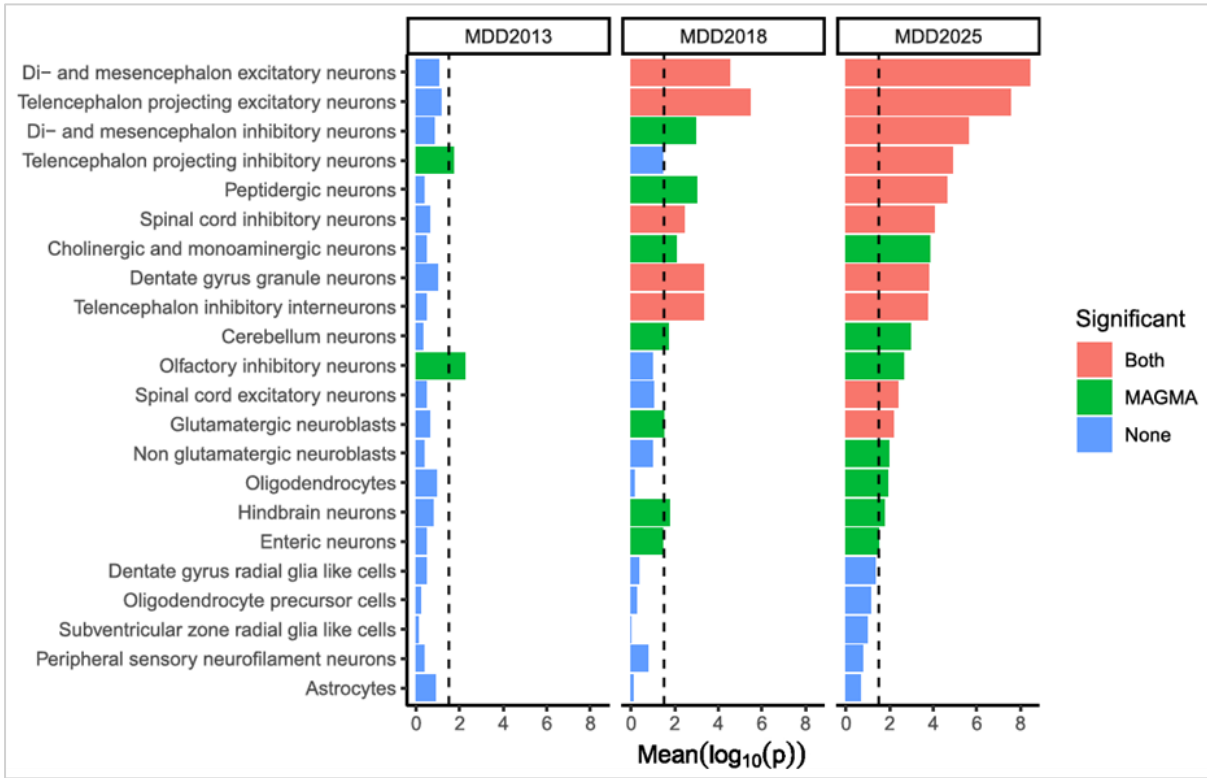


Figure 17: Brain cell category enrichment analysis. Highest enrichment was found for excitatory and inhibitory neurons in the diencephalon, mesencephalon, and telencephalon. Significance of the enrichment is indicated for MAGMA gene set enrichment test (green), partitioned LDSC (blue), and both (red). Columns represent the results of each test using summary statistics from MDD2013, MDD2018, and this PGC study. The dotted line shows threshold of nominal (uncorrected) statistical significance. Adams et al. (2025)¹⁸⁴

Research into the genetic basis of MDD plays a critical role in advancing our understanding of its aetiology and treatment, as evidenced by the identification of potential new drug targets in several studies. For example, a 2024 study by Meng et al. identified 53 novel loci associated with MDD, one of which mapped to the gene *NDUFAF3*, the main target of a first-line drug for treating

type 2 diabetes, highlighting the potential of drug repurposing¹⁹¹. Levey et al. (2021) also identified several drugs of interest that overlapped with associated genes. Riluzole, used to treat amyotrophic lateral sclerosis and currently in trials for combination therapy for treatment resistant depression, cyclothiazide, an allosteric modulator of glutamatergic AMPA receptors, and felbamate, and anti-seizure medication¹⁷⁰.

3.7 Shared genetic factors with psychiatric disorders

The high co-occurrence of MDD with other psychiatric phenotypes can be partially explained by an overlapping genetic basis²⁶. A strong genetic correlation was found between MDD and depressive symptoms measured as a continuous trait, suggesting that subthreshold depressive symptoms could have a similar aetiology as MDD^{26,192}. MDD and anxiety disorders also have a high genetic correlation of up to 90%, and anxiety disorders share several genetic loci previously associated with depression and neuroticism amongst other disorders^{35,193–196}. The genetic correlation between the personality trait irritability and depression has shown to be up to 0.7 in adolescents and 0.56 in adults, and neuroticism and depression have a genetic correlation of up to 0.56-0.79^{41–43,45,197–200}. Suicidality and MDD has a high genetic correlation of 0.59-0.78^{201,202}. Other psychiatric disorders like attention-deficit/hyperactivity disorder (ADHD), bipolar disorder, and schizophrenia also shared a genetic basis with MDD, with genetic correlations of 0.473, 0.343, and 0.357, respectively (**Figure 18**)^{26,185}. Interestingly, MDD was found to be negatively genetically correlated with educational attainment and cognitive performance¹⁸². Mendelian randomization studies even found educational attainment to reduce the risk of depression, whereas BMI increased the risk²⁰³.

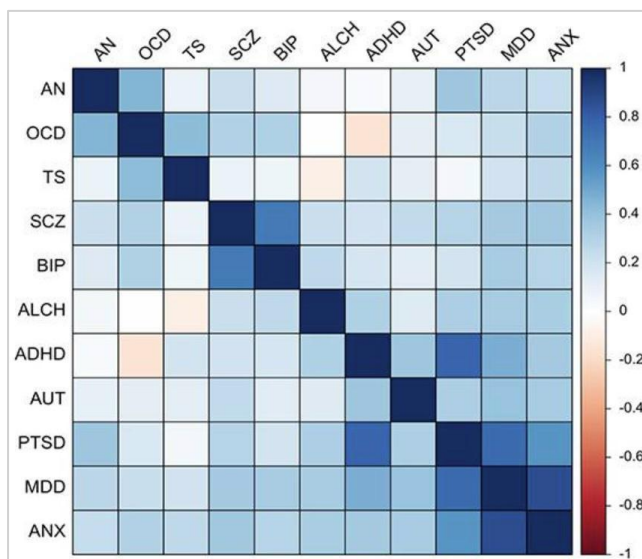


Figure 18: Genetic correlations between eleven psychiatric disorders. Genetic correlations estimated using LDSC. ADHD, attention-deficit/hyperactivity disorder; OCD, obsessive-compulsive disorder; TS, Tourette syndrome; PTSD, post-traumatic stress disorder; AN, anorexia nervosa; AUT, autism spectrum disorder; ALCH, problematic alcohol use; ANX, anxiety; MDD, major depressive disorder; BIP, bipolar disorder; SCZ, schizophrenia. Grotzinger et al. (2022)²⁰⁴

4 THE PLEIOTROPIC ROLE OF *RBFOX1* IN PSYCHIATRIC DISORDERS

RBFOX1 is a highly pleiotropic gene involved in multiple psychiatric and neurodevelopmental disorders, including MDD. It encodes a splicing factor that regulates networks of genes involved in essential processes like neuronal differentiation, migration, and excitation^{182,205–207}. However, the mechanisms behind its diverse effects were not fully understood.

4.1 Functions of *RBFOX1*

RBFOX1, also known as *Ataxin 2-Binding Protein 1 (A2BP1)*, encodes a splicing factor regulating the alternative splicing of developmental and tissue-specific genes by binding to the (U)GCAUG splicing enhancer element (**Figure 19**)^{206,208–210}. While the C-terminal domain of *RBFOX1* was found to be sufficient to activate its inclusion when tethered downstream of an exon, both the RNA recognition motif and the C-terminal were necessary for exon repression when tethered upstream of it²¹¹. *RBFOX1* is located at human chromosome 16p13.3 (chromosome number, p/q denotes the long/short arm of each chromosome, and band number)²⁰⁶. It spans 2.4 MB, being one of the largest genes in the human genome²⁰⁶. It is subject to complex regulatory processes, including autoregulatory processes, with multiple starting points for transcription and translation, leading to various structurally distinct, tissue-specific isoforms^{212,213}.

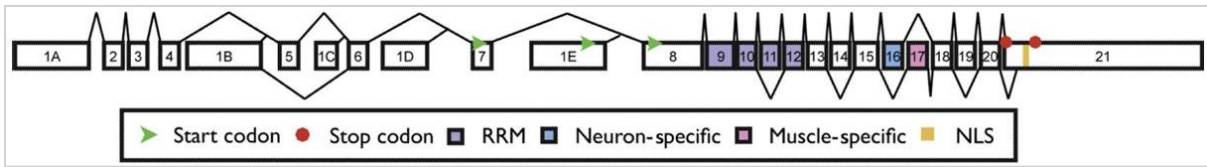


Figure 19: Structure of the *RBFOX1* gene. *RBFOX1* contains several transcriptional and translational start sites, multiple isoforms, and alternate endings, enabling the spatiotemporal expression of structurally distinct, tissue-specific protein isoforms. NLS, nuclear localization sequence; RRM, RNA recognition motif. Bill et al. (2013)²⁰⁶

RBFOX1 is predominantly expressed in brain, heart, and skeletal muscle tissues in both men and women (**Figure 20**)²¹⁴. Studies in mice showed that its ortholog *Rbfox1* (also known as *A2bp1*) was expressed as early as stage E11 in the embryonic development as well as in adult mice in the cerebellum, cerebral cortex, brain stem, and thalamus/ hypothalamus, whereas it was expressed in the heart at all stages²¹⁵.

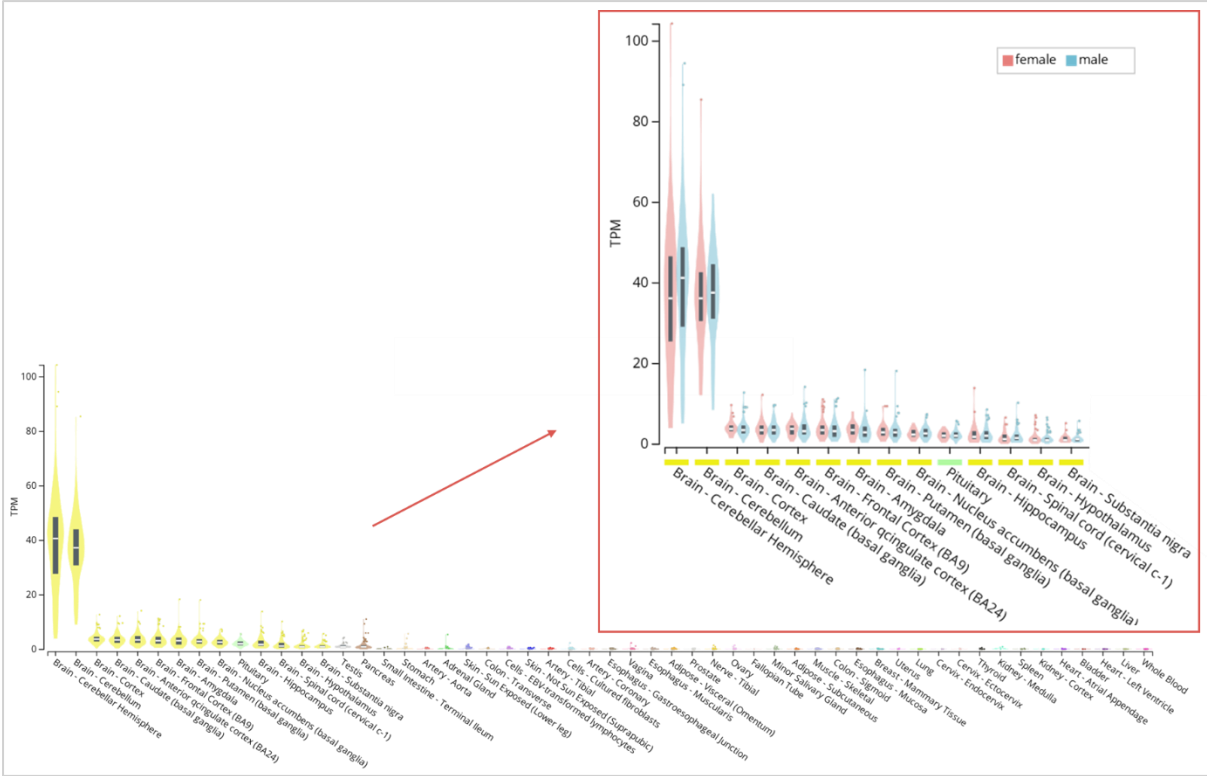


Figure 20: Bulk tissue gene expression for RBFOX1. RBFOX1 (ENSG00000078328.23) is predominantly expressed in Brain, skeletal muscle, and heart tissues, both in men and women. GTEx portal^[24]

RBFOX1 regulates a wide range of alternative splicing events, and the network of its target genes is involved in multiple essential neurological processes, including neuronal differentiation, migration, maintenance, and neuronal excitation^{205,216–219}. RBFOX1 splicing targets include other splicing factors and RNA-binding proteins, as well as transcription factors^{205,216}. Furthermore, RBFOX1 splicing targets were found enriched in pathways involved in cell proliferation, cell signalling, cytoskeletal organization, cell adhesion, gene regulation, and neuronal development²⁰⁵. *RBFOX1* is alternatively spliced into nuclear and cytoplasmic isoforms with distinct roles, as was discovered by Lee et al. (2016)²²⁰. While the nuclear isoform functions as a splicing regulator, the cytoplasmic isoform regulates mRNA stability and translation, especially of genes involved in the cortical development and ASD²²⁰.

Importantly, the effects of *RBFOX1* are not limited to alternative splicing; differential expression analyses revealed a downstream transcriptional network regulated by *RBFOX1* that is involved in pathways implicated in ASD and other neurodevelopmental diseases²⁰⁵. Overlapping motifs were found in both the splicing and the transcriptional network of *RBFOX1*, suggesting a coordinated and complex interplay of both networks during the neurodevelopment²⁰⁵. Interestingly, other motifs belonging to different factors were found to be enriched in introns of differentially spliced genes and promoters of differentially expressed genes, suggesting the involvement of further regulatory factors in the *RBFOX1* network²⁰⁵.

4.2 The role of *RBFOX1* in stress and inflammation

Several studies suggest that *RBFOX1* plays a role in stress responses and the regulation of inflammation. In mice, prolonged cellular depolarization led to the skipping of *Rbfox1* exon19, which affects its subcellular localization and splicing of target genes²²¹. This induced a reversal of some alternative splicing changes caused by depolarization²²¹. In *Drosophila*, increased *Rbfox1* levels were found under stress conditions²²². In humans and higher primates, *RBFOX1* was found to interact with the transcriptional corepressor Lysine Specific Demethylase 1 (LSD1) to regulate the stress response²²². Additionally, overexpression of *RBFOX1* has been shown to protect against inflammation²²³. These findings suggest that *Rbfox1* is involved in adaptive mechanisms to chronic stress, which may play a crucial role in the development of neurodevelopmental and psychiatric diseases^{206,224}. Disruptions in these adaptive mechanisms caused by mutations in *RBFOX1* could impact the neurodevelopment, even if the regular functions of *RBFOX1* remain unaffected²⁰⁶. Bill et al. (2013) suggest a model for *RBFOX1* dysregulation in ASD, in which *RBFOX1* haploinsufficiency contributes to ASD both through alterations in its direct effects on RNA splicing and mRNA stability, as well as through perturbations in the adaptive response to cellular stress (**Figure 21**)²⁰⁶.

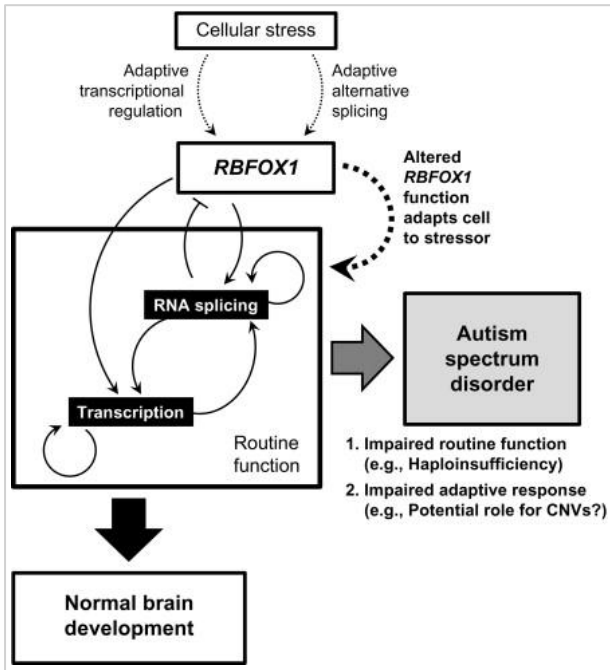


Figure 21: Proposed model for *RBFOX1* dysregulation in autism spectrum disorder. *RBFOX1* regulates RNA splicing, mRNA stability, and transcription during neurodevelopment, ensuring normal neuronal development. Disruption of *RBFOX1*, such as through haploinsufficiency, can lead to autism spectrum disorder (ASD). Additionally, *RBFOX1* may help the brain to adapt to cellular stress by responding to environmental changes. Impairments in this process, including structural variations (CNVs) that affect *RBFOX1*, can increase the risk of ASD, particularly when combined with environmental stress during critical developmental stages. Bill et al. (2013)²⁰⁶

This may also hint to why psychiatric disorders like MDD are best understood as a result of gene-environment interactions rather than each of these factors in isolation (see chapters 1.3 and 1.4). Genetic variants alone are insufficient to cause psychiatric disorders like MDD; typically, depressive episodes are triggered through interactions with environmental factors (see chapter 1.4). For instance, early life stress is a major risk factor for developing depression during later life stages. Extending the by Bill et al. (2013)²⁰⁶ proposed model to MDD would suggest that mutations

in *RBFOX1* that would not affect its regular functions are limiting it from helping the cells to adapt to stressors. Hence, without external stressors, MDD would not be developed, and in return the external stressors are also insufficient to cause MDD by themselves. This theory could be extended to include more *RBFOX1* regulated genes and other risk genes, which, if mutated similarly to *RBFOX1*, may not be able compensate for the lack of adaptation caused by *RBFOX1* mutations.

4.3 The role of *RBFOX1* in clinical phenotypes

RBFOX1 was initially identified for its interaction with the causal protein ataxin-2 in the neurodegenerative disease spinocerebellar ataxia type 2 (SCA2), and is now recognized as a risk factor for various neurodevelopmental and psychiatric disorders^{206,225}. For over a decade, both inherited and *de novo* copy number variants (CNVs), as well as haploinsufficiency of *RBFOX1*, have been associated with disorders such as global neurodevelopmental delay, epilepsy, macroencephaly and microencephaly, Alzheimer's disease, and ASD^{206–208,226–230}. However, as *RBFOX1* CNVs do not cause haploinsufficiency in isolation, and some CNVs are inherited from unaffected individuals, other genetic and environmental factors likely also contribute to these disorders²⁰⁶. Bill et al. (2013) suggest that when *RBFOX1* levels are reduced due to haploinsufficiency, other splicing factors could compete with *RBFOX1* for binding, leading to alterations in the splicing of some targets²⁰⁶.

In addition to CNVs, common variants (such as SNPs) have associated *RBFOX1* to a range of psychiatric disorders, including ASD, MDD, schizophrenia, and alcohol use disorders^{207,231}. *RBFOX1* has also been implicated in neuroticism and aggression^{232–234}. The important role of *RBFOX1* in psychiatric disorders was highlighted in a 2019 study by the cross-disorder group of the PGC (see chapter 3.4), where *RBFOX1* was identified as the second most pleiotropic locus, with associations to seven out of the eight psychiatric disorders tested (**Figure 22**)¹⁸⁵.

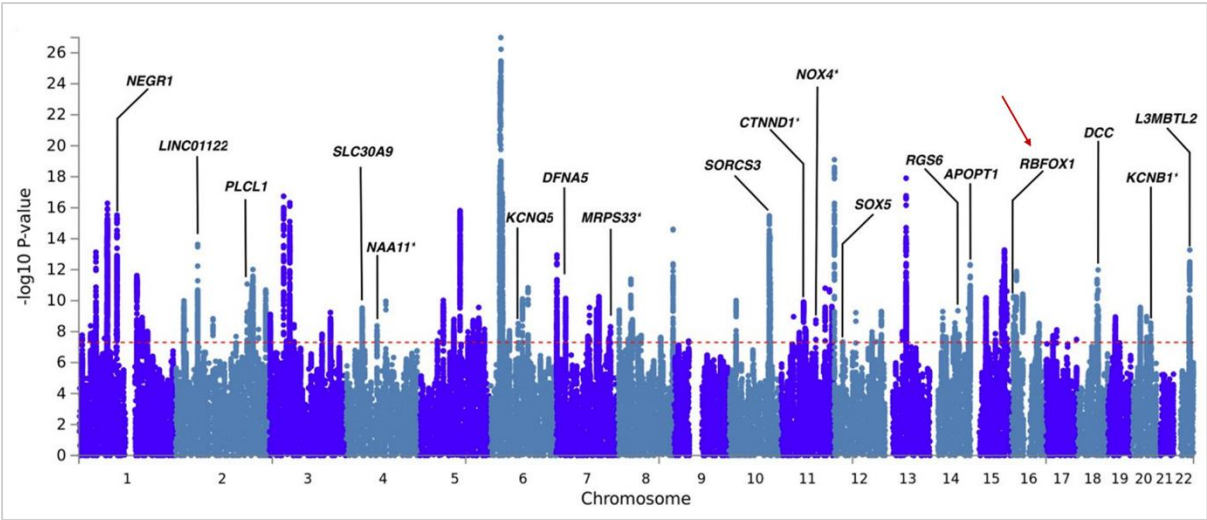


Figure 22: *RBFOX1* is mapped as a candidate gene in a cross-disorder study. Manhattan plot displaying the cross-disorder meta-analysis results highlighting candidate genes mapped to top pleiotropic regions, including *RBFOX1*. Candidate genes not previously implicated in individual disorder GWAS are marked with an asterisk. Lee et al. (2019)¹⁸⁵

The involvement of common variants *RBFOX1* in psychiatric phenotypes was confirmed by recent GWAS and gene-based analyses, where it was found associated with ADHD, anxiety disorders, MDD, neuroticism, schizophrenia, as well as a cross-disorder meta-analysis, among others (Figure 23)^{203,207,235–239}.

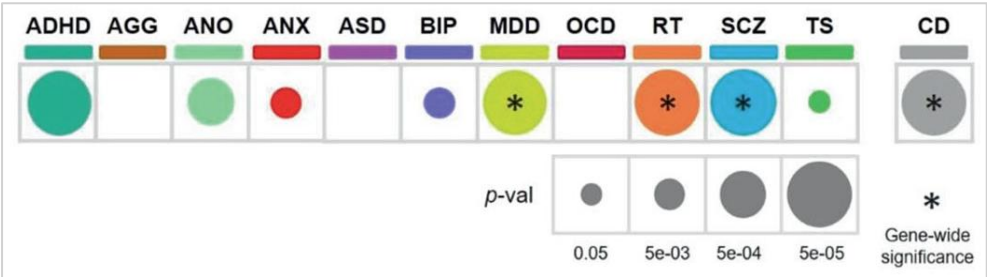


Figure 23: Genetic risk variants in *RBFOX1* in different psychiatric conditions and traits. Common single-nucleotide variants in *RBFOX1* showed a gene-based association with ADHD, ANO, ANX, BIP, MDD, RT, SCZ, TS, as well as in the cross-disorder meta-analysis. ADHD attention-deficit/hyperactivity disorder, AGG aggression, ANO anorexia, ANX anxiety, ASD autism spectrum disorder, BIP bipolar disorder, MDD major depressive disorder, OCD obsessive-compulsive disorder, RT risk tolerance behaviour, SCZ schizophrenia, TS Tourette’s syndrome, CD cross-disorder meta-analysis. O’Leary et al. (2022)²⁰⁷

Notably, serum levels of *RBFOX1* together with *TCF4* were proposed as potential biomarkers for MDD²⁴⁰. *RBFOX1* and *TCF4* were increased in drug-naïve patients of MDD in comparison to both healthy controls and drug-treated patients, with no observable sex differences²⁴⁰.

Another link between MDD and *RBFOX1* was found in brain organoids of 16p11.2 deletion syndrome²⁴¹. In these organoids, reduced *RBFOX1* mRNA and protein levels and disruptions in *RBFOX1*-regulated genes were observed²⁴¹. Interestingly, the 16p11.2 genomic region is

associated with MDD (see chapter 3), schizophrenia susceptibility, and neurodevelopmental traits, strengthening the link of *RBFOX1* with MDD and other phenotypes²⁴¹. In this region, hemideletions are also associated with macrocephaly and obesity, while hemiduplications relate to microcephaly and low body mass index (BMI)^{174,241}.

An increasing number of studies investigate the different mechanisms through which *RBFOX1* influences different neurodevelopmental and psychiatric disorders. For example, Chung et al. (2024) found significantly lower cytoplasmic levels of RBFOX1 protein in parvalbumin-expressing interneurons (PVIs) in schizophrenia compared to healthy controls²⁴². One important target of the cytoplasmic *RBFOX1* isoform is the *vesicle associated membrane protein 1* (*Vamp1*) gene, which regulates GABA release from PVIs, and VAMP1 levels were found to be reduced in a subset of the schizophrenia cohort^{242,243}. Alterations in the *RBFOX1-VAMP1* pathway in PVIs of schizophrenia patients may lower GABA release, disrupt network synchrony, and reduce gamma oscillation power without markedly altering the overall network activity²⁴². This aligns with findings of Wamsley et al. (2018), who discovered different alternative splicing networks regulated by *RBFOX1* in somatostatin-expressing interneurons (SST+) and PVIs²⁴⁴. Interestingly, *Rbfox1* knockout mice showed reduced inhibitory synaptic transmission in the cortex, and they were more prone to spontaneous seizures²⁴⁴.

RBFOX1 has also been linked to cognitive symptoms of MDD, such as working memory impairments and disruptions in emotion processing^{207,243,245}. Elevated levels of RBFOX1 were shown to impair BDNF dependent LTP by increasing TrkB.T1 receptor levels²⁴³. This could partially explain the implication of *RBFOX1* in working memory problems, which has been observed before²⁴⁵. *RBFOX1* was also shown to play a role in neuronal reactivity to emotional stimuli along with reduced prefrontal processing in cognitive control tasks, and enhanced fear expression and avoidance behaviour²⁰⁷. This implicates *RBFOX1* in heightened emotional reactivity as is observed in MDD, as well as impaired impulse control and aggression²⁰⁷.

4.4 The role of *Rbfox1* in behavioural alterations in animal models

There are several animal models of *Rbfox1* used to investigate its functions as a splicing factor and mRNA stabilizer, as well as the phenotypic consequences of *Rbfox1* mutations. Male mice with a homozygous neuron-specific deletion of *Rbfox1* were found hyperactive compared to heterozygous and wildtype mice in several behavioural tests, and showed increased exploration²⁰⁷. Moreover, the homozygous *Rbfox1* knockout mice were less aggressive and displayed impairments in social behaviour, fear acquisition and extinction, but not associative learning or spatial working memory²⁰⁷. These behavioural alterations are also features of ASD, implicating *RBFOX1* in the development of this disorder. This gene has also been related to aggressive behaviour in other animals, such as *Drosophila melanogaster* and dogs^{246,247}.

Interestingly, in a study of our group in a murine model for frustration after expected reward, *Rbfox1* showed upregulation of expression in the ventral striatum^{246,247}. Additionally, *Rbfox1* knockout mice presented increased neuronal excitability and seizures^{219,244}. These promising findings demonstrate the value of *Rbfox1* animal models in investigating psychiatric disorders.

5 ZEBRAFISH AS AN ANIMAL MODEL IN PSYCHIATRY

Zebrafish (*Danio rerio*) has emerged as a powerful model organism for psychiatric and drug research due to their behavioural complexity, conserved neurobiology, cost-effectiveness, high genetic homology to humans and rapid development. Their similarities in genetics, neurotransmitter systems, and disease-associated genes make zebrafish a valuable model for studying a wide range of psychiatric phenotypes.

The primary goals of research into psychiatric pathologies include identifying the genetic basis of disorders, characterizing their molecular pathology, and developing and improving therapeutic strategies²⁴⁸. As most psychiatric disorders do not have measurable pathobiological markers, diagnoses typically rely on behavioural and cognitive phenotypes²⁴⁹. Core behaviours, such as certain social behaviours, anxiety, and responses to fear or stress, seem to be evolutionarily conserved across different species, albeit with varying complexity²⁵⁰. Therefore, while it is not possible to model the complete range of human behaviours, interactions, and schemas in their whole complexity, certain elements of neurodevelopmental and psychiatric disorders can be studied using animal models. Additionally, specific hypotheses under controlled conditions can be tested in animal models, which would be ethically or practically impossible in humans²⁵¹.

Traditionally, the most commonly used animals in psychiatric research are rodents, particularly mice and rats²⁵². As mammals, their physiology and brain morphology is similar to humans, and there are well-established behavioural models for psychiatric research (see chapter 5)²⁵². However, due to their behavioural complexity, conserved neurobiology, cost-effectiveness, rapid development, genetic tractability, and other reasons, zebrafish have also become increasingly popular in psychiatric research²⁵³.

5.1 Life stages of zebrafish

Zebrafish are small freshwater teleost fish from South-East Asia with a lifespan of three to five years in laboratory conditions, reaching adulthood as indicated by sexual maturity around three months of age^{250,254}. Like humans, zebrafish are a diurnal species relying on vision as their main sensory modality²⁵⁵. Their short life span along with their high reproductive rate, with one female fish being able to lay several hundred eggs each week, are key advantages as an animal model²⁵⁰.

Zebrafish undergo several different life stages: embryonic pre-hatching (0–72 hours post fertilization, hpf) and post-hatching (72–120 hpf); larval (1–29 dpf; free-swimming from 5 dpf) to juvenile (30–89 dpf), and adult zebrafish (90 dpf – two years), and aging/aged zebrafish (from two years) (**Figure 24**)²⁵⁰.

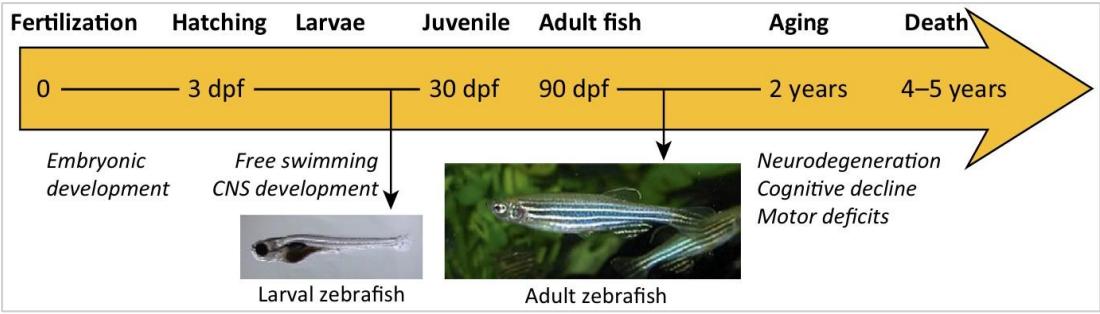


Figure 24: Outline of the life cycle of zebrafish. Embryonic pre-hatching (0–72 hpf), larval (3–29 dpf), juvenile (30–89 dpf), adult (90 dpf–2 years), and aged zebrafish (>2 years). Stewart et al. (2014)²⁵⁶

During embryonic and larval stages, zebrafish are transparent, allowing for *in vivo* monitoring and manipulation of neurodevelopmental processes (**Figure 25**, chapter 5.4). Both during larval and adult stages, zebrafish behaviour can be tested in different established paradigms including locomotion, anxiety, and social behaviours (chapter 5.5).

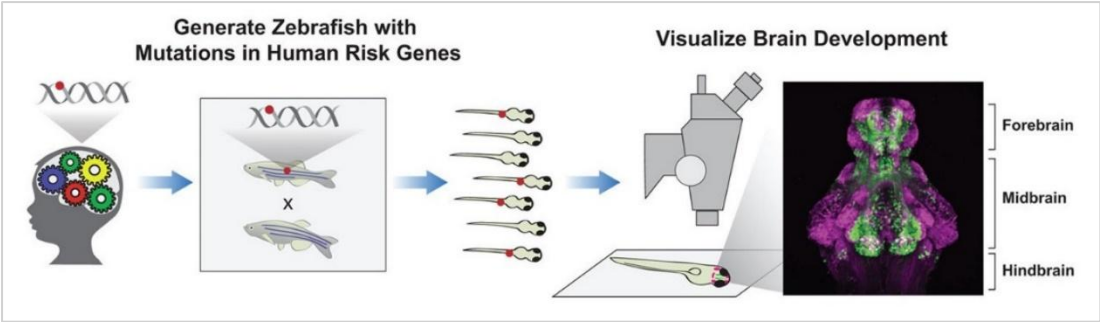


Figure 25: Schematic representation of the functional analysis of risk genes in neuropsychiatric disorders using a zebrafish model system. Genetic variants associated with neurodevelopmental and psychiatric disorders are generated in zebrafish. Transparent zebrafish embryos and larvae allow for visualization of brain development in zebrafish carrying mutations in risk genes. A representative high-resolution image of a control larval zebrafish brain (ventral view) is shown with labelled excitatory glutamatergic (magenta) and inhibitory GABAergic (green) neurons. The major subdivisions of a vertebrate brain (forebrain, midbrain, hindbrain) are shown. Adapted from Ijaz et al. (2016)²⁵⁷

5.2 Genetics of zebrafish

Due to the teleost-specific genome duplication event, which occurred 320-350 million years ago, zebrafish possess over 26000 protein-coding genes on 25 chromosomes²⁵⁸. The zebrafish genome is highly homologous to humans, with ~70% of human genes having at least one zebrafish orthologue^{258,259}. Remarkably, 84 % of human genes associated with disease possess zebrafish counterparts²⁵⁸.

Zebrafish, like all animal models, can be used in “forward genetics”, where random mutations are generated to discover new genes involved in a particular phenotype, or in “reverse genetics”, where a gene of interest is mutated or silenced to study the resulting phenotype (**Figure 26**)^{250,260}.

There are several methods to apply forward genetics in zebrafish, including Ethyl-Nitroso Urea (ENU) chemical mutagenesis, viral vector-mediated insertional mutagenesis, and radiation^{250,260}. “Reverse genetics” involve methods like the ectopic expression of a gene, gene silencing, and precise modification or knock out of the gene²⁵⁰.

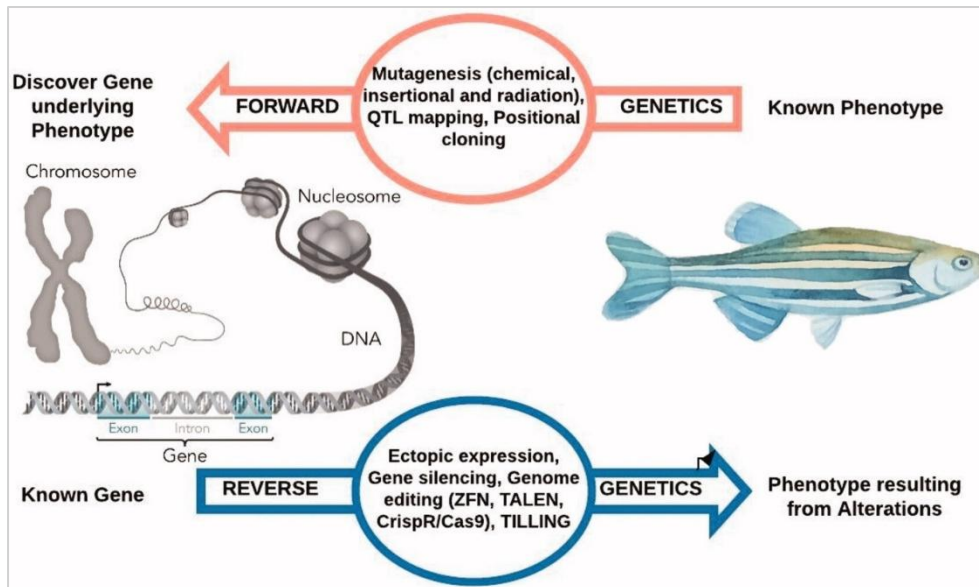


Figure 26: Forward and reverse genetics in zebrafish. Spontaneous mutations can be induced using various methods to study the gene or genes underlying a displayed phenotype (forward genetics). Mutating a known gene to knock it out, knock it down, or silence it can be used to study the phenotype resulting from the genetic alteration (reverse genetics). Clustered regularly interspaced short palindromic repeats, CRISPR; Targeting Induced Local Lesions in Genomes, TILLING; Transcription activator-like effector nucleases, TALEN; QTL, quantitative trait loci; Zink Finger Nucleases (ZFN). From Cheresiz et al. (2020)²⁶⁰

Behavioural differences have been reported between zebrafish strains, which may affect the outcome of experiments²⁵⁰. One limitation of zebrafish as an animal model is the lack of inbred strains with a clear breeding history, which can lower the reproducibility of experiments due to unaccounted genetic variability²⁶¹. However, if genetic variability is carefully considered when designing experiments, for example by using an adequate sample size, zebrafish studies can yield offer valuable insights to the field.

Interestingly, zebrafish lines commonly used for research do not possess sex chromosomes, instead, their sex determination is influenced by various factors, including chromosome 3, 4, and 5, a region on chromosome 16, as well as environmental factors^{258,262,263}. However, the precise mechanisms of sex determination are not yet fully understood and may vary between zebrafish strains. The sex of the fish can therefore only be determined morphologically, which can be difficult due to the visual similarity between males and females, particularly during larval, juvenile, and early adulthood stages. This should be considered when designing and analysing experiments.

5.3 Brain morphology and neurotransmitter systems in zebrafish

The brain morphology of zebrafish is highly similar to mammalian brains, both in terms of structural organization and cell morphology, even though the brain organization is less complex (**Figure 27**)²⁵⁰. While many basic functions of the different brain regions in zebrafish are already present during larval stages, the complexity and specialization increase as the fish mature. In zebrafish, the forebrain comprises the telencephalon and diencephalon, which are responsible for sensory processing, locomotion, and higher cognitive functions²⁶⁴. Although zebrafish lack a neocortex, the telencephalon is considered homologous to the cerebral cortex and limbic structures in mammals (**Figure 27**)²⁵³. Its functions also parallel those of the hippocampus and amygdala in mammals, regulating learning processes, spatial memory, navigation, and social behaviours^{265–267}. As zebrafish mature, the telencephalon becomes more complex, supporting more advance cognition and behavioural processes, although basic functions are already established during larval stages^{267,268}. The diencephalon in zebrafish is involved in relaying and processing sensory signals, particularly in regions of the thalamus^{269,270}. The midbrain, which includes the optic tectum, is critical for visual processing and regulating locomotion, as well as processing sensory stimuli^{271,272}. As in all vertebrates, the cerebellum is essential for motor coordination and socio-emotional functions such as anxiety and exploration²⁷³. The hindbrain, including the medulla oblongata and cerebellum, plays an important role in locomotion, escape behaviours, and processing of sensory cutaneous information, among other functions^{274–276}.

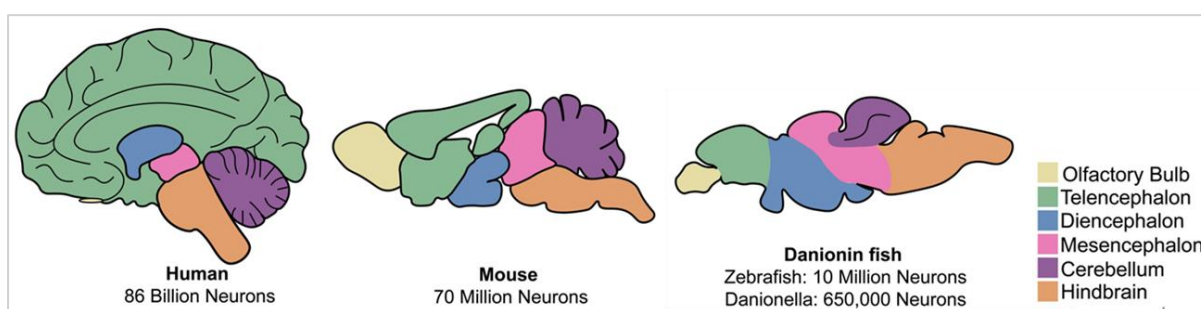


Figure 27: Comparison of anatomy for human, mouse, and zebrafish brain. Both mammalian and non-mammalian vertebrates share similar brain anatomy, though region size and organization vary between species. Adapted from Haynes et al. (2022)²⁵⁹

Similar to brain morphology, the brain neurochemistry of zebrafish is highly similar to humans, with zebrafish possessing all major neurotransmitter and neuromediator systems²⁵⁹. The serotonergic and dopaminergic systems in zebrafish are highly conserved, resembling those in mammals²⁶⁵. In zebrafish, serotonergic nuclei are primarily located in the raphe nuclei of the hindbrain as well as in the medulla oblongata, from where they project primarily to the forebrain and midbrain (**Figure 28**)²⁷⁷. Additional serotonergic neurons are present in the forebrain, specifically in the telencephalon and olfactory bulb, as well as in the diencephalon, including the

hypothalamus, pineal gland, pretectal area, and posterior tuberculum^{277,278}. The serotonergic system in zebrafish regulates fear, anxiety, and stress-related behaviours²⁷⁹. It is also involved in aggression and locomotion although findings regarding the role of serotonin in these behaviours are inconsistent^{277,280}.

Dopaminergic neurons in zebrafish are primarily located in the diencephalon, particularly in the ventral diencephalon, which is considered analogous to the substantia nigra in mammals, as well as in the olfactory bulb and pretectum (**Figure 28**)²⁸¹. Dopaminergic neurons are also present in parts of the telencephalon, and potentially in parts of the hindbrain²⁸¹. The dopaminergic system is involved in the regulation of learning, locomotion, social behaviour, and stereotypy^{282–287}.

GABA and glutamate are the primary inhibitory and excitatory neurotransmitters in vertebrates and key modulators of neuronal excitability^{288,289}. In zebrafish, GABAergic neurons are present in the olfactory bulb, telencephalon, tectum stratum, and in the diencephalon, including the hypothalamus, thalamus, preoptic region, and posterior tuberculum, as well as in the parts of the hindbrain, including the cerebellum and medulla oblongata (**Figure 28**)^{290–293}. The GABAergic system is involved in the modulation of anxiety and motor control, among others^{294,295}.

Glutamatergic neurons in zebrafish are present in the forebrain, specifically in the telencephalon, the optic tectum, and in the posterior tuberculum of the diencephalon, as well as to a lesser extent in the hindbrain (**Figure 28**)²⁹⁶. In the CNS, glutamatergic signalling plays an important role in developmental processes, adaptation to the environment, and memory and learning²⁶⁵. Glutamatergic NMDA receptors have been shown to be involved in long-term potentiation (LTP) in the telencephalon of zebrafish, and are involved in the regulation of neuronal migration in the cerebellum^{297,298}.

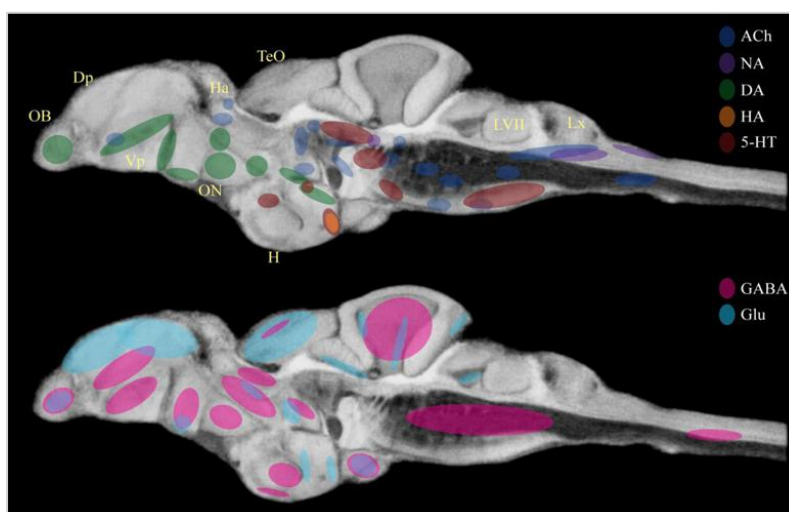


Figure 28: Main neurotransmitter systems in adult zebrafish brains.

ACh - acetylcholine (dark blue), NA noradrenergic (purple), DA dopamine (green), HA histamine (orange), 5-HT serotonin (red); GABA γ -aminobutyric acid (pink), Glu glutamate (light blue). The highlighted brain regions include olfactory bulb (OB), dorsal pallium (Dp), ventral pallium (Vp), habenula (Ha), optic nerve (ON), facial lobe (LVII) and vagal lobe (LX). Stewart et al. (2015)²⁵³

Due to the high homology of neurotransmitter receptors, transporters, and enzymes, zebrafish respond to all major classes of neurotropic drugs, including certain antidepressants and anxiolytics, making them a valid animal model for drug research^{250,299}.

5.4 Brain imaging in zebrafish

As they are transparent during embryonic and larval stages, their brain activity can be monitored *in vivo*. For instance, zebrafish larvae expressing a neuronal calcium marker, typically GCaMP6, can be recorded under a light-sheet microscope, and their single-cell as well as whole brain activity can be analysed (**Figure 29**)²⁵⁵. This technique can provide valuable insights into how genetic mutations affect neuronal activity and connectivity, and help to elucidate their role in psychiatric conditions²⁵⁵. Using this technique, a previous study of our group could shed light on the neurobiological mechanisms underlying the contribution of the *YWHAZ* gene in neurodevelopmental disorders, including ASD²⁵⁵.

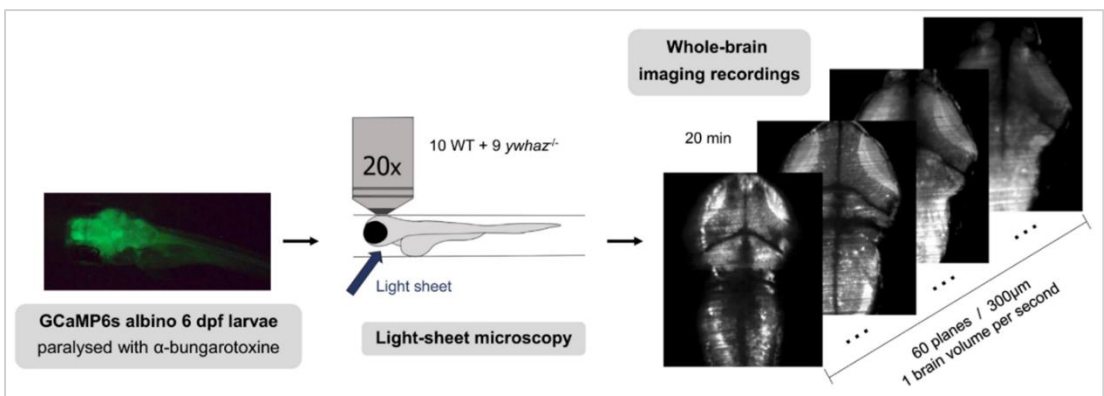


Figure 29: Whole-brain imaging recordings in zebrafish larvae expressing GCaMP6s pan-neuronally. Mutant zebrafish larvae expressing GCaMP6s pan-neuronally are paralysed with α-bungarotoxin and recorded using a light-sheet microscope. Adapted from Antón-Galindo et al. (2022)²⁵⁵

5.5 Behavioural paradigms in zebrafish

Zebrafish exhibit well-defined, context-specific behaviours, despite having a lower neurological complexity than humans. They are highly social animals, living in large shoals of up to hundreds of animals in the wild. This makes them suitable animal models for anxious behaviours and locomotion, as well as social behaviours like shoaling, social preference, and aggression, paralleling common behavioural test in mice (**Figure 30**)²⁵⁰. Popular paradigms for anxious behaviour include the open field tests, in which the animal is placed in a novel, stimulus free environment, where their movement is tracked. Their locomotion is measured for potential hyperactivity or hypoactivity, stereotypic movements, as well as potential thigmotaxis, i.e., the preference to stay closer to the walls, which can indicate anxiety. A more specific test for anxiety is the light-dark or black and white test, in which the animal is placed in a half black and half white

chamber (**Figure 30**)²⁵⁰. Their locomotion as well as their exploration on the light side is measured, as sticking to the dark side can be an indicator of anxiety. Social models include the social preference test, which exists in several variations. Typically, the tested animal can choose to stay close to a physically but not visually separated compartment containing one or more animal, or an empty compartment (**Figure 30**)²⁵⁰. One variation of this test includes a second step, in which the animal can choose between the former group of animals or a new group of animals, testing their preference for social novelty. Another test which is more specific to group behaviour in zebrafish is the shoaling test. Here, a group of typically five fish are placed in a novel, stimulus free arena (**Figure 30**)²⁵⁰. Their movement is tracked to measure locomotion, thigmotaxis, as well as grouping behaviour, i.e., the distance between the animals in the group. Another test for social interactions is the aggression test. While in rodents the aggression test typically involves another animal, in zebrafish this is not necessary. Here, this test is commonly conducted using a tank with a transparent wall, in front of which a mirror is placed at a 21° angle (**Figure 30**)²⁵⁰. The fish, which does not recognise itself, can choose to attack the “other fish”, and the number of attacks and duration of this aggressive behaviour is counted.

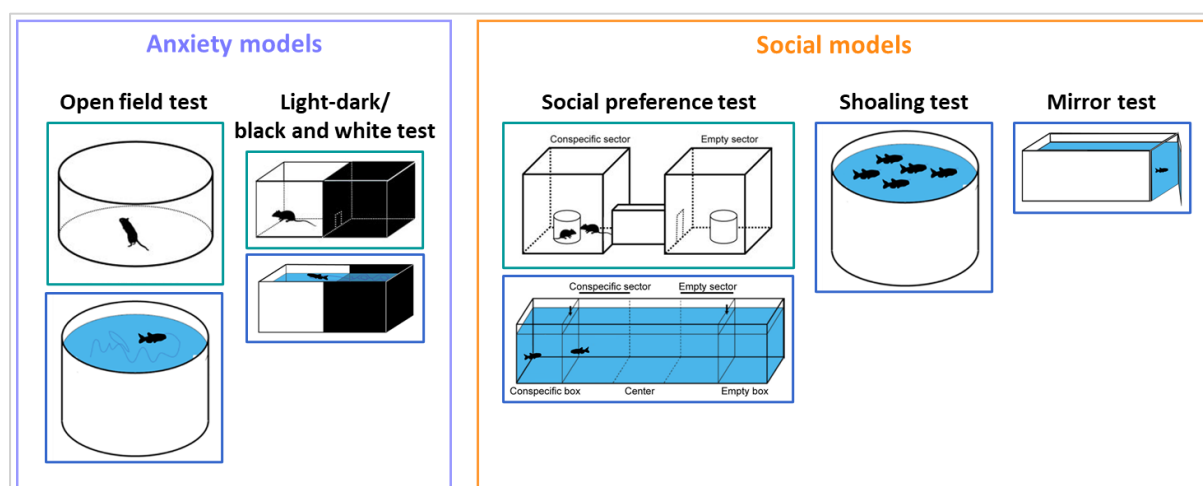


Figure 30: Selection of behavioural paradigms in zebrafish and mice. Behavioural paradigms testing anxious (purple) and social behaviour (orange) in mice (green) and zebrafish (blue). Anxiety models: Open field and light-dark/ black and white test, social models: social preference test, shoaling test, and mirror test for measuring aggression. Adapted from Kalueff et al. (2014)²⁵⁰

To model a psychiatric phenotype in an animal, it is important to ensure its predictive and construct validity, i.e., the similarity between the observable characteristics between the animal model and the human condition, and the alignment with the theoretical understanding of the disease or condition. When selecting an animal model, it is important to select the least complex model possible and always comply to ethical and legal animal welfare guidelines³⁰⁰. Furthermore, the model should be replicable by the same or other groups if the same conditions are met. Like with all animal models, certain batch effects can lower the reproducibility, which must be taken

into account when planning the experiments and deciding on an appropriate sample size. However, if the context of the observed behaviour is considered when interpreting the results of behavioural studies, and, if possible, the assessed behaviour is tested in several different paradigms, animal studies can produce solid and reliable results, advancing the knowledge in the field.

OBJECTIVES

The overall objective of this work is to characterize the underlying mechanisms through which *RBFOX1* contributes to the aetiology of psychiatric phenotypes, with a focus on major depressive disorder (MDD) and comorbid traits. To obtain a comprehensive overview, we followed a multi-faceted approach investigating the effects of *RBFOX1* at the levels of genetics, transcriptomics, neurotransmission, brain activity, and behaviour.

The specific objectives of this work are the following:

Chapter 1. The influence of the *RBFOX1* regulatory network on MDD and comorbid traits.

- 1.1 Assess the association of *RBFOX1* with MDD, anxiety, irritability, neuroticism, and suicide attempt
- 1.2 Evaluate the contribution of *RBFOX1*-regulated genes to the shared genetic basis of MDD and comorbid traits
- 1.3 Investigate the biological mechanisms through which *RBFOX1*-regulated genes affect MDD and comorbid traits
- 1.4 Explore the contribution of *RBFOX1*-regulated genes to rare diseases with symptoms of depression, anxiety, neuroticism or irritability
- 1.5 Highlight the more relevant genes from the *RBFOX1* regulatory network contributing to MDD and comorbid traits using integrative analyses

Chapter 2. The effect of *rbfox1* on behaviour in zebrafish.

- 2.1 Characterize the role of *rbfox1* on zebrafish behaviour using two different mutant lines
- 2.2 Compare the observed behavioural alterations between the two *rbfox1* mutant zebrafish lines and with *Rbfox1* knockout mice
- 2.3 Assess potential sex differences in the behaviour of *rbfox1* mutant and wildtype fish

Chapter 3. Pharmacological interventions in *rbfox1* mutant zebrafish.

- 3.1 Detect the effect of *rbfox1* on basal levels of serotonin and tryptophan in different brain regions
- 3.2 Evaluate to which extent the behavioural changes in *rbfox1* mutant zebrafish can be rescued by different doses of fluoxetine, an SSRI targeting the serotonergic system
- 3.3 Evaluate to which extent the behavioural changes in *rbfox1* mutant zebrafish can be rescued by quinpirole and diazepam, medication targeting the dopaminergic and GABAergic systems, respectively

Chapter 4. Central nervous system alterations in *rbfox1* mutant zebrafish.

- 4.1 Assess alterations in neurotransmission by quantifying levels of serotonin, dopamine, GABA, and glutamate in different brain regions
- 4.2 Examine changes in neuronal activity in different brain regions in developmental stages
- 4.3 Identify genes and pathways regulated by *rbfox1* by assessing transcriptomic changes in gene expression and splicing in different brain regions

THESIS SUPERVISORS' REPORT ON THE CONTRIBUTION OF THE PHD CANDIDATE TO THE ARTICLES INCLUDED IN THIS DOCTORAL THESIS

Thesis title: “The role of *RBFOX1* and its regulatory network in major depression and comorbid psychiatric phenotypes”

Author: Maja Rebecca Adel

Directors: Dra. Noèlia Fernàndez-Castillo and Dr. Florian Freudenberg

The undersigned, Noèlia Fernàndez-Castillo and Florian Freudenberg, acting as PhD supervisors of the thesis by Maja Rebecca Adel presented as a compendium of 4 research articles, hereby inform about the precise contribution of the candidate in each publication.

Chapter 1. The influence of the *RBFOX1* regulatory network on MDD and comorbid traits.

Article 1

Title: The *RBFOX1* regulatory gene network contributes to major depressive disorder, anxiety, irritability and neuroticism

Authors: Maja R. Adel, Ester Antón-Galindo, Florian Freudenberg, Bru Cormand, Noèlia Fernàndez-Castillo

Journal: To be submitted

Contribution of the PhD candidate:

Data curation and preparation of summary statistics. Gene-based analyses, identification of gene overlap, enrichment statistical tests. Gene-set analyses on biological functions and pathways and network construction. Gene expression prediction and retrieval of genes involved in rare disorders with related symptoms. Interpretation of the results, integrative analyses and gene ranking. Writing the first draft of the manuscript, figures and tables, and participating in the final edits.

Chapter 2. The effect of *rbfox1* on behaviour in zebrafish.

Article 2

Title: Pleiotropic contribution of *rbfox1* to psychiatric and neurodevelopmental phenotypes in two zebrafish models

Authors: Ester Antón-Galindo, Maja R. Adel, Judit García-González, Adele Leggieri, Laura López-Blanch, Manuel Irimia, William H. J. Norton, Caroline H. Brennan, Noèlia Fernández-Castillo[#] and Bru Cormand[#]

[#] equally supervised this work

Journal: Translational psychiatry

Impact factor (2023): 5.8. First decile of the category “PSYCHIATRY”

Contribution of the PhD candidate:

Coordinating the crossing and genotyping of zebrafish mutant lines. Behavioural experiments, tracking of the fish, and analyses. Participated in writing the first draft of the manuscript and tables, and in the final edits.

Chapter 3. Pharmacological interventions in *rbfox1* mutant zebrafish.

Article 3

Title: Decreased Brain Serotonin in *rbfox1* Mutant Zebrafish and Partial Reversion of Behavioural Alterations by the SSRI Fluoxetine

Authors: Maja R. Adel, Ester Antón-Galindo, Edurne Gago-Garcia, Angela Arias-Dimas, Concepció Arenas, Rafael Artuch, Bru Cormand and Noèlia Fernández-Castillo.

Journal: Pharmaceuticals

Impact factor (2023): 4.3. First quartile of the category “PHARMACOLOGY & PHARMACY”.

Contribution of the PhD candidate:

Coordinating the crossing and genotyping of zebrafish mutant line. Collection and dissection of zebrafish brains for quantification of neurotransmitter and amino acid levels and data analysis. Fluoxetine acquisition and preparation. Set up of drug treatment and behavioural

experiments. Tracking of the fish and data analyses. Interpretation of the results. Writing the first draft of the manuscript, figures and tables, and participation in the final edits.

Chapter 4. Central nervous system alterations in *rbfox1* mutant zebrafish.

Article 4

Title: Alterations in neurotransmission, neuronal activity, gene expression and splicing in *rbfox1* mutant zebrafish

Authors: Maja R. Adel*, Ester Antón-Galindo*, Javier G Orlandi, Gustavo Castro, Tahnee Mackensen, Luis P Iñiguez, Eloi Casals, Anna Esteve-Codina, Oriol Vidal Casasayas, Marc Guasch, Angela Arias-Dimas, Concepció Arenas, Manuel Irimia, Pablo Loza-Alvarez, Rafael Artuch, Bru Cormand, Noèlia Fernàndez-Castillo

*equally contributed

Journal: To be submitted

Contribution of the PhD candidate:

Coordinating the crossing and genotyping of zebrafish mutant line. Collection and dissection of zebrafish brains for RNAseq and quantification of neurotransmitter levels. Data analyses on neurotransmitter levels. Improvement of the protocols for the whole-brain imaging recordings and analysis. Setting up and performing the analyses of whole-brain imaging recordings, extraction of fluorescence signals, definition of brain areas and statistical analysis of brain activity. RNA extraction of samples for transcriptomic analyses. Gene expression differential expression and splicing analyses and enrichment of biological functions and pathways. Writing the first draft of the manuscript, figures and tables, and participating in the final edits.

Barcelona, 31st of March 2025

Dra. Noèlia Fernàndez-Castillo

Dr. Florian Freudenberg

RESULTS

Chapter 1. The influence of the *RBFOX1* regulatory network on MDD and comorbid traits.

The *RBFOX1* regulatory gene network contributes to major depressive disorder, anxiety, irritability and neuroticism

Authors

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Keywords

RBFOX1, depression, anxiety, irritability, neuroticism, regulatory network

ABSTRACT

Major depressive disorder (MDD) is a complex psychiatric disorder frequently comorbid with anxiety disorders, suicide attempts, and personality traits such as irritability and neuroticism. *RBFOX1*, which encodes a splicing factor, is a known risk gene for MDD, and has also been associated with anxiety and neuroticism. However, the working mechanisms of how *RBFOX1* influences these conditions are not yet understood. Here, we aim to shed light on the effects of *RBFOX1* and its regulated genes on MDD, anxiety, irritability, neuroticism, and suicide attempt using genome-wide association study (GWAS) data from previous research. We demonstrate that *RBFOX1* is associated with MDD, anxiety, and neuroticism, and that genes associated with all tested phenotypes, except suicide attempts, are significantly enriched among *RBFOX1*-regulated genes. These genes show a substantial overlap between MDD and comorbid traits and are involved in neuronal development, synaptic processes, and neurotransmitter regulation. Our network analysis reveals that *RBFOX1* functions as a central hub, integrating and coordinating neighbouring nodes. Through differential gene expression prediction and the study of rare disorders presenting with depression, anxiety, irritability, or neuroticism, alongside our previous analyses, we highlight 19 top-ranked genes regulated by *RBFOX1*, including *CADM2*, *CTSB*, *MAPT*, *NSF*, *PAX6*, *SCAI*, *SNCA*, *SP4*, and *TCF4*. Our findings underscore the key role of *RBFOX1* and its regulated gene network in the genetic architecture of MDD and comorbid traits. These insights may contribute to improving the prevention and treatment of MDD in the future.

1 INTRODUCTION

With over 300 million people worldwide affected, major depressive disorder (MDD) is one of the main contributors to disability and mortality¹⁻³. It is a complex disorder characterized by symptoms such as anhedonia, feelings of guilt, sleep disturbances, weight fluctuations, and cognitive changes^{1,4}. MDD has an estimated heritability of 30-50% with a polygenic architecture^{1,3}. While the precise mechanisms through which genetic and environmental factors interact in the development of MDD are still not fully understood, growing evidence from genome-wide association studies (GWAS) underscores the significance of genetic factors in understanding its complex aetiology¹. Anxiety disorders are among the most common comorbidities of MDD, with 45%-85% of MDD patients reported to have at least one comorbid anxiety disorder⁵⁻⁷. This comorbidity is further associated with increased suicidality and greater antidepressant resistance in MDD patients⁸⁻¹⁰. Similarly, personality traits such as irritability and neuroticism are consistent predictors of higher MDD rates and reduced subjective well-being. Irritability not only predicts depression and anxiety, among other psychiatric disorders, but also correlates with depression severity¹¹⁻¹⁵. Likewise, higher neuroticism scores are associated with

an increased risk of various psychiatric disorders, including anxiety and MDD, with evidence suggesting a causal effect on neuroticism^{3,16–19}. MDD is also a known risk factor for suicide attempts and completed suicide. Suicidal ideation was reported in up to 46% of MDD patients, and, depending on additional risk factors, the incidence of suicide attempts in MDD patients range from 3% up to 30%^{20–22}.

The comorbidity of MDD and anxiety disorders can be partially attributed to shared genetic factors, with a genetic correlation of up to 90%^{23,24}. Anxiety disorders share several genetic *loci* previously associated with depression, neuroticism, and other conditions^{8,25,26}. Similarly, irritability and depression exhibit overlapping genetic and potentially environmental risk factors, with a genetic correlation of up to 70% in adolescents and 56% in adults^{11,13,27,28}. Like irritability, neuroticism and depression have a genetic correlation of ranging from 56% to 79%^{18,19,29,30}. Suicidality, another common symptom of MDD, also has a strong genetic link to MDD, with a genetic correlation of 59–78%^{31,32}.

While major psychiatric disorders frequently co-occur and share genetic risk, both through direct associations and a common general psychopathology factor, the search for common pathogenic pathways and their master regulators remains inconclusive^{8,33}. One potential master regulator of shared pathways in psychiatric phenotypes is the splicing factor *RBFOX1* (*RNA Binding Fox-1 Homolog 1*). *RBFOX1* is a highly pleiotropic gene identified in multiple studies as a risk *locus* for major depression, as well as other psychiatric and neurodevelopmental disorders^{2–4,17,34–36}. It has also been associated with anxiety sensitivity, and it has been implicated in the causal relationship between MDD, anxiety, and neuroticism^{17,25}. Further evidence from animal models supports a contribution of *RBFOX1* to psychiatric phenotypes. Male *Rbfox1* knockout mice exhibit pronounced hyperactivity and alterations in social behaviour³⁷. These behavioural changes, along with thigmotaxis, have also been observed in two independent lines of *rbfox1* mutant zebrafish³⁸. Interestingly, in one of the lines, the acute administration of the selective serotonin reuptake inhibitor (SSRI) fluoxetine, a commonly prescribed antidepressant medication, partially reversed hyperactivity and alterations in social behaviours³⁹.

RBFOX1 is a splicing factor that regulates the alternative splicing of gene networks involved in brain development, however, its cytoplasmatic isoform has also been shown to increase target mRNA stability and translation by binding to its 3'UTR^{4,40–43}. *RBFOX1* is mostly expressed in the brain, heart, and skeletal muscle and is most active during neuronal development, maturation, and maintenance^{44,40,45}. The importance of *RBFOX1* in the regulation of splicing and gene expression has been highlighted in multiple studies. In mouse studies, hundreds of genes have been identified as regulated by *RBFOX1*^{41,45}. Notably, the influence of *RBFOX1* extends beyond the regulation of alternative splicing events, as differential expression analyses have revealed a

downstream transcriptional network regulated by RBFOX1 implicated in pathways associated with autism spectrum disorder (ASD) and other neurodevelopmental diseases⁴⁶.

In this study, we aimed to investigate the *RBFOX1* regulatory network under the hypothesis that genes regulated directly or indirectly by this pleiotropic splicing regulator may contribute to MDD and its related traits.

2 METHODS

2.1 Human orthologs of RBFOX1-regulated genes

Rbfox1 regulated genes were obtained from two publications from Lee et al. (2016) and Wamsley et al. (2018), which showed a certain overlap in genes found differentially expressed or spliced in mice^{41,45}. Lee et al. (2016) used RNAi knockdown using *Rbfox1* and *Rbfox3* siRNAs, microarray analysis, RNA sequencing (RNASeq), and iCLIP in hippocampal and cortical cultures derived from postnatal day 0 mice to identify genes regulated by *Rbfox1* in terms of both expression and splicing⁴¹. Wamsley et al. (2018) performed RNASeq on cortical interneurons in three different *Rbfox1* mouse lines, i.e., *Dlx6aCre*, *SSTCre*, *Tac1Cre*, with different *Rbfox1* deletions⁴⁵. Using the *Dlx6aCre* line, they investigated a broad population of GABAergic interneurons during embryonic development, and the *SSTCre* and *Tac1Cre* were used for somatostatin-positive and tachykinin-expressing interneurons during postnatal stages, respectively⁴⁵.

From the 2718 different genes identified in these studies, 2515 human *RBFOX1* orthologs were retrieved using Biomart and manual curation (Supplementary Table S1).

2.2 Gene-based analysis

Summary statistics for MDD were obtained from Howard et al. (2019)⁴⁷. GWAS summary statistics on anxiety, irritability, and neuroticism were obtained using UKBiobank data (<https://www.nealelab.is/uk-biobank>), which also contained information from the X chromosome (Table 3). Summary statistics on suicide attempt were obtained from Mullins et al. (2022)⁴⁸.

Table 3: GWAS summary statistics used in the gene-based analysis

	Source	Individuals	Predicted SNP heritability	Standard error
Major depressive disorder (MDD)	Howard et al. (2019) ⁴³	246,363 cases + 561,190 controls	0.089	0.003

Anxiety: worrier/ anxious feelings	UKBiobank	199,463 cases + 152,370 controls	0.098	0.005
Irritability	UKBiobank	97,000 cases + 250,000 controls	0.063	0.003
Neuroticism score	UKBiobank	293,006 individuals	0.088	0.004
Suicide attempt	Mullins et al. (2022) ⁴⁴	29,782 suicide attempt cases (20% completed suicide) + 519,961 controls	0.075	0.006

A gene-based analysis using the respective summary statistics on MDD, anxiety, irritability, neuroticism, and suicide attempt was performed using MAGMA version 1.09b and version 1.10.⁴⁹. After correcting for multiple testing at 5% FDR, 2016 genes were identified associated for MDD, 1148 for anxiety, 629 for irritability, 1534 for neuroticism and 200 for suicide attempt, (Supplementary Tables S2-S6). The obtained Entrez IDs were subsequently converted to gene symbols using the SynGO ID conversion tool⁵⁰. After converting the entrez IDs to gene symbols, 2009 genes were associated to MDD, 1145 to anxiety, 200 to suicide attempt, 627 to irritability, and 1529 to neuroticism.

2.3 Enrichment of RBFOX1-regulated genes and overlap

The enrichment of RBFOX1 regulated genes in the genes associated with MDD, anxiety, suicide attempt, irritability, and neuroticism, was tested using a Fisher’s F test and conducted in R (R version 4.4.2). Subsets including only those RBFOX1 regulated genes associated with each phenotype were created. The overlap between those subsets was identified using PyCharm version 2024.2.4 (Community Edition) with Python version 3.13.

2.4 Gene-set enrichment analyses of Gene Ontology (GO) and KEGG pathways

Enrichment analyses of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were performed on the RBFOX1 regulated genes associated with MDD, anxiety, irritability, and neuroticism, using the WEB-based GENE Set Analysis Toolkit (WebGestalt) 2024⁵¹. For the GO analysis the category “geneontology - Biological Process noRedundant” was chosen, and for all analyses the reference set “genome protein-coding” was selected. The redundancy removal method “weighted set cover” was used, and the multiple test adjustment was set to Benjamini Hochberg with an false discover rate (FDR) cut-off of 0.05 (5% FDR).

2.5 Network construction of RBFOX1-regulated genes in selected phenotypes

A network of RBFOX1 regulated genes showing association with MDD and at least one more trait was created with the stringAPP in Cytoscape (version 3.10.3), which uses the STRING database to compute a protein-protein interaction network⁵²⁻⁵⁴. For this full STRING network, a confidence

score of 0.4 was chosen, and no tissue or cell compartment filters were applied. Nodes not connected to the main network were removed from the figure. Parameters for centrality, connectivity, statistics of neighbouring nodes, and network group stress were calculated with the stringAPP in Cytoscape.

2.6 Predicted gene expression correlates in each selected phenotype

The predicted differential expression of genes associated with MDD, anxiety, irritability, and neuroticism was assessed using MetaXcan and the GWAS summary statistics described above (Supplementary Tables S2-S6). Prediction models were constructed using the SNPs located within ± 1 Mb from the transcription start site (TSS) of each gene and were trained with the RNA-seq data of 13 brain tissues from GTEx v8⁵⁵⁻⁶⁰. SNP covariate matrices were generated using the EUR 1000 Genomes project (Phase 3) data⁶¹. MetaXcan consists of S-PrediXcan and S-MultiXcan for tissue-level and cross-tissue expression prediction, respectively^{59,60}. For S-PrediXcan, the analyses were conducted using the brain tissues “Amygdala”, “Anterior cingulate cortex BA24”, “Caudate basal ganglia”, “Cerebellar Hemisphere”, “Cerebellum”, “Cortex”, “Frontal Cortex BA9”, “Hippocampus”, “Hypothalamus”, “Nucleus accumbens basal ganglia”, “Putamen basal ganglia”, “Substantia nigra”, as well as “Spinal cord cervical c-1”. Values were corrected for multiple testing using the Bonferroni-method: For S-PrediXcan, a threshold of $0.05/13 = 0.003846$ was applied, for S-MultiXcan a threshold of $0.05/14164 = 3.53008\text{e-}06$ was applied.

For MDD, 990 genes were predicted to be differentially expressed in brain, and 736, 604 and 852 genes for anxiety, irritability and neuroticism, respectively (Supplementary Figure 1A; Supplementary Tables S7-S11). For MDD, 450 genes that were associated in the gene-based analysis were also predicted to be differentially expressed, and 273, 163 and 356 genes for anxiety, irritability, and neuroticism, respectively.

2.7 RBFOX1-regulated genes in rare disorders with similar symptomology as selected phenotypes

To find genes related to rare disorders with a similar symptomology as the selected disorders or traits (MDD, anxiety, irritability, and neuroticism), we used the Online Mendelian Inheritance in Man® (OMIM) database. We searched for the terms “depression”, “depressive”, “anxiety”, “irritability”, “irritable”, “neuroticism”, and “neurotic” using the OMIM API and PyCharm version 2024.1.2 (Community Edition) with Python version 3.13. We retrieved information from the sections “geneMap”, “text:description”, “text:clinicalFeatures”, “clinicalSynopsis”, and focused on entries with the prefix “#” (descriptive entries, usually of a phenotype, and does not represent a unique locus). Entries were excluded if the search term appeared in the “miscellaneous” section,

or if the status was not “live”. Associated genes and *loci* were excluded if they appeared in entries with the prefix “%” (entries describing a confirmed mendelian phenotype or phenotypic locus for which the underlying molecular basis is not known). We then manually excluded entries of non-rare disorders from the analysis. The enrichment of RBFOX1 regulated genes in rare disorder with a similar symptomology as the selected phenotypes was tested using a Fisher’s F test was conducted in R (R version 4.4.2).

2.8 Gene ranking of RBFOX1-regulated genes with pleiotropic effect in MDD and comorbid traits

We ranked the RBFOX1 regulated genes by their total number of occurrences in the gene lists obtained for each of the three analyses: gene-based association, predicted differential expression, and rare disorder with similar symptomology. The prioritized genes were ranked using a simple weighted sum method: 2 x (total occurrence in MDD gene lists) + 1 x (total occurrence in anxiety gene lists) + 1 x (total occurrence in irritability gene lists) + 1 x (total occurrence in neuroticism gene lists). The rationale was to add more weight to the genes identified to contribute to MDD, as this study mainly focuses on the contribution of RBFOX1 regulated genes to this disorder and the comorbidity with the other traits.

3 RESULTS

3.1 *RBFOX1* and its regulated genes are associated with MDD and comorbid traits

To better understand the relationship between *RBFOX1* and its regulated genes with MDD, anxiety, irritability, neuroticism, and suicide attempt, we first conducted a gene-based analysis using GWAS summary statistics of the different phenotypes. This analysis identified *RBFOX1* as significantly associated with MDD ($p = 9.29\text{e-}15$, $p_{\text{adj}} = 1.87\text{e-}11$), anxiety ($p = 1.45\text{e-}07$, $p_{\text{adj}} = 1.97\text{e-}05$), and neuroticism ($p = 4.64\text{e-}10$, $p_{\text{adj}} = 1.13\text{e-}07$), and nominally associated with irritability ($p = 4.78\text{e-}03$, $p_{\text{adj}} = 6.65\text{e-}02$) and suicide attempt ($p = 8.13\text{e-}03$, $p_{\text{adj}} = 1.30\text{e-}01$).

Next, we examined 2,515 RBFOX1-regulated genes and found that a substantial number were associated with MDD, anxiety, irritability, and neuroticism (Figure 31A, Supplementary Table S12). Specifically, RBFOX1-regulated genes were significantly enriched among those associated with MDD (315 out of 2,009 MDD-associated genes, $p = 9.64\text{e-}06$) (Supplementary Table S12), anxiety (200 out of 1,145 genes, $p = 5.31\text{e-}07$), irritability (112 out of 627 genes, $p = 6.80\text{e-}05$), and neuroticism (262 out of 1,529 genes, $p = 4.71\text{e-}08$) (Figure 31A, Supplementary Table S12).

However, no significant enrichment was observed for suicide attempt-associated genes (31 out of 200 genes, $p = 0.13$), leading us to exclude this trait from further analyses.

To assess the extent of the influence of *RBFOX1* and its regulated genes across the different phenotypes, we examined the genetic overlap of *RBFOX1*-regulated genes in MDD, anxiety, irritability, and neuroticism (Figure 31B). Of the *RBFOX1*-regulated genes that are associated with MDD, 137 were also associated with at least one additional trait (anxiety, irritability, or neuroticism), and 23 were associated with MDD and all three traits. Interestingly, six of these shared genes encode transcription factors, namely *CAMTA1*, *PAX6*, *SOX6*, *SP4*, *TCF4*, and *TEF* (Supplementary Tables S2-S6). The genetic overlap between MDD, anxiety, and neuroticism was particularly pronounced, with 57 shared genes (Figure 31B).

To further explore the biological pathways through which *RBFOX1* and its regulated genes influence these phenotypes, we searched for enriched gene sets and pathways. Among the 12 biological processes from GO that showed enrichment of *RBFOX1*-regulated genes associated with MDD, three were shared with the other three traits, namely “regulation of neuron projection development” (GO:0010975), “regulation of trans-synaptic signaling” (GO:0099177), and “synapse organization” (GO:0050808) (Figure 31C; Supplementary Tables S13-S16). Additionally, the biological processes “axonogenesis” (GO:0007409) and “cell junction assembly” (GO:0034329) were shared between *RBFOX1*-regulated genes associated with MDD, irritability, and neuroticism. Furthermore, of the 40 KEGG pathways enriched in *RBFOX1*-regulated genes associated with MDD, six were shared with neuroticism, including “Long-term depression” (hsa04730), “Cholinergic synapse” (hsa04725), “Dopaminergic synapse” (hsa04728), and “Glutamatergic synapse” (hsa04724) (Figure 31C; Supplementary Tables S17-S19; overlapping GO and KEGG pathways in Supplementary Table S20).

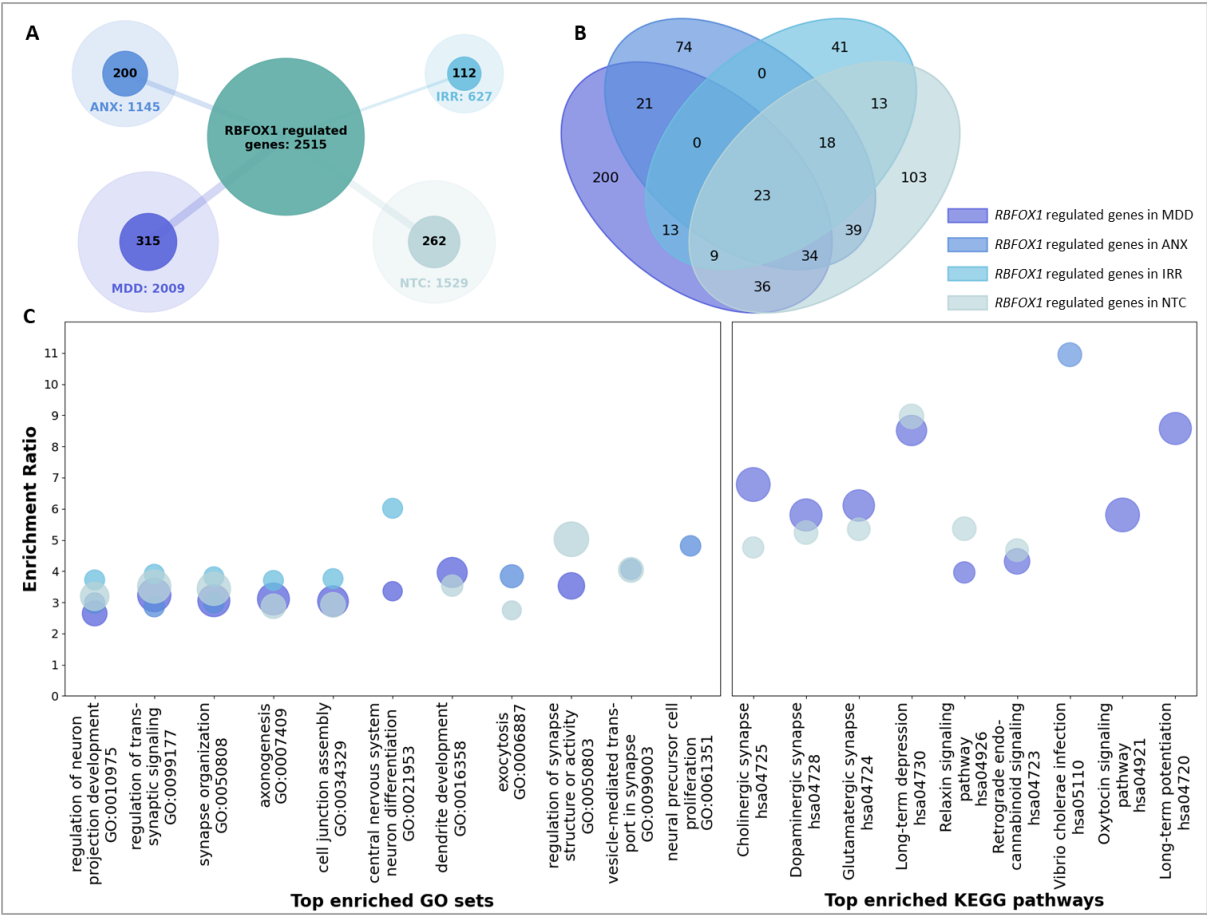


Figure 31: RBFOX1 and its regulated genes are associated with MDD, anxiety, irritability and neuroticism. A: Number of RBFOX1 regulated genes associated with the different phenotypes; B: Overlap of RBFOX1 regulated genes associated with MDD, anxiety, irritability, and neuroticism; C: Overlap of biological processes from GO and KEGG pathways enriched in RBFOX1 regulated genes associated with each phenotype. Marker size represents the $-\log_{10}$ of the FDR corrected value; MDD: major depressive disorder, ANX: anxiety, IRR: irritability, NTC: neuroticism

To analyse the connections between RBFOX1 regulated genes associated with MDD and other traits, as well as to confirm that these associations are not downstream consequences by one or a few of the Rbfox1-regulated genes we, constructed an undirected protein-protein interaction network (Figure 32). The resulting network containing 67 nodes (out of 137 input genes; Supplementary Table S21) and 98 edges is not tightly interconnected (network statistics in Figure 32; Supplementary Table S22). Among the 23 RBFOX1-regulated genes associated with all phenotypes, 13 are present in this network. One of these 13 genes, *CELF2*, is a direct neighbour of *RBFOX1*. Additionally, seven of these 13 genes, including *CADM2* and *TCF4*, are indirectly linked to *RBFOX1* through at least one intermediary node. *RBFOX1* ranks as one of the most central nodes in the network, as is reflected in all centrality measures (Supplementary Table S22). Notably, it is also among the most highly connected nodes, as reflected in the connectivity metrics (Supplementary Table S22). *RBFOX1* plays a bridging role in connecting the other nodes in this network, which are otherwise not closely interconnected, as evidenced by neighbourhood

connectivity measures (Supplementary Table S22). Furthermore, its importance in preserving the robustness of the network is highlighted by its role in facilitating alternative pathways, as indicated by stress measures (Supplementary Table S22).

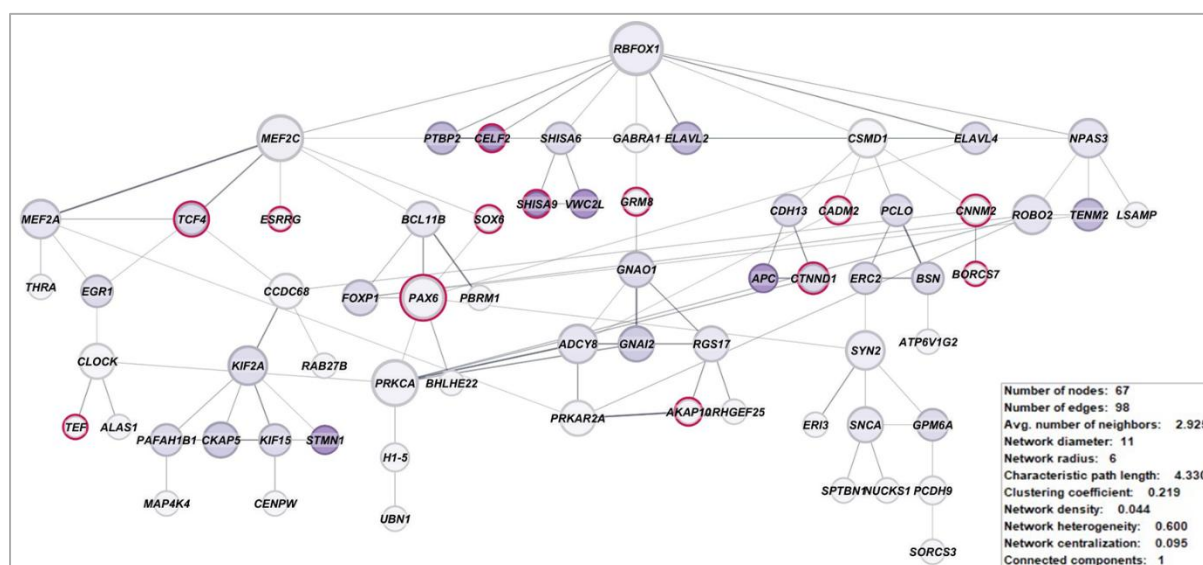


Figure 32: Network of RBFOX1-regulated genes associated with MDD and at least another trait. Circled in red are 13 genes that are associated with all phenotypes; Network created using Cytoscape stringApp. Node size $\propto$ number of undirected edges, node colour intensity $\propto$ clustering coefficient, edge weight $\propto$ string db score.

3.2 Predicted differential expression in brain tissues of RBFOX1-regulated genes in MDD and comorbid traits

We then estimated the expression of RBFOX1-regulated genes in brain tissues using MetaXcan and identified 118 genes predicted to be differentially expressed in MDD, 89 in anxiety, 61 in irritability and 97 in neuroticism (Supplementary Figure 1A). A total of 79 RBFOX1-regulated genes were shared across at least two phenotypes, and 18 of them were shared across at least three phenotypes. Approximately half of the genes predicted to be differentially expressed were also found associated with the same phenotype in the gene-based analyses (64 with MDD, 41 with anxiety, 24 with irritability and 52 with neuroticism).

Remarkably, four RBFOX1-regulated genes, *CTSB*, *NSF*, *SCAI*, and *SP4*, were predicted to be differentially expressed across all phenotypes. All four genes were also associated with at least three phenotypes in the gene-based analysis (Supplementary Figure 1B, Supplementary Table S11). Among them, *SP4* was predicted to be upregulated in the nucleus accumbens for all four phenotypes and was also associated with all of them in the gene-based analyses (Supplementary Tables S2-S6 and S7-S10). On the other hand, *CTSB* was predicted to be downregulated in the cerebellum for MDD but upregulated in the caudate basal ganglia, cortex, frontal cortex area BA9, nucleus accumbens, and putamen basal ganglia for anxiety and

irritability, as well as in the caudate basal ganglia, cortex, frontal cortex area BA9, and putamen basal ganglia for neuroticism (Supplementary Tables S7-S10). Additionally, S-MultiXcan analysis predicted upregulation of *CTSB* in multiple tissues in anxiety, irritability, and neuroticism (Supplementary Tables S7-S11). Furthermore, *CSTB* was associated with anxiety, irritability, and neuroticism in the gene-based analysis (Supplementary Tables S2-S6).

3.3 **RBFOX1 regulated genes in rare disorders with similar symptomology as selected phenotypes**

We analysed entries of rare disorders presenting with symptoms of depression, anxiety, irritability, and neuroticism, as catalogued in the OMIM database, and investigated whether *RBFOX1* and its regulated genes in the genes were present among the genes responsible for these disorders. OMIM entries with the symptoms “depression/depressive” were linked to 212 unique genes, “anxiety” was linked to 142 unique genes, and “irritability/irritable”, and “neuroticism/neurotic” to 157 and 4 genes, respectively (Supplementary Tables S23-S27).

RBFOX1-regulated genes were significantly enriched among the causal genes for rare disorders presenting with depression (41 out of 212 genes, $p = 3.15e-03$) or with anxiety (33 out of 142, $p = 3.09e-04$) (Supplementary Table S28). However, no significant enrichment was observed for irritability or neuroticism (20 out of 157 and 0 out of 4, respectively, $p > 0.05$). *RBFOX1* itself was not linked to any rare disorder presenting with the symptoms considered. In total, 74 *RBFOX1*-regulated genes related to at least one of the symptoms, including 18 genes linked to both depression and at least one additional symptom, such as *TCF4* (Figure 33). Also, *HTT* and *JPH3* were shared between anxiety, depression, and irritability, but none of them were common across all four symptoms.

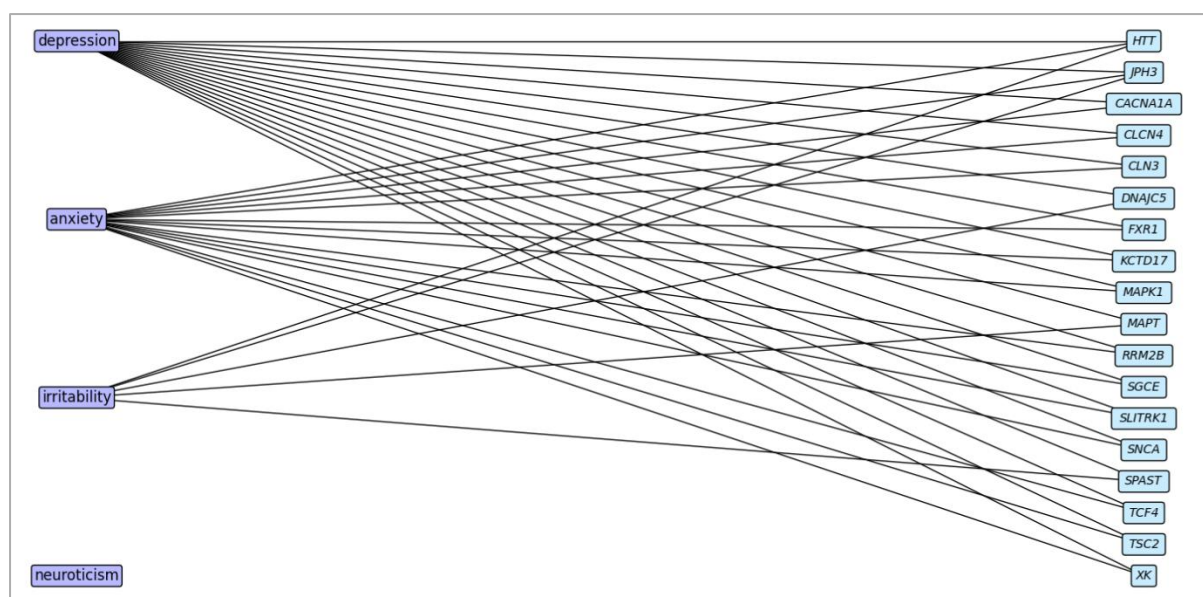


Figure 33: Subset of RBFOX1 regulated genes causal for rare disorders presenting with the symptom “depression” and at least another symptom. Entries with rare disorders presenting with this symptomology were found in the OMIM database, non-rare disorders were excluded from the analysis.

3.4 Top ranked RBFOX1-regulated genes

Based on the findings from our previous analyses of RBFOX1-regulated genes and their contributions to MDD, anxiety, irritability, and neuroticism (gene-based associations, predicted differential expression, and links to rare disorders presenting with these phenotypes), we ranked the 791 genes identified in at least one of these analyses. The ranking was weighted for MDD (see Methods and Supplementary Table S29). We highlight 19 RBFOX1-regulated genes, all of which are associated with at least three phenotypes, and nine are associated with all four phenotypes, including *CADM2*, *TCF4*, and *PAX6* (Figure 34, Table 2, Supplementary Table S29). Among these, *SP4*, *SCAI*, *CTSB*, and *NSF* were predicted to be differentially expressed in all phenotypes. Additionally, *MAPT*, *TCF4*, and *SNCA* were found in the OMIM database as connected to both depression and irritability, and *PAX6* was related to depression.

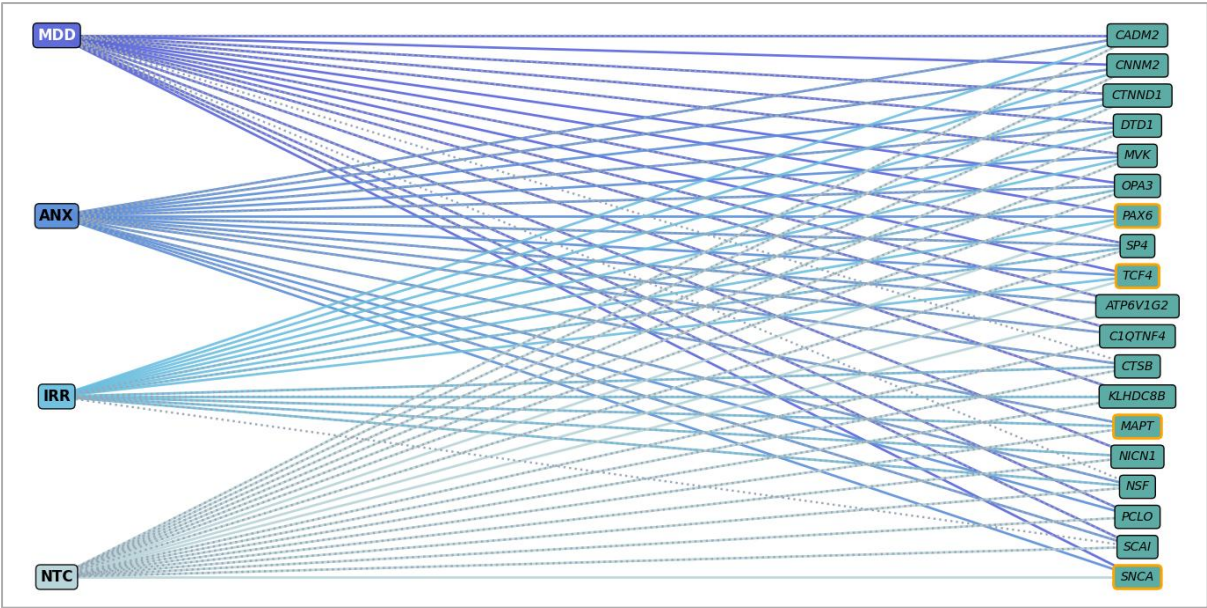


Figure 34: Top ranked RBFOX1 regulated genes. Associated genes from the gene-based analysis are marked by edges of the respective phenotype colours, predicted differential expression is marked by grey dashed edges between the phenotypes and associated genes, genes found to be responsible for rare disorders presenting with the selected symptoms in the OMIM database are marked by orange label borders of the respective genes; MDD: major depressive disorder, ANX: anxiety, IRR: irritability, NTC: neuroticism

Table 2: Top ranked RBFOX1 regulated genes contributing to MDD and comorbid traits

Gene symbol	Gene Name	MDD	Anxiety	Irritability	Neuroticism	Weighted Ranking*	Gene network
SP4	Sp4 transcription factor	Assoc, Expr	Assoc, Expr	Assoc, Expr	Assoc, Expr	10	
MAPT	microtubule associated protein tau	Rare dis	Assoc, Expr	Assoc, Expr, Rare dis	Assoc, Expr	9	
SCAI	suppressor of cancer cell invasion	Assoc, Expr	Assoc, Expr	Expr	Assoc, Expr	9	
DTD1	D-aminoacyl-tRNA deacylase 1	Assoc, Expr	Assoc, Expr	Assoc	Assoc, Expr	9	
CADM2	cell adhesion molecule 2	Assoc, Expr	Assoc, Expr	Assoc	Assoc, Expr	9	Yes
CTSB	cathepsin B	Expr	Assoc, Expr	Assoc, Expr	Assoc, Expr	8	
NSF	N-ethylmaleimide sensitive factor, vesicle fusing ATPase	Expr	Assoc, Expr	Assoc, Expr	Assoc, Expr	8	
OPA3	outer mitochondrial membrane lipid metabolism regulator OPA3	Assoc	Assoc, Expr	Assoc, Expr	Assoc, Expr	8	
NICN1	nicotin 1, tubulin polyglutamylase complex subunit	Assoc, Expr		Assoc, Expr	Assoc, Expr	8	
C1QTNF4	C1q and TNF related 4	Assoc, Expr	Assoc, Expr		Assoc, Expr	8	
KLHDC8B	kelch domain containing 8B	Assoc, Expr		Assoc, Expr	Assoc, Expr	8	
CTNND1	catenin delta 1	Assoc, Expr	Assoc	Assoc	Assoc, Expr	8	Yes
MVK	mevalonate kinase	Assoc, Expr	Assoc	Assoc	Assoc, Expr	8	
TCF4	transcription factor 4	Assoc, Rare dis	Assoc, Rare dis	Assoc	Assoc	8	Yes
CNNM2	cyclin and CBS domain divalent metal cation transport mediator 2	Assoc	Assoc, Expr	Assoc	Assoc, Expr	7	Yes
PCLO	piccolo presynaptic cytomatrix protein	Assoc, Expr	Assoc		Assoc, Expr	7	Yes
ATP6V1G2	ATPase H+ transporting V1 subunit G2	Assoc, Expr	Assoc, Expr		Assoc	7	Yes
SNCA	synuclein alpha	Assoc, Rare dis	Assoc, Rare dis		Assoc	7	Yes
PAX6	paired box 6	Assoc, Rare dis	Assoc	Assoc	Assoc	7	Yes

Assoc: associated in the gene base analyses; Expr: predicted differential expression in MetaXcan analyses; Rare dis: causal gene for a rare disorder presenting with symptomatology of the trait.

* Weight calculated as: $2 \times (\text{total occurrence in MDD gene lists}) + 1 \times (\text{total occurrence in anxiety gene lists}) + 1 \times (\text{total occurrence in irritability gene lists}) + 1 \times (\text{total occurrence in neuroticism gene lists})$.

4 DISCUSSION

In this study, we explored the contribution of *RBFOX1* regulatory network to MDD and comorbid traits, including anxiety, irritability, and neuroticism, and suicidal behaviour. We inspected a comprehensive list of genes regulated by *RBFOX1* (2,515 genes) and our results show a contribution of these genes to MDD, anxiety, irritability, and neuroticism. The *RBFOX1*-regulated genes associated with these phenotypes are mainly involved in neuron projection and synaptic processes across all conditions, which may, at least in part, explain the underlying mechanisms and high comorbidity between MDD and these traits. We highlight 19 genes as the most pleiotropic *RBFOX1*-regulated genes, identified through gene-based association analyses, predicted differential expression in brain, or links to rare disorders presenting with symptoms of depression, anxiety, irritability, or neuroticism.

RBFOX1 itself was found associated with MDD, anxiety, and neuroticism, and nominally associated with irritability and suicide attempts in our gene-based analyses. Additionally, we found that genes associated with MDD, anxiety, irritability, and neuroticism, but not suicide attempts, are enriched in *RBFOX1*-regulated genes. This provides novel insights into the link between irritability and *RBFOX1* through its regulated genes, a connection that, to our knowledge, has not been established before. The lack of a conclusive connection between suicide attempts and *RBFOX1* or its regulated genes may suggest that the pathways mediating suicidal behaviour are not primarily regulated by *RBFOX1*^{31,32}. However, it is important to note that the GWAS of suicide attempts used for this study might have limited power, with a sample size of 29,782 suicide attempt cases and 519,961 controls, and only 200 genes associated at 5% FDR in the gene-based analysis. Thus, further studies with larger sample sizes are needed to clarify the potential role of *RBFOX1* and its regulated network in suicidal behaviour. Given this consideration, we focused our study on MDD and the other three comorbid traits with well-established connections to *RBFOX1*.

RBFOX1-regulated genes associated with MDD, anxiety, irritability, and neuroticism showed considerable overlap, with 23 genes associated with all four phenotypes. Notably, six transcription factors, namely *CAMTA1*, *PAX6*, *SOX6*, *SP4*, *TCF4*, and *TEF*, were among the shared genes, along with *CELF2*, which is involved in pre-mRNA alternative splicing. This suggests that *RBFOX1* influences large gene subnetworks beyond its directly regulated genes. This emphasizes the role of *RBFOX1* as an overarching regulatory element with far-reaching direct and indirect influences on gene splicing and expression. Our findings contribute to the growing body of research highlighting the role of *RBFOX1* in the shared genetic architecture of MDD and anxiety, as well as the bidirectional causal relationship of MDD and neuroticism, in addition to the well-established connection to MDD^{2-4,16,17,25,35,36}.

The significant overlap of *RBFOX1*-regulated genes found associated across the investigated phenotypes suggests that they are partially caused by shared biological processes and pathways regulated by *RBFOX1*. This is further supported by our gene-set enrichment analyses (GO and KEGG), which revealed that pathways related to neuronal projection and synaptic processes are implicated both in MDD and its comorbid traits. Additionally, MDD and neuroticism share enrichment in pathways related to neurotransmitter systems, including cholinergic, dopaminergic, and glutamatergic synapses, as well as oxytocin and endocannabinoid signalling. This aligns with previous studies demonstrating that *RBFOX1* plays an important role in neuronal development, neuronal maturation and excitability, the regulation of cell proliferation, as well as cytoskeletal organization, cell adhesion, and synaptic functions^{40,42}.

Previously reported *RBFOX1*-regulated genes include ion channels, neurotransmitter receptors, and cell adhesion molecules, emphasizing its central role in neurogenesis and neuronal function⁴. Disturbances in the neurodevelopment, which are often influenced by environmental risk factors, are linked to the development of psychiatric disorders, with early life stressors being among the most significant non-genetic risk factors for depression^{62,63}. Our findings suggest that *RBFOX1* might contribute to depression by affecting neurodevelopmental processes. During brain development, anatomical and functional changes are correlated, which is well exemplified by the role of monoaminergic signalling in depression⁶². We found that *RBFOX1*-regulated genes in MDD are enriched in dopaminergic and serotonergic synapses, two neurotransmitter systems known to influence neurodevelopment and to contribute to MDD and other psychiatric disorders^{1,62,64}. Interestingly, the glutamatergic and GABAergic synapse were also enriched in the *RBFOX1*-regulated genes associated with MDD, as well as long-term potentiation and depression, all of which have been related to MDD before¹. Dysregulation of the glutamatergic and GABAergic systems have also been implicated in MDD through multiple studies, and drugs targeting these systems are gaining prominence in the treatment of treatment-resistant depression^{1,64–66}. Altogether, this findings underscore the role of *RBFOX1* and its regulated genes in synaptic signalling and organization, highlighting how perturbations in *RBFOX1*-mediated regulation *RBFOX1* can impact neurotransmission and neuroplasticity, ultimately contributing to MDD and its comorbid traits. To further understand the role of *RBFOX1*-regulated genes in the comorbidity between MDD and other phenotypes, we constructed a protein-protein interaction network consisting of *RBFOX1* and its regulated genes associated with MDD and at least one other trait. The resulting network contained 67 nodes, with 13 genes associated with MDD and all three phenotypes. Interestingly, *RBFOX1*-regulated genes in this network are not tightly interconnected, suggesting that they participate in diverse biological functions and processes. The moderate network heterogeneity implies that some *RBFOX1*-regulated genes may be more highly regulated or influenced by *RBFOX1* than others. *RBFOX1* is one of the most central and

well-connected nodes in this network, playing a crucial role in maintaining network robustness and connecting different parts of it. This indicates that *RBFOX1* plays a broad regulatory role across a wide range of transcripts, taking a hub-like role in integrating and coordinating different cellular functions related to mRNA processing and stability. These findings furthermore confirm that the observed effects are not being driven by one, or even a few, of the *Rbfox1*-regulated genes. As we used the default confidence score of 0.4 for predicted interactions, not all nodes are connected to *RBFOX1*, which could be due to several reasons. On one hand, the *RBFOX1*-regulated genes identified in the study might not all directly interact with *RBFOX1* in a physical sense, or their interactions might not be included in the STRING database.

On the other hand, *RBFOX1*-regulated genes were originally identified in mice, and some of the human orthologs might not share the exact same functional context or direct interactions. Also, lowering the confidence score cut-off would lead to a network that includes more connections between *RBFOX1* and the other nodes; however, here we focused on interactions with a higher confidence score to ensure the validity of our analysis.

In our study, several *RBFOX1* regulated genes were predicted to be differentially expressed in MDD and related traits. In MDD, 118 genes were identified, aligning with findings from a previous TWAS of MDD, in which 72 of these genes were also predicted to be differentially expressed⁶⁷. Among the 18 genes shared between MDD and at least two other traits, *KLHDC8B* and *NICN1* ranked highly in our study. The *KLHDC8B* gene was predicted to be upregulated in the cerebellum in our analyses of MDD, irritability, and neuroticism, and in spinal cord in the MDD TWAS study⁶⁷. *NICN1* was upregulated in the cortex, frontal cortex, and nucleus accumbens in MDD and irritability, and in the cortex in neuroticism in our study, but downregulated in hypothalamus in the TWAS of MDD. Another top-ranked gene, *PAX6*, was associated with MDD and all comorbid traits but not identified in MetaXcan analyses. However, it was found to be upregulated in caudate nucleus in the TWAS of MDD⁶⁷. The four genes, *CTSB*, *NSF*, *SCAI*, and *SP4*, that overlapped between MDD and all traits in our study, were not identified in the MDD TWAS.

Our analysis of rare disorders presenting with symptoms of depression, anxiety, irritability, or neuroticism revealed enrichment of *RBFOX1*-regulated genes in depression and anxiety. Many of these genes were also implicated in MDD and related traits in previous analyses, and in the overlap between them. Our approach linking rare genetic disorders to complex psychiatric phenotypes has previously been applied in studies of ADHD, supporting the relevance of Mendelian disorder-associated genes in the broader genetic architecture of psychiatric conditions⁶⁸.

In summary, integrating gene-based associations with the different phenotypes, prediction of differential expression, and links to rare diseases in the OMIM database, we identified 19

RBFOX1-regulated genes as key contributors to the shared genetic basis of MDD, anxiety, irritability, and neuroticism. Among these, we highlight the transcription factors *SP4*, *TCF4*, and *PAX6*. *SP4* has been associated with MDD, and its activity is repressed in a murine chronic stress model, leading to increased 5-HT1A expression^{69,70}. Similarly, *TCF4* is associated with MDD, schizophrenia and insomnia, and, together with other genes including *RBFOX1*, with the causal link between MDD and neuroticism^{17,71,72}. RBFOX1 and TCF4 have recently been proposed as potential biomarkers for MDD, with *RBFOX1* potentially involved in neuroinflammation⁴. In our study, we found a link between *RBFOX1* and inflammation in anxiety, but not in depression. Interestingly, *PAX6* is also linked to depression through neuroinflammation, as decreased Pax6 levels were found to be associated with increased neuroinflammation in a murine stress model of depression⁷³.

Since our study is based on results from orthologs identified in experiments conducted in mice and cell cultures, future studies should aim to replicate these findings in RBFOX1-regulated genes found through human transcriptomic analyses^{41,45}. Another important direction for future research is to explore potential sex differences in the effects of the *RBFOX1* regulatory network. This is particularly relevant as MDD is twice as prevalent in women compared to men, and there are reports suggesting the existence of sex-dependent etiological pathways underlying MDD^{1,74,75}. Our study highlights a main role of *RBFOX1* and its regulated gene network in MDD, anxiety, irritability and neuroticism, by influencing neurodevelopmental and synaptic processes. Furthermore, it provides novel insights into the shared genetic basis of MDD and its comorbid traits.

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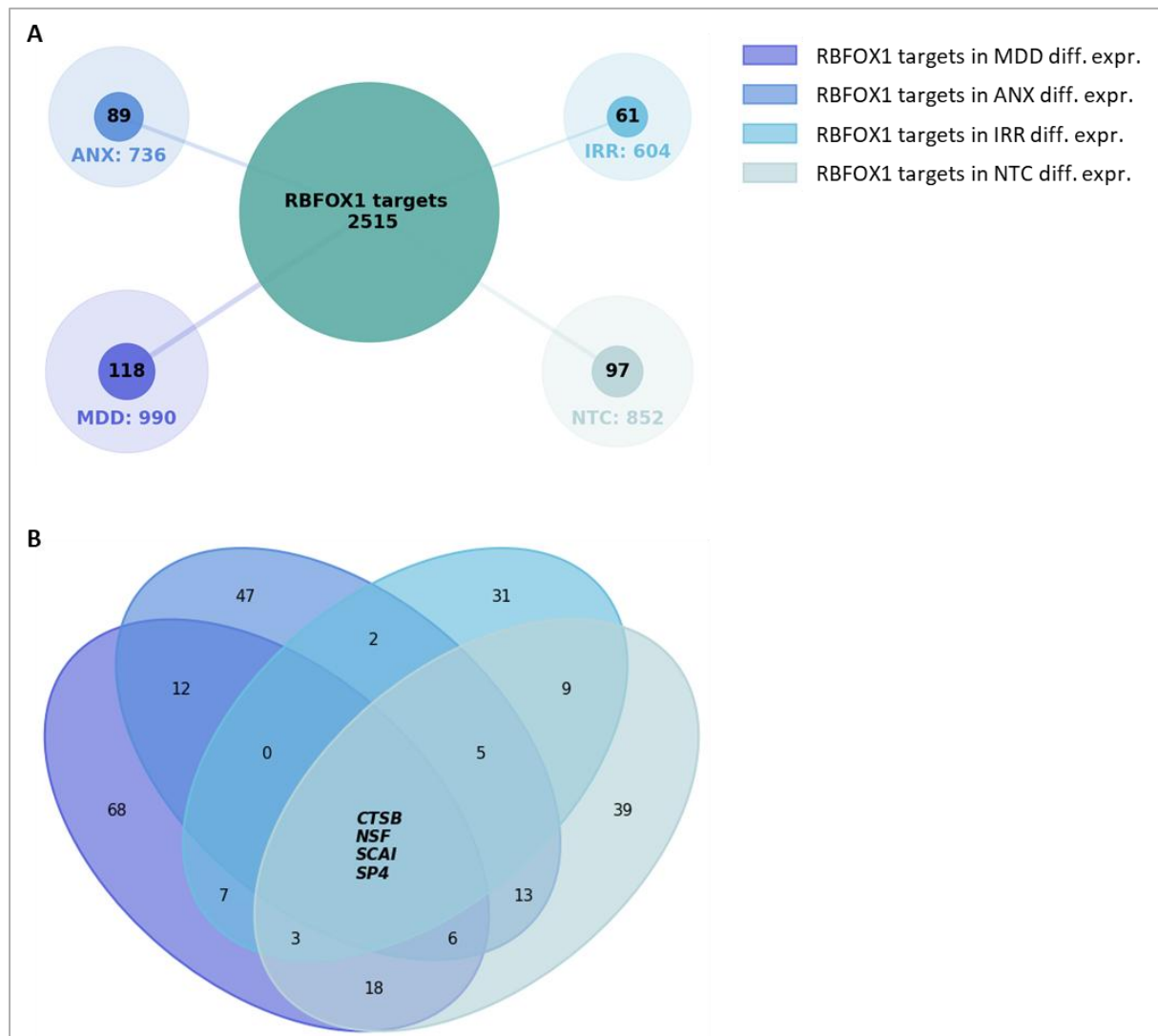
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6 SUPPLEMENTARY MATERIAL

6.1 Supplementary Figures



Supplementary Figure 1: RBFOX1 regulated genes predicted to be differentially expressed in MDD, anxiety, irritability and neuroticism. A: Number of RBFOX1 regulated genes predicted to be differentially expressed in each phenotype using S-PrediXcan and S-MultiXcan. B: Overlap of RBFOX1 regulated genes predicted to be differentially expressed (diff. expr.) in each phenotype; MDD: major depressive disorder, ANX: anxiety, IRR: irritability, NTC: neuroticism

6.2 Supplementary Tables

The following supplementary tables in **bold** were included as summaries of the results in this thesis.

Supplementary Table 1: Human homologs of RBFOX1 regulated genes identified in previous studies by Lee et al. (2016) and Wamsely et al. (2018)

Supplementary Table 2: Results from gene-based association study on major depressive disorder

Supplementary Table 3: Results from gene-based association study on anxiety: worrier/anxious feelings

Supplementary Table 4: Results from gene-based association study on irritability

Supplementary Table S5: Results from gene-based association study of neuroticism score

Supplementary Table S6: Results from gene-based association study on suicide attempt

Supplementary Table S7: Predicted differentially expressed genes for major depressive disorder

Supplementary Table S8: Predicted differentially expressed genes for anxiety: worrier/anxious feelings

Supplementary Table S9: Predicted differentially expressed genes for irritability

Supplementary Table S10: Predicted differentially expressed genes for neuroticism score

Supplementary Table S11: Summary of predicted differentially expressed genes for MDD, anxiety, irritability and neuroticism

Supplementary Table S12: Enrichment of RBFOX1 regulated genes in genes associated with MDD, ANX, SCD, IRR, and NTC

Supplementary Table S13: Biological Processes from GO enriched in RBFOX1 regulated genes associated with major depressive disorder

Supplementary Table S14: Biological Processes from GO enriched in RBFOX1 regulated genes associated with anxiety

Supplementary Table S15: Biological Processes from GO enriched in RBFOX1 regulated genes associated with irritability

Supplementary Table S16: Biological Processes from GO enriched in RBFOX1 regulated genes associated with neuroticism

Supplementary Table S17: Enriched KEGG pathways in RBFOX1 regulated genes associated with major depressive disorder

Supplementary Table S18: Enriched KEGG pathways in RBFOX1 regulated genes associated with anxiety

Supplementary Table S19: Enriched KEGG pathways in RBFOX1 regulated genes associated with neuroticism

Supplementary Table S20: Overlap of GO and KEGG pathways identified in RBFOX1 regulated genes associated with MDD, ANX, SCD, IRR, and NTC

Supplementary Table S21: RBFOX1 regulated genes associated with MDD and at least another trait used for network construction with Cytoscape STRING

Supplementary Table S22: Results from network analysis of Cytoscape STRING network with RBFOX1 regulated genes associated with MDD and at least another trait

Supplementary Table S23: Genes found in entries with the symptom "depression" in the OMIM database

Supplementary Table S24: Genes found in entries with the symptom "anxiety" in the OMIM database

Supplementary Table S25: Genes found in entries with the symptom "irritability" in the OMIM database

Supplementary Table S26: Genes found in entries with the symptom "neuroticism" in the OMIM database

Supplementary Table S27: Summary of genes found to be linked to rare disorders presenting with symptoms of depression, anxiety, irritability, or neuroticism in the OMIM database

Supplementary Table S28: Enrichment of RBFOX1 regulated genes in OMIM genes related to depression, anxiety, irritability, and neuroticism

Supplementary Table 29: Summary of results obtained for all analyses and rank of RBFOX1 regulated genes

Supplementary Table 11: Summary of predicted differentially expressed genes for MDD, anxiety, irritability and neuroticism

RBFox1 regulated genes		MDD		Anxiety		Irritability		Neuroticism	
Entrez ID	Gene Symbol	DE all	DE z-score	DE all	DE z-score	DE all	DE z-score	DE all	DE z-score
1508	CTSB	y		y	3.02	y	3.12	y	2.02
4905	NSF	y		y		y		y	-4.63
286205	SCAI	y	2.88	y		y		y	2.66
6671	SP4	y		y		y		y	
468	ATF4	y		y				y	-3.72
114900	C1QTNF4	y		y				y	
253559	CADM2	y		y				y	
92675	DTD1	y		y				y	
10211	FLOT1	y		y				y	
200942	KLHDC8B	y	4.68			y	6.05	y	
84276	NICN1	y				y		y	
10180	RBM6	y		y				y	
80351	TNKS2	y				y		y	
1394	CRHR1			y	-1.57	y	-2.11	y	-3.50
81631	MAP1LC3B			y		y		y	
4137	MAPT			y		y	0.35	y	-0.32
80207	OPA3			y		y		y	
9726	ZNF646			y		y		y	
53	ACP2	y						y	
60312	AFAP1	y						y	
534	ATP6V1G2	y		y					
10645	CAMKK2	y						y	
1010	CDH12	y		y					
1500	CTNND1	y						y	
2060	EPS15	y				y			
57488	ESYT2	y						y	
388650	FAM69A	y						y	
2781	GNAZ	y						y	
348980	HCN1	y						y	
51440	HPCAL4	y						y	
3400	ID4	y		y					
3895	KTN1	y						y	
9113	LATS1	y				y			
4598	MVK	y						y	
28512	NKIRAS1	y				y			
27020	NPTN	y						y	
10562	OLFM4	y		y					
51633	OTUD6B	y				y			
164091	PAQR7	y		y					
27445	PCLO	y						y	
8396	PIP4K2B	y		y					

23126	POGZ	y	y		
5576	PRKAR2A	y		y	
58155	PTBP2	y	y		
54870	QRICH1	y		y	
5898	RALA	y			y
9522	SCAMP1	y	y		
205564	SENP5	y			y
8604	SLC25A12	y			y
254428	SLC41A1	y			y
23315	SLC9A8	y	y		
646424	SPINK8	y		y	
81603	TRIM8	y	y		
51699	VPS29	y	y		
340481	ZDHHC21	y			y
114800	CCDC85A		y	y	
54805	CNNM2		y	-4.34	y
9337	CNOT8		y		y
81545	FBXO38		y		y 4.21
10989	IMMT		y	y	
83892	KCTD10		y		y
9856	KIAA0319		y		y
84542	KIAA1841		y		y
9480	ONECUT2		y		y
56098	PCDHGC4		y		y
79834	PEAK1		y		y
5711	PSMD5		y		y
6176	RPLP1		y		y
23428	SLC7A8		y		y -4.44
399979	SNX19		y		y
57463	AMIGO1			y	y
146433	IL34			y	y
84456	L3MBTL3			y	y
286343	LURAP1L			y	y
10873	ME3			y	y
4325	MMP16			y	y
5364	PLXNB1			y	y
5500	PPP1CB			y	y
55206	SBNO1			y	y
10152	ABI2	y			
104	ADARB1	y			
84920	ALG10	y			
155185	AMZ1	y			
490	ATP2B1	y			
94032	CAMK2N2	y			
157922	CAMSAP1	y			
203238	CCDC171	y			

1000	CDH2	y	
55743	CHFR	y	
5067	CNTN3	y	
64506	CPEB1	y	
9419	CRIP1	y	
1434	CSE1L	y	
8722	CTSF	y	
9420	CYP7B1	y	-0.58
7458	EIF4H	y	
2009	EML1	y	
27436	EML4	y	
26122	EPC2	y	
2048	EPHB2	y	
84253	GARNL3	y	
9453	GGPS1	y	
84662	GLIS2	y	
2849	GPR26	y	
23438	HARS2	y	
9454	HOMER3	y	
11077	HSF2BP	y	
85442	KNDC1	y	
55323	LARP6	y	
80774	LIMD2	y	
54839	LRRC49	y	
7798	LUZP1	y	
4131	MAP1B	y	
4208	MEF2C	y	
56947	MFF	y	
84879	MFSD2A	y	
25834	MGAT4C	y	
65009	NDRG4	y	
4828	NMB	y	
64710	NUCKS1	y	
56666	PANX2	y	
5136	PDE1A	y	
5037	PEBP1	y	
54858	PGPEP1	y	
55012	PPP2R3C	y	
5052	PRDX1	y	
112464	PRKCDBP	y	
112476	PRRT2	y	
9991	PTBP3	y	
5874	RAB27B	y	
10156	RASA4	y	
65055	REEP1	y	
55288	RHOT1	y	

389432	SAMD5	y	
79634	SCRN3	y	
84251	SGIP1	y	
64089	SNX16	y	
6752	SSTR2	y	
134429	STARD4	y	
9399	STOML1	y	
29966	STRN3	y	
6804	STX1A	y	
23336	SYNM	y	
84640	USP38	y	
64856	VWA1	y	
285525	YIPF7	y	
153527	ZMAT2	y	
88	ACTN2		y
3267	AGFG1		y
282679	AQP11		y
155066	ATP6V0E2		y
56970	ATXN7L3		y
8927	BSN		y
283152	CCDC153		y
5218	CDK14		y
56171	DNAH7		y
22979	EFR3B		y
84624	FNDC1		y
8790	FPGT		y
2585	GALK2		y
10014	HDAC5		y
55364	IMPACT		y
9371	KIF3B		y
3909	LAMA3		y
10162	LPCAT3		y
145748	LYSMD4		y
5595	MAPK3		y
79772	MCTP1		y
51642	MRPL48		y
25961	NUDT13		y
5108	PCM1		y
9601	PDIA4		y
23089	PEG10		y
5179	PENK		y
23262	PPIP5K2		y
56341	PRMT8		y
54899	PXK		y
26575	RGS17		y
6048	RNF5		y

6342	SCP2	y	
23513	SCRIB	y	
12	SERPINA3	y	
54847	SIDT1	y	
23094	SIPA1L3	y	
57181	SLC39A10	y	
6711	SPTBN1	y	
342667	STAC2	y	
6863	TAC1	y	
85461	TANC1	y	
26115	TANC2	y	
9878	TOX4	y	
9618	TRAF4	y	
493829	TRIM72	y	
83719	YPEL3	y	-3.79
10058	ABCB6		y
100	ADA		y
107	ADCY1		y
122416	ANKRD9		y
374569	ASPG		y
127703	C1orf216		y
10153	CEBPZ		y
1767	DNAH5		y
100132074	FOXO6		y
170384	FUT11		y
2563	GABRD		y
2587	GALR1		y
3038	HAS3		y
11014	KDELR2		y
11275	KLHL2		y
29967	LRP12		y
130574	LYPD6		y
8972	MGAM		y
25821	MTO1		y
4814	NINJ1		y
115209	OMA1		y
221692	PHACTR1		y
5339	PLEC		y
25913	POT1		y
5798	PTPRN		y
11159	RABL2A		y
389075	RESP18		y
79641	ROGDI		y
6691	SPINK2		y
6764	ST5		y
29091	STXBP6		y

54806	AHI1	y
200539	ANKRD23	y
94134	ARHGAP12	y
9254	CACNA2D2	y
1281	COL3A1	y
1314	COPA	y
11316	COPE	y
79877	DCAKD	y
63951	DMRTA1	y
26052	DNM3	y
55862	ECHDC1	y
2118	ETV4	y
23401	FRAT2	y
79068	FTO	y
3760	KCNJ3	y
56992	KIF15	y
10314	LANCL1	y
55915	LANCL2	y
84894	LINGO1	y
5602	MAPK10	y
5601	MAPK9	y
8685	MARCO	y
90523	MLIP	y
54892	NCAPG2	y
79954	NOL10	y
29763	PACSIN3	y
23207	PLEKHM2	y
5368	PNOC	y
5433	POLR2D	y
5447	POR	y
23362	PSD3	y
255426	RASGEF1C	y
57758	SCUBE2	y
80031	SEMA6D	y
55512	SMPD3	y
23321	TRIM2	y
7280	TUBB2A	y
23032	USP33	y
55759	WDR12	y

**DE all genes predicted to be differentially expressed in any brain tissue, DE z-score Smultixcan z-score.*

Supplementary Table 22: Results from network analysis of Cytoscape STRING network with RBFOX1 regulated genes associated with MDD and at least another trait

query term	display name	Centrality measures				Connectivity measures		Neighbourhood connectivity measures				Stress
		AverageShortestPathLength	ClosenessCentrality	Radiality	Eccentricity	Degree	NumberOfUndirectedEdges	BetweennessCentrality	ClusteringCoefficient	TopologicalCoefficient	NeighborhoodConnectivity	
PAX6	PAX6	3.121212121	0.32038835	0.764309764	7	7	7	0.278003108	0.047619048	0.176470588	3.714285714	2122
RBFOX1	RBFOX1	3.121212121	0.32038835	0.764309764	6	9	9	0.249189329	0.111111111	0.151515152	3.666666667	1672
SYN2	SYN2	3.727272727	0.268292683	0.696969697	8	5	5	0.224498834	0.1	0.230769231	3.6	1656
PRKCA	PRKCA	3.227272727	0.309859155	0.752525253	7	7	7	0.228451178	0.047619048	0.178571429	4.285714286	1650
MEF2C	MEF2C	3.090909091	0.323529412	0.767676768	6	7	7	0.202087542	0.095238095	0.183673469	4.285714286	1598
CDC68	CDC68	3.848484848	0.25984252	0.683501684	8	4	4	0.231693862	0	0.25	3.25	1554
CSMD1	CSMD1	3.181818182	0.314285714	0.757575758	6	5	5	0.198795649	0	0.2	4	1252
TCF4	TCF4	3.424242424	0.292035398	0.730639731	7	4	4	0.172991453	0.333333333	0.321428571	4.75	1208
KIF2A	KIF2A	4.651515152	0.214983713	0.594276094	9	5	5	0.16969697	0.3	0.333333333	3.2	1132
CNNM2	CNNM2	3.727272727	0.268292683	0.696969697	7	3	3	0.107964258	0	0.333333333	3.333333333	726
ROBO2	ROBO2	3.439393939	0.290748899	0.728956229	7	5	5	0.055716136	0.2	0.28	4.6	610
ADCY8	ADCY8	3.5	0.285714286	0.722222222	7	5	5	0.063970474	0.2	0.292307692	4.2	582
CLOCK	CLOCK	3.696969697	0.270491803	0.7003367	6	4	4	0.088601399	0	0.25	3	576
NPAS3	NPAS3	3.787878789	0.295964126	0.735690236	7	5	5	0.068106708	0.2	0.252631579	5	570
BCL11B	BCL11B	3.333333333	0.3	0.740740741	6	4	4	0.068614349	0.166666667	0.303571429	4.75	560
MEF2A	MEF2A	3.439393939	0.290748899	0.728956229	7	5	5	0.081476301	0.2	0.261538462	3.8	560
PRKAR2A	PRKAR2A	3.666666667	0.272727273	0.703703704	7	4	4	0.064107744	0	0.270833333	4.25	510
PCLO	PCLO	3.787878788	0.264	0.69023569	6	3	3	0.076899767	0.333333333	0.416666667	3.666666667	490
SNCA	SNCA	4.606060606	0.217105263	0.599326599	9	4	4	0.06013986	0.166666667	0.333333333	2.5	438
GPM6A	GPM6A	4.606060606	0.217105263	0.599326599	9	3	3	0.05967366	0.333333333	0.416666667	3.666666667	436
ELAVL4	ELAVL4	3.424242424	0.292035398	0.730639731	7	3	3	0.058124838	0.333333333	0.4	6.333333333	418
SHISA6	SHISA6	4.045454545	0.247191011	0.661616162	7	3	3	0.05967366	0.333333333	0.4	4.333333333	376
ERC2	ERC2	4.03030303	0.248120301	0.663299663	7	3	3	0.054390054	0.333333333	0.416666667	3.666666667	376
SOX6	SOX6	3.409090909	0.293333333	0.732323232	6	2	2	0.030730381	0	0.545454545	7	350
GNAI2	GNAI2	3.893939394	0.256809339	0.678451178	8	4	4	0.042900803	0.5	0.357142857	5	328
EGR1	EGR1	3.803030303	0.262948207	0.688552189	7	3	3	0.045431235	0.333333333	0.444444444	4.333333333	308
CADM2	CADM2	3.651515152	0.273858921	0.705387205	7	2	2	0.031849262	0	0.5	5	240
GNAO1	GNAO1	4.227272727	0.23655914	0.641414141	8	4	4	0.025107485	0.333333333	0.388888889	3.75	220
PCDH9	PCDH9	5.560606061	0.179836512	0.493265993	10	2	2	0.03030303	0	0.5	2	220
RGSI7	RGSI7	4.454545455	0.224489796	0.616161616	8	4	4	0.034747475	0.166666667	0.375	2.75	216
FOXP1	FOXP1	3.681818182	0.271604938	0.702020202	7	4	4	0.018251748	0.333333333	0.365384615	4.75	204
PAFAH1B1	PAFAH1B1	5.590909091	0.178861789	0.48989899	10	3	3	0.03030303	0.333333333	0.6	3.333333333	202

KIF15	KIF15	5.590909091	0.178861789	0.48989899	10	3	3	0.03030303	0.333333333	0.6	3.333333333	202
CTNND1	CTNND1	3.757575758	0.266129032	0.693602694	7	3	3	0.03010878	0.333333333	0.407407407	4	200
GABRA1	GABRA1	3.924242424	0.254826255	0.675084175	7	2	2	0.029391349	0	0.5	5.5	190
HIST1H1B	H1-5	4.181818182	0.239130435	0.646464646	8	2	2	0.03030303	0	0.5	4	188
CDH13	CDH13	3.803030303	0.262948207	0.688552189	7	3	3	0.028904429	0.333333333	0.428571429	3.333333333	160
BSN	BSN	4.424242424	0.226027397	0.61952862	7	3	3	0.03030303	0.333333333	0.5	2.333333333	156
GRM8	GRM8	4.484848485	0.222972973	0.612794613	8	2	2	0.014972805	0	0.5	3	102
AKAP10	AKAP10	4.53030303	0.220735786	0.607744108	8	2	2	0.016627817	0	0.5	4	84
ODZ2	TENM2	3.742424242	0.267206478	0.695286195	7	3	3	0.003167573	0.666666667	0.466666667	4.666666667	66
ELAVL2	ELAVL2	3.803030303	0.262948207	0.688552189	7	3	3	0.003496503	0.666666667	0.5	5	38
CKAP5	CKAP5	5.560606061	0.179836512	0.493265993	10	4	4	0.001864802	0.5	0.464285714	3.25	16
PTBP2	PTBP2	4.075757576	0.24535316	0.658249158	7	3	3	2.33E-04	0.666666667	0.518518519	4.666666667	2
ESRRG	ESRRG	4.075757576	0.24535316	0.658249158	7	1	1	0	0	0	7	0
BHLHE22	BHLHE22	4.106060606	0.243542435	0.654882155	8	1	1	0	0	0	7	0
CELF2	CELF2	4.090909091	0.244444444	0.656565657	7	2	2	0	1	0.666666667	6	0
LSAMP	LSAMP	4.363636364	0.229166667	0.626262626	8	1	1	0	0	0	5	0
THRA	THRA	4.424242424	0.226027397	0.61952862	8	1	1	0	0	0	5	0
ER13	ER13	4.712121212	0.21221865	0.587542088	9	1	1	0	0	0	5	0
STMN1	STMN1	5.621212121	0.177897574	0.486531987	10	2	2	0	1	0.9	4.5	0
PBRM1	PBRM1	4.318181818	0.231578947	0.631313131	7	1	1	0	0	0	4	0
TEF	TEF	4.681818182	0.213592233	0.590909091	7	1	1	0	0	0	4	0
ALAS1	ALAS1	4.681818182	0.213592233	0.590909091	7	1	1	0	0	0	4	0
RAB27B	RAB27B	4.833333333	0.206896552	0.574074074	9	1	1	0	0	0	4	0
ARHGEF25	ARHGEF25	5.439393939	0.183844011	0.506734007	9	1	1	0	0	0	4	0
SPTBN1	SPTBN1	5.590909091	0.178861789	0.48989899	10	1	1	0	0	0	4	0
NUCKS1	NUCKS1	5.590909091	0.178861789	0.48989899	10	1	1	0	0	0	4	0
APC	APC	4.318181818	0.231578947	0.631313131	7	2	2	0	1	0.75	3	0
C10orf32	BORCS7	4.712121212	0.21221865	0.587542088	8	1	1	0	0	0	3	0
ATP6V1G2	ATP6V1G2	5.409090909	0.18487395	0.51010101	8	1	1	0	0	0	3	0
CENPW	CENPW	6.575757576	0.152073733	0.38047138	11	1	1	0	0	0	3	0
MAP4K4	MAP4K4	6.575757576	0.152073733	0.38047138	11	1	1	0	0	0	3	0
SHISA9	SHISA9	5.015151515	0.19939577	0.553872054	8	2	2	0	1	0.833333333	2.5	0
VWC2L	VWC2L	5.015151515	0.19939577	0.553872054	8	2	2	0	1	0.833333333	2.5	0
UBN1	UBN1	5.166666667	0.193548387	0.537037037	9	1	1	0	0	0	2	0
SORCS3	SORCS3	6.545454545	0.152777778	0.383838384	11	1	1	0	0	0	2	0

Supplementary Table 27: Summary of genes found to be linked to rare disorders presenting with symptoms of depression, anxiety, irritability, or neuroticism in the OMIM database

depression	anxiety	irritability	neuroticism	depression	anxiety	irritability	neuroticism
DCTN1	PTPN22	ST3GAL5	SERPING1	IFT140	SATB2	DNAJC5	
TWNK	FCGR2A	IBA57	MPZ	MYO7A	SMAD4	D2HGDH	
TOR1A	FCGR2B	DHCR7	PARK7	USH2A	UGT1A1	TRMU	
CACNA1A	CTLA4	OTC	NRXN1	PDZD7	SPECC1L	HBB	
SGCE	TREX1	SLC19A3		CLRN1	THRB	AMACR	
POLG	DNASE1	DDC		IL1RAPL1	HMBS	TMEM237	
STUB1	SGCE	HTT		RNU12	ANKRD11	TPK1	
PAH	FMR1	SAMHD1		VPS13A	SDHD	EARS2	
COLEC11	PQBP1	FOXG1		GFAP	SNCA	PNPT1	
SLC20A2	STUB1	NDUFS1		CLN3	FMO3	TOE1	
CSF1R	HTT	NDUFAF4		CLN6	ACO2	SNX10	
HTT	WFS1	NHLRC2		PHOX2B	NFIX	ATP5F1A	
C19orf12	MSTO1	PPT1		MCCC2	GATAD2B	SLC1A2	
ELP4	PHIP	ACOX1		HLA-DQA1	TTC19	MRPS34	
DNMT1	TET3	ARG1		GNAS	PDGFB	STUB1	
MASP1	PAH	SLC25A20		ELP1	ELP4	NDUFA2	
FGF14	CLCN4	TREX1		FXN	DENND5A	NDUFAF5	
TBP	nan	ASAH1		ZEB2	YY1	FOXRED1	
FMR1	USP27X	ALPL		CBS	TCF20	DEGS1	
ATP1A3	ZFX	GLDC		XYLT2	DEAF1	LYRM7	
PRNP	MECP2	PSEN2		PINK1	ADNP	NALCN	
GBA1	ATP1A3	AVPR2		LRRK2	RNF125	SQSTM1	
ADH1C	CACNA1A	NSDHL		MAOA	SOX5	IFNAR2	
ATXN2	PRRT2	PSAT1		BTK	FXR1	CNP	
ATXN8OS	IFNG	SUCLA2		XK	SCN2A	YIF1B	
ATXN3	TSC2	GALC		GLA	CDH2	SLC25A46	
MAPT	LARP7	PTH		TAFAZZIN	RIMS2	COX16	
PSEN1	PPM1D	DAGLA		EBP	CNOT1	AARS1	
WFS1	TLK2	STXBP2		FGD1	PRKACB	RBL2	
AMACR	KPTN	SLC6A3		F8	EIF5A	ABHD16A	
CSNK1D	SETD2	COG4		PHEX	EDEM3	FBP2	
PDGFB	SLC7A6OS	FADD		OFD1	CDC42BPB	ASNS	
nan	ADCY5	SEPSECS		KCND3	SCN11A	NPTX1	
EDNRA	SMC5	DNMT1		CYP2D6	PTPA	EIF2B2	
XPR1	SDHB	GRN		SLC38A8	SCAF4	DNM1	
SLC7A6OS	PRNP	ST3GAL3		SCN1A	MSL2	POLR1A	
ENAM	PINK1	SLC25A1		KANSL1		BCKDHB	
C9orf72	PARK7	UBA5		PDE11A		THAP11	
CISD2	RLIM	FDFT1		PRKAR1A		ABCB1	
TBC1D7	GABRA3	NAXD		GGCX		SLC6A2	
PPT1	MED12	IFIH1		TCF4		C1S	
MMACHC	KANSL1	VPS53		ACAD9		GUCY2C	
PRDX1	GCH1	SLC1A4		SERPINA6		NTN1	

CLCN4	SRCAP	CARS1	ACKR1	XIAP
ATXN10	SLITRK1	SDHB	ATP1A2	POLG2
TREX1	HDC	LSM11	HLA-DRB1	FGF10
TBX1	RRM2B	RNU7-1	TP63	HLA-DQA1
USP8	RAD21	SLC25A19	EXT1	SI
PANK2	KMT2B	GCSH	KIF21A	ADAMTSL2
JPH3	DNAJC12	HLA-DQB1	PAX6	CRLF1
NOTCH3	YWHAG	PRNP	MUC1	MPZ
LMNB1	ARID2	TH	COL4A1	
KCNJ2	NFIB	HMGCL	KMT2D	
MECP2	NR3C1	SAR1B	AAGAB	
GCH1	KCTD17	BCKDHA	PLCD1	
SPAST	PDGFRB	WDR73	COL2A1	
COL11A1	WAC	MOCS2	SEPTIN9	
DNAJC5	GPR88	HLCS	NF1	
SLC4A1	FLT4	SMPD1	FGFR1	
SNCA	MED12L	OSTM1	AP2S1	
FMO3	EIF2AK1	PAH	CACNA1C	
PLA2G6	SOX6	QDPR	CASR	
TNNC1	MAPK1	PTS	RRM2B	
DOCK6	UQCRC1	PCK1	SLITRK1	
KCNT1	SPEN	CYP27B1	IFNG	
PDGFRB	POLG2	SUOX	TSC2	
COX15	PRR12	TCN2	PRSS56	
TTC19	AARS1	FAH	RP1L1	
CTSF	NSD2	VDR	CCDC8	
ADAMTS18	WARS2	TSEN54	CYP2B6	
KMT2B	TSC1	PRF1	RAD21	
MSTO1	MAGEL2	nan	GRM1	
SLC18A2	KMT2E	ASL	PNPT1	
COG4	NAA60	ASS1	RTEL1	
MYF5	ATXN3	FIG4	ARCN1	
TRRAP	ITM2B	HRAS	TGM3	
COASY	NKX2-1	EIF2AK3	DNAJC12	
KCTD17	HLA-DQB1	SLC46A1	EED	
PER3	PPP2R2B	FBP1	ALPK3	
TRNT1	PTEN	GCDH	GDAP2	
FLT4	SDHC	GCH1	AARS2	
TET3	POLG	CPS1	ARMC5	
JAM2	ZBTB20	LPL	GRID2	
CEP85L	ALDH5A1	ABCC8	CHCHD2	
MAPK1	GM2A	CAV3	CACNA1G	
UQCRC1	SPART	SHANK3	FXR1	
POLG2	FGFR3	JPH3	CARS1	
AARS1	TBX1	SLC6A8	SDHB	
WARS2	VPS13A	SLC16A2	SDHA	

HMBS	CLN3	FMR1	TSC1
NAA60	ATM	ATRX	ATXN8
AIP	PHOX2B	OCRL	ENPP1
NOTCH2	HLA-DQA1	ATP7A	MAGEL2
GJA5	TMCO1	NDP	GFRA1
CPOX	GNAS	LDB3	EIF2B4
OVOL2	ELP1	CLN8	COQ7
HLA-DQB1	ZEB2	RNASEH2C	PTPA
PPP2R2B	ABCC9	SLC2A1	PTRHD1
PER2	JPH3	MAPT	RAB32
PRKCG	ACSL4	GPC3	ABCB1
COLEC10	XK	NFU1	MSL2
EFHC1	HCFC1	AQP2	RPS6KA3
WNT10A	CNTNAP2	HPD	CNTN2
FAM20C	PDE11A	PSEN1	ARX
OPLAH	PRKAR1A	SPAST	ACO2
CHRNA4	TCF4	CLCN1	MYORG

Supplementary Table 29: Summary of results obtained for all analyses and rank of RBFOX1 regulated genes

RBFOX1 regulated genes		MDD			ANX			IRR			NTC			SA	ST	NW
ENTREZ ID	GENE SYMBOL	GB	DE	RD	GB	DE	RD	GB	DE	RD	GB	DE	RD	GB		
6671	SP4	y	y		y	y		y	y		y	y				
286205	SCAI	y	y		y	y			y		y	y		y		
253559	CADM2	y	y		y	y		y			y	y				y
92675	DTD1	y	y		y	y		y			y	y				
4137	MAPT			y	y	y		y	y	y	y	y				
4598	MVK	y	y		y			y			y	y			y	
6925	TCF4	y		y	y		y	y			y				y	y
114900	C1QTNF4	y	y		y	y					y	y				
1500	CTNND1	y	y		y			y			y	y				y
1508	CTSB		y		y	y		y	y		y	y				
200942	KLHDC8B	y	y					y	y		y	y				
84276	NICN1	y	y					y	y		y	y				
4905	NSF		y		y	y		y	y		y	y				
80207	OPA3	y			y	y		y	y		y	y				
5080	PAX6	y		y	y			y			y				y	y
27445	PCLO	y	y		y						y	y		y		y
54805	CNNM2	y			y	y		y			y	y				y
534	ATP6V1G2	y	y		y	y					y					y
6622	SNCA	y		y	y		y				y					y
3895	KTN1	y	y								y	y			y	
9726	ZNF646				y	y		y	y		y	y			y	
10645	CAMKK2	y	y		y							y			y	
23126	POGZ	y	y		y	y									y	
55553	SOX6	y			y		y	y			y				y	y
80031	SEMA6D	y			y			y			y	y		y	y	
5576	PRKAR2A	y	y					y	y					y		y
54870	QRICH1	y	y					y	y					y		
58155	PTBP2	y	y		y	y								y		y
60312	AFAP1	y	y								y	y				
1394	CRHR1				y	y		y	y		y	y				
348980	HCN1		y		y			y			y	y				
81631	MAP1LC3B				y	y		y	y		y	y				
80351	TNKS2	y	y						y			y				
1000	CDH2		y		y		y	y			y					
5874	RAB27B	y	y		y						y					y
64710	NUCKS1	y	y		y						y					y
389432	SAMD5	y	y					y			y					
3064	HTT	y		y			y			y						
81603	TRIM8	y	y		y	y										
8722	CTSF	y	y	y												
53	ACP2	y	y									y			y	
773	CACNA1A	y		y			y								y	
4208	MEF2C	y	y					y							y	y

10180	RBM6	y	y	y	y	y	y
23261	CAMTA1	y	y	y	y	y	y
23438	HARS2	y	y	y	y	y	y
57488	ESYT2	y	y	y	y	y	y
340481	ZDHHC21	y	y	y	y	y	y
8927	BSN	y	y	y	y	y	y
10562	OLFM4	y	y	y	y	y	y
468	ATF4	y	y	y	y	y	y
51440	HPCAL4	y	y	y	y	y	y
83892	KCTD10	y	y	y	y	y	y
56992	KIF15	y	y	y	y	y	y
5364	PLXNB1	y	y	y	y	y	y
205564	SENP5	y	y	y	y	y	y
254428	SLC41A1	y	y	y	y	y	y
373	TRIM23	y	y	y	y	y	y
2104	ESRRG	y	y	y	y	y	y
2918	GRM8	y	y	y	y	y	y
7008	TEF	y	y	y	y	y	y
9625	AATK	y	y	y	y	y	y
9870	KIAA0317	y	y	y	y	y	y
10659	CELF2	y	y	y	y	y	y
11216	AKAP10	y	y	y	y	y	y
25913	POT1	y	y	y	y	y	y
54858	PGPEP1	y	y	y	y	y	y
56884	FSTL5	y	y	y	y	y	y
119032	C10orf32	y	y	y	y	y	y
153527	ZMAT2	y	y	y	y	y	y
388336	SHISA6	y	y	y	y	y	y
9113	LATS1	y	y	y	y	y	y
112476	PRRT2	y	y	y	y	y	y
164091	PAQR7	y	y	y	y	y	y
8396	PIP4K2B	y	y	y	y	y	y
9522	SCAMP1	y	y	y	y	y	y
2048	EPHB2	y	y	y	y	y	y
6326	SCN2A	y	y	y	y	y	y
8672	EIF4G3	y	y	y	y	y	y
9856	KIAA0319	y	y	y	y	y	y
26115	TANC2	y	y	y	y	y	y
29855	UBN1	y	y	y	y	y	y
55193	PBRM1	y	y	y	y	y	y
64478	CSMD1	y	y	y	y	y	y
84251	SGIP1	y	y	y	y	y	y
84465	MEGF11	y	y	y	y	y	y
157922	CAMSAP1	y	y	y	y	y	y
211	ALAS1	y	y	y	y	y	y
3009	HIST1H1B	y	y	y	y	y	y

5101	PCDH9	y	y	y	y	y
27086	FOXP1	y	y	y	y	y
81545	FBXO38		y	y	y	y
10211	FLOT1	y	y		y	
2781	GNAZ	y			y	y
146433	IL34		y	y	y	y
84542	KIAA1841		y	y	y	y
84456	L3MBTL3			y	y	y
29763	PACSIN3	y			y	y
23428	SLC7A8		y	y	y	y
399979	SNX19		y	y	y	y
1012	CDH13	y	y		y	y
1525	CXADR	y	y		y	
1958	EGR1	y	y		y	y
1993	ELAVL2	y	y		y	y
2686	GGT7	y		y	y	
2771	GNAI2	y	y		y	y
3796	KIF2A	y	y		y	y
5578	PRKCA	y	y		y	y
6854	SYN2	y	y		y	y
8492	PRSS12	y	y		y	
22899	ARHGEF15	y	y		y	
22986	SORCS3	y	y		y	y
26051	PPP1R16B	y		y	y	
54715	RBFOX1	y	y		y	y
57624	NYAP2	y	y		y	
58476	TP53INP2	y		y	y	
64067	NPAS3	y	y		y	y
79033	ERI3	y	y		y	y
80323	CCDC68	y	y		y	y
83698	CALN1	y	y		y	
120892	LRRK2		y	y	y	
284069	FAM171A2	y	y		y	
407738	FAM19A1	y	y		y	
57338	JPH3		y	y	y	
114800	CCDC85A	y		y	y	
646424	SPINK8	y	y		y	
1010	CDH12	y	y	y		
5218	CDK14	y	y	y		
51642	MRPL48	y	y	y		
26575	RGS17	y	y	y		y
6711	SPTBN1	y	y	y		y
5052	PRDX1	y	y			
104	ADARB1	y	y			
94032	CAMK2N2	y	y			
203238	CCDC171	y	y			

9419	CRIP1	y	y						
1434	CSE1L	y	y						
9420	CYP7B1	y	y						
9453	GGPS1	y	y						
2849	GPR26	y	y						
11077	HSF2BP	y	y						
55323	LARP6	y	y						
80774	LIMD2	y	y						
54839	LRRC49	y	y						
7798	LUZP1	y	y						
84879	MFSD2A	y	y						
25834	MGAT4C	y	y						
4828	NMB	y	y						
56666	PANX2	y	y						
55012	PPP2R3C	y	y						
9991	PTBP3	y	y						
64089	SNX16	y	y						
9399	STOML1	y	y						
6092	ROBO2	y				y		y	y
7249	TSC2		y		y			y	
8087	FXR1		y		y			y	
8910	SGCE		y		y			y	
9244	CRLF1	y				y		y	
9254	CACNA2D2		y			y	y	y	
9354	UBE4A	y				y		y	
9448	MAP4K4	y			y			y	y
9575	CLOCK	y				y		y	y
10989	IMMT			y	y	y		y	
23030	KDM4B	y				y		y	
26031	OSBPL3	y				y		y	
26052	DNM3			y		y	y	y	
26059	ERC2	y				y		y	y
27303	RBMS3	y				y		y	
27351	DESI1		y		y	y		y	
51455	REV1	y	y					y	
56987	BBX		y		y	y		y	
57451	ODZ2	y				y		y	y
79734	KCTD17		y		y			y	
79834	PEAK1			y		y	y	y	
84614	ZBTB37	y				y		y	
1996	ELAVL4	y			y			y	y
3908	LAMA2	y			y			y	
729993	SHISA9	y				y		y	y
57463	AMIGO1				y	y	y		
388650	FAM69A		y				y		
23401	FRAT2	y					y		

5602	MAPK10		y			y	y	
4325	MMP16				y	y		y
27020	NPTN	y						y
5447	POR				y		y	y
5711	PSMD5			y			y	y
5898	RALA	y						y
57758	SCUBE2		y				y	y
8604	SLC25A12	y						y
115	ADCY9		y		y		y	
2719	GPC3				y		y	y
2775	GNAO1	y					y	y
2823	GPM6A	y					y	y
3251	HPRT1		y		y		y	
4607	MYBPC3	y					y	
4842	NOS1	y					y	
4858	NOVA2		y		y		y	
5037	PEBP1	y					y	
5048	PAFAH1B1	y					y	y
6323	SCN1A		y				y	
6764	ST5				y	y	y	
7067	THRA	y					y	y
8140	SLC7A5	y					y	
8476	CDC42BPA	y					y	
8609	KLF7	y					y	
9358	ITGBL1	y					y	
9403	SEPT15	y					y	
9793	CKAP5	y					y	y
10777	ARPP21		y		y		y	
23080	AVL9	y					y	
23314	SATB2		y		y		y	
27319	BHLHE22	y					y	y
27324	TOX3		y		y		y	
51320	MEX3C	y					y	
57582	KCNT1		y				y	
58499	ZNF462	y					y	
84640	USP38		y				y	
129401	NUP35		y		y		y	
266727	MDGA1	y					y	
339416	ANKRD45	y					y	
387103	CENPW	y					y	y
402117	VWC2L	y					y	y
80331	DNAJC5		y				y	
6683	SPAST		y				y	
10058	ABCB6	y				y		
2060	EPS15		y			y		
130574	LYPD6	y				y		

8972	MGAM	y		y			
28512	NKIRAS1		y		y		
51633	OTUD6B		y		y		
2045	EPHA7	y			y		
10152	ABI2		y		y		
26091	HERC4	y			y		
55676	SLC30A6	y			y		
56479	KCNQ5	y			y		
64168	NECAB1	y			y		
64919	BCL11B	y			y		y
1183	CLCN4		y		y		
1201	CLN3		y		y		
5594	MAPK1		y		y		
50484	RRM2B		y		y		
114798	SLITRK1		y		y		
7504	XK		y		y		
22979	EFR3B	y			y		
3400	ID4		y		y		
23315	SLC9A8		y		y		
85461	TANC1	y			y		
51699	VPS29		y		y		
114	ADCY8	y		y			y
324	APC	y		y			y
2554	GABRA1	y		y			y
3925	STMN1	y		y			y
4045	LSAMP	y		y			y
4205	MEF2A	y		y			y
8821	INPP4B	y		y			
9315	NREP	y		y			
10677	AVIL	y		y			
115557	ARHGEF25	y		y			y
253980	KCTD13	y		y			
1314	COPA				y	y	y
1390	CREM			y		y	y
1600	DAB1			y		y	y
2009	EML1		y				y
2911	GRM1		y				y
3752	KCND3		y				y
4154	MBNL1	y					y
5530	PPP3CA	y					y
5601	MAPK9				y	y	y
5789	PTPRD	y					y
6908	TBP		y				y
8028	MLLT10			y		y	y
8567	MADD	y					y
9201	DCLK1	y					y

10181	RBM5		y		y	y
10631	POSTN	y				y
10904	BLCAP		y		y	y
10915	TCERG1	y				y
23029	RBM34	y				y
23262	PIIP5K2		y	y		y
23369	PUM2	y				y
23499	MACF1			y	y	y
27436	EML4	y				y
29966	STRN3	y				y
55605	KIF21A		y			y
55700	MAP7D1	y				y
65009	NDRG4	y				y
80036	TRPM3	y				y
80146	UXS1	y				y
89797	NAV2	y				y
94134	ARHGAP12			y	y	y
113451	ADC	y				y
128989	C22orf25	y				y
145748	LYSMD4		y	y		y
255928	SYT14		y		y	y
339829	CCDC39	y				y
3097	HIVEP2	y				y
83719	YPEL3		y	y		y
196385	DNAH10	y				y
388121	TNFAIP8L3	y				y
9337	CNOT8		y		y	
11316	COPE				y	y
79877	DCAKD				y	y
63951	DMRTA1			y		y
79068	FTO				y	y
3760	KCNJ3				y	y
10314	LANCL1				y	y
286343	LURAP1L			y		y
10873	ME3			y		y
9480	ONECUT2		y			y
56098	PCDHGC4		y			y
5500	PPP1CB			y		y
255426	RASGEF1C				y	y
6176	RPLP1		y			y
55206	SBNO1			y		y
23032	USP33				y	y
55759	WDR12				y	y
758	MPPED1			y	y	
5595	MAPK3		y		y	
5764	PTN		y		y	

5798	PTPRN		y	y
6096	RORB	y		y
6643	SNX2	y		y
6857	SYT1		y	y
7704	ZBTB16	y		y
7903	ST8SIA4	y		y
8828	NRP2	y		y
10231	RCAN2	y		y
10325	RRAGB	y		y
10687	PNMA2		y	y
11275	KLHL2		y	y
23007	PLCH1	y		y
23558	WBP2	y		y
26468	LHX6		y	y
29999	FSCN3	y		y
50809	HP1BP3	y		y
51555	PEX5L	y		y
54557	SGTB		y	y
54861	SNRK		y	y
57452	GALNTL1	y		y
63916	ELMO2	y		y
79969	ATAT1	y		y
80034	CSRNP3	y		y
85455	DISP2	y		y
131034	CPNE4	y		y
132864	CPEB2	y		y
150726	FBXO41	y		y
164633	CABP7	y		y
219539	YPEL4	y		y
285237	C3orf38		y	y
4884	NPTX1		y	y
127703	C1orf216		y	y
3038	HAS3		y	y
11014	KDELRL2		y	y
221692	PHACTR1		y	y
79641	ROGDI		y	y
29091	STXBP6		y	y
88	ACTN2	y	y	
3267	AGFG1	y	y	
155066	ATP6V0E2	y	y	
283152	CCDC153	y	y	
8790	FPGT	y	y	
9371	KIF3B	y	y	
5108	PCM1	y	y	
9601	PDIA4	y	y	
57181	SLC39A10	y	y	

9878	TOX4	y	y
9049	AIP	y	
83636	C19orf12	y	
6900	CNTN2	y	
1436	CSF1R	y	
8726	EED	y	
2157	F8	y	
2259	FGF14	y	
2674	GFRA1	y	
2717	GLA	y	
25974	MMACHC	y	
7053	TGM3	y	
51095	TRNT1	y	
9213	XPR1	y	
84920	ALG10	y	
155185	AMZ1	y	
490	ATP2B1	y	
55743	CHFR	y	
5067	CNTN3	y	
64506	CPEB1	y	
7458	EIF4H	y	
26122	EPC2	y	
84253	GARNL3	y	
84662	GLIS2	y	
9454	HOMER3	y	
85442	KNDC1	y	
4131	MAP1B	y	
56947	MFF	y	
5136	PDE1A	y	
112464	PRKCDBP	y	
10156	RASA4	y	
65055	REEP1	y	
55288	RHOT1	y	
79634	SCRN3	y	
6752	SSTR2	y	
134429	STARD4	y	
6804	STX1A	y	
23336	SYNM	y	
64856	VWA1	y	
285525	YIPF7	y	
108	ADCY2	y	
351	APP	y	
529	ATP6V1E1	y	
785	CACNB4	y	
793	CALB1	y	
814	CAMK4	y	

862	RUNX1T1	y
947	CD34	y
1131	CHRM3	y
1310	COL19A1	y
1762	DMWD	y
1955	MEGF9	y
1983	EIF5	y
2840	GPR17	y
2847	MCHR1	y
2890	GRIA1	y
2903	GRIN2A	y
2975	GTF3C1	y
3024	HIST1H1A	y
3184	HNRNPB	y
3416	IDE	y
3798	KIF5A	y
4150	MAZ	y
4254	KITLG	y
4293	MAP3K9	y
4306	NR3C2	y
4638	MYLK	y
4673	NAP1L1	y
4676	NAP1L4	y
4701	NDUFA7	y
4744	NEFH	y
4947	OAZ2	y
5110	PCMT1	y
5128	CDK17	y
5569	PKIA	y
5579	PRKCB	y
5604	MAP2K1	y
5867	RAB4A	y
6196	RPS6KA2	y
6252	RTN1	y
7070	THY1	y
7276	TTR	y
7408	VASP	y
7905	REEP5	y
8460	TPST1	y
8507	ENC1	y
8531	CSDA	y
8535	CBX4	y
8543	LMO4	y
8725	URI1	y
9232	PTTG1	y
9331	B4GALT6	y

9474	ATG5	y
9495	AKAP5	y
9530	BAG4	y
9537	TP53I11	y
9541	CIR1	y
9592	IER2	y
9612	NCOR2	y
9693	RAPGEF2	y
9735	KNTC1	y
9745	ZNF536	y
9936	CD302	y
10000	AKT3	y
10098	TSPAN5	y
10120	ACTR1B	y
10125	RASGRP1	y
10190	TXNDC9	y
10243	GPHN	y
10439	OLFM1	y
10724	MGEA5	y
10808	HSPH1	y
10963	STIP1	y
11130	ZWINT	y
22931	RAB18	y
22954	TRIM32	y
23105	FSTL4	y
23112	TNRC6B	y
23193	GANAB	y
27145	FILIP1	y
29128	UHRF1	y
29767	TMOD2	y
51090	PLLP	y
51278	IER5	y
55117	SLC6A15	y
55184	DZANK1	y
55239	OGFOD1	y
57118	CAMK1D	y
57120	GOPC	y
57419	SLC24A3	y
57540	PTCHD2	y
57795	FAM5B	y
64084	CLSTN2	y
64405	CDH22	y
64754	SMYD3	y
64897	C12orf43	y
79056	PRRG4	y
79623	GALNT14	y

79947	DHDDS	y		
80233	C17orf70	y		
80725	SRCIN1	y		
80856	KIAA1715	y		
81551	STMN4	y		
84435	GPR123	y		
84447	SYVN1	y		
84678	KDM2B	y		
84833	USMG5	y		
84918	LRP11	y		
89853	FAM125B	y		
89927	C16orf45	y		
91404	SESTD1	y		
116135	LRRC3B	y		
121256	TMEM132D	y		
121551	BTBD11	y		
121601	ANO4	y		
124274	GPR139	y		
131408	FAM131A	y		
134391	GPR151	y		
135152	B3GAT2	y		
140688	C20orf112	y		
142891	SAMD8	y		
146183	OTOA	y		
150465	TTL	y		
161253	REM2	y		
164832	LONRF2	y		
256586	LYSMD2	y		
285382	C3orf70	y		
375567	VWC2	y		
401474	SAMD12	y		
404672	GTF2H5	y		
646486	FABP12	y		
100499483	C9orf174	y		
288	ANK3	y		y
2118	ETV4		y	y
2521	FUS	y		y
3190	HNRNPK	y		y
4133	MAP2	y		y
4430	MYO1B		y	y
4771	NF2	y		y
4952	OCRL		y	y
5339	PLEC		y	y
5971	RELB		y	y
6248	RSC1A1		y	y
6616	SNAP25	y		y

6863	TAC1	y	y
7392	USF2	y	y
8761	PABPC4	y	y
9910	RABGAP1L	y	y
9912	ARHGAP44	y	y
10057	ABCC5	y	y
10150	MBNL2	y	y
10522	DEAF1	y	y
23077	MYCBP2	y	y
23207	PLEKHM2	y	y
23215	PRRC2C	y	y
23426	GRIP1	y	y
23513	SCRIB	y	y
23543	RBFOX2	y	y
25766	PRPF40B	y	y
25961	NUDT13	y	y
30819	KCNIP2	y	y
51132	RLIM	y	y
51317	PHF21A	y	y
54806	AHI1	y	y
54899	PXK	y	y
55023	PHIP	y	y
57703	CWC22	y	y
64750	SMURF2	y	y
79772	MCTP1	y	y
84079	ANKRD27	y	y
84894	LINGO1	y	y
90523	MLIP	y	y
91746	YTHDC1	y	y
115209	OMA1	y	y
116931	MED12L	y	y
122416	ANKRD9	y	y
151050	KANSL1L	y	y
285175	UNC80	y	y
346171	ZFP57	y	y
8448	DOC2A	y	y
55860	ACTR10	y	y
200539	ANKRD23	y	
1281	COL3A1	y	
55862	ECHDC1	y	
55915	LANCL2	y	
8685	MARCO	y	
54892	NCAPG2	y	
79954	NOL10	y	
5368	PNOC	y	
5433	POLR2D	y	

23362	PSD3	y
55512	SMPD3	y
23321	TRIM2	y
7280	TUBB2A	y
732	C8B	y
766	CA7	y
1398	CRK	y
1808	DPYSL2	y
1960	EGR3	y
2257	FGF12	y
3360	HTR4	y
3747	KCNC2	y
4088	SMAD3	y
4094	MAF	y
4130	MAP1A	y
4326	MMP17	y
4782	NFIC	y
5028	P2RY1	y
5046	PCSK6	y
5198	PFAS	y
5581	PRKCE	y
5649	RELN	y
6091	ROBO1	y
7201	TRHR	y
7323	UBE2D3	y
7465	WEE1	y
8825	LIN7A	y
9468	PCYT1B	y
9771	RAPGEF5	y
9901	SRGAP3	y
10768	AHCYL1	y
10801	SEPT9	y
10827	FAM114A2	y
23108	RAP1GAP2	y
23180	RFTN1	y
23344	ESYT1	y
25771	TBC1D22A	y
26019	UPF2	y
26999	CYFIP2	y
27336	HTATSF1	y
54768	HYDIN	y
55090	MED9	y
55450	CAMK2N1	y
55604	LRRC16A	y
55626	AMBRA1	y
55740	ENAH	y

57084	SLC17A6	y
64806	IL25	y
79012	CAMKV	y
79789	CLMN	y
79968	WDR76	y
80143	SIKE1	y
85397	RGS8	y
90161	HS6ST2	y
91752	ZNF804A	y
92400	RBM18	y
118856	MMP21	y
127829	ARL8A	y
146691	TOM1L2	y
169841	ZNF169	y
196500	PIANP	y
255252	LRRC57	y
284110	GSDMA	y
285016	FAM150B	y
342918	C19orf81	y
347731	LRRTM3	y
399664	MEX3D	y
400745	SH2D5	y
497190	CLEC18B	y
642968	FAM163B	y
100113407	TMEM170B	y
100127206	KIAA1024L	y
9719	ADAMTSL2	y
1644	DDC	y
1717	DHCR7	y
2255	FGF10	y
2653	GCSH	y
3455	IFNAR2	y
4023	LPL	y
134353	LSM11	y
29078	NDUFAF4	y
85358	SHANK3	y
6567	SLC16A2	y
100	ADA	y
107	ADCY1	y
374569	ASPG	y
10153	CEBPZ	y
1767	DNAH5	y
100132074	FOXO6	y
170384	FUT11	y
2563	GABRD	y
2587	GALR1	y

29967	LRP12	y
25821	MTO1	y
4814	NINJ1	y
11159	RABL2A	y
389075	RESP18	y
6691	SPINK2	y
794	CALB2	y
1404	HAPLN1	y
1742	DLG4	y
2740	GLP1R	y
2824	GPM6B	y
2977	GUCY1A2	y
3842	TNPO1	y
4311	MME	y
4697	NDUFA4	y
4703	NEB	y
4880	NPPC	y
7306	TYRP1	y
8522	GAS7	y
8541	PPFIA3	y
9517	SPTLC2	y
9678	PHF14	y
10039	PARP3	y
11080	DNAJB4	y
23295	MGRN1	y
26289	AK5	y
27112	FAM155B	y
51571	FAM49B	y
57188	ADAMTSL3	y
80228	ORAI2	y
124739	USP43	y
158135	TTLL11	y
7915	ALDH5A1	y
2556	GABRA3	y
51574	LARP7	y
4781	NFIB	y
5567	PRKACB	y
6391	SDHC	y
4089	SMAD4	y
7532	YWHAG	y
26137	ZBTB20	y
282679	AQP11	y
56970	ATXN7L3	y
56171	DNAH7	y
84624	FNDC1	y
2585	GALK2	y

10014	HDAC5	y
55364	IMPACT	y
3909	LAMA3	y
10162	LPCAT3	y
23089	PEG10	y
5179	PENK	y
56341	PRMT8	y
6048	RNF5	y
6342	SCP2	y
12	SERPINA3	y
54847	SIDT1	y
23094	SIPA1L3	y
342667	STAC2	y
9618	TRAF4	y
493829	TRIM72	y
381	ARF5	y
1655	DDX5	y
3786	KCNQ3	y
5176	SERPINF1	y
5336	PLCG2	y
5469	MED1	y
5910	RAP1GDS1	y
6117	RPA1	y
7026	NR2F2	y
7200	TRH	y
7334	UBE2N	y
7855	FZD5	y
8766	RAB11A	y
9275	BCL7B	y
9728	SECISBP2L	y
9777	TM9SF4	y
11279	KLF8	y
22880	MORC2	y
22936	ELL2	y
23211	ZC3H4	y
23237	ARC	y
23416	KCNH3	y
25999	CLIP3	y
27069	GHITM	y
51760	SYT17	y
54466	SPIN2A	y
55013	CCDC109B	y
55327	LIN7C	y
57446	NDRG3	y
57679	ALS2	y
57698	KIAA1598	y

79731	NARS2	y
79830	ZMYM1	y
80335	WDR82	y
84630	TTBK1	y
84705	GTPBP3	y
91653	BOC	y
92521	SPECC1	y
93273	LEMD1	y
114826	SMYD4	y
116328	C8orf34	y
222553	SLC35F1	y
493911	PHOSPHO2	y

MDD major depressive disorder, ANX anxiety, IRR irritability, NTC neuroticism, SA suicide attempt, GB associated in gene-based analysis, DF predicted to be differentially expressed, RD gene causal for a rare disorder with the respective symptom, ST RBFOX1 splicing target, NW present in network

Chapter 2. The effect of *rbfox1* on behaviour in zebrafish.

ARTICLE OPEN



Pleiotropic contribution of *rbfox1* to psychiatric and neurodevelopmental phenotypes in two zebrafish models

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RBFOX1 is a highly pleiotropic gene that contributes to several psychiatric and neurodevelopmental disorders. Both rare and common variants in *RBFOX1* have been associated with several psychiatric conditions, but the mechanisms underlying the pleiotropic effects of *RBFOX1* are not yet understood. Here we found that, in zebrafish, *rbfox1* is expressed in spinal cord, mid- and hindbrain during developmental stages. In adults, expression is restricted to specific areas of the brain, including telencephalic and diencephalic regions with an important role in receiving and processing sensory information and in directing behaviour. To investigate the contribution of *rbfox1* to behaviour, we used *rbfox1*^{sa15940}, a zebrafish mutant line with TL background. We found that *rbfox1*^{sa15940} mutants present hyperactivity, thigmotaxis, decreased freezing behaviour and altered social behaviour. We repeated these behavioural tests in a second *rbfox1* mutant line with a different genetic background (TU), *rbfox1*^{del19}, and found that *rbfox1* deficiency affects behaviour similarly in this line, although there were some differences. *rbfox1*^{del19} mutants present similar thigmotaxis, but stronger alterations in social behaviour and lower levels of hyperactivity than *rbfox1*^{sa15940} fish. Taken together, these results suggest that mutations in *rbfox1* lead to multiple behavioural changes in zebrafish that might be modulated by environmental, epigenetic and genetic background effects, and that resemble phenotypic alterations present in *Rbfox1*-deficient mice and in patients with different psychiatric conditions. Our study, thus, highlights the evolutionary conservation of *rbfox1* function in behaviour and paves the way to further investigate the mechanisms underlying *rbfox1* pleiotropy on the onset of neurodevelopmental and psychiatric disorders.

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INTRODUCTION

RNA Binding Fox-1 Homolog 1 (*RBFOX1*, also referred to as *A2BP1* or *FOX1*) encodes an RNA splicing factor that is specifically expressed in brain, heart and muscle in human adults (GTEx, <https://gtexportal.org/home/gene/RBFOX1>). This gene regulates the expression and splicing of large gene networks and plays an important role in neurodevelopment [1, 2]. Rare genetic variations, including point mutations and copy number variants, have been reported in *RBFOX1* in patients with neurodevelopmental disorders such as autism spectrum disorder (ASD) [3–6], and *RBFOX1* haploinsufficiency results in a syndrome characterised by impaired neurodevelopment [7, 8]. In addition, transcriptomic analysis of brains from autistic individuals revealed decreased levels of *RBFOX1* and dysregulation of *RBFOX1*-dependent alternative splicing [6, 9]. *RBFOX1* has not only been related to neurodevelopmental conditions, but increasing evidence points to both rare and common variants

in this gene as contributors to several psychiatric and neurological disorders [5, 6, 10–12]. Interestingly, common variants in *RBFOX1* were found significantly associated with the cross-trait phenotype of the most recent genome-wide association studies (GWAS) meta-analysis of psychiatric disorders [13] and *RBFOX1* was identified as the second most pleiotropic locus in a previous cross-disorder GWAS meta-analysis, showing association of common variants with attention-deficit/hyperactivity disorder (ADHD), ASD, bipolar disorder (BIP), major depression (MD), obsessive-compulsive disorder, schizophrenia (SCZ) and Tourette's syndrome (TS) [14]. Finally, *Rbfox1*^{-/-} mutant mice present a heightened susceptibility to seizures and neuronal hyperexcitability [15], and *Rbfox1* neuron-specific knockout mice show pronounced hyperactivity, stereotyped behaviour, impairments in fear acquisition and extinction, reduced social interest and lack of aggression [6], behaviours that are related to different psychiatric disorders. All these data

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suggest a major role for *RBFOX1* in psychopathology, although the mechanisms underlying its pleiotropic effects are not well understood.

In the last years, zebrafish have become a powerful model to study psychiatric disorders thanks to their high genetic similarity to human and their well-defined behavioural phenotypes, which can be easily assessed in the laboratory and compared to human psychiatric phenotypes [16–18]. *RBFOX1* has two orthologous genes in zebrafish, *rbfox1* (*a2bp1*, NCBI gene ID: 449554) and *rbfox1l* (*a2bp1l*, NCBI gene ID: 407613). While the human gene *RBFOX1* is expressed in both the brain and skeletal and cardiac muscle (GTEx, <https://gtexportal.org/home/gene/RBFOX1>), *rbfox1* is mainly expressed in brain – but is also transcribed in heart –, and *rbfox1l* is exclusively expressed in skeletal and cardiac muscles at early developmental stages [19, 20] and shows a low and restricted expression in only some neuronal populations of the adult zebrafish brain [21]. In this study we focused on *rbfox1*, a gene that encodes a major protein isoform with 84% identity to the human protein [19], given its strong brain expression during development. To date, the expression of *rbfox1* at later stages has not been investigated nor its role in zebrafish neurodevelopment and behaviour.

Genetic studies in humans have pointed to a pleiotropic contribution of *RBFOX1* to several psychiatric conditions. Here, we have characterised the effect of altered *rbfox1* function on zebrafish behaviour, and our data help describe mechanisms underlying its pleiotropic effects on the onset of neurodevelopmental and psychiatric disorders.

MATERIAL AND METHODS

Zebrafish strains, care and maintenance

Adult zebrafish and larvae (*Danio rerio*) were maintained at 28.5 °C on a 14:10 light-dark cycle following standard protocols. All experimental procedures were approved by the Animal Welfare and Ethical Review board of the Generalitat de Catalunya. Behavioural experiments were performed using two different *rbfox1* mutant strains with different genetic backgrounds. *rbfox1*^{sa15940}, on the Tübingen Long-fin (TL) background, is a transgenic line obtained from the European Zebrafish Resource Center of the Karlsruhe Institute of Technology (KIT). This line contains an intronic point mutation at the -2 position of a 3' acceptor splicing site of *rbfox1* before the second/third exon of *rbfox1* annotated isoforms (A>T, Chr3:28068329, GRCz11) (Supplementary Fig. 1). The second line, *rbfox1*^{del19}, on the Tübingen (TU) background, was created using CRISPR/Cas9 genetic engineering and causes a frameshift deletion of 19 bp within exon 2 or 3 of *rbfox1* annotated isoforms (Chr3:28068264–28068282, GRCz11) (Supplementary Fig. 2). Homozygous mutant fish (HOM, *rbfox1*^{sa15940/sa15940} and *rbfox1*^{del19/del19}), heterozygous (HZ, *rbfox1*^{sa15940/+} and *rbfox1*^{del19/+}) and wild-type (WT, TL *rbfox1*^{+/+} and TU *rbfox1*^{+/+}) fish were used for all behavioural experiments. For both *rbfox1*^{sa15940} and *rbfox1*^{del19} lines, homozygous, heterozygous and wild-type fish were obtained from heterozygous crosses to ensure a common genetic background.

Gene expression analysis using Real-Time quantitative PCR (RT-qPCR)

Total RNA was extracted from the whole brain of 7 TL *rbfox1*^{+/+}, 7 TL *rbfox1*^{sa15940/+} and 7 TL *rbfox1*^{sa15940/sa15940} adult zebrafish and from 5 pools of 10 whole larvae of each genotype *rbfox1*^{+/+}, *rbfox1*^{sa15940/+} and *rbfox1*^{sa15940/sa15940} to perform RT-qPCR. Primers were designed to amplify cDNA from all the *rbfox1*, *rbfox1l*, *rbfox2*, *rbfox3a* and *rbfox3b* protein-coding isoforms described in the GRCz11 Ensembl database except for the *rbfox1*-203 isoform (http://www.ensembl.org/Danio_rerio/, Supplementary Table 1). In the case of *rbfox1* primers, the forward primer binds to the exon situated after the intronic point mutation in the *rbfox1*^{sa15940} line and the reverse primer binds to the subsequent exon. Results were normalised to the expression levels of reference housekeeping genes: the ribosomal protein *L13a* (*rpl13a*) and the eukaryotic translation elongation factor 1 alpha 1a (*eef1a1a*) housekeeping genes in the case of adult brains, and the ubiquitin-conjugating enzyme *E2A* (*ube2a*) in the case of larval samples, as the expression level of this gene is more stable through developmental stages (Supplementary Table 1). The relative expression of the genes and the fold change were calculated using the 2^{-ΔΔCT} comparative method [22, 23].

In situ hybridisation (ISH)

A specific mRNA probe targeting *rbfox1* (NCBI Reference Sequence: NM_001005596) was prepared and ISH experiments were performed in larvae (28 h post fertilisation (hpf), 2-, 3-, 4-, and 5-days post fertilisation (dpf)) and dissected adult brains of WT fish from TL and TU lines. Further details are described in the Supplementary Methods.

Generation of a *rbfox1* zebrafish loss-of-function line using CRISPR/Cas9

We used the CRISPR/Cas9 technology to generate stable *rbfox1* loss-of-function mutants (Supplementary Fig. 2). Briefly, we designed 20 bp sequences (crRNA) targeting *rbfox1* next to a PAM sequence (Supplementary Table 2). scRNA, tracrRNA and Cas9 were purchased, and 1 nL of injection solution was injected into the cell of single-cell stage zebrafish embryos. After 24 hpf, the injection efficiency and crRNA efficacy were assessed and injected embryos (called F₀ thereafter) with high injection efficiency were raised to adulthood. F₀ were then crossed with WT zebrafish, generating F₁ animals heterozygous for different mutations. DNA extraction and PCR followed by DNA Sanger sequencing analysis of F₁ at 24 hpf identified the batches of F₁ siblings that were more likely to contain a high proportion of frameshift mutations and the selected batches were raised to adulthood. F₁ was screened to select a frameshift mutation and two siblings (one male and one female) that carried the same 19 bp frameshift mutation were in-crossed to generate F₂ offspring that were 25% wild type, 50% heterozygous and 25% homozygous for the 19 bp mutation. The genotype of each F₂ zebrafish was assessed to grow the animals and establish the mutant line. Further details of the method are described in the Supplementary Methods.

Behavioural tests

A battery of behavioural tests was performed on adult zebrafish (3–6 months-old) using mixed groups of both sexes: open field test, shoaling test, visually-mediated social preference (VMSP) test, black and white test, and aggression test (Supplementary Fig. 3). All the experiments were performed with homozygous mutant fish (*rbfox1*^{sa15940/sa15940} and *rbfox1*^{del19/del19}), heterozygous (*rbfox1*^{sa15940/+} and *rbfox1*^{del19/+}) and wild-type (TL *rbfox1*^{+/+} and TU *rbfox1*^{+/+}) fish. In this first batch of experiments, the proportion of females in the tanks for both lines ranged from 50 to 70%. A second batch of experiments was performed with the *rbfox1*^{sa15940} fish segregating them by genotype and sex. Sex was phenotypically determined as it cannot be genotyped in zebrafish [24, 25]. In all instances, all fish were genotyped, sized-matched and 13 individuals were randomly selected per condition. These fish were then housed together in a single tank per condition for one week prior to and during the behavioural experiments. For each experiment, fish were placed in a different tank once tested to avoid retesting them and when the experiment was finished, fish were housed together in the original tank. All tanks were kept in close proximity within the facility and were subjected to identical environmental and housing conditions. To prevent any potential housing bias, caretakers responsible for the fish were blind to both the genotype and experimental details.

Experiments were completed between 9:00 and 18:00 and recorded using StreamPix 7 software (Norpix) and a digital camera. Fish were left for 30 min to habituate to the testing room before the experiment. To determine the appropriate number of individuals in each group, we utilised GPower 3.1 software [26]. The calculation was based on data from previous experiments conducted within the same experimental setup, aiming to guarantee sufficient statistical power to detect differences between the groups in the behavioural tests without using more animals than necessary. During the experiments, genotypes were alternated to prevent any bias resulting from the time of day or other possible confounders and all individuals were tested in the same setup and testing tanks. Most of the measures were performed automatically using a tracking system and analysed using software, ensuring blinding of the data. However, for a few measures that required manual quantification, a retrospective blinding process was implemented to ensure that there was no bias during the manual quantification of phenotypes. Further details of the tests are described in the Supplementary Methods.

Statistical methods

Statistical analysis of RT-qPCR and behavioural data were performed with GraphPad Prism 8 (GraphPad Software, La Jolla California USA). The data sets were assessed for normality using D'Agostino-Pearson and

Shapiro-Wilk normality test and either a one-way ANOVA test followed by a Tukey's *post-hoc* test or a Kruskal-Wallis test with Dunn's correction for multiple testing were used to compare between multiple groups. Statistical analysis of the visually-mediated social preference test (VMSP) was performed by a two-way ANOVA with Sidak's *post-hoc* test or a Kruskal-Wallis test followed by a Dunn's correction for multiple testing. Standard deviation (SD) is indicated in the figures for each group of data. In the behavioural tests, the median of the individual speed was used instead of the mean as it was more representative of non-normal data caused by a high degree of freezing behaviour.

RESULTS
***rbfox1* expression is restricted to neurons during development and is localised to specific forebrain, midbrain and hindbrain areas in adulthood**

During early development (28 hpf), *rbfox1* is expressed in spinal cord and hindbrain lateral neurons (Fig. 1A). At later developmental stages (2–5 dpf) *rbfox1* expression is widespread in the mid- and hindbrain (Fig. 1A). These findings are in line with previous published data [27]. Furthermore, we found that during

development *rbfox1* is also expressed in the heart, in line with what has previously been described elsewhere [19].

In adult fish, *rbfox1* is expressed along the entire rostro-caudal brain axis. In the pallial region of the forebrain, *rbfox1* is expressed in the glomerular (GL), external (ECL) and internal (ICL) cellular layers of the olfactory bulbs (Fig. 1B – a, a'). More caudally, *rbfox1* is expressed in the dorsal telencephalic area (D) and in the dorsal (Vd), lateral (Vl) and ventral (Vv) nuclei of ventral telencephalic area (Fig. 1B – a, b). In the diencephalon, *rbfox1*-expressing cells have been detected in the ventral habenular nucleus (HaV), and in the anterior (A) and ventromedial (VM) thalamic nuclei (Fig. 1B – c). *rbfox1* is also expressed in the periventricular layer of the thalamic and hypothalamic areas including the ventral part of the periventricular pretectal nucleus (PPv), the central posterior thalamic nucleus (CP), the periventricular nucleus of posterior tuberculum (TPp), the anterior tubular nucleus (ATN), and the ventral zone of the periventricular hypothalamus (Hv) (Fig. 1B – d, d'). In the midbrain, *rbfox1* has been detected in the periventricular grey zone (PGZ) and in the torus longitudinalis (TL) (Fig. 1B – d, d', e). Finally, in the

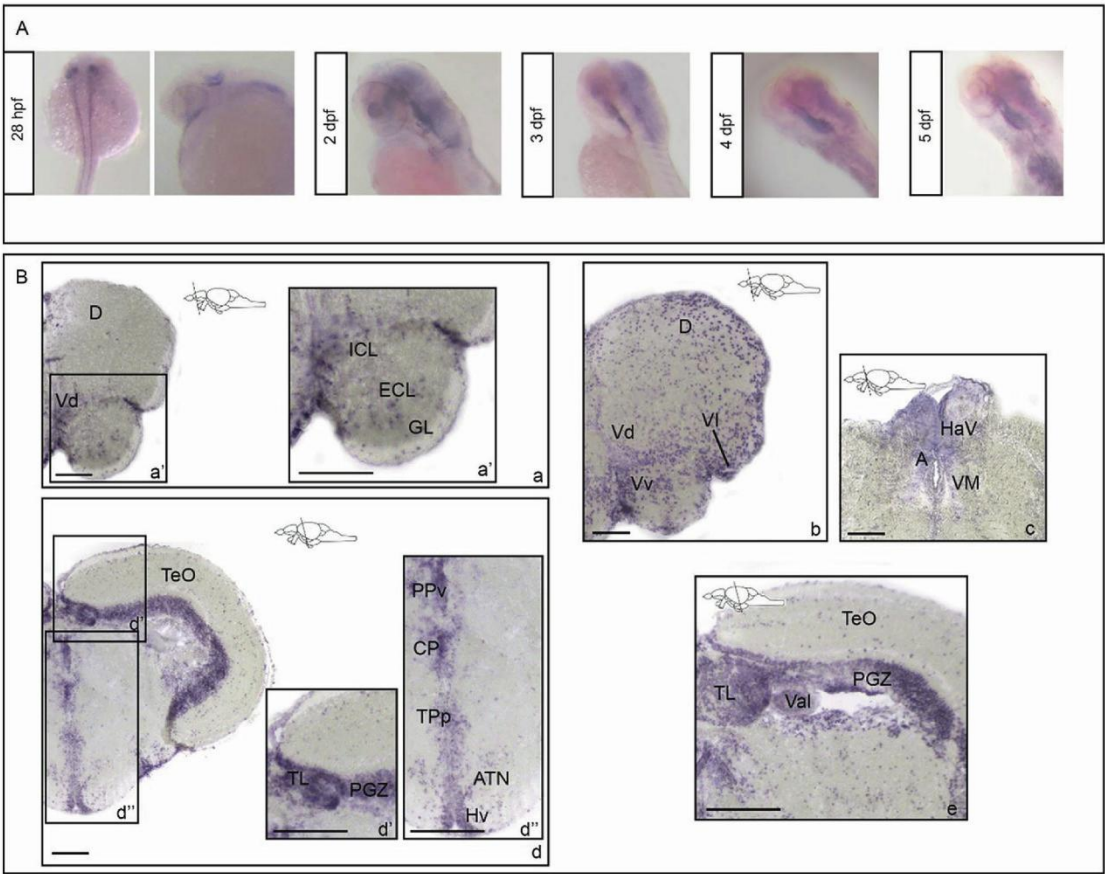


Fig. 1 *rbfox1* shows restricted neuronal expression during development and is localised to specific forebrain, midbrain and hindbrain areas during adulthood. *rbfox1* in situ hybridisation on (A) zebrafish whole mount larvae and (B) adult zebrafish brains, TL background. **A** *rbfox1* whole mount in situ hybridisation on zebrafish larvae at 28 h post fertilisation, 3-, 4- and 5-days post fertilisation. **B** *rbfox1* in situ hybridisation on adult zebrafish brains, (a–c) forebrain and (d–e) midbrain transverse sections. **A** anterior thalamic nuclei; CP, central posterior thalamic nucleus; D, dorsal telencephalic area; GL, glomerular cellular layer; HaV, ventral habenular nucleus; Hv, ventral zone of periventricular hypothalamus; ICL, internal cellular layer; PGZ, periventricular grey zone; PPv, ventral part of the periventricular pretectal nucleus; TeO, optic tectum; TL, torus longitudinalis; TPp, periventricular nucleus of posterior tuberculum; ATN, anterior tubular nucleus; Vd, dorsal nucleus of ventral telencephalic area; Vl, lateral nucleus of ventral telencephalic area; VM, ventromedial thalamic nuclei; Vv, ventral nucleus of ventral telencephalic area. Scale bars: 100 µm (a, b, c, d, e); 200 µm (a', d', d'').

hindbrain *rbfox1* expression is observed in the lateral division of the valvula cerebelli (Val) (Fig. 1B – e).

No differences were observed in *rbfox1* expression between TU and TL backgrounds (Supplementary Figure 4) at either larval or adult stages.

***rbfox1*^{sa15940/sa15940} zebrafish do not express the complete *rbfox1* mRNA sequence and do not show alterations in the expression of the other *rbfox* genes**

The first mutant line that we characterised, *rbfox1*^{sa15940} (A > T, Chr3:28068329, GRCz11), has an intronic point mutation at the -2 position of the 3' acceptor splicing site before the second/third exon of all but one of the annotated *rbfox1* zebrafish isoforms (Supplementary Fig. 1), which would cause that this exon is skipped during the splicing process and a shorter and aberrant *rbfox1* mRNA sequence produced. This mutation would cause the deletion of 81 aminoacids, which represent 22 to 54% of the original Rbfox1 protein isoforms, leading to a significant change in conformation that may affect the functionality of the mutant protein. By RT-qPCR, we observed a strongly decreased level of the expression of the *rbfox1* exon situated after the intronic point mutation in both homozygous and heterozygous *rbfox1*^{sa15940} mutant adult brains (93% and 43% respectively) compared to WT (mean HZ = 0.47; mean HOM = 0.07, WT vs. HOM: $p = 0.0002$, Fig. 2A). We also observed a decreased level of the expression of this *rbfox1* exon in both homozygous and heterozygous *rbfox1*^{sa15940} mutant 3dpf larvae (80% and 38% respectively) compared to WT (mean HZ = 0.62; mean HOM = 0.20, WT vs. HOM: $p = 0.0036$, Fig. 2D). These results suggest that this mutant line can be used to examine the effect of *rbfox1* function in zebrafish.

We explored the expression levels of the four other *rbfox* zebrafish genes in both adult brains and 3dpf larvae. We observed no differences in the expression of *rbfox1l*, *rbfox2*, *rbfox3a* and *rbfox3b* in brain between WT and mutant *rbfox1*^{sa15940} adult fish (Fig. 2B) and in the expression of *rbfox1l*, *rbfox2* and *rbfox3a* between WT and mutant *rbfox1*^{sa15940} larvae (Fig. 2E). The expression of *rbfox3b* was slightly reduced in homozygous mutant *rbfox1*^{sa15940} larvae compared to WT and HZ (WT vs. HOM: $p = 0.049$, HZ vs. HOM: $p = 0.011$, Fig. 2E). We also found that *rbfox1l* expression in the WT adult brain was very low compared to the expression of the other *rbfox* genes in this tissue, as its Cq (quantification cycle value) is much higher in the RT-qPCR analysis (Fig. 2C). Unlike in adult brains, the expression levels of all the *rbfox* genes in whole WT 3dpf larvae were similar, with Cq ranging between 22 and 26 (Fig. 2F).

Finally, the expression levels of the *rbfox* genes at 6dpf could not be assessed as all the reference genes considered (*rpl13a*, *eef1a1a*, *ube2a* and *tmem50a*) did not show stable expression among individuals and genotypes at this larval stage.

***rbfox1*^{sa15940} mutation in zebrafish produces behavioural alterations**

We performed a battery of five behavioural tests (open field test, shoaling test, VMSP test, black and white test and aggression test) (Supplementary Fig. 3) in TL WT *rbfox1*^{+/+}, heterozygous (HZ) *rbfox1*^{sa15940/+}, and homozygous (HOM) *rbfox1*^{sa15940/sa15940} adult fish, to investigate whether this *rbfox1* mutation affects behaviour (Fig. 3 and Supplementary Fig. 5).

In this mutant line, all HZ and HOM individuals spend less than 20% of the time in the centre of the open field arena and show thigmotaxis, a behaviour that could be related to anxiety or stereotypies, whereas TL WT fish do not show preference to swim close to the walls of the arena (Fig. 3A and Supplementary Fig. 5A). In addition, HZ and HOM fish spend less time freezing than TL WT fish (WT vs. HZ, $p = 0.0068$; WT vs. HOM, $p = 0.0001$; Fig. 3A) and show hyperactivity, as they swim longer distances (WT vs. HZ, $p = 0.0027$; WT vs. HOM, $p = 0.0002$; Fig. 3A). They also present a

higher swimming speed than TL WT individuals (WT vs. HZ, $p = 0.0026$; WT vs. HOM, $p = 0.0002$; Supplementary Fig. 5A).

In the VMSP we did not observe differences in social preference between genotypes for this line (Fig. 3B, C, and Supplementary Fig. 5B, C). In the first step, all the genotypes prefer to stay close to the group of stranger fish rather than in the opposite corner (1st strangers vs. Opposite area: WT, $p < 0.0001$; HZ, $p < 0.0001$; HOM, $p = 0.0005$; Fig. 3B) and in the second step all the genotypes show an equal preference for both stimulus groups (1st strangers vs. 2nd strangers: WT, $p > 0.99$; HZ, $p = 0.90$; HOM, $p = 0.61$; Fig. 3C). However, in the first step of the test, mutant fish again showed hyperactivity, reflected by more distance travelled (HZ vs. HOM, $p = 0.0282$; WT vs. HOM, $p = 0.0487$; Fig. 3B) and a higher speed of HOM fish compared to TL WT individuals (WT vs. HOM, $p = 0.0130$; Supplementary Fig. 5B).

In the shoaling test, we observed thigmotaxis in *rbfox1*^{sa15940} mutant fish (Supplementary Fig. 5D) and we found differences in the mean interindividual distance (IID), which was higher in HZ and HOM compared to TL WT fish (WT vs. HZ, $p = 0.0194$; WT vs. HOM, $p = 0.0005$; Fig. 3D). No differences were found in the time spent in the white chamber of the black and white test, but HOM fish cross more times the limit between areas, a sign of hyperactivity (WT vs. HOM, $p = 0.0334$; Fig. 3E). Finally, no differences were observed between mutants and WT fish in the aggression test, but HOM fish are significantly more aggressive than HZ fish, as they spend more time exhibiting aggressive behaviour against a mirror (HZ vs. HOM, $p = 0.0083$; Fig. 3F).

Taken together, these results show behavioural alterations in *rbfox1*^{sa15940} mutants in the TL genetic background that include hyperactivity, thigmotaxis and alterations in social behaviour.

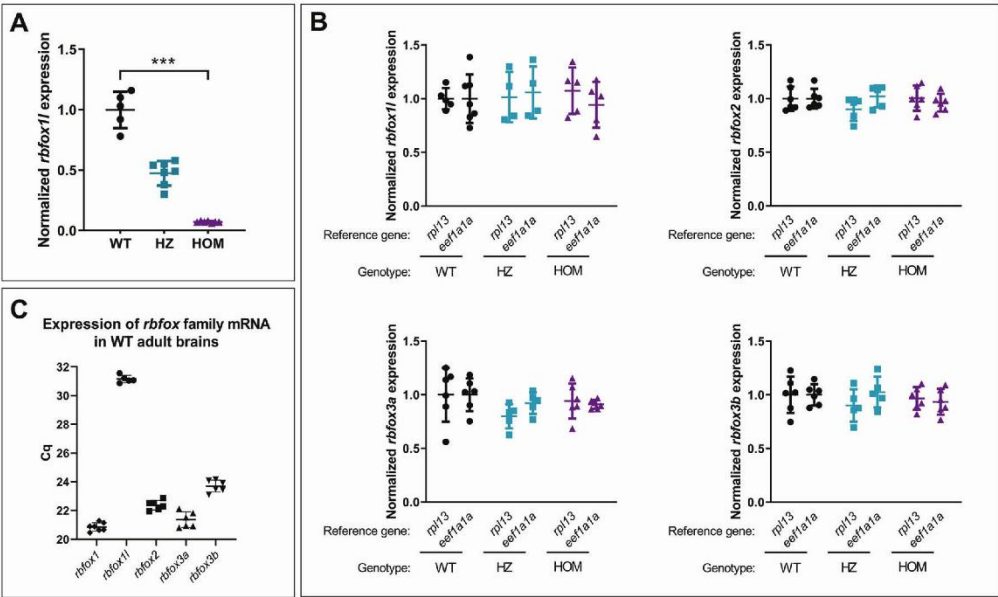
We also investigated potential sex differences in the effects of this *rbfox1*^{sa15940} mutation. In a second batch of experiments, fish were segregated by sex before and during the behavioural tests. We did not observe any significant variations in behaviour between males and females within the mutant groups, and observed only slight differences in locomotion during the initial step of the VMSP among the WT fish, where WT females travelled shorter distances and exhibited lower swimming speeds compared to WT males (Supplementary Fig. 6). In this second batch, encompassing analyses of males, females, and combined groups, the mutant fish did not show hyperactivity or distinct thigmotaxis. However, alterations in social behaviour were evident in both VMSP and shoaling tests (Supplementary Fig. 7A, B and C). Furthermore, we observed behavioural differences between the TL WT groups from the first and second batches (Supplementary Fig. 7D), which could potentially account for the different outcomes obtained in these two batches.

***rbfox1*^{del19} mutation in zebrafish affects behaviour similarly**

We then repeated the battery of behavioural tests in a second *rbfox1* mutant line with a TU genetic background, *rbfox1*^{del19}, to investigate if *rbfox1*-deficiency affects behaviour also in this line. This line was created by using the CRISPR/Cas9 genome editing technique, causing a frameshift deletion of 19 bp in exon 2 that disrupts the *rbfox1* coding sequence and produces a premature stop codon (Supplementary Fig. 2 and 8). We observed behavioural differences between *rbfox1*^{del19} mutants and TU WT fish in all the tests performed, although some of the behavioural changes differed from those obtained for the *rbfox1*^{sa15940} line (Fig. 4 and Supplementary Fig. 9).

Similar to findings in *rbfox1*^{sa15940}, *rbfox1*^{del19} mutants tend to spend less time in the centre than TU WT fish, being significant for HZ fish (WT vs. HZ, $p = 0.0467$, Fig. 4A) and present with thigmotaxis (Supplementary Fig. 9A). However, we also observed differences in behaviour in the open field test between *rbfox1*^{del19} and *rbfox1*^{sa15940} lines: we did not find differences in locomotor activity (nor in distance travelled or speed) and freezing behaviour between genotypes in the *rbfox1*^{del19} line (Fig. 4A and Supplementary Fig. 9A).

Relative expression of *rbfox* family mRNA in adult brains



Relative expression of *rbfox* family mRNA in 3dpf larvae

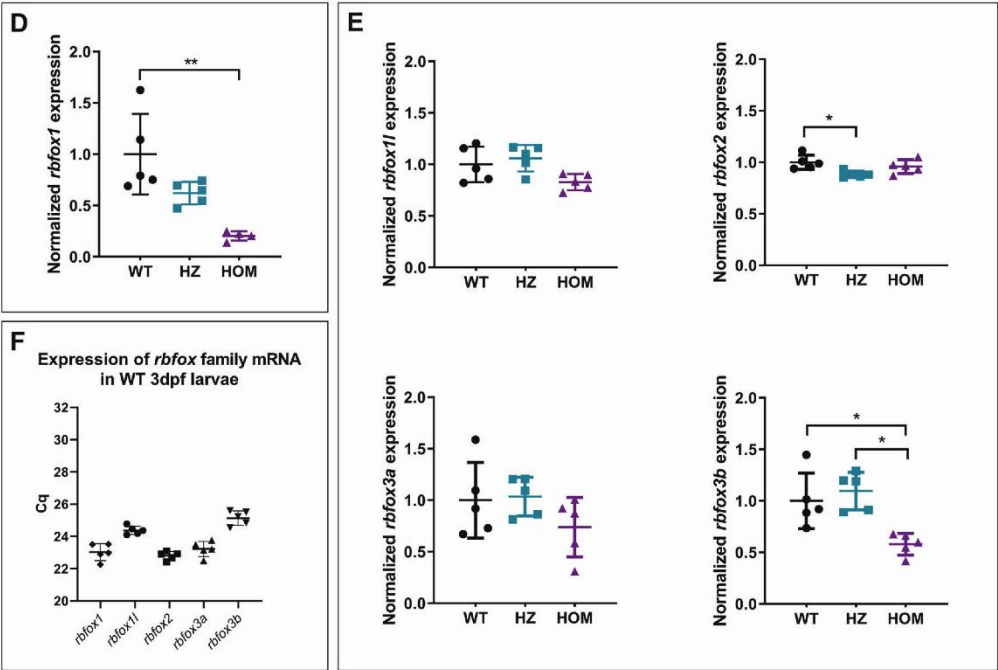


Fig. 2 Relative expression of *rbfox* family mRNAs in adult brains and 3dpf larvae from the *rbfox1^{sa15940}* line. Adult brains: Relative brain expression of (A) *rbfox1*, (B) *rbfox1*, *rbfox2*, *rbfox3a* and *rbfox3b* mRNA in adult brains. mRNA expression is normalised to the average expression of the mRNA in wild-type fish and to the reference housekeeping genes *ribosomal protein L13a* (*rp13*) (A, B) or the *eukaryotic translation elongation factor 1 alpha 1a* (*eef1a1a*) (B). Kruskal-Wallis test followed by Dunn's multiple comparison test. $n = 5-7$ WT, $5-7$ HZ, $5-7$ HOM. Mean $\pm$ SD. C Brain expression of the *rbfox* family mRNAs in adult wild-type zebrafish brains represented as the quantification cycle value for each gene. $n = 7$ WT, 7 HZ, 7 HOM. Mean $\pm$ SD. 3dpf larvae: Relative brain expression of (D) *rbfox1*, (E) *rbfox1*, *rbfox2*, *rbfox3a* and *rbfox3b* mRNA in 3 dpf whole larvae. mRNA expression is normalised to the average expression of the mRNA in wild-type fish and to the reference housekeeping gene *ubiquitin-conjugating enzyme E2A* (*ube2a*). Kruskal-Wallis test followed by Dunn's multiple comparison test. $n = 5$ WT, 5 HZ, 5 HOM. Mean $\pm$ SD. F Brain expression of the *rbfox* family mRNAs in 3 dpf wild-type larvae represented as the quantification cycle value for each gene. $n = 5$ WT, 5 HZ, 5 HOM. Mean $\pm$ SD. Each point represents the results from a pool of 10 larvae.

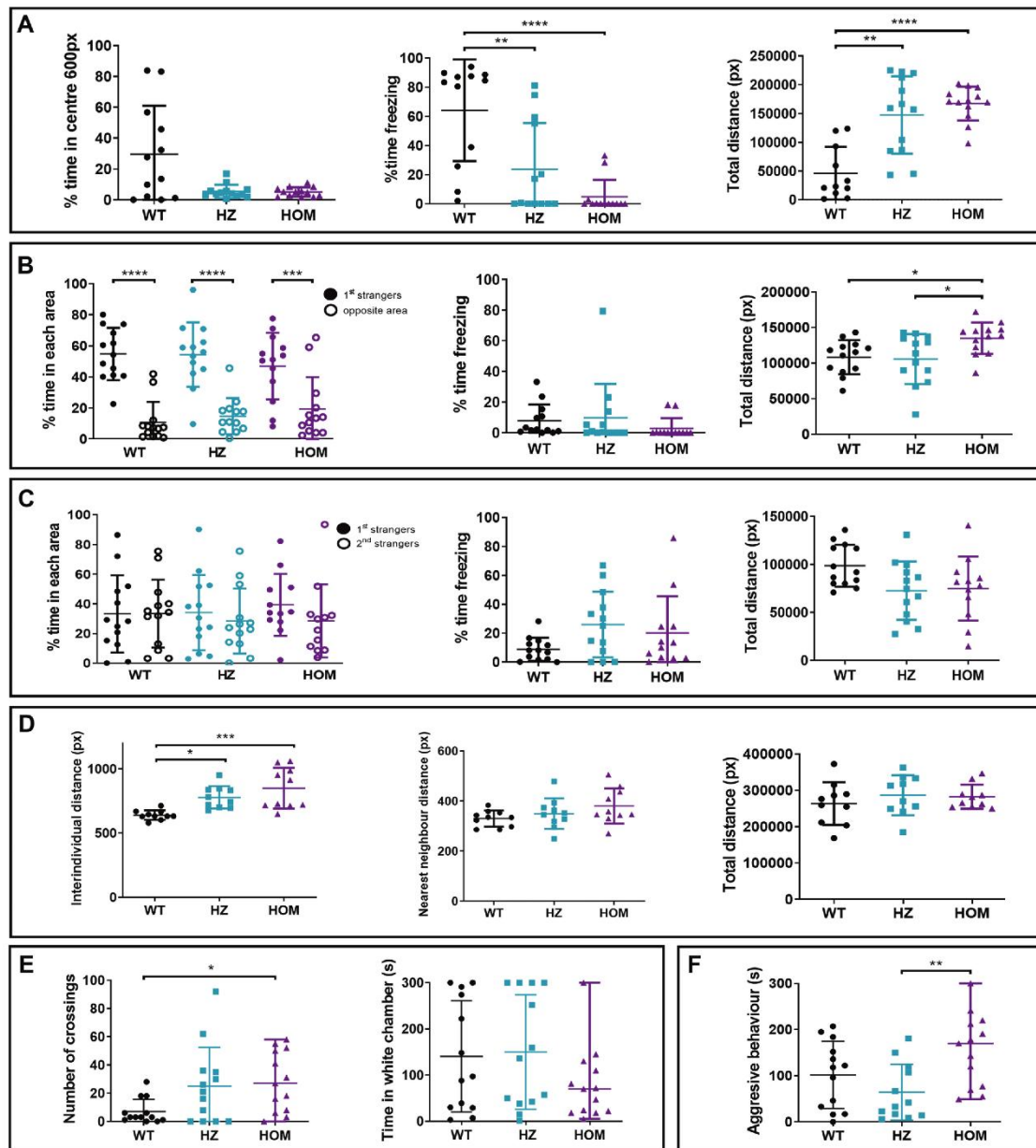


Fig. 3 Behavioural alterations observed in the *rbfox1*^{sa15940} line. **A** Open field test. Time spent in the centre of the arena, time spent freezing and total distance travelled during the open field test. One-way ANOVA followed by Tukey's multiple comparison test. **B** Visually-mediated social preference test (VMSP). Social preference step. Time spent in the area close to the 1st strangers and in the opposite area, time spent freezing and total distance travelled during the social preference step of the VMSP test. Two-way ANOVA followed by Sidak's multiple comparison test. **C** Visually-mediated social preference test (VSNP). Preference for social novelty step. Time spent in the areas close to the 1st or 2nd strangers, time spent freezing and total distance travelled during the preference for social novelty step of the VMSP test. Two-way ANOVA followed by Sidak's multiple comparison test. **D** Shoaling test. Mean of interindividual distance, nearest neighbour distance, cluster score and total distance travelled during the shoaling test. One-way ANOVA followed by Tukey's multiple comparison test and Kruskal-Wallis followed by Dunn's multiple comparisons test. **E** Black and white test. Number of crossings between areas and time spent in the white area of the tank during the black and white test. Kruskal-Wallis followed by Dunn's multiple comparisons test. **F** Mirror test. Time spent exhibiting an aggressive behaviour against the mirror. For all the experiments except for the shoaling test: HOM, *rbfox1*^{sa15940/sa15940} fish; HZ, *rbfox1*^{sa15940/+} fish; WT, wild-type TL. *n* = 13 WT, 13 HZ and 13 HOM for all tests except for the shoaling test. For the shoaling test: *n* = 2 groups of 5 individuals per genotype. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. Mean ± SD.

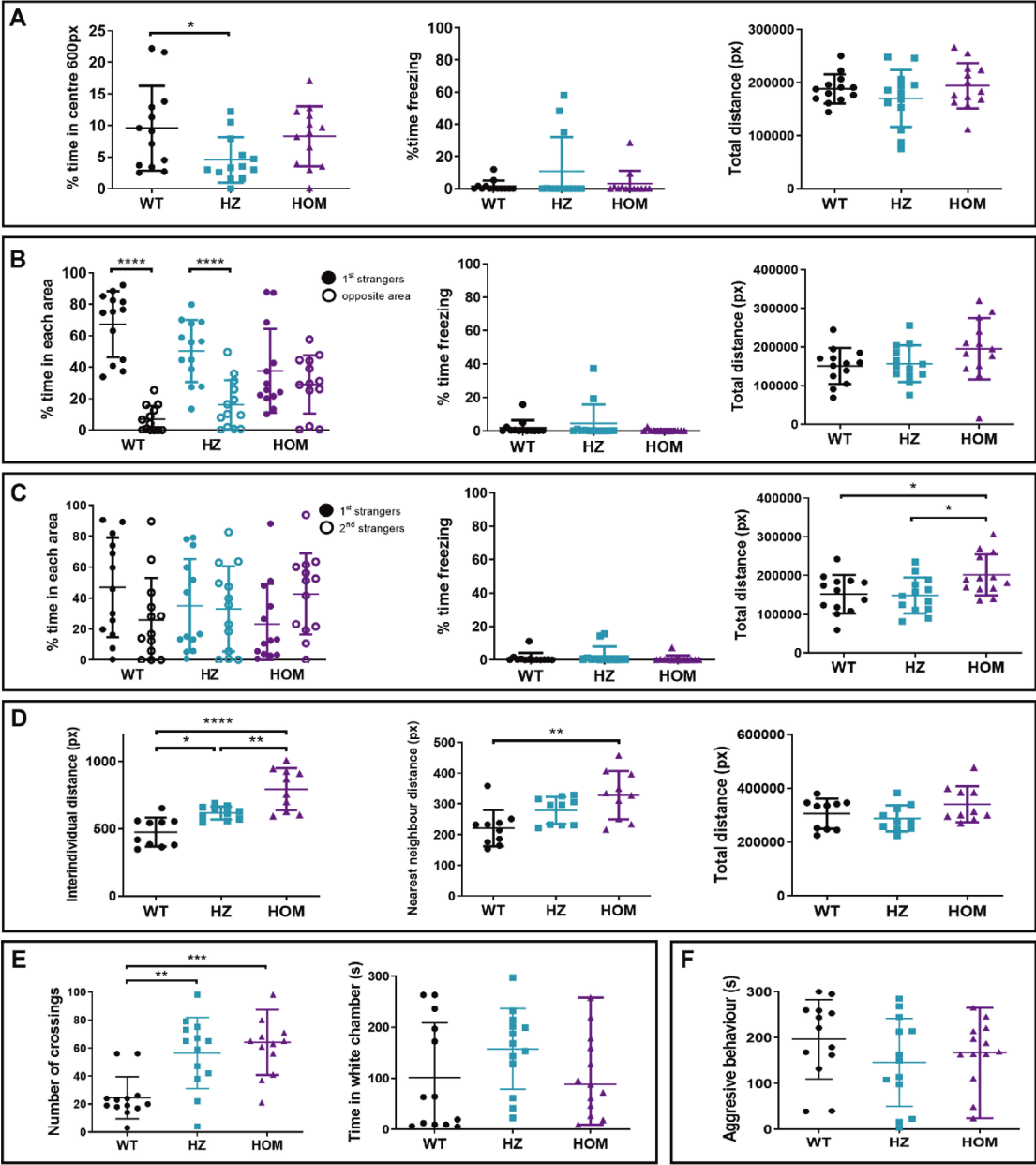


Fig. 4 Behavioural alterations observed in the *rbfox1*^{del19} line. **A** Open field test. Time spent in the centre of the arena, time spent freezing and total distance travelled during the open field test. One-way ANOVA followed by Tukey's multiple comparison test. **B** Visually-mediated social preference test (VMSP). Social preference step. Time spent in the area close to the 1st strangers and in the opposite area, time spent freezing and total distance travelled during the social preference step of the VMSP test. Two-way ANOVA followed by Sidak's multiple comparison test. **C** Visually-mediated social preference test (VMSP). Preference for social novelty step of the VMSP test. Time spent in the areas close to the 1st or 2nd strangers, time spent freezing and total distance travelled during the preference for social novelty step of the VMSP test. Two-way ANOVA followed by Sidak's multiple comparison test. **D** Shoaling test. Mean of interindividual distance, nearest neighbour distance, cluster score and total distance travelled during the shoaling test. One-way ANOVA followed by Tukey's multiple comparison test and Kruskal-Wallis followed by Dunn's multiple comparisons test. **E** Black and white test. Number of crossings between areas and time spent in the white area of the tank following the black and white test. Kruskal-Wallis followed by Dunn's multiple comparisons test. **F** Mirror test. Time spent exhibiting an aggressive behaviour against the mirror. For all the experiments except for the shoaling test: HOM, *rbfox1*^{del19/del19} fish; HZ, *rbfox1*^{del19/+} fish; WT, wild-type TU. *n* = 13 WT, 13 HZ and 13 HOM for all tests except for the shoaling test. For the shoaling test: *n* = 2 groups of 5 individuals per genotype. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. Mean ± SD.

Table 1. Summary of behavioural alterations in *rbfox1*^{sa15940} and *rbfox1*^{del19} mutant zebrafish and *Rbfox1* depleted mice [6].

Behavioural alterations	Zebrafish TL (♀, ♂)	Zebrafish TU (♀, ♂)	Mice (♂)
Hyperactivity	Total distance and swimming speed increased in HZ and HOM fish in the Open Field test Total distance and swimming speed increased in HOM fish in the VMSP test Number of crossings increased in HOM fish in the Black and White test	Total distance and swimming speed increased in HOM fish in the VMSP test Number of crossings increased in HZ and HOM fish in the Black and White test	Total distance increased in HOM mice in the Open field test with and without the presence of a novel object in the centre Total distance increased in HOM mice in the Light-dark box test Total distance increased in HOM mice in the Marble-burying test
Increased thigmotaxis behaviour	Thigmotaxis increased in HZ and HOM fish in the shoaling and Open Field test	Thigmotaxis increased in HZ fish in the Open Field test Thigmotaxis increased in HZ and HOM fish in the Shoaling test	Thigmotaxis increased in HOM mice in the Open Field test Thigmotaxis decreased in HOM mice in the Novel Object Exploration paradigm
Decreased freezing behaviour	Decreased freezing in HZ and HOM fish in the Open Field	No differences found between the genotypes	Decreased freezing in HOM mice in the fear conditioning test in all stages Decreased freezing in HZ mice in the fear conditioning test in extinction phase
Decreased social interest	Increased interindividual distance in HZ and HOM fish in the Shoaling test	Increased interindividual distance in HZ and HOM fish in the Shoaling test Increased nearest neighbour distance in HOM fish in the Shoaling test HOM fish spend less time near the 1st strangers in first step of the VMSP paradigm	Less social interest in social interaction test in the HOM mice
Altered aggressive behaviour	No difference between mutant and control fish in aggressive behaviour in the mirror test, but aggression is increased in HOM fish compared to HZ fish	No differences found between the genotypes	Lack of aggressive behaviour in HOM mice in the escalated aggression test

HOM homozygous individuals for the respective *rbfox1* mutation, HZ heterozygous individuals for the respective *rbfox1* mutation, VMSP visually-mediated social preference test, WT wild-type individuals, ♀ female individuals, ♂ male individuals.

In the preference step of the VMSP test, TU WT and HZ *rbfox1*^{del19} fish show a preference to stay close to stranger fish, whereas HOM *rbfox1*^{del19} fish show no social preference (1st strangers vs. Opposite area: HOM, $p = 0.6979$; Fig. 4B) and spend significantly less time than TU WT fish near strangers and more in the opposite area (WT vs. HOM, $p = 0.0057$; Supplementary Fig. 9B). In the social novelty preference step, we observed similar behaviour in both *rbfox1*^{del19} and *rbfox1*^{sa15940} lines: none of the genotypes show preference for a group of strangers (Fig. 4C and Supplementary Fig. 9C). In line with the *rbfox1*^{sa15940} results, HOM *rbfox1*^{del19} fish present hyperactivity in the two steps of the VMSP test, reflected by a higher speed (WT vs. HOM, $p = 0.0339$; Supplementary Fig. 9B) and a further distance travelled (WT vs. HOM, $p = 0.0380$; Fig. 4C) than TU WT.

We found similar results in both *rbfox1* HOM lines in the shoaling and black and white tests: mutant *rbfox1*^{del19} fish present impaired social behaviour (IID: WT vs. HZ, $p = 0.0235$; WT vs. HOM, $p < 0.0001$; HZ vs. HOM, $p = 0.0047$; NND: WT vs. HOM, $p < 0.0001$; Fig. 4D) and thigmotaxis (Supplementary Fig. 9D) and HZ and HOM *rbfox1*^{del19} performed a higher number of crossings between areas than WT (WT vs. HZ, $p = 0.0040$; WT vs. HOM, $p = 0.0006$; Fig. 4E). Finally, in contrast to HOM *rbfox1*^{sa15940} fish, HOM *rbfox1*^{del19} fish were not more aggressive than HZ fish (Fig. 4F).

In summary, both *rbfox1*^{sa15940} and *rbfox1*^{del19} mutants show hyperactivity, thigmotaxis and impaired social behaviour. However, each *rbfox1* line presents particularities: *rbfox1*^{sa15940} mutants show alterations in freezing behaviour and trends of aggression while *rbfox1*^{del19} mutants have stronger social impairments. The behavioural differences reported between the two

rbfox1 mutant lines might be explained by environmental effects and genetic background differences that modulate *rbfox1* effect on behaviour. Indeed, we can see that some behavioural aspects are different between the two WT lines, as we observe strong differences in the freezing behaviour (Supplementary Fig. 10). Finally, even though discrepancies are reported, the effect of mutations in *rbfox1* on behaviour in these two zebrafish models is in line with previous results found in *Rbfox1*-deficient mice [6], as summarised in Table 1.

DISCUSSION

In this study we have investigated the role of *rbfox1* in neurodevelopmental and psychiatric disorders by studying the behavioural effects of *rbfox1* function in zebrafish. This gene has previously been reported to be highly pleiotropic, contributing to several psychiatric disorders [13, 14, 28]. In addition, we have validated zebrafish *rbfox1*^{sa15940} and *rbfox1*^{del19} HOM lines as models of neurodevelopmental and psychiatric conditions.

First, *rbfox1* shows a restricted expression in brain and heart across developmental stages that suggests an important role of this gene during brain zebrafish development, in line with previous findings. Indeed, a study in human neural progenitor cells demonstrated that *RBFOX1* regulates splicing and expression of large gene networks implicated in neuronal development and maturation [29], and another study showed that *Rbfox1* controls synaptic transmission in the mouse brain [15, 30]. Also, previous studies in mice have shown that specific *Rbfox1* deficiency in the central nervous system leads to impairments in neuronal migration, axon extension, dendritic arborisation and synapse

network formation, suggesting that loss of *Rbfox1* function contributes to the pathophysiology of neurodevelopmental disorders [31–33]. Finally, several point mutations and copy number variations (CNVs) in *RBFOX1* have been described in patients with neurodevelopmental disorders, such as ASD and ADHD [4–6, 10, 34]. We therefore hypothesise that altered *rbfox1* function may affect brain maturation in zebrafish and therefore lead to impaired neuronal function and transmission during adulthood, with implications in the sensory response to the environment and in behaviour.

In addition, *rbfox1* specific expression is found mainly in forebrain areas in adult WT zebrafish, including the dorsal and ventral telencephalon, thalamus and periventricular hypothalamus. Interestingly, these areas are involved in receiving and processing sensory information, stress, and in directing behaviour, especially social behaviour and emotion [35–38]. Given the important role of *Rbfox1* in controlling splicing and expression in neurons, *rbfox1* mutations may induce an impaired neuronal function in these areas with an impact on sensory processing, stress and behaviour in zebrafish.

Interestingly, both *rbfox1*^{sa15940} and *rbfox1*^{del19} HOM lines present alterations in behaviour. *rbfox1*^{sa15940} mutants present hyperactivity, thigmotaxis – a behaviour related to anxiety and stereotypes –, decreased freezing behaviour and altered social behaviour. *rbfox1*^{del19} mutants present similar thigmotaxis, but stronger alterations in social behaviour and lower levels of hyperactivity than *rbfox1*^{sa15940} fish. These results are in line with the behavioural alterations observed in a neuron-specific *Rbfox1* KO mouse line that presents decreased *Rbfox1* expression, as *Rbfox1* KO mice show a pronounced hyperactivity, thigmotaxis and reduced social interest [6]. All these behavioural phenotypes can be assimilated to phenotypic alterations observed in patients with psychiatric or neurodevelopmental conditions. For example, social impairment is a symptom of ASD, hyperactivity of ADHD, and thigmotaxis is considered an anxiety-like behaviour in mouse and zebrafish.

We found differences in behaviour between *rbfox1*^{sa15940} and *rbfox1*^{del19} lines. On one hand, *rbfox1*^{sa15940} is a hyperactive line that presents with thigmotaxis and slight social impairments. On the other hand, *rbfox1*^{del19} fish present also with thigmotaxis, show only hyperactivity in one of the tests performed, and present stronger social impairments than *rbfox1*^{sa15940} fish. The phenotypic variations observed between the two zebrafish lines are likely attributable to genetic background disparities and/or environmental influences, although we cannot rule out differential effects of the two *rbfox1* mutations (e.g., exon skipping in the *rbfox1*^{sa15940} line versus a frameshift deletion in the *rbfox1*^{del19} line). Another factor that may contribute to the behavioural differences found between lines is the tank effect: each genotype was segregated in a separate tank and therefore behaviour could be influenced by the conspecifics present in the tank. In addition, behavioural differences between WT TL and TU strains have been previously reported, WT TL fish being considered more anxious and sensitive to anxiogenic stimuli than TU WT fish [39]. Our results are in line with these reported phenotypes, as we found that TL WT presents a strong freezing behaviour, especially in the open field test, that is not present in TU WT fish. In addition, *Rbfox1* KO mice present behavioural alterations not described in the zebrafish lines such as, lack of aggressive behaviour, and behaviours that could not be tested in our zebrafish lines for practical reasons such as deficit in the acoustic startle response and impairments in fear acquisition [6]. Given the differences observed between the WT zebrafish lines, we hypothesised that the *rbfox1* mutations alter behaviour differently depending on other environmental and genetic effects.

Moreover, when separating *rbfox1*^{sa15940} fish by sex in a second batch of experiments, the results obtained were different from the first batch, although social behaviour was shown to be altered as well. Indeed, the WT fish from the two batches behave differently

in some tests, being more active in the second batch. These differences might be explained again by the influence of the environment and the genetic background. The fish used in this second batch come from a new generation of *rbfox1*^{sa15940} fish that were bred with a different TL WT strain and it is known that zebrafish strains are not completely inbred and genetically well-defined as it is the case with laboratory mice [40], which might lead to variations in the genetic background between these two batches. In addition, housing fish in sex-separated groups before and during the experiments has been described to affect behaviour [41].

These results suggest that, on one side, environmental effects might play a role when assessing behavioural effects of a genetic variation and, on the other side, that the effects of variants in other genes may contribute to the final phenotype, in agreement with a recent proposed genetic model for complex psychiatric disorders composed by ‘hub’ and ‘peripheral’ genes [42–45]. Our results show that the damaging effect of a mutation in *rbfox1* may be modulated by genetic and environmental effects and therefore lead to different phenotypes, which is also in line with the different diagnosis of patients with rare CNVs or point mutations in the *RBFOX1* gene as well as the contribution of common variants to different psychiatric disorders [6, 10, 11, 14, 46].

To conclude, all these results show that altered function of *rbfox1* in zebrafish and mice leads to behavioural alterations that can be related to different neurodevelopmental and psychiatric disorders. Thus, our data contribute to a better understanding of the involvement of *RBFOX1* in psychiatric disorders and point to a pleiotropic contribution of this gene that can be modulated by other environmental and genetic factors. In addition, we have validated two new *rbfox1* HOM zebrafish lines to be used as models for psychiatric disorders, in which further experiments can be performed to unravel the molecular mechanisms that link *RBFOX1* with psychiatric phenotypes.

DATA AVAILABILITY

More detailed data from the current study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

N.F.-C. and B.C. conceived and coordinated the study. N.F.-C. and B.C. designed the experimental approaches for the behavioural experiments. E.A.-G. designed and conducted the behavioural experiments, contributed to the characterisation of the mutant lines and wrote the paper. M.R.A. conducted the second batch of behavioural experiments and the larvae qPCR experiments. J.G.-G. designed and performed the CRISPR/Cas9 experiment. A.L. designed and conducted the ISH experiments and contributed to the characterisation of the mutant lines. L.L.-B. and M.I. contributed to the behavioural experiments. W.H.J.N. contributed to the design of the behavioural experiments. CH.B. supervised the CRISPR/Cas9, ISH and qPCR experiments. All authors discussed and commented on the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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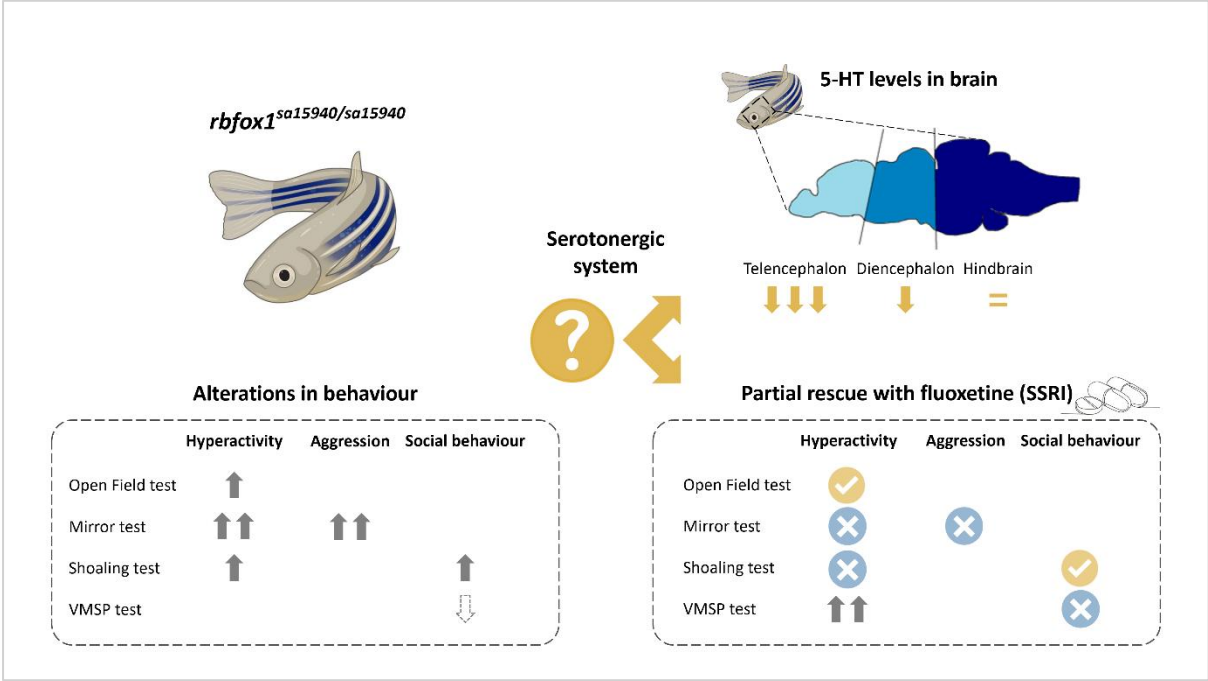
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Chapter 3. Pharmacological interventions in *rbfox1* mutant zebrafish.

Decreased Brain Serotonin in *rbfox1* Mutant Zebrafish and Partial Reversion of Behavioural Alterations by the SSRI Fluoxetine

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Graphical abstract: Decreased Brain Serotonin in *rbfox1* Mutant Zebrafish and Partial Reversion of Behavioural Alterations by the SSRI Fluoxetine



Article

Decreased Brain Serotonin in *rbfox1* Mutant Zebrafish and Partial Reversion of Behavioural Alterations by the SSRI Fluoxetine

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Abstract: *RBFOX1* functions as a master regulator of thousands of genes, exerting a pleiotropic effect on numerous neurodevelopmental and psychiatric disorders. A potential mechanism by which *RBFOX1* may impact these disorders is through its modulation of serotonergic neurotransmission, a common target for pharmacological intervention in psychiatric conditions linked to *RBFOX1*. However, the precise effects of *RBFOX1* on the serotonergic system remain largely unexplored. Here we show that homozygous *rbfox1*^{sa15940} zebrafish, which express a shorter, aberrant *rbfox1* mRNA, have significantly reduced serotonin levels in telencephalon and diencephalon. We observed that the acute administration of fluoxetine partially reverses the associated behavioural alterations. The hyperactive phenotype and altered shoaling behaviour of the *rbfox1*^{sa15940/sa15940} zebrafish could be reversed with acute fluoxetine exposure in the Open Field and the Shoaling test, respectively. However, in the other paradigms, hyperactivity was not diminished, suggesting a distinct intrinsic motivation for locomotion in the different paradigms. Acute fluoxetine exposure did not reverse the alterations observed in the aggression and social novelty tests, suggesting the involvement of other neurological mechanisms in these behaviours. These findings underscore the importance of investigating the intricate working mechanisms of *RBFOX1* in neurodevelopmental and psychiatric disorders to gain a better understanding of the associated disorders along with their pharmacological treatment.

Keywords: *RBFOX1*; psychiatric disorders; zebrafish; fluoxetine; serotonin



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1. Introduction

The *RNA Binding Fox-1 Homolog 1 (RBFOX1)* gene encodes a neuronal splicing factor with a pleiotropic effect on numerous neurological, neurodevelopmental, and psychiatric disorders [1–7]. While functioning primarily as a splicing regulator, *RBFOX1* has also been shown to stabilise target mRNA levels in the cytoplasm, and it controls extensive gene networks implicated in various neurodevelopmental and psychiatric disorders [1,2,8]. *RBFOX1* copy number variants have been associated with epilepsy, autism spectrum disorder (ASD), schizophrenia (SCZ), mild to severe global developmental delay, and intellectual disability [3,4,7]. It has been identified as a candidate gene for aggression in human and rodent studies, directly and through the regulation of other aggression-related genes [4,5]. Furthermore, in a recent cross-disorder study investigating the genetic architecture of eleven psychiatric disorders, including attention-deficit/hyperactivity disorder (ADHD), ASD, anxiety disorders, bipolar disorder, major depressive disorder (MDD), obsessive-compulsive disorder (OCD), and SCZ, among others, *RBFOX1* emerged as an independent

associated locus in genome-wide association study (GWAS) across all eleven psychiatric traits [9]. It was also identified in a subset of internalising disorders and as a higher-order transdiagnostic psychopathology factor. This aligns with a previous GWAS meta-analysis across eight psychiatric disorders where *RBFOX1* was recognised as the second most pleiotropic locus [6]. Recently, the serum levels of *RBFOX1*, along with transcription factor 4 (TCF4), have been proposed as potential biomarkers for depression due to their substantially increased levels in unmedicated patients with MDD [10].

Given its significant role in neurodevelopment and psychiatric disorders, the study of *RBFOX1* has garnered considerable interest in recent years. Various animal models have been developed to investigate the behavioural consequences of alterations in *RBFOX1*. Both zebrafish and mouse models with *rbfox1* mutations exhibit behavioural alterations consistent with the symptomatology of *RBFOX1*-associated disorders [5,7,11]. In the case of zebrafish, *rbfox1* mutant lines (*rbfox1*^{sa15940} and *rbfox1*^{del19}) have been studied for behavioural alterations. The *rbfox1* mutant line *rbfox1*^{sa15940} used in this paper has a point mutation leading to the in-frame skipping of the second exon (or third in isoform *rbfox1*-206), resulting in the expression of a shorter, aberrant mRNA, whereas the *rbfox1*^{del19} line has a deletion of 19 bp, which is also located in the second exon, leading to a frameshift mutation and a premature stop codon [11]. Both lines exhibit hyperactivity and alterations in social behaviour, specifically social novelty preference and/or shoaling behaviour [11]. Additionally, *rbfox1*^{sa15940} mutants display altered aggressive behaviour [11]. *Rbfox1* knock-out mice also demonstrate hyperactivity and altered aggressive and social interaction behaviour, along with stereotyped behaviour and impairments in fear acquisition and fear extinction [7].

Zebrafish serve not only as a valuable tool for studying the contributions and mechanisms of genes in complex psychiatric disorders but also for exploring potential pharmacological treatments. The substantial homology of receptors and enzymes between zebrafish and humans allows for comparable influences of pharmacological interventions, rendering them a time- and cost-efficient model for medical research [12–14]. Selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine, are among the recommended pharmacological treatments for various *RBFOX1*-associated disorders, including MDD, OCD, and anxiety, making them one of the most commonly prescribed classes of medication worldwide for these disorders [14–18]. Fluoxetine has been studied extensively in zebrafish, both with regard to its clinical applications and its potential aquatic toxicity [14,19–22]. As an SSRI, its primary mechanism involves binding to the serotonin (5-hydroxytryptamine, 5-HT) transporter on the presynaptic membrane, thereby preventing 5-HT reuptake. This process elevates 5-HT concentrations in the synaptic cleft, prolonging the activation of postsynaptic receptors [14].

In zebrafish, 5-HT is involved in regulating fear, anxiety, and stress-related behaviours, exhibiting noteworthy variations among zebrafish strains [22]. Additionally, aggressive behaviour and locomotion are influenced by 5-HT, where higher levels are associated with both decreased locomotion and reduced aggression [23,24]. Considering the role of 5-HT in locomotion and aggressive behaviour, we hypothesise that alterations in serotonergic neurotransmission could be implicated in the observed behavioural changes in *rbfox1*^{sa15940/sa15940} zebrafish [11]. However, the effects of *rbfox1* on serotonergic changes and the subsequent behavioural alterations have been largely unexplored. In this study, we investigate the contribution of 5-HT neurotransmission to the *rbfox1*^{sa15940/sa15940} phenotype in adult zebrafish by characterising neurotransmitter levels in the brain and examining the effects of the SSRI fluoxetine on behaviour.

2. Results

2.1. Neurotransmitter Alterations in *rbfox1*^{sa15940/sa15940} Zebrafish

Basal serotonin (5-HT) and tryptophan (Trp) levels were assessed in *rbfox1*^{sa15940/sa15940} and WT adult zebrafish across three distinct brain regions: telencephalon, diencephalon, and hindbrain (Figure 1A). Phenylalanine (Phe) levels served as a normalisation reference.

Prior to normalisation to Phe, 5-HT levels in *rbfox1^{sa15940/sa15940}* zebrafish were significantly lower than in WT zebrafish in the telencephalon and diencephalon ($p_{\text{adj}} < 0.0001$ and $p_{\text{adj}} = 0.0336$, respectively; 5-HT mean $\pm$ SD WT: 0.1140 ± 0.0646 , *rbfox1^{sa15940/sa15940}*: 0.0300 ± 0.0283 and 5-HT mean $\pm$ SD WT: 0.4297 ± 0.1560 , *rbfox1^{sa15940/sa15940}*: 0.2571 ± 0.1765 , respectively) but not the hindbrain ($p_{\text{adj}} = 0.3316$; 5-HT mean $\pm$ SD WT: 0.0324 ± 0.0852 , *rbfox1^{sa15940/sa15940}*: 0.0240 ± 0.0292) (Supplementary Figure S1A). Both Trp and Phe levels did not show significant differences in any region, except for Phe levels in the telencephalon, which were slightly higher in *rbfox1^{sa15940/sa15940}* zebrafish ($p_{\text{adj}} = 0.0104$; telencephalon: Phe mean $\pm$ SD WT: 1.8180 ± 1.1610 , *rbfox1^{sa15940/sa15940}*: 2.5170 ± 0.5780 and Trp mean $\pm$ SD WT: 0.8592 ± 0.5599 , *rbfox1^{sa15940/sa15940}*: 0.8255 ± 0.1139 ; diencephalon: Phe mean $\pm$ SD WT: 3.6900 ± 2.7970 , *rbfox1^{sa15940/sa15940}*: 4.3300 ± 1.7290 and Trp mean $\pm$ SD WT: 1.1610 ± 0.4104 , *rbfox1^{sa15940/sa15940}*: 1.2110 ± 0.8592 ; hindbrain: Phe mean $\pm$ SD WT: 5.4290 ± 2.7690 , *rbfox1^{sa15940/sa15940}*: 5.4880 ± 1.5500 and Trp mean $\pm$ SD WT: 1.7530 ± 0.4270 , *rbfox1^{sa15940/sa15940}*: 1.5880 ± 0.2946) (Supplementary Figure S1B,C). After normalisation to Phe, both 5-HT and Trp concentrations were significantly lower in *rbfox1^{sa15940/sa15940}* zebrafish compared to WT fish in the telencephalon ($p_{\text{adj}} < 0.0001$ and $p_{\text{adj}} = 0.0006$, respectively; 5-HT mean $\pm$ SD WT: 0.0764 ± 0.0528 , *rbfox1^{sa15940/sa15940}*: 0.0118 ± 0.0100 , Trp mean $\pm$ SD WT: 0.5045 ± 0.1467 , *rbfox1^{sa15940/sa15940}*: 0.3384 ± 0.0562) (Figure 1B,C). In the diencephalon, basal 5-HT levels were significantly lower in *rbfox1^{sa15940/sa15940}* zebrafish than in WT fish ($p_{\text{adj}} = 0.0336$; 5-HT mean $\pm$ SD WT: 0.1978 ± 0.1368 , *rbfox1^{sa15940/sa15940}*: 0.0787 ± 0.0768), while Trp levels did not show a significant difference between the groups ($p_{\text{adj}} = 0.0652$, Trp mean $\pm$ SD WT: 0.4090 ± 0.1636 , *rbfox1^{sa15940/sa15940}*: 0.2985 ± 0.0466). In the hindbrain, no differences were detected between the two genotypes in 5-HT or Trp levels ($p_{\text{adj}} = 0.3316$ and $p_{\text{adj}} = 0.1932$, respectively; 5-HT mean $\pm$ SD WT: 0.0142 ± 0.0421 , *rbfox1^{sa15940/sa15940}*: 0.0055 ± 0.0078 , Trp mean $\pm$ SD WT: 0.4455 ± 0.3392 , *rbfox1^{sa15940/sa15940}*: 0.3007 ± 0.0951).

2.2. Partial Reversal of Behavioural Alterations in *rbfox1^{sa15940/sa15940}* Zebrafish with Fluoxetine

Given the significantly lower basal levels of 5-HT in the telencephalon and diencephalon of *rbfox1^{sa15940/sa15940}* zebrafish compared to WT fish, we hypothesised that acute exposure to an SSRI like fluoxetine could potentially rescue some of the previously observed behavioural alterations in the *rbfox1^{sa15940/sa15940}* line, including hyperactivity, aggressive, and altered social behaviour [11].

2.2.1. Open Field

In the Open Field paradigm, *rbfox1^{sa15940/sa15940}* zebrafish exhibited behavioural alterations consistent with prior findings, displaying pronounced hyperactivity [11]. Adult *rbfox1^{sa15940/sa15940}* and WT zebrafish of mixed sexes were exposed to either 0 $\mu\text{g/L}$ (control group), 50 $\mu\text{g/L}$ (C1), or 500 $\mu\text{g/L}$ (C2) fluoxetine for one hour before being tested in the Open Field paradigm. Hyperactivity observed in *rbfox1^{sa15940/sa15940}* zebrafish could be reversed by acute exposure to the higher of the two tested fluoxetine concentrations (C2, 500 $\mu\text{g/L}$) for one hour (Figure 2).

The total swimming distance was significantly higher in the *rbfox1^{sa15940/sa15940}* control group than in the WT control group ($p_{\text{adj}} = 0.0126$). Zebrafish in the *rbfox1^{sa15940/sa15940}* C2 group had a significantly lower total swimming distance than the *rbfox1^{sa15940/sa15940}* control group ($p_{\text{adj}} = 0.0126$), with no significant difference from the WT control group ($p_{\text{adj}} = 0.1058$). The swimming distance in the WT C1 group was significantly higher than in the WT control group ($p = 0.0492$).

Swimming speed in the *rbfox1^{sa15940/sa15940}* control group was significantly higher than in the WT control group ($p_{\text{adj}} = 0.0116$), whereas zebrafish in the *rbfox1^{sa15940/sa15940}* C2 group had a significantly lower swimming speed than the *rbfox1^{sa15940/sa15940}* control group ($p_{\text{adj}} = 0.0116$).

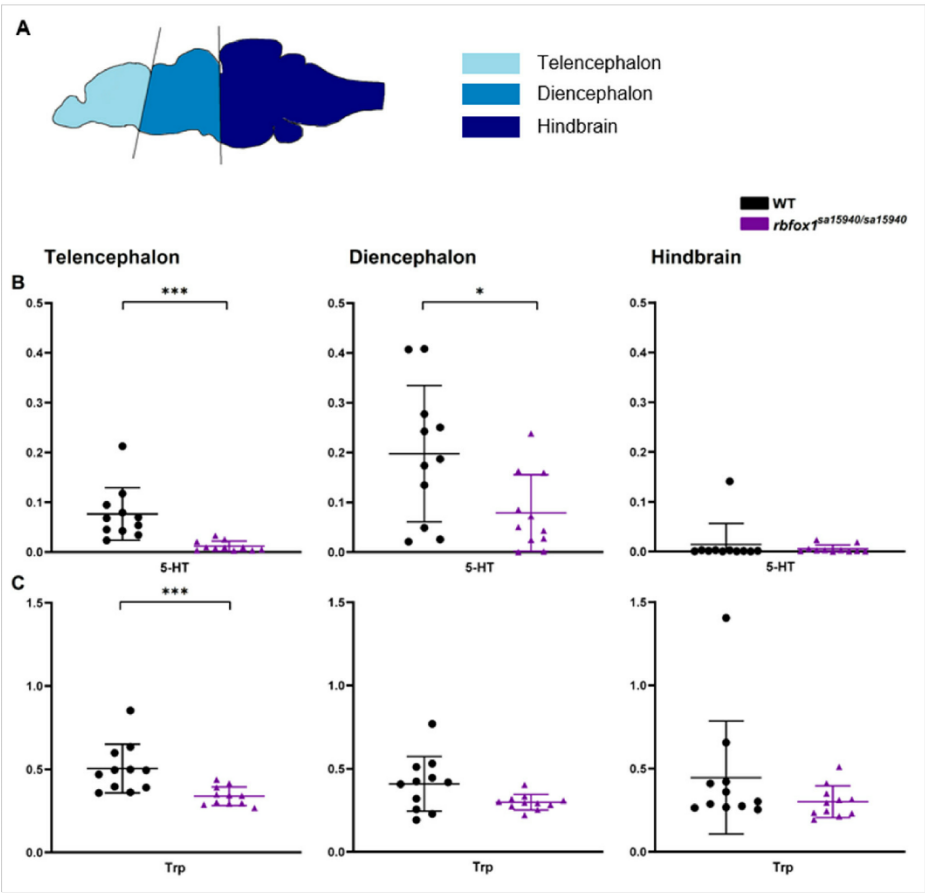


Figure 1. Basal serotonin and tryptophan levels in wildtype and *rbfox1^{sa15940/sa15940}* zebrafish. (B) Serotonin (5-HT) and (C) Tryptophan (Trp) levels of adult zebrafish were measured via ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC/MS-MS) in the telencephalon, diencephalon, and hindbrain (A) and normalised to phenylalanine concentration of the respective sample; WT = wildtype, *rbfox1^{sa15940/sa15940}* = homozygous mutants, 5-HT = Serotonin, Trp = Tryptophan; N = 11 per group; two-tailed Mann–Whitney test; mean $\pm$ SD; * $p_{\text{adj}} < 0.05$, *** $p_{\text{adj}} < 0.001$.

Fish in the *rbfox1^{sa15940/sa15940}* group exposed to C2 of fluoxetine spent significantly more time freezing than the *rbfox1^{sa15940/sa15940}* control group ($p_{\text{adj}} = 0.0140$), but no more than the WT control group ($p_{\text{adj}} = 0.4120$). There was no notable difference in freezing behaviour between the *rbfox1^{sa15940/sa15940}* control group and the WT controls ($p_{\text{adj}} = 0.1718$). There were no significant differences detected in the time spent in the centre of the arena between the groups.

Acute exposure to either of the fluoxetine concentrations did not significantly alter the behaviour of the WT fish apart from the total distance measure in the WT C1 group. In the tested behaviours, no significant differences were found between the *rbfox1^{sa15940/sa15940}* zebrafish exposed to C1 of fluoxetine and the WT or *rbfox1^{sa15940/sa15940}* control groups.

Consequently, we used only the C2 fluoxetine concentration (500 $\mu\text{g/L}$) for subsequent tests.

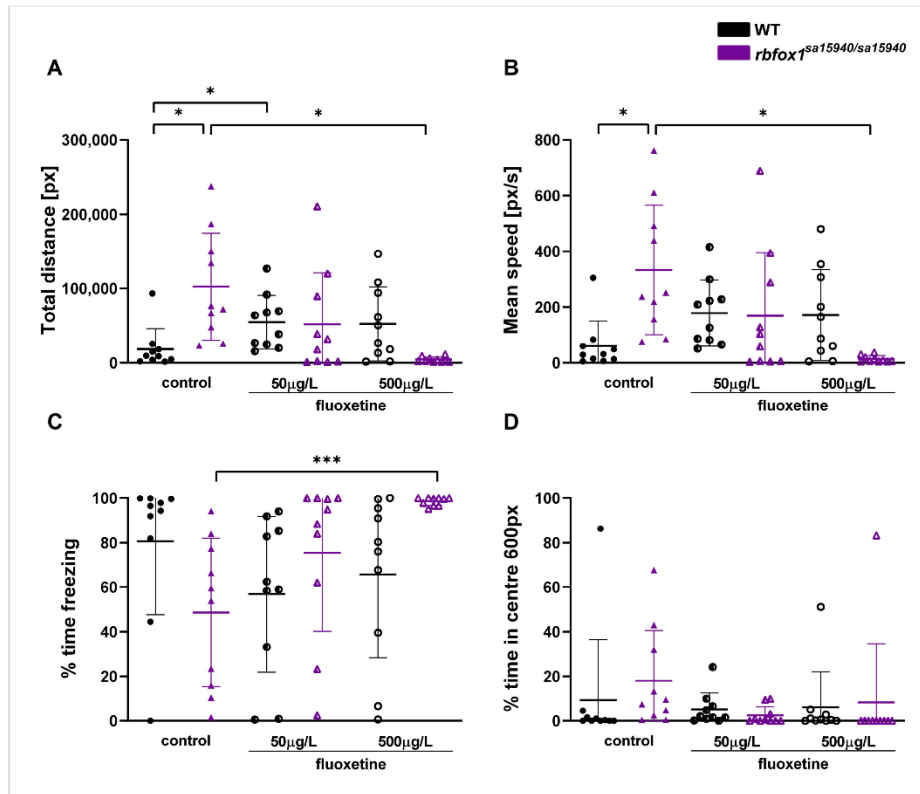


Figure 2. Behaviour of wildtype and *rbfox1*^{sa15940/sa15940} zebrafish after exposure to fluoxetine in the Open Field. Adult zebrafish were tested in the Open Field paradigm after exposure to water (control group) or to different concentrations of fluoxetine for one hour prior to testing. (A) Total swimming distance; (B) mean speed; (C) time spent freezing; (D) time spent in the inner 50% of the arena; WT = wildtype, *rbfox1*^{sa15940/sa15940} = homozygous mutants, control = 0 µg/L fluoxetine, N = 10 per group; permutation two-way ANOVA with interaction followed by *post hoc* permutational analysis using Benjamini and Hochberg correction; mean ± SD; * $p_{\text{adj}} < 0.05$, *** $p_{\text{adj}} < 0.001$.

2.2.2. Aggressive Behaviour of *rbfox1*^{sa15940/sa15940} Zebrafish under the Influence of Fluoxetine

The Mirror test serves as a paradigm for measuring aggressive behaviour (Figure 3). Here, the *rbfox1*^{sa15940/sa15940} control group and the *rbfox1*^{sa15940/sa15940} fluoxetine group exhibited significantly more aggressive behaviour than the WT control group ($p_{\text{adj}} = 0.0038$ and $p_{\text{adj}} = 0.0038$, respectively). The *rbfox1*^{sa15940/sa15940} fluoxetine group exhibited significantly more aggressive behaviour than the WT fluoxetine group ($p_{\text{adj}} = 0.0038$). There was no significant difference in the time spent in the 25% of the arena close to the mirror between groups. Both the *rbfox1*^{sa15940/sa15940} control group and the *rbfox1*^{sa15940/sa15940} fluoxetine group spent significantly less time freezing than the WT control group ($p_{\text{adj}} = 0.0035$ and $p_{\text{adj}} = 0.0035$, respectively). The *rbfox1*^{sa15940/sa15940} fluoxetine group spent significantly less time freezing than the WT fluoxetine group ($p_{\text{adj}} = 0.0035$). Furthermore, the *rbfox1*^{sa15940/sa15940} control group and the *rbfox1*^{sa15940/sa15940} fluoxetine group swam a significantly longer distance than the WT control group ($p_{\text{adj}} = 0.0040$ and $p_{\text{adj}} = 0.0040$, respectively). The *rbfox1*^{sa15940/sa15940} fluoxetine group swam a significantly longer distance than the WT fluoxetine group ($p_{\text{adj}} = 0.0035$).

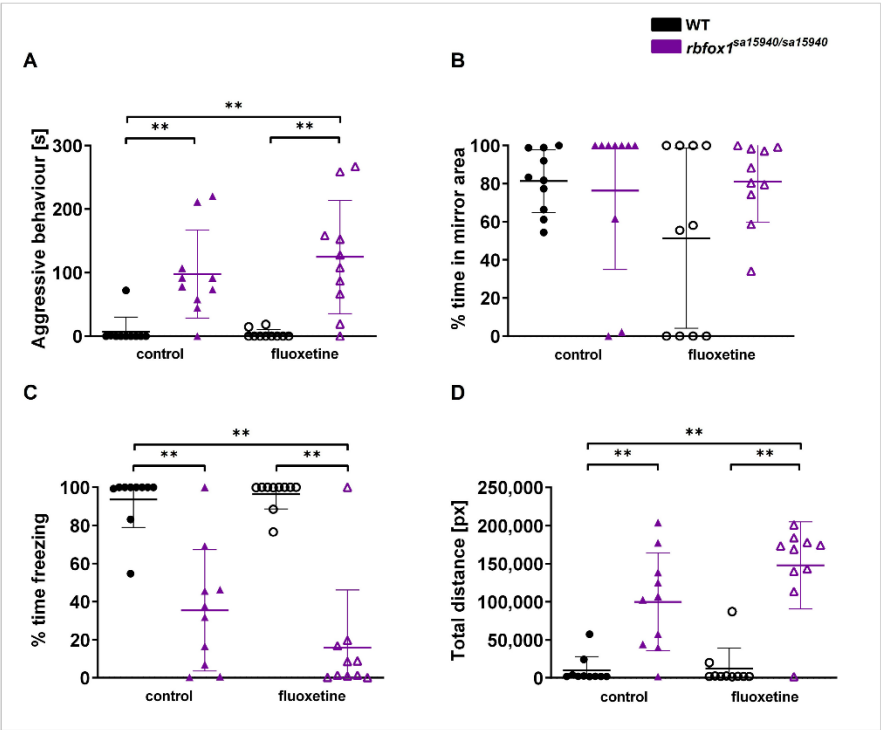


Figure 3. Aggressive behaviour in wildtype and *rbfox1*^{sa15940/sa15940} zebrafish after exposure to 500 µg/L fluoxetine. Zebrafish were tested in the Mirror test to assess aggressive behaviour after exposure to water (control group) or to 500 µg/L fluoxetine for one hour prior to testing. (A) Time displaying aggressive behaviour; (B) time spent in 25% of the arena close to the mirror; (C) time spent freezing; (D) total swimming distance; WT = wildtype, *rbfox1*^{sa15940/sa15940} = homozygous mutants, control = 0 µg/L fluoxetine, fluoxetine = 500 µg/L fluoxetine; N = 10 per group; permutation two-way ANOVA with interaction followed by *post hoc* permutational analysis using Benjamini and Hochberg correction; mean ± SD; ** *p*_{adj} < 0.01.

2.2.3. Social Behaviour of *rbfox1*^{sa15940/sa15940} Zebrafish under the Influence of Fluoxetine

In the Open Field paradigm, exposure to 500 µg/L of fluoxetine was found to reverse some of the behavioural alterations observed in the *rbfox1*^{sa15940/sa15940} zebrafish. We, therefore, sought to determine if these effects extended to paradigms for social behaviour, including the VMSP paradigm in which we had not observed previously differences in this *rbfox1*^{sa15940} mutant line [11].

Social Novelty Preference Test

The Visually Mediated Social Preference (VMSP) paradigm comprises two parts. Part 1 assesses social preference, measuring whether the tested fish prefers to swim closer to a group of three unfamiliar zebrafish (strangers 1) (Figure 4A). In part 2, the social novelty preference step, another group of three novel zebrafish (strangers 2) is introduced, and it is measured whether the tested fish prefers to swim closer to the first or second group (Figure 4B). In part 1, no significant differences were found between the tested groups regarding the time spent freezing and the total distance. Both the WT and the *rbfox1*^{sa15940/sa15940} control group, as well as the *rbfox1*^{sa15940/sa15940} fish exposed to fluoxetine, spent significantly more time close to strangers 1 than the opposite area (*p* = 0.0020, *p* = 0.0078, and *p* = 0.0117, respectively). Although not significant, WT zebrafish exposed to fluoxetine

displayed a similar tendency to spend more time next to strangers 1 ($p_{\text{adj}} = 0.1309$). In part 2, neither the WT control group nor the WT fish exposed to fluoxetine exhibited a clear preference for either group of strangers. In contrast, both the *rbfox1*^{sa15940/sa15940} control group and the *rbfox1*^{sa15940/sa15940} fluoxetine group demonstrated a significant preference for the group of strangers 1 ($p = 0.0195$ and $p = 0.0371$, respectively). Additionally, the *rbfox1*^{sa15940/sa15940} fluoxetine group spent significantly less time freezing than the WT control and WT fluoxetine groups ($p_{\text{adj}} = 0.0065$ and $p_{\text{adj}} = 0.0070$, respectively). The *rbfox1*^{sa15940/sa15940} fluoxetine group also swam a significantly longer distance than the WT control, the *rbfox1*^{sa15940/sa15940} control, and the WT fluoxetine groups ($p_{\text{adj}} = 0.0010$, $p_{\text{adj}} = 0.0298$, and $p_{\text{adj}} = 0.0298$, respectively).

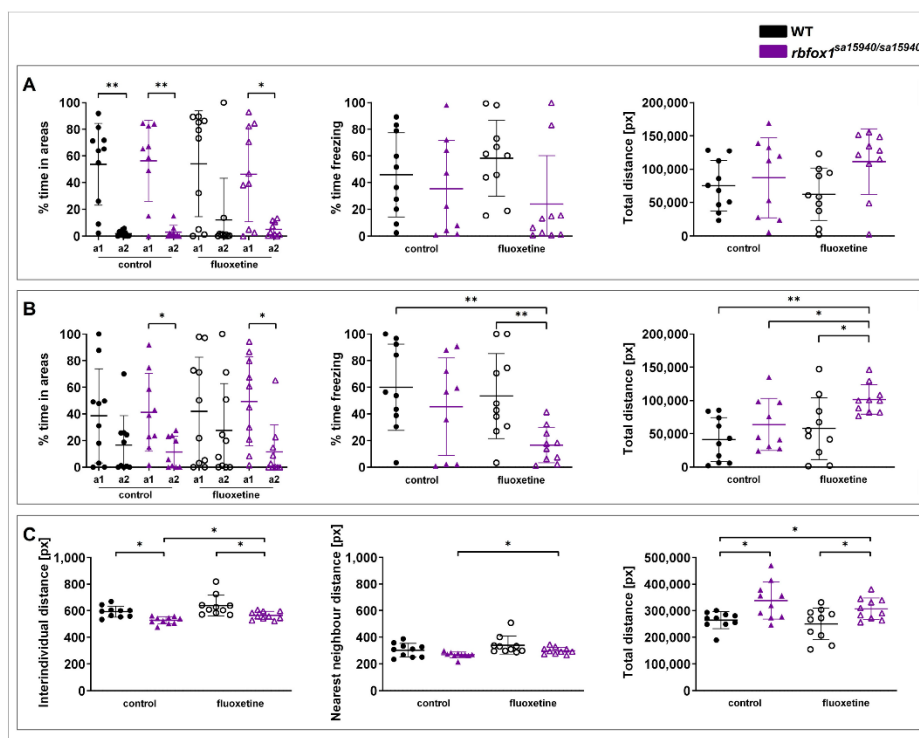


Figure 4. Social behaviour in wildtype and *rbfox1*^{sa15940/sa15940} zebrafish after exposure to 500 µg/L fluoxetine. Zebrafish were tested in the Visually Mediated Social Preference (VMSP) and the Shoaling paradigm to assess social behaviour after being exposed to water (control group) or to 500 µg/L fluoxetine for one hour prior to testing. (A) VMSP part 1 time spent in the zone close to the first group of strangers (a1) versus the opposite area (a2), time spent freezing, and total swimming distance; (B) VMSP part 2 time spent in the zone close to the first group of strangers (a1) versus the area close to the second group of strangers (a2), time spent freezing, and total swimming distance; (C) shoaling interindividual distance, nearest neighbour distance, and total swimming distance; WT = wildtype, *rbfox1*^{sa15940/sa15940} = homozygous mutants, control = 0 µg/L fluoxetine, fluoxetine = 500 µg/L fluoxetine; N = 10 per group for all groups except the *rbfox1*^{sa15940/sa15940} control group in the VMSP (N = 9); permutation two-way ANOVA with interaction followed by *post hoc* permutational analysis using Benjamini and Hochberg correction except for % time in areas: Wilcoxon matched-pairs signed rank test; mean ± SD; * $p/p_{\text{adj}} < 0.05$, ** $p/p_{\text{adj}} < 0.01$.

Shoaling

The Shoaling paradigm measures the behaviour of zebrafish in a group (Figure 4C). Here, the *rbfox1*^{sa15940/sa15940} control zebrafish had a slightly lower interindividual distance (IID) than the WT control group ($p_{\text{adj}} = 0.0118$), a difference not observed in the *rbfox1*^{sa15940/sa15940} fluoxetine group ($p_{\text{adj}} = 0.1193$).

The *rbfox1*^{sa15940/sa15940} fluoxetine group had a slightly higher IID than the *rbfox1*^{sa15940/sa15940} control group ($p_{\text{adj}} = 0.0222$) and a slightly lower IID than the WT fluoxetine group ($p_{\text{adj}} = 0.0118$).

The *rbfox1*^{sa15940/sa15940} fluoxetine group had a slightly higher nearest neighbour distance (NND) than the *rbfox1*^{sa15940/sa15940} control group ($p_{\text{adj}} = 0.0400$), with no significant difference to the WT control group ($p_{\text{adj}} = 0.8470$).

Both the *rbfox1*^{sa15940/sa15940} control and the *rbfox1*^{sa15940/sa15940} fluoxetine group swam a significantly longer distance than the WT control group ($p_{\text{adj}} = 0.0375$ and $p_{\text{adj}} = 0.0417$, respectively). The total distance of the *rbfox1*^{sa15940/sa15940} fluoxetine group was significantly higher than the total distance of the WT fluoxetine group ($p_{\text{adj}} = 0.0417$).

Consistent with our observations in the Open Field paradigm, acute exposure to fluoxetine did not significantly alter the behaviour of the WT fish in any of the tested social paradigms with respect to time spent freezing, total swimming distance, IID, or NND.

3. Discussion

RBFOX1 has been linked to numerous neurodevelopmental and psychiatric disorders, whose treatment usually includes the commonly prescribed SSRI fluoxetine [1,3,8,11,25,26]. This is the first study investigating the link between this gene and serotonergic neurotransmission. In this study, we examined the impact of a *rbfox1* mutation on 5-HT levels in adult zebrafish and explored the extent to which observed behavioural alterations in our *rbfox1*^{sa15940/sa15940} model can be attributed to serotonergic functions. To achieve this, we measured levels of 5-HT and its precursor tryptophan in the brains of adult zebrafish, revealing significantly lower 5-HT concentrations in the telencephalon and diencephalon, along with lower concentrations of tryptophan in the telencephalon in *rbfox1*^{sa15940/sa15940} compared to WT fish. Subsequently, we investigated the effect of the SSRI fluoxetine and observed a partial reversal of the hyperactivity and altered shoaling behaviour of the *rbfox1*^{sa15940/sa15940} zebrafish when acutely treated with fluoxetine. Our study suggests that alterations in 5-HT neurotransmission contribute, to a certain extent, to the phenotype observed in *rbfox1* mutant zebrafish.

3.1. Serotonin Imbalances Associated with the *rbfox1*^{sa15940} Mutation

In our UPLC/MS-MS analysis, we found reduced levels of 5-HT in the telencephalon and diencephalon, as well as reduced levels of tryptophan in the telencephalon in *rbfox1*^{sa15940/sa15940} compared to WT fish. In adult zebrafish, serotonergic nuclei are primarily located in the diencephalon and hindbrain. Unlike mammals, zebrafish also have serotonergic nuclei along the spinal cord [23,27,28]. Serotonergic nuclei, as described by Lillesaar et al. (2011), are located in the diencephalon within the pineal gland, the boundary region of the thalamus and pretectum, the posterior tuberculum, and the hypothalamus [23]. Additionally, serotonergic nuclei in the hindbrain are found in the superior and inferior raphe populations, the reticular formation of the Medulla oblongata, and the area postrema [23]. These nuclei project primarily to different fore- and midbrain areas, with only a few projections reaching some hindbrain regions [23,27]. The neuronal terminals of the projecting neurons are primarily located in the forebrain and diencephalon, which could explain the decreased 5-HT levels in the telencephalon and diencephalon in *rbfox1* mutant zebrafish, but not the hindbrain [23,27,28]. The alterations in the 5-HT levels may have various explanations. One possibility is impaired 5-HT synthesis; we observed reduced basal levels of the 5-HT precursor tryptophan in the telencephalon of *rbfox1*^{sa15940/sa15940} zebrafish suggesting insufficient availability of tryptophan, which, in turn, could impede

5-HT synthesis. However, as the observed differences in Trp levels only become significant after normalisation to Phe, effects other than the availability of Trp could be the true reason for the lowered 5-HT levels.

3.2. Influence of Fluoxetine on Zebrafish Behaviour

In zebrafish, 5-HT is involved in modulating various behaviours, including locomotion, stress, fear, anxiety, appetite, aggression, and social behaviour [14,23]. For this study, we selected the Open Field, the VMSP, the Shoaling, and the Mirror tests, as they have been used in previous investigations of two lines of *rbfox1* mutant zebrafish [11]. Since neither of the two *rbfox1* mutant zebrafish lines used in the aforementioned study by Antón-Galindo et al. (2024) exhibited increased anxiety behaviour in the Black and White test, we omitted this test from the present study. Given the absence of a standardised protocol for testing fluoxetine in zebrafish, we initially examined two different concentrations in the Open Field paradigm [29,30]. We observed an effect only at the higher of the two doses (500 µg/L), which was subsequently chosen for the remaining experiments.

The Open Field paradigm assesses locomotion and thigmotaxic behaviour in a novel environment. We noted pronounced freezing behaviour in WT fish, consistent with prior findings from this strain [11]. The *rbfox1*^{sa15940/sa15940} control group displayed hyperactive behaviour, as previously observed, potentially resulting from the deficiency in 5-HT [11,23,24]. Fluoxetine reduced hyperactive behaviour and increased freezing in *rbfox1*^{sa15940/sa15940} zebrafish, prompting them to exhibit behaviour more akin to WT fish. Exposing WT fish to 50 µg/L fluoxetine led to a significant increase in locomotion; however, this effect was not significant in the WT group exposed to 500 µg/L fluoxetine. Similarly to the Open Field test, the *rbfox1*^{sa15940/sa15940} control group displayed hyperactive behaviour in the Shoaling test, which was slightly decreased in the *rbfox1*^{sa15940/sa15940} fluoxetine group. In contrast to the Open Field test, this hyperactive behaviour was not fully rescued by fluoxetine. Reduced swimming activity in zebrafish exposed to fluoxetine has been reported before, however, these results are not consistent across studies [14]. Interestingly, one study found that 5-HT that is released from the brainstem and spinal cord reduced fictive locomotion in adult zebrafish [24]. An increase in 5-HT concentration induced by fluoxetine in the brains of the *rbfox1*^{sa15940/sa15940} zebrafish could therefore directly impact their locomotion, diminish hyperactive behaviour, and contribute to restoring a wildtype-like phenotype in these paradigms. As the WT fish in this study exhibited strong freezing behaviour under control conditions and after exposure to fluoxetine, it remains unclear whether fluoxetine would lead to a decrease in locomotion in the WT fish.

In contrast to the Open Field and Shoaling tests, exposure to fluoxetine during the second part of the VMSP and the aggression test did not result in a decrease in the total swimming distance in *rbfox1*^{sa15940/sa15940} zebrafish compared to the WT control group. Furthermore, there was no observable increase in freezing in the *rbfox1*^{sa15940/sa15940} fluoxetine group. This discrepancy may be attributed to distinct extrinsic and intrinsic motivations influencing locomotor behaviour in the different tests. The Open Field exposes a fish to a novel arena devoid of external stimulations, while in the Shoaling test, the tested group of fish is familiar with each other as they have been housed in the same tank. However, in the VMSP and the Mirror test, the tested fish encounters groups of strangers and does not recognise itself in the mirror, respectively. The sight of unfamiliar peers may agitate the fish, leading to hyperactive behaviour. In these tests, intrinsic freezing behaviour might be concealed by a general state of arousal induced by the external stimulus of encountering unfamiliar fish, as opposed to the controlled conditions of the Open Field and Shoaling tests [31]. Another possible explanation could be the involvement of additional neurotransmitters in the behaviour observed in the VMSP and Mirror tests, which may not be the primary targets of the SSRI fluoxetine.

In the first part of the VMSP test, WT zebrafish exposed to fluoxetine spent slightly less time with the group of unfamiliar fish. This could be a consequence of decreased social interest following acute exposure to fluoxetine, as demonstrated previously [14]. However, the observed effect in this study was relatively small and may be a statistical artefact.

In the social novelty preference step of the VMSP test, *rbfox1*^{sa15940/sa15940} zebrafish of both the control and the fluoxetine group displayed a slight decrease in social novelty preference, as they spent significantly more time close to the first group of unfamiliar fish. In the Shoaling test, the *rbfox1*^{sa15940/sa15940} control group had a significantly lowered interindividual distance, which was rescued in the *rbfox1*^{sa15940/sa15940} fluoxetine group. The nearest neighbour distance was also slightly increased in the *rbfox1*^{sa15940/sa15940} fluoxetine group compared to the *rbfox1*^{sa15940/sa15940} control group. This could indicate that fluoxetine helps to re-establish a WT-like phenotype in the *rbfox1*^{sa15940/sa15940} fish in these paradigms. Considering the lack of preference for the group of unfamiliar fish in part 1 of the VMSP in the WT fish that were exposed to fluoxetine, these findings could also indicate that fluoxetine lowered the social interest in these groups. However, the observed effects are quite small.

In the aggression test, *rbfox1*^{sa15940/sa15940} zebrafish displayed increased aggressive behaviour both in the control condition and after exposure to fluoxetine. *RBFOX1* has been identified as a strong candidate gene for aggression in human and rodent studies, both directly and through the regulation of the expression of other genes associated with aggression [5,11,25]. *rbfox1*^{sa15940/sa15940} zebrafish from this line have previously shown increased aggression [11]. One possible explanation for increased aggression in *rbfox1*^{sa15940/sa15940} zebrafish could lie in their altered 5-HT levels. Interestingly, innately high levels of 5-HT and acute exposure to fluoxetine have been linked to reduced aggressive behaviour in wildtype fish [21,23]. We found 5-HT levels to be reduced in the *rbfox1*^{sa15940/sa15940} zebrafish, which could be a reason for their increased aggression. However, the exposure to fluoxetine did not lower the displayed aggressive behaviour in the tested *rbfox1*^{sa15940/sa15940} zebrafish. This could stem from various reasons. Although serotonergic functions have been linked to aggression, the aggressive behaviour in *rbfox1*^{sa15940/sa15940} zebrafish may involve other underlying neurological mechanisms, possibly interacting with the serotonergic system, which conceals the effects of fluoxetine on the aggressive behaviour. For instance, the dopaminergic system, in conjunction with the 5-HT system, has been implicated in zebrafish aggression in the Mirror test and in the dyadic interaction of two fish, while in rats and humans, the interaction of the dopaminergic and noradrenergic systems plays a role in aggressive behaviour [32]. Given that *RBFOX1* exerts pleiotropic effects on numerous genes, it is plausible that other *rbfox1* targets outside the serotonergic system, and therefore largely unaffected by the acute fluoxetine exposure, are involved in the aggressive behaviour [5,25]. As both the WT control group and the WT group exposed to fluoxetine spent most of the time freezing, the potential effects of fluoxetine on their behaviour could not be discerned. It is important to note that findings about altered aggressive behaviour differ among *rbfox1* animal models: The *rbfox1*^{sa15940} line used in this study has displayed increased aggression before; however, a *rbfox1* mutant zebrafish line with a TU background did not exhibit altered aggressive behaviour, and male mice in a *Rbfox1* knockdown model even showed decreased aggression [7,11]. This underscores the influence of the genetic background, indicating varied genetic interactions between *rbfox1* and other aggression-related genes. Furthermore, while both zebrafish and *rbfox1* mutant mice were tested for aggressive behaviour, the tests themselves might not be directly comparable. Zebrafish were subjected to the Mirror test, assessing aggressive behaviour toward a perceived stranger in a novel environment, while mice were tested in a resident-intruder test [7]. Therefore, the underlying motivation for aggressive behaviour may vary between these paradigms.

Our study suggests that behavioural changes in *rbfox1*^{sa15940/sa15940} zebrafish may also be influenced by mechanisms other than serotonergic neurotransmission, as not all the phenotypic alterations in mutant zebrafish could be reversed upon exposure to fluoxetine.

Further studies should explore the involvement of neurotransmission systems beyond the serotonergic pathway.

3.3. Limitations of This Study

A limitation of this study is the inability to weigh the extracted brain areas for the UPLC/MS-MS analysis to normalise the neurotransmitters to tissue weight. Additionally, normalisation to total protein content was not feasible since protein measures were low and not reliable. To address this, we normalised the neurotransmitter levels to the corresponding Phe concentration, a method employed previously [33]. In our samples, Phe exhibited no differences between genotypes, maintaining similar levels across regions (proportional to their sizes). Only a slight increase in Phe levels was observed in telencephalon of *rbfox1^{sa15940/sa15940}*. Even prior to normalisation to Phe, 5-HT levels were significantly lower in *rbfox1^{sa15940/sa15940}* zebrafish compared to WT in the telencephalon and diencephalon, indicating robustness of the observed differences in 5-HT between genotypes.

We quantified 5-HT and Trp levels in 3-month-old zebrafish, and behaviour was assessed in 4–5-month-old fish. Although we do not expect differences in adults since there are no significant changes in maturation during this period, we cannot discard them. Furthermore, we were not able to analyse the levels of 5-HT and Trp in the brains of the zebrafish after exposure to fluoxetine and behavioural testing, as, for technical reasons, the brains could not be obtained under the same conditions as the brains of the naïve fish.

Generally, there appears to be a pronounced difference between acute and chronic exposure to fluoxetine, with the latter yielding more consistent and anxiolytic effects [19,30,31]. A limitation of our study lies in that we could only test acute exposure to fluoxetine. Acutely, fluoxetine operates by blocking the serotonin transporter (SERT), thereby limiting the 5-HT reuptake from the synaptic cleft and increasing 5-HT concentrations in the brain [14,19,26,31]. It is possible that in the tested doses of fluoxetine in this study, its acute effects are only measurable in the *rbfox1^{sa15940/sa15940}* zebrafish, given their lower base levels of 5-HT in the telencephalon and diencephalon. Additionally, unlike many studies on fluoxetine in zebrafish that induce acute stress before testing, our study examined the impact of fluoxetine on the “baseline behaviour” of WT and *rbfox1^{sa15940/sa15940}* zebrafish. This difference in approach may explain the lack of similar effects on WT fish compared to other groups. Furthermore, our sample size might have been insufficient to uncover all the effects of the *rbfox1* mutation and/or fluoxetine on the tested behaviours. Adhering to the “three R principle”, we selected the smallest possible sample size based on previous studies, which introduces a potential limitation.

Pooling results from male and female zebrafish is another potential limitation. Many of the psychiatric conditions associated with *RBFOX1* exhibit different prevalence in men and women, possibly involving distinct mechanisms. However, as previous analyses did not reveal behavioural differences between the sexes in the employed paradigms, both male and female zebrafish were tested, and the results were pooled [11]. Thirty to 70% of the tested zebrafish appeared to be female, although it is important to note that the determination of zebrafish sex was based on the morphological assessment by the experimenter, which may be prone to errors due to the visual similarity of male and female zebrafish at this age.

Finally, we did not explore the mechanisms by which the *rbfox1* mutation presented in this study alters serotonin neurotransmission and behaviour. This should be investigated and clarified in future studies.

4. Materials and Methods

4.1. Zebrafish Lines and Husbandry

The zebrafish line employed for behavioural experiments, *rbfox1^{sa15940}* on a Tübingen Long-fin background (TL), was obtained from the European Zebrafish Resource Center of the Karlsruhe Institute of Technology. As described previously [11], this line carries a point mutation at the −2 position of a 3′ acceptor splicing site of the *rbfox1* gene in

the intron before the second or third exon of all annotated isoforms except one (A > T, Chr3:28068329, GRCz11), which results in an exon skipping that leads to a shorter aberrant *rbfox1* mRNA [11]. The expression of the other annotated *rbfox* genes in zebrafish is not affected by this mutation [11]. Homozygous *rbfox1* mutant zebrafish (*rbfox1*^{sa15940/sa15940}) and wildtype fish (WT) of mixed sexes (estimated percentage of female fish between 30 and 70 per test) were used for all experiments. Zebrafish were maintained in tanks at 28.5 °C under a 14:10 h light–dark cycle following standard procedures. Ethical approval for all experiments was obtained from the Animal Welfare and Ethical Review Board of the Generalitat de Catalunya.

4.2. Quantification of Basal Serotonin and Tryptophan Levels

We quantified in the brain the levels of serotonin (5-HT) in the brain, its precursor tryptophan (Trp), and phenylalanine (Phe) used as a reference for normalisation, as previously reported [33]. For that, a total of 11 WT and 11 *rbfox1*^{sa15940/sa15940} zebrafish were euthanised at 3 months of age through immersion in ice water. Their brains were extracted in a Petri dish containing 1X phosphate-buffered saline (PBS) solution using a Leica EZ4D microscope and separated into four areas: telencephalon, diencephalon, hindbrain (Figure 1A), and optic tectum; however, the latter was not used for the analysis. The telencephalon, diencephalon, and hindbrain samples were put into previously frozen 1.5 mL reaction tubes and immediately put into dry ice. Until analysis, they were stored at −80 °C. Samples were sent to Hospital de Joan de Déu to analyse the concentrations of 5-HT, the 5-HT precursor tryptophan, and the amino acid phenylalanine with ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) [34].

The frozen pellets were resuspended in 80 µL of phosphate-buffered saline. Cells were lysed via sonication, using cycles of 10 s (40% amplitude, on ice), and frozen at −80 °C until analysis. After thawing, sample aliquots were vortexed with 25 µL of the internal standard solution and 150 µL of methanol/0.1% formic acid to precipitate the protein samples. They were subsequently centrifuged for 10 min at 10 °C, and amino acid levels were measured in the supernatants by UPLC-MS/MS, as previously described [34]. For 5-HT, 10 µL of the cell supernatants were mixed with 10 µL of the internal standard solution (dopamine D4 as internal standard; Sigma-Aldrich REF 73483). Then, samples were centrifuged at 600 × g for 10 min at 10 °C. Chromatographic conditions; column: CORTECS C18 2.1 × 150, 1.6 µm; mobile phase: water/formic acid (99.5%) and acetonitrile/formic acid (0.5%). All samples were analysed using a Waters ACQUITY UPLC H-class coupled to a Waters Xevo TQD triple quadrupole mass spectrometer using positive electrospray ionisation in the multiple reaction monitoring modes. Together with phenylalanine, tryptophan was also analysed by the same method. The results were normalised to phenylalanine concentration, a reliable technique established by the group [33].

4.3. Drugs

The fluoxetine used in these experiments was solid fluoxetine hydrochloride (SIGMA-ALDRICH, F132-10MG). A fluoxetine stock solution of 10 mg/L in deionised water was previously prepared following adequate safety protocols. On the day of testing, this solution was diluted with water from the zebrafish system to a concentration of 50 µg/L and 500 µg/L fluoxetine for the Open Field experiment, or 500 µg/L for the Visually Mediated Social Preference, the Shoaling, and the Mirror test.

4.4. Behavioural Experiments

Zebrafish aged 4–5 months were tested in four paradigms as described by Antón-Galindo et al. (2024) [11], namely the Open Field, the Visually Mediated Social Preference (VMSP), the Shoaling, and the Mirror tests. Around one week prior to testing, fish were randomly assigned to testing groups based on genotype and kept in separate housing tanks, with the caretakers being blind to the experimental design. Tanks were maintained under

uniform environmental and housing conditions. The sample size required for behavioural experiments was calculated using GPower 3.1 [35].

Before testing, fish were placed in 1 L habituation tanks in the testing room for 55–65 min, exposed to the respective fluoxetine concentration (50 µg/L and 500 µg/L for the Open Field experiment, or 500 µg/L fluoxetine for the Visually Mediated Social Preference, the Shoaling, and the Mirror test), or water from the zebrafish system for the control group. For the Open Field, VMSP, and Mirror test, two fish were placed together in habituation tanks, as isolation might cause stress to zebrafish [36]. For the Shoaling test, five fish were habituated in their corresponding testing group. The testing order was structured to alternate between control groups (WT and *rbfox1*^{sa15940/sa15940} at 0 µg/L fluoxetine) and fish groups exposed to fluoxetine (WT and *rbfox1*^{sa15940/sa15940} at 50 µg/L and at 500 µg/L fluoxetine for the Open Field, and only at 500 µg/L for the Shoaling test, VMSP, and Mirror test). To ensure that there were no traces of fluoxetine left in the arena when starting a new round of testing, the water in the arena was renewed before the first control group of each round of testing.

Fish were recorded during testing with a digital camera and Streampix7 software (Norpix, Single Camera version). Testing arenas were surrounded by white material to reduce distractions and reflection. After testing, fish were placed into a 3 L tank and euthanised in groups of 15–20 fish at a time via immersion in a 1000 ppm Lidocaine solution for >10 min. After the Open Field experiments, the fish were not euthanised using Lidocaine, but by immersion in ice water right after testing.

Tracking analysis of the behavioural experiments was conducted as described by Antón-Galindo et al. (2024) [11]. Tracking utilised idtrackerai scripts (https://gitlab.com/polavieja_lab/idtrackerai, downloaded on 4 January 2021) and the trajectorytools module in Python (<https://github.com/fjhheras/trajectorytools> accessed on 4 January 2021) [37]. In the Mirror test, the time displaying aggressive behaviour was stopped manually.

4.4.1. Open Field

In the Open Field test, WT and *rbfox1*^{sa15940/sa15940} zebrafish were tested under two fluoxetine concentrations (50 µg/L or 500 µg/L) and compared to a control group in water to establish the adequate concentration for the other three paradigms. Each fish was introduced into a circular arena (43 cm diameter, water depth ~6–7 cm) novel to them and recorded for five minutes [11]. Analysed parameters included swimming speed, total distance, time spent in the inner 50% of the arena, and time freezing. A total of 10 zebrafish per group were tested between 10:00 h and 14:00 h.

4.4.2. Mirror Test

To assess aggressive behaviour, the Mirror test was employed, following Norton et al. (2011) [38]. WT and *rbfox1*^{sa15940/sa15940} zebrafish were tested at either 0 µg/L (control) or 500 µg/L fluoxetine. The arena comprised a single white chamber (24 cm × 12 cm, ~10 cm water depth) with a transparent wall at one of the shorter ends. Attached to one of the corners of the transparent wall was a mirror angled at 22°, in which the tested fish could see but not recognise itself. Fish were recorded for five minutes, with analysed parameters including swimming speed, total distance, time freezing, time displaying aggressive behaviour, and time spent in the 25% of the arena close to the mirror. A total of 10 zebrafish per group were tested between 9:00 h and 18:00 h.

4.4.3. Social Novelty Preference Test

In the VMSP test following Carreño Gutierrez et al. (2019) [39], WT and *rbfox1*^{sa15940/sa15940} zebrafish were tested at either 0 µg/L (control) or 500 µg/L fluoxetine. The VMSP arena had five chambers separated by transparent walls, with the central chamber (20 cm × 14 cm, ~10 cm water depth) being flanked by two smaller neighbouring chambers (10 cm × 7 cm each, ~10 cm water depth) on opposite sides (described in Figure 3A,B of Carreño Gutierrez et al. (2019) [39]). Each fish was recorded in two consecutive rounds of test-

ing: In the first round, the social preference step, three unfamiliar zebrafish (strangers 1) were placed in one of the corner chambers. The tested fish was placed in the central chamber and recorded for five minutes. Immediately afterwards, in the social novelty preference step, three more unfamiliar zebrafish (strangers 2) were placed in the corner diagonally opposing the chamber of the first group of fish, and the tested fish was recorded for another five minutes. Total distance, time freezing, time spent in the quadrant close to the group of strangers 1, and time spent in the diagonally opposite quadrant (part 1 of the experiment), which contained the group of strangers 2 in part 2 of the experiment, were analysed. A total of 10 zebrafish were tested between 9:00 h and 18:00 h, except for the *rbfox1*^{sa15940/sa15940} control group, where only 9 zebrafish were tested.

4.4.4. Shoaling

In the Shoaling test, WT and *rbfox1*^{sa15940/sa15940} zebrafish were tested at either 0 µg/L (control) or 500 µg/L fluoxetine. Five zebrafish were placed in the same arena as the Open Field, and, after a five-minute habituation period, they were recorded for ten minutes [40]. Analysed parameters included swimming speed, total distance, nearest neighbour distance (NND), and inter-individual distance (IID). A total of 10 zebrafish were tested between 10:00 h and 14:00 h.

4.5. Statistical Methods and Analysis

In comparing the levels of 5-HT and tryptophan across brain areas, the relative concentrations of 5-HT and tryptophan (normalised to phenylalanine) were compared between WT and *rbfox1*^{sa15940/sa15940} for each brain region. Two-tailed Mann–Whitney tests were conducted using GraphPad Prism (GraphPad Prism version 8.0.2 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com, downloaded on 23 February 2022).

For the behavioural experiments involving fluoxetine, statistical analyses were carried out using GraphPad Prism (GraphPad Prism version 8.0.2 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com), as well as RStudio (R version 4.3.1). For each analysis, a permutation two-way ANOVA with interaction followed by post hoc permutational analysis using Benjamini and Hochberg correction was performed, except for the comparison of the percentage of time spent in the areas in the VMSP. In this case, we performed a Wilcoxon matched-pairs signed rank test. Only significant differences after Benjamini and Hochberg correction were considered and indicated (*p*_{adj}), except for the comparison of the percentage of time spent in the areas in the VMSP, where no correction for multiple testing was applied. In the Open Field test, *rbfox1*^{sa15940/sa15940} and WT fish exposed to two fluoxetine concentrations (C1 = 50 µg/L and C2 = 500 µg/L) were compared to the WT control group. Additionally, *rbfox1*^{sa15940/sa15940} zebrafish exposed to C1 and C2 of fluoxetine were compared to the *rbfox1*^{sa15940/sa15940} control group. For the other tests, *rbfox1*^{sa15940/sa15940} and WT fish exposed to 500 µg/L fluoxetine were compared to the WT control group, and *rbfox1*^{sa15940/sa15940} zebrafish exposed to 500 µg/L fluoxetine were compared to the *rbfox1*^{sa15940/sa15940} control group and the WT fluoxetine group. In the VMSP, the time spent in two areas of the arena containing unfamiliar fish was compared within each group.

5. Conclusions

In conclusion, our study underscores the important role of *RBFOX1* in psychiatric phenotypes, specifically hyperactivity and aggression, and emphasises the involvement of serotonergic neurotransmission in these traits. We highlight the changes observed in 5-HT associated with *rbfox1*, providing partial insight into the behavioural changes in *rbfox1* mutant zebrafish. Additionally, we highlight the pharmacological rescue of hyperactive behaviour in specific contexts by targeting serotonin reuptake. Future research should explore the influence of *RBFOX1* on other neurotransmitters or targets to understand the behavioural changes not accounted for by the identified serotonergic imbalance.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ph17020254/s1>, Supplementary Figure S1: Raw levels of serotonin, tryptophan, and phenylalanine in wildtype and *rbfox1^{sa15940/sa15940}* zebrafish.

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Annex to article 3

Pharmacological interventions in a *rbfox1* zebrafish model targeting the dopaminergic and GABAergic Systems

1 BACKGROUND

1.1 Quinpirole and the dopaminergic system in zebrafish

The dopaminergic system is a highly conserved neurotransmitter system involved in the regulation of locomotor activity, as well as stereotypy, learning, and social behaviour^{1–6}. In humans it has been linked to numerous neurological and psychiatric conditions like attention-hyperactivity deficit disorder, major depressive disorder, Parkinson's disease, schizophrenia, Tourette's syndrome, amongst others^{3,7}. In vertebrates, dopaminergic receptors can be categorized into two major groups, namely D1 and D2 classes of receptors⁸. D1 and D5 receptors belong to the D1-class, whereas D2, D3, and D4 receptors belong to the D2-class.

Quinpirole is a selective D2 and D3 receptor agonist and is used in research for neurological disorders like schizophrenia, anxiety, and obsessive-compulsive-disorder, among others¹. Quinpirole has been shown to alter the behaviour of both adult and larval zebrafish, however, the results have not always been consistent. In zebrafish larvae, both an increase as well as a decrease in locomotion have been reported in response to acute exposure to quinpirole^{1,3,9,10}. Behavioural alterations in adult zebrafish include a decrease in locomotor activity, an increase in anxiety-like behaviour, increased stereotypic swimming patterns, and impairments in learning and memory tests¹.

1.2 Diazepam and the GABAergic system in zebrafish

γ -Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system of vertebrates, acting through phasic and tonic inhibition at postsynaptic and extrasynaptic sites, respectively^{11,12}. GABA regulates the excitability of neural networks mainly through GABA_A receptors, and perturbations in GABA signalling have been linked to a range of neurological diseases in humans¹². The GABAergic system has been reported to be involved in psychiatric disorders such as major depressive disorder, schizophrenia, and autism spectrum disorder, among others, however, reports about the involvement of GABA in these disorders vary^{13,14}. Mammals have 19 different GABA_A receptor subunits, whereas in zebrafish 23 putative GABA_A receptor subunits have been identified¹¹. Nevertheless, the properties of GABA_A receptor subunits appear to be conserved among vertebrates, with $\alpha 1$ being the major isoform¹¹. In zebrafish, GABA_A receptors are involved in locomotion, as well as anxiety-like behaviour^{15,16}.

Diazepam is a commonly used anxiolytic drug with additional anaesthetic, anticonvulsant, muscle relaxing, and sedative effects. This drug exerts its effects by interacting with four different GABA_A receptor subunits^{17,18}. Diazepam has been reported to alleviate anxiety-like behaviour in

zebrafish and reduce locomotor activity, the latter possibly due to light sedation, with the effect of diazepam depending on the dosage and genetic background of the fish^{18–20}.

1.3 The effect of *rbfox1* on zebrafish behaviour

The *RNA Binding Fox-1 Homolog 1 (RBFOX1)* gene encodes a splicing factor with a pleiotropic effect on numerous neurological and psychiatric disorders in humans, and has been a popular target for behavioural studies in murine and zebrafish models^{21–27}. Behavioural alterations in mice and zebrafish include hyperactivity, as well as alterations in social behaviour and aggression^{25,28,29}. A previous study of the group using the mutant zebrafish line *rbfox1*^{sa15940} has recently described that the *rbfox1* mutant fish have lowered levels of serotonin, and that the administration of fluoxetine, a selective serotonin reuptake inhibitor, could partially reverse the behavioural alterations of the *rbfox1* mutant fish²⁹.

In this study, we explore the effects of quinpirole and diazepam, two drugs affecting the dopaminergic and GABAergic systems, respectively, on the behaviour of *rbfox1* mutant and wildtype zebrafish.

2 MATERIAL AND METHODS

2.1 Zebrafish lines and husbandry

For these experiments we used a *rbfox1*^{sa15940} zebrafish line on a Tübingen Long-fin background (TL), which was obtained from the European Zebrafish Resource Center of the Karlsruhe Institute of Technology. This line has an intronic point mutation at the acceptor site of the 3rd exon of the gene *rbfox1*. Zebrafish were maintained in standard conditions. Ethical approval for all experiments was obtained from the Animal Welfare and Ethical Review Board of the Generalitat de Catalunya.

2.2 Quinpirole

The effect of acute exposure to quinpirole on zebrafish behaviour was tested at a concentration of 0.25mg/L quinpirole hydrochloride [Sigma-Aldrich, REF: Q102-10MG] after 1h exposure as previously described by Antón-Galindo et al. (2022)³⁰.

Possible rescue of behavioural alterations observed in homozygous mutant zebrafish (*rbfox1*^{sa15940/sa15940}) compared to wildtype zebrafish was evaluated using the Open Field test²⁸. The tested *rbfox1*^{sa15940/sa15940} and wildtype fish were exposed to either standard zebrafish water (control group) or 0.25mg/L quinpirole hydrochloride (testing group) for 60-65 minutes prior to testing. Thus, four groups of fish were used, considering genotype and treatment, and two rounds of testing with quinpirole were conducted. In the first round of experiments, each group contained 10 fish for testing, with the fish being 5-6 months old. Each group contained 70-90% fish identified as female. In the second round of experiments, each group contained 13 fish, except for the *rbfox1*^{sa15940/sa15940} un-treated group, which had 12 fish. The fish were 5-6 months old, with 30-40% of them being identified as female.

2.3 Diazepam

The effect of acute exposure to diazepam on zebrafish behaviour was tested at a concentration of 0.16mg/L diazepam [Sigma-Aldrich, REF: D0899-100MG] after a 1-hour exposure. This concentration has been tested in previous studies, with short-, medium- and long-term exposure³¹⁻³³.

The possible rescue of behavioural alterations observed in *rbfox1*^{sa15940/sa15940} compared to wildtype zebrafish was measured using the Open Field test as described by Antón-Galindo et al. (2024)²⁸. The tested *rbfox1*^{sa15940/sa15940} and wildtype fish were exposed to either standard zebrafish water (un-treated control group) or 0.16mg/L diazepam for 60-65 minutes prior to testing. Each

group (four in total) contained 12 fish for testing, with the fish being ~5 months old, and a percentage of 30-40% fish were identified as female.

2.4 Statistical analyses

The first experiments with quinpirole as well as the experiments with diazepam were analysed using a permutation two-way ANOVA with interaction followed by *post hoc* permutational analysis. Benjamini and Hochberg correction for multiple testing was used, and only significant differences after correction were considered and indicated (p_{adj}).

The mean and standard deviation were calculated using GraphPad Prism 8 (GraphPad Software, La Jolla California USA). Wildtype and *rbfox1*^{sa15940/sa15940} fish exposed to the respective drugs (testing groups) were compared to the wildtype control group, and the *rbfox1*^{sa15940/sa15940} testing group was compared to the *rbfox1*^{sa15940/sa15940} control group and the wildtype testing group.

3 RESULTS

3.1 The effects of acute exposure to quinpirole on locomotion in adult zebrafish

First round of testing the behavioural effects of acute exposure to quinpirole

Adult zebrafish of both sexes were tested in the Open Field test after an acute exposure to 0.25mg/L quinpirole for one hour prior to testing. There were no significant differences in the total distance travelled, mean speed, or time spent freezing between wildtype and *rbfox1^{sa15940/sa15940}* zebrafish, neither in the treated versus non-treated groups, nor between the genotypes ($p_{\text{adj}} > 0.05$) (Figure 35A-C).

Hyperactivity observed previously in *rbfox1^{sa15940/sa15940}* zebrafish compared to wildtypes^{29,34} could not be detected, as in these experiments wildtype fish show high levels of activity (mean $\pm$ SD of un-treated groups: total distance = 102795 ± 53566 px in wildtypes and 71428 ± 71614 px in *rbfox1^{sa15940/sa15940}* zebrafish, mean speed = 357.3 ± 185.8 px/s in wildtypes and 238.6 ± 238.8 px/s in *rbfox1^{sa15940/sa15940}*) and lower freezing behaviour (mean $\pm$ SD of un-treated groups: freezing = $41.12 \pm 31.62\%$ in wildtypes and $62.35 \pm 39.37\%$ in *rbfox1^{sa15940/sa15940}* zebrafish) compared to wildtype behaviour in previous studies: The total distance travelled by the wildtype zebrafish in the un-treated group was more than twice as high as in Antón-Galindo et al. (2024) and Adel et al. (2024), whereas the time spent freezing was almost half as low^{28,29}.

Interestingly, in the non-treated groups before testing, the *rbfox1^{sa15940/sa15940}* zebrafish spent significantly less time in the centre of the arena than the wildtype fish ($p_{\text{adj}} = 0.0180$) (Figure 35D). This effect was not visible in the group exposed to quinpirole, there was no significant differences between the wildtype and *rbfox1^{sa15940/sa15940}* zebrafish ($p_{\text{adj}} > 0.05$). Between the wildtype fish of the un-treated control group and the wildtype fish exposed to quinpirole, as well as between the *rbfox1^{sa15940/sa15940}* fish of the un-treated control group and the *rbfox1^{sa15940/sa15940}* fish exposed to quinpirole, there was no significant difference in the time spent in the centre ($p_{\text{adj}} > 0.05$).

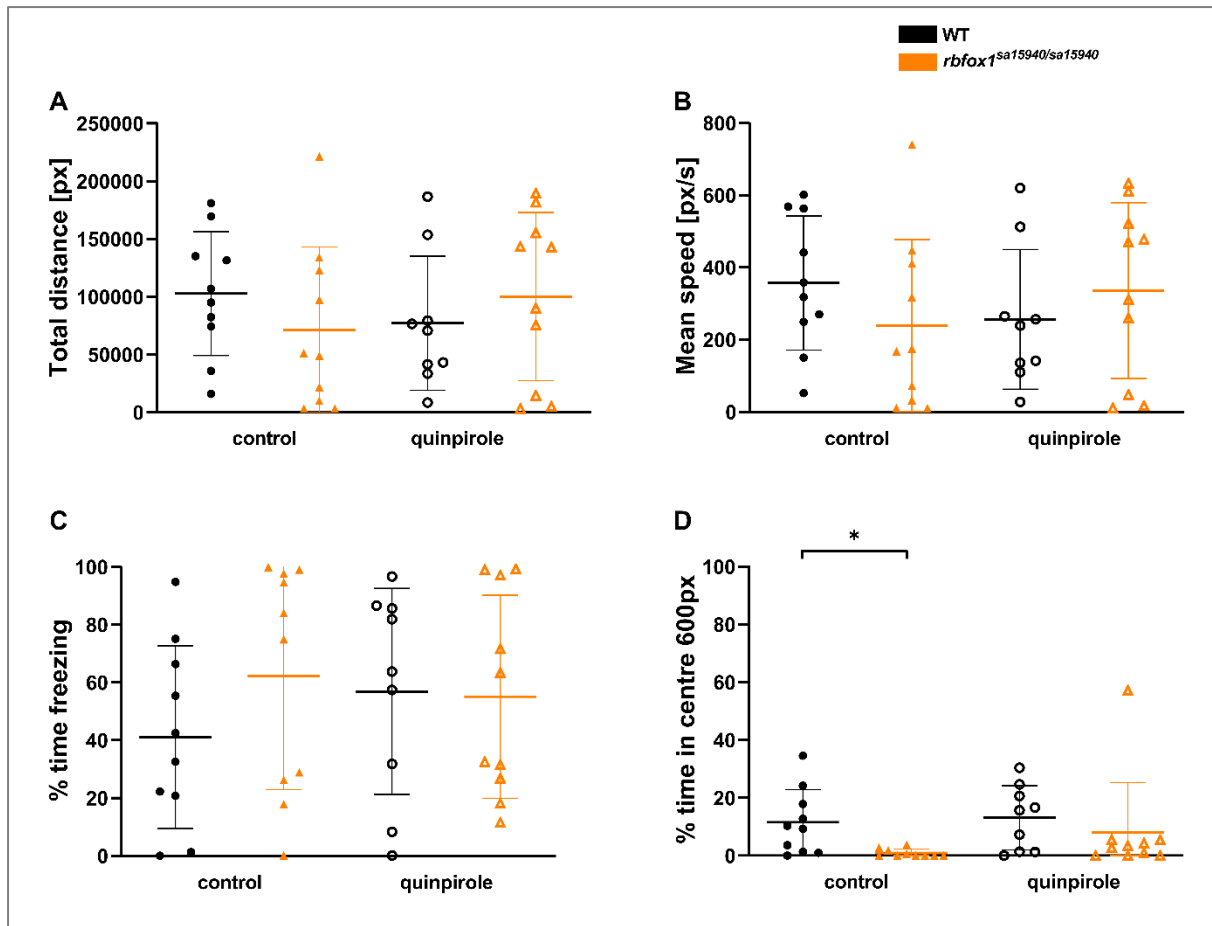


Figure 35: Behaviour of adult zebrafish in the Open Field test after 1h exposure to 0.25mg/L quinpirole. First round of experiments. A: Total distance travelled, B: Mean speed, C: Percentage of time spent freezing, D: Percentage of time spent in the inner half of the arena; control = exposure to zebrafish water, quinpirole = exposure to 0.25mg/L quinpirole. $N = 13$ per group, except for the *rbfox1*^{sa15940/sa15940} un-treated group, which had 12 fish; permutation two-way ANOVA with interaction followed by post hoc permutational analysis using Benjamini and Hochberg correction; mean $\pm$ SD; * $p_{adj} < 0.05$.

Repetition of testing the behavioural effects of acute exposure to quinpirole

After the negative results observed in the previous round of experiments and the fact that wildtype fish showed hyperactivity compared to previous studies of our group, we aimed at repeating the experiments on quinpirole exposure with the same concentrations and conditions.

Similar to the first round of testing, there was no significant differences in the total distance travelled, mean speed, or time spent freezing between wildtype and *rbfox1*^{sa15940/sa15940} zebrafish, neither in the treated group versus non-treated control group, nor between the genotypes, ($p_{adj} > 0.05$) (Figure 36). In this round of testing, there was also no significant difference between the groups in the time spent in the centre ($p_{adj} > 0.05$).

The unusual hyperactivity of wildtype fish observed in the first round of experiments was not observed in this second round (mean $\pm$ SD of un-treated groups: total distance = 34298 ± 28199

px in wildtypes and 73149 ± 57932 px in *rbfox1*^{sa15940/sa15940} zebrafish, mean speed = 113.7 ± 93.45 px/s in wildtypes and 241.3 ± 191.8 px/s in *rbfox1*^{sa15940/sa15940}, freezing = $85.41 \pm 15.32\%$ in wildtypes and $71.44 \pm 27.88\%$ in *rbfox1*^{sa15940/sa15940} zebrafish), as the activity was similar to previous studies^{28,29}. The *rbfox1*^{sa15940/sa15940} showed increased activity compared to wildtype in the non-treated group, but did not reach significance and were considerably lower to previous studies.

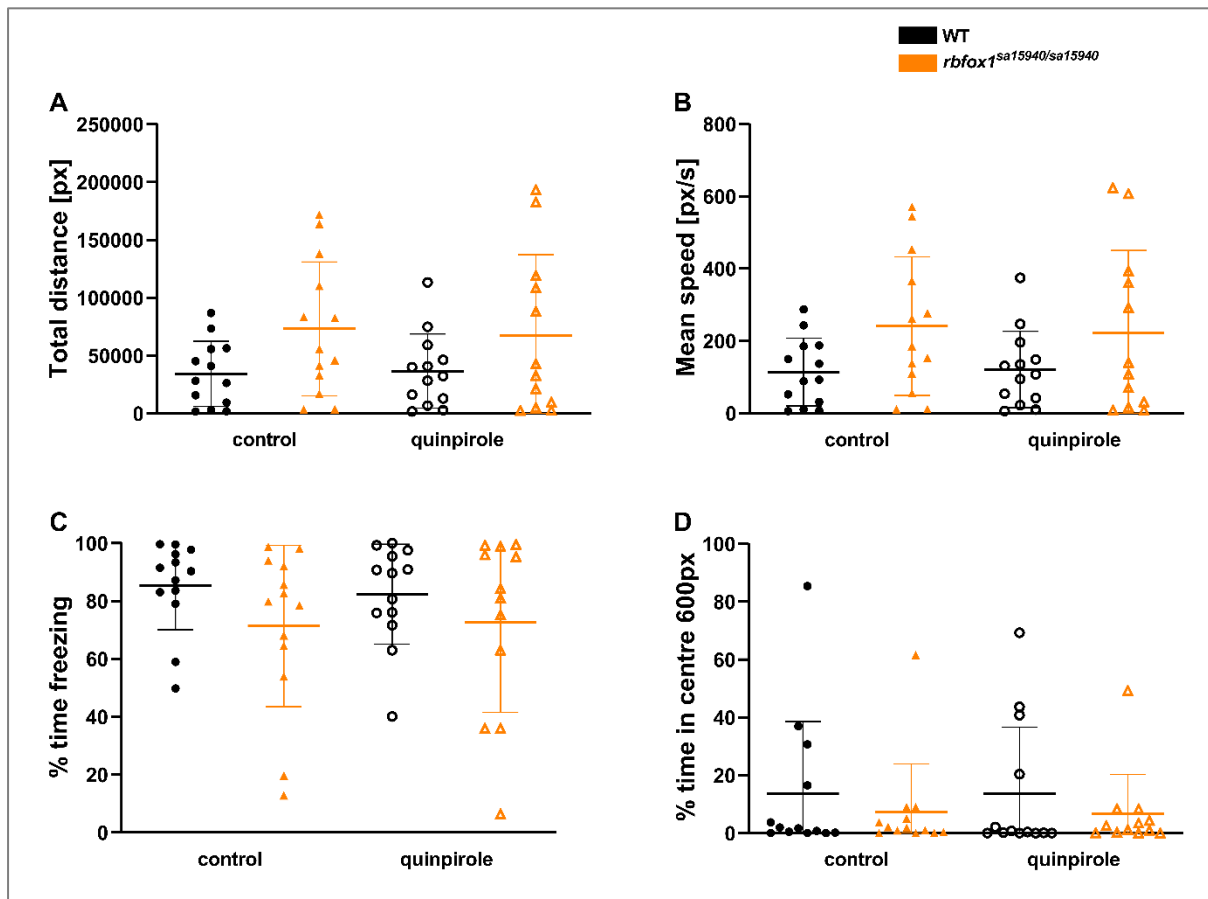


Figure 36: Repetition of behavioural experiments (2nd round) in adult zebrafish in the Open Field test after 1h exposure to 0.25mg/L quinpirole. A: Total distance travelled, B: Mean speed, C: Percentage of time spent freezing, D: Percentage of time spent in the inner half of the arena; control = exposure to zebrafish water, quinpirole = exposure to 0.25mg/L quinpirole. N = 10 per group; permutation two-way ANOVA with interaction followed by post hoc permutational analysis using Benjamini and Hochberg correction; mean ± SD.

3.2 The effects of acute exposure to diazepam on locomotion in adult zebrafish

Adult zebrafish of both sexes were tested in the Open Field test after an acute exposure to 16mg/L diazepam for one hour prior to testing. There were no significant differences between wildtype and *rbfox1*^{sa15940/sa15940} zebrafish, neither in the treated versus un-treated control groups, nor between the genotypes, in the total distance travelled, mean speed, time spent freezing, or time spent in the centre ($p_{\text{adj}} > 0.05$) (Figure 37).

In this case we observed again unusual hyperactive behaviour in the non-treated wildtype fish. The hyperactivity was even more pronounced, with almost a 1.5-fold increase in the total distance travelled than in the first round of quinpirole experiments (Figure 35). The *rbfox1*^{sa15940/sa15940} also showed increased activity compared to the wildtype non-treated group similarly to previous studies (Antón-Galindo et al., 2024²⁸ and Adel et al., 2024²⁹), but did not reach significance (mean ± SD of un-treated groups: total distance = 151479 ± 85045px in wildtypes and 200542 ± 66850px in *rbfox1*^{sa15940/sa15940} zebrafish, mean speed = 495.0 ± 278.2px/s in wildtypes and 657.1 ± 215.8px/s in *rbfox1*^{sa15940/sa15940}, freezing = 34.38 ± 38.55% in wildtypes and 14.12 ± 22.98% in *rbfox1*^{sa15940/sa15940} zebrafish).

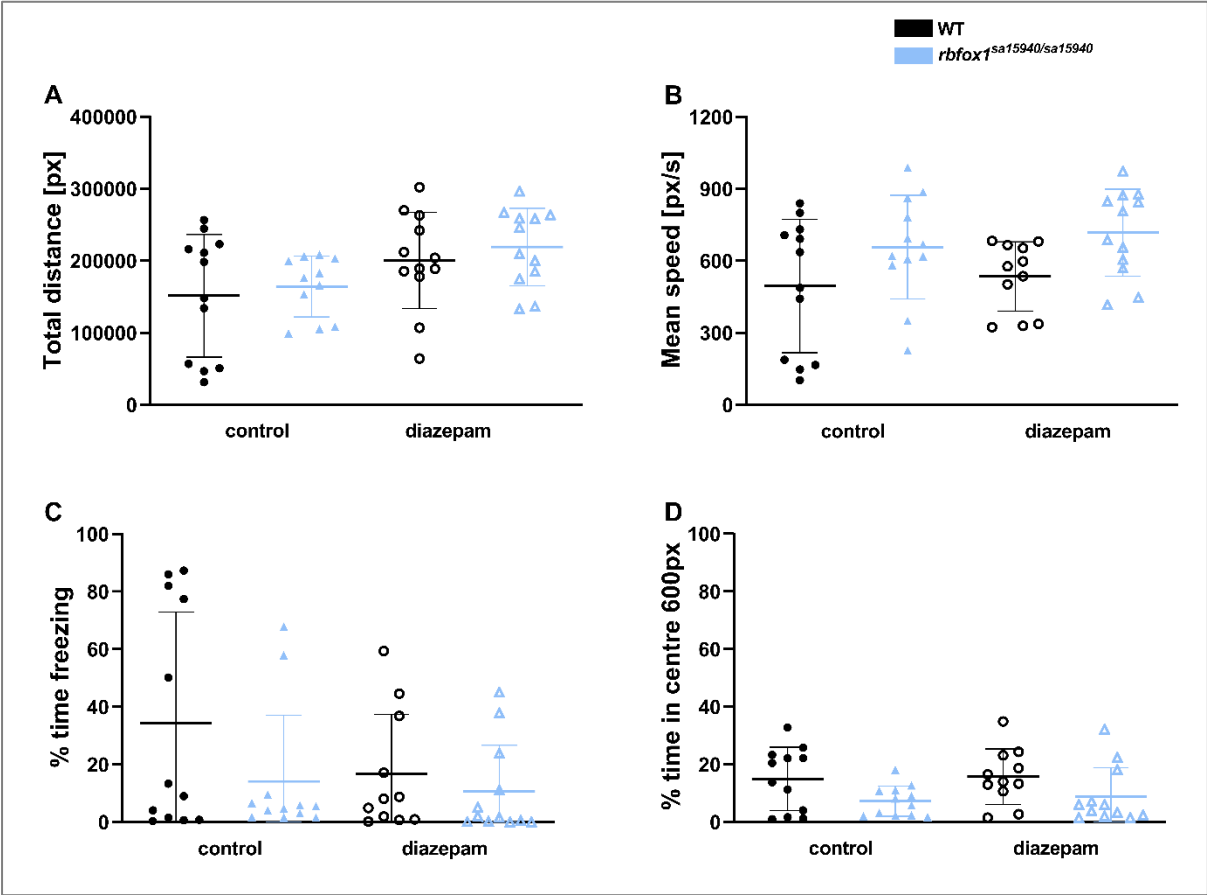


Figure 37: Behaviour of adult zebrafish in the Open Field test after 1h exposure to 16mg/L diazepam. A: Total distance travelled, B: Mean speed, C: Percentage of time spent freezing, D: Percentage of time spent in the inner half of the arena; control = exposure to zebrafish water, quinpirole = exposure to 16mg/L diazepam. N = 12 per group; permutation two-way ANOVA with interaction followed by post hoc permutational analysis using Benjamini and Hochberg correction; mean ± SD.

4 DISCUSSION AND CONCLUSIONS

In this study we aimed to elucidate the effects of two pharmacological treatments targeting the dopaminergic and GABAergic systems, quinpirole and diazepam, respectively, on the behaviour of our *rbfox1*^{sa15940} mutant zebrafish line. Unfortunately, we could not observe differences caused by this treatment on the behaviour of the zebrafish, neither with quinpirole nor diazepam, in either genotype. It is important to mention that differences in the activity between mutant and wildtype fish observed in previous results could not be replicated in these experiments, as the wildtype behaviour was inconsistent across the experiments. Significant findings were only observed in the first round of testing with quinpirole, in which treated *rbfox1*^{sa15940/sa15940} zebrafish spent more time in the centre than the untreated *rbfox1*^{sa15940/sa15940} group. These results could not be replicated in the second round of testing. This could also be partially due to the fact that in the second round, *rbfox1*^{sa15940/sa15940} zebrafish did not significantly spend less time in the centre than the wildtype fish.

Several considerations could explain the observed negative findings, both in replicating the behaviour in wildtype and mutant zebrafish, and when testing the effect of the pharmacological treatments. The behaviour of the wildtype zebrafish in non-treated groups was unusual and not consistent between experiments, as they displayed hyperactivity in the first round of experiments with quinpirole and diazepam, but normal levels of activity in the second round of experiments with quinpirole. Furthermore, although *rbfox1*^{sa15940/sa15940} zebrafish showed hyperactivity in both non-treated and treated groups, this behaviour did not reach significant levels, as it did in previous studies^{28,29}. Additionally, in the experiments with diazepam, *rbfox1*^{sa15940/sa15940} zebrafish showed increased hyperactivity in response to the treatment, however, this did not reach significant levels either. Inconsistency in the behaviour both in wildtype and *rbfox1*^{sa15940/sa15940} fish could be the main reason to explain the negative results to these treatments.

On one hand, even though the fish were kept in the same facility at all times and in principle under the same conditions, including light conditions, water quality, and stocking density, slight variations in the facility, e.g., due to different handling of the fish by different caretakers, are factors we could not control for, but might potentially influence the behaviour of the fish³⁵. Furthermore, even though the zebrafish belong to the same strain, slight variations in the genetic background of the fish could result in different behavioural outcomes. To preserve genetic homogeneity, the fish used for these studies are inbred, and after three generations wildtype fish with the same genetic background and same facility from another group are introduced to avoid inbreeding depression. Still, the inbreeding could result in small differences in the genetic background resulting from increased homozygosity or genetic drift, whereas the introduction of the wildtypes can introduce different genetic variants which can also affect the behaviour, as

zebrafish maintenance is not standardized across the field³⁶. It is furthermore important to note that recent health reports have found the pathogen *Pseudoloma neurophilia* in the zebrafish facility, which could also affect the reproducibility of the behavioural experiments, especially after inbreeding of several generations, however, it is hard to estimate the extent to which this could have played a role during this study³⁷. Another limiting factor of this study is the fact that the sex of the tested fish could only be determined morphologically, which at this testing age is potentially unreliable. Although in previous studies we could not find sex-differences in the tested behaviours, we cannot exclude the possibility that this might have been an influencing factor as well, both on the individual level and in housing group dynamics.

Additionally, the different breeding batches may have slight differences in health or metabolic states that influence their behaviour. Another factor that can influence the behaviour is the stress of social isolation the fish underwent during their genotyping period. During the genotyping, the fish were kept in individual tanks with visual access to the other fish to minimize stress. And while all fish were genotyped several weeks prior to the experiments to avoid this stress affecting the experiments, there is a possibility that some fish might be more affected by this than others. Similarly, different hierarchies of the groups of fish held in the same tanks could potentially also contribute to the behavioural differences in the fish of different batches. Zebrafish are social animals and their behaviour in Open Field tests may be influenced by prior interactions with other fish, both during housing and prior to testing. If social dynamics such as dominance hierarchies, differences between proactive and reactive individuals, or recent interactions, which we could not monitor, vary between housing groups, this could influence their locomotion and anxiety-like behaviour during testing^{35,38,39}.

It is hard to conclusively estimate to which extent variation in the behaviour of zebrafish and failure to replicate findings of behavioural experiments is to be expected, as there is a reported bias for publishing positive results^{40,41}. One study found that more than 50% of scientists had problems replicating their own results in biological studies, and up to 70% could not replicate findings from other groups, however, these reports include different types of studies, not just behavioural experiments⁴¹.

The inconsistency with the effects of quinpirole on the behaviour of the zebrafish is also reflected in the literature, where the effects of quinpirole on zebrafish behaviour are not clearly established. Intraperitoneally injected quinpirole has been shown to affect locomotor activity and anxiety-like behaviour, and exposure to quinpirole during larval stages has been shown to lead to anxiety-like behaviour and erratic swimming patterns during adulthood^{42,43}. However, another study found no effect of quinpirole on the behaviour of AB wildtype zebrafish³⁰. Interestingly, in one study quinpirole has been shown to not increase dopamine levels in the

brain, but significantly increase glutamate and decrease serotonin¹. However, in another study quinpirole did not alter the expression of GABA-related genes⁴⁴.

Similar to quinpirole, the effects of diazepam on zebrafish are not entirely consistent among studies. In one study testing the anxiolytic effect of diazepam on 5dpf old zebrafish larvae in the black-white test, no alleviating effect of diazepam on anxious behaviour was found¹⁷. However, the same study found a lowered swim speed in the with diazepam treated groups, potentially due to altered motor function and/or a mild sedative effect of diazepam on the zebrafish larvae¹⁷. These findings contradict findings from previous groups, potentially due to genetic variation in the zebrafish as well as procedural and environmental differences in the different groups^{17,45,46}. Similar to zebrafish larvae, experiments with adult zebrafish have shown varying effects of diazepam on anxiety-like behaviour: While some studies report an anxiolytic effect of diazepam in zebrafish in the novel-tank diving and the black-white test, other studies could not replicate these results^{18–20}.

The fact that there is no clearly established protocol for testing quinpirole or diazepam in adult zebrafish poses a general limitation for studies using zebrafish to test these drugs. Zebrafish are an increasingly popular model animal for pharmacological testing; however, this testing is often conducted in larvae. As drug response in zebrafish varies across life stages, protocols established for zebrafish in larval stages might not be translatable to adult fish⁴⁷. In addition to this limitation, we could not replicate findings from previous studies, where we found the wildtype fish to exhibit more freezing behaviour, whereas the *rbfox1*^{sa15940/sa15940} fish displayed hyperactivity and alterations in social behaviour, which could partially be reversed with acute exposure to fluoxetine^{28,29}.

One way of gaining more clarity about the behavioural inconsistency we found in this study could be to increase the sample size of the testing groups to increase the statistical power and reduce in-group variability. Furthermore, more concentrations of the different drugs could be tested to see whether they have different effects at different concentrations.

In conclusion, the effect of quinpirole and diazepam could not be properly tested as inconsistent behaviour was observed both in mutant and in wildtype zebrafish in non-treated groups. Further studies are required to evaluate the effect of these drugs on *rbfox1*^{sa15940} mutant zebrafish line.

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Chapter 4. Central nervous system alterations in *rbfox1* mutant zebrafish.

Alterations in neurotransmission, neuronal activity, gene expression and splicing in *rbfox1* mutant zebrafish

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ABSTRACT

The splicing factor *RBFOX1* exerts a pleiotropic effect on numerous neurodevelopmental and psychiatric disorders. In previous studies of two different lines of zebrafish, mutations in *rbfox1* lead to hyperactivity, increased thigmotaxis, and social impairments. These behavioural alterations could be partially reversed by acute exposure to the SSRI fluoxetine. However, the biological mechanisms underlying these behavioural changes are not yet understood. In this study we aim to shed light on the effects of *rbfox1* on neurotransmission, neural activity, and transcriptomic alterations during development and adulthood using a *rbfox1*^{sa15940} mutant zebrafish line. We found a multifaceted disruption of neurotransmission and neural activity, as well as pronounced transcriptomic changes in *rbfox1*^{sa15940} mutant zebrafish. Using UPLC/MS-MS analyses, we detected lower basal levels of the neurotransmitters serotonin, dopamine, GABA, and glutamate, as well as the serotonin precursor tryptophan, in the forebrain of *rbfox1*^{sa15940} mutant zebrafish. *In vivo* whole-brain imaging revealed increased neuronal activity and enhanced synchronicity in midbrain and hindbrain regions of *rbfox1*^{sa15940} mutant zebrafish. Next generation sequencing analyses identified 1062 differentially expressed and 331 differentially spliced genes, with significant enrichment in processes related to cell development in the telencephalon and DNA repair in diencephalon and hindbrain. These transcriptomic changes likely contribute to the observed disruptions in neurotransmission and neural activity. Our results highlight the crucial role of *rbfox1* in regulating the intricate interactions between neurotransmission, neuronal activity, and transcriptomic changes contributing to neurodevelopmental disorders and psychiatric conditions.

1 INTRODUCTION

The *RBFOX1* (*RNA Binding Fox-1 Homolog 1*) gene has a pleiotropic effect on numerous neurodevelopmental and psychiatric disorders. It has been found associated with major depressive disorder (MDD), anxiety, attention-deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), bipolar disorder, and schizophrenia, among others, and has been highlighted in a cross-disorder meta-analysis of different psychiatric disorders^{1–7}. Furthermore, it has been related to epilepsy, and is known to regulate neuronal excitability and susceptibility to seizures^{8–10}. *RBFOX1* encodes a tissue-specific splicing factor with additional cytosolic mRNA stabilizing functions, and is predominantly expressed in brain, heart, and skeletal muscle tissues^{11,12}. It is a regulator for large alternative splicing networks involved in essential neurological and neurodevelopmental processes like neuronal differentiation, migration, maintenance, and excitation^{13–17}. Additionally, *RBFOX1* was found to regulate a downstream network of differentially expressed genes implicated in ASD and other neurodevelopmental

disorders, suggesting an integrated role of both splicing and differential expression regulation in these conditions¹³.

Preclinical studies in different animal models were able to shed light on some of the contributions of *RBFOX1* to psychiatric phenotypes. In mice, the deficiency of this gene leads to hyperactivity and alterations in social behaviour in males, as well as reduced inhibitory synaptic transmission in the cortex^{2,8,17}. Furthermore, increased neuronal excitability, and increased susceptibility to spontaneous seizures were observed in one *Rbfox1* knockout mouse model^{2,8,17}. This aligns with previous studies from our group in two different *rbfox1* mutant zebrafish lines with distinct genetic backgrounds¹⁸. The first line, *rbfox1*^{sa15940}, which is also used in this study, has a Tübingen longfin, also called Tüpfel long fin, (TL) background and a point mutation leading to an exon-skipping event, resulting in a shorter, aberrant mRNA¹⁸. The second line, *rbfox1*^{del19}, has a frameshift mutation leading to a premature stop codon on a Tübingen (TU) background. While both lines showed hyperactivity, indicated by increased locomotion and decreased freezing, as well as thigmotaxis, these behaviours were more prominent in the *rbfox1*^{sa15940} line¹⁸. Both lines also displayed impairments in social behaviours, most notably in reduced interest in social novelty and increased interindividual distance in the shoaling test, both of which were more pronounced in the *rbfox1*^{del19} line¹⁸. Additionally, the *rbfox1*^{sa15940} line displayed increased aggression, which was absent in the *rbfox1*^{del19} line¹⁸.

We furthermore found lowered levels of serotonin and the serotonin precursor tryptophan in the forebrain, i.e., the telencephalon and diencephalon, of homozygous *rbfox1*^{sa15940} mutant zebrafish¹⁹. Exposure to fluoxetine, a selective serotonin reuptake inhibitor commonly prescribed as an antidepressant, partially rescued some of these behavioural alterations. Fluoxetine decreased hyperactivity in the open field test and improved social behaviour by increasing the nearest neighbour distance and reducing inter-individual distance in the homozygous *rbfox1*^{sa15940} mutant zebrafish¹⁹. Interestingly, it did not rescue other alterations in behaviour such as aggression and reduced social novelty interest¹⁹. This suggests that distinct underlying mechanisms and neurotransmitter systems may be driving these behavioural changes, which may not be fully responsive to serotonin modulation alone.

Given the established role of *RBFOX1* in neurodevelopment and its impact on psychiatric phenotypes, as well as the behavioural and neurotransmitter changes observed in the *rbfox1*^{sa15940} mutant line, we aim to further investigate how this mutation affects neurotransmission, neural activity, and transcriptomic changes during development and adulthood.

2 MATERIAL AND METHODS

2.1 Zebrafish husbandry

All zebrafish (*Danio rerio*) used in this study were kept at 28.5 °C in a 14:10 light cycle following standard protocols. The zebrafish used were wildtype, heterozygous (*rbfox1^{sa15940/+}*), and homozygous (*rbfox1^{sa15940/sa15940}*) for the mutation *rbfox1^{sa15940}* with a Tübingen Long-fin (TL) background. The *rbfox1^{sa15940}* mutation contains an intronic point mutation at the -2 position of the acceptor splicing site of the intron before the second or third exon of all annotated isoforms except one (A > T, Chr3:28068329, GRCz11), which results in an exon skipping that leads to a shorter aberrant *rbfox1* mRNA¹⁸. For all experiments male and female zebrafish were used. The neurotransmitter and transcriptomics experiments were performed in adult three months old fish, and the *in vivo* brain imaging in larvae at 6 days post fertilization (dpf). All experiments were approved by the Animal Welfare and Ethical Review board of the Generalitat de Catalunya.

2.2 Neurotransmitter measures in adult *rbfox1* zebrafish

2.2.1 Brain extraction

To quantify the levels of the neurotransmitters and precursors, the telencephalon, diencephalon and hindbrain of wildtype, *rbfox1^{sa15940/+}*, and *rbfox1^{sa15940/sa15940}* zebrafish were extracted and dissected as described by Adel et al. (2024)¹⁹. Three months old zebrafish of the three different genotypes (n = 11 in each group) were sacrificed by immersion in ice water, and their brains were immediately extracted and dissected in a petri dish containing 1X phosphate-buffered saline solution and immediately frozen at -80 °C until further analysis, as described before¹⁹.

2.2.2 UPLC-MS/MS

Several neurotransmitters and precursors (amino acids) were quantified in telencephalon, diencephalon and hindbrain: serotonin, dopamine, gamma-aminobutyric acid (GABA), glutamate, tyrosine, and tryptophan. The levels of these neurotransmitters and amino acids were measured with ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) as previously described²⁰. Phenylalanine (Phe) was also quantified with UPLC-MS/MS for normalisation. For this analysis, frozen pellets were resuspended in 80 µL of phosphate-buffered saline. Cells were lysed by sonication in 10-second cycles at 40 % amplitude on ice. The samples were frozen at -80 °C until analysis. Before measurement, samples were thawed and centrifuged for 10 min at 10 °C. Amino acid levels were measured in the supernatants by UPLC-MS/MS, as previously described²⁰. For GABA analysis, the sample was first incubated with 4 mol/L HCL for 3 hours, and afterwards hydrolysed with 12 mol/L NaOH, using the same

column and mobile phase previously reported²⁰. For dopamine and serotonin, 10 µL of the cell supernatants were mixed with 10 µL of the internal standard solution (dopamine D4 as internal standard; Sigma-Aldrich REF 73483). Then, samples were centrifuged at 600 x g for 10 min at 10 °C. Chromatographic conditions; Column: CORTECS C18 2.1 x 150, 1.6 µm. Mobile phase: water/formic acid (99.5 %) and acetonitrile/formic acid (0.5 %). All samples were analysed using a Waters ACQUITY UPLC H-class coupled to a Waters Xevo TQD triple-quadrupole mass spectrometer using positive electrospray ionization in the multiple reaction monitoring mode.

2.2.3 Statistical analysis

For technical reasons the neurotransmitter levels could not be normalised to the tissue weight. Therefore, they were normalised to the Phe concentration, as reported before²¹. After normalisation to Phe, the relative concentrations of the neurotransmitters were compared between wildtype and *rbfox1*^{sa15940/+} zebrafish and between wildtype and *rbfox1*^{sa15940/sa15940} zebrafish using the Kruskal-Wallis test and corrected for multiple comparison using Dunn's test. The results of the serotonin levels in wildtype and *rbfox1*^{sa15940/sa15940} zebrafish have been previously published, but as this study also includes *rbfox1*^{sa15940/+} zebrafish, the statistical analyses were redone to account for the extra testing group¹⁹.

2.3 Whole-brain imaging *in vivo* in zebrafish larvae

Whole-brain imaging *in vivo* was performed in 6 dpf old zebrafish larvae. The sample size was 12 per genotype, wildtype, *rbfox1*^{sa15940/+}, and *rbfox1*^{sa15940/sa15940}, based on prior studies of the group and literature^{22–24}.

2.3.1 Whole-brain imaging recordings using light sheet microscopy

The imaging was performed using a custom-build Light Sheet Fluorescence Microscopy (LSFM) system in a configuration known as inverted Selective Plane Illumination Microscopy (iSPIM) as described before by Antón-Galindo et al. (2022), with some minor alterations^{22,25}. To avoid movement during the recording, larvae were paralyzed for 8-10 minutes in a 1 mg/mL α-bungarotoxin (Thermofisher), and 0.3 % agarose was added to the E3 medium solution in the fluorinated ethylene-propylene (FEP) tube (0.7 mm inner diameter), where the fish were placed for the recording. Before and during the recording the larvae were kept at 28/29 °C, and recordings of wildtype, *rbfox1*^{sa15940/+} and *rbfox1*^{sa15940/sa15940} larvae were alternated.

Whole-brain imaging was conducted using a custom-built LSFM system configured as iSPIM²⁶. The light sheet was generated by passing the **laser beam** from an ArKr laser (Innova 70C Spectrum, Coherent) through an acousto-optic tuneable filter (AOTFnc-400.650-TN, AAOptoelectronics), which selected the wavelength (488 nm) and controlled the beam power.

The Gaussian beam from the laser was expanded using a Galilean telescope (AC254-050-A-ML and AC254-150-A-ML, Thorlabs) before being directed to the illumination arm of the iSPIM system. This system includes a cylindrical lens ($f = 75$ mm, LJ1703RM-A, Thorlabs) that forms the light sheet, a relay lens (AC254-150-A-ML and AC254-200-A-ML, Thorlabs), and an illumination objective (0.30 NA, 3.5 mm WD, Nikon CFI Plan Fluorite). Precise alignment of the optical elements in their conjugated planes was ensured. A galvanometric mirror (GVS002, Thorlabs) was placed after the cylindrical lens to scan the light sheet perpendicularly to the detection axis. The light sheet at the sample plane had a thickness of **6.10 μm** across the entire field of view, covering a **360 μm** volume of the brain.

The detection path included a **20X objective** (NA = 1, WD = 2 mm, Olympus XLUMPLFLN), a 200 mm tube lens (TTL-200-A, Thorlabs), and a relay lens ($M = 0.5$, AC508-200-A-ML and AC508-100-A-ML, Thorlabs). An electrically tunable lens (ETL, Optotune, EL-16-40-TC-VIS-20D) was placed at the Fourier plane of the relay lens, used to compensate for the light sheet's displacement caused by the galvanometric mirror, ensuring the generated image remained in focus²⁷. A GFP filter (Semrock, FF01-525/45-25) was placed before the sCMOS camera (Hamamatsu Orca-Flash4.0 v3). The total magnification was **10x**, with a field of view of 600 μm and a pixel size of **0.8125 μm** .

The chamber containing a zebrafish embryo was mounted onto a platform connected to a motorized xyz stage (NSA12, Newport). The FEP tube capillary was attached to a stepper motor (L4018S1204-M6, Nanotec) using a screw, enabling precise sample positioning and alignment with the light sheet. Manual adjustments were made for the x, y, and z positions of the detection objective and sample orientation. The region of interest (ROI) was selected once the entire brain of the larva was visible with symmetrical display of both hemispheres (indicating the larva's horizontal position). Once the ROI was selected, fluorescence signal acquisition was performed for **15 minutes** at a scanning speed of **one volume per second**, with **3 ms exposure time** per frame. Each volume comprised **60 optical sections** with a depth of **360 μm** , with **6.10 μm spacing** between planes. The voxel size was **0.8125 μm x 0.8125 μm x 6.10 μm** (x, y, z).

2.3.2 Image segmentation and extraction of calcium signals

The $\Delta F/F$ traces were extracted following the CalmAn pipeline in MATLAB²⁸. Motion correction of the volumetric data was performed before by separating it into time series for each image plane. If motion correction could not be performed, the videos were discarded. Individual planes were processed following a constrained non-negative matrix factorization method (CNMF)²⁹. To avoid signal detection outside the brain, a mask was applied, and ROIs with a size exceeding 2x the mean were filtered out. Finally, the data of each plane was combined into a single dataset per animal ($n = 12$ per genotype).

2.3.3 Single cell, collective burst, and connectivity analysis

Analysis of the fluorescent data was performed using Netcal (www.itsnetcal.com) to obtain single cell and collective burst statistics³⁰. OASIS deconvolution was applied to deconvolute $\Delta F/F$ traces to inferred spikes³¹. With the Z-brain atlas as a reference, the MATLAB Volume Segmenter was used to separate the brain into four areas: forebrain, midbrain, cerebellum, and medulla oblongata (Figure 38)³².

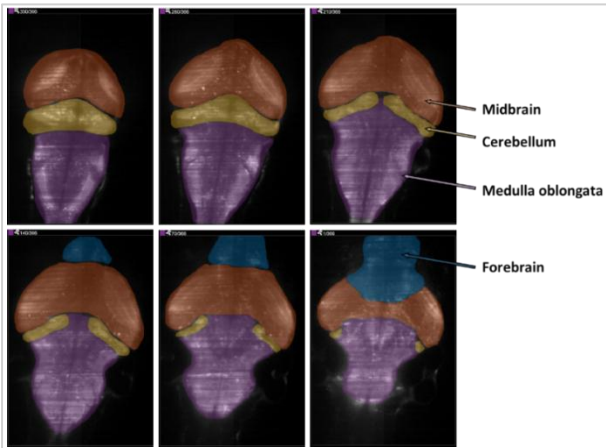


Figure 38: Delimitation of the brain areas. The forebrain, midbrain, cerebellum, and medulla oblongata were defined using the MATLAB volume segmenter. Exemplary picture.

Single cell analysis

The number of active neurons was determined through the above-mentioned CNMF procedure after correction for overlapping ROIs and inactive cells. ROIs were merged if more than 20 % of their volume overlapped and if the Pearson correlation between their fluorescence traces was above 0.7. Neurons were deemed inactive if not more than one spike was found after the deconvolution process.

As previously described, a single cell burst was defined as a set of consecutive spikes within 1 s, based on the imaging framerate (Antón-Galindo et al. 2022)²². The number of active cells, number of spikes, and the fraction of spikes within bursts, i.e., the relative number of total spikes belonging to a single cell burst, were subsequently analysed.

Collective burst analysis

A rise-detection method was used for defining area-wide bursts. For this analysis, the derivate of the firing rate within each neuron was computed using finite differences and averaged across the population, defining the population rise activity. If a substantial increase of activity within the defined area was detected, i.e., if both the average activity and the rise activity overcame a given threshold, which was set based on percentiles of each distribution, a collective burst was identified. Burst statistics including burst duration and amplitude, as well as the bursting rate were then calculated.

2.4 Transcriptomic analyses in adult *rbfox1* zebrafish

RNA sequencing was performed in different brain regions of wildtype and *rbfox1*^{sa15940/sa15940} adult zebrafish. Three months old zebrafish of mixed sexes, six per genotype, were sacrificed by immersion in ice water. Their brains were extracted and dissected into telencephalon, diencephalon, and hindbrain in a petri dish containing 1X phosphate-buffered saline solution and immediately frozen at -80 °C until further analysis, as described before¹⁹. The RNA was extracted from the different brain areas using an RNA extraction kit (Qiagen RNeasy Lipid Tissue Mini Kit, reference number 74804) following the kit protocol, including the DNase digestion and optional centrifugation steps, and stored at -80 °C. Next generation sequencing was performed at the Centro Nacional de Análisis Genómico (CNAG) Barcelona.

Reads were mapped to the *Danio rerio* transcriptome (GRCz11) using Salmon (v1.5.1)³³. The count tables with raw counts and TPMs were imported into R using the R package tximport (v1.32.0)^{33,34} (<https://paperpile.com/c/AIFPYn/P7z4+ys51O>). Only genes with > 20 read counts across all replicates in at least one treatment group (i.e., genotype-region) were kept. Differential gene expression analysis was done with the R package DESeq2 (v 1.44.00) with a model of ~ Genotype for testing effect of the *rbfox1*^{sa15940/sa15940} disregarding the brain region or group (genotype-region) + batch for comparing *rbfox1*^{sa15940/sa15940} vs wildtype in between regions. For differential splicing analyses, reads were mapped with vast-tools align v.2.5.1 to *Danio rerio* genome (danRer10) for alternative splicing analysis³⁵. Events were considered for pair-wise comparisons if quality of the percentage of splice-in (PSI) quality was VLOW or above in at least 80 % of samples, then for each group the event should not be constitutive (> 90 PSI) neither cryptic (< 10 PSI) in more than 50 % of the grouped samples. Filtered events were then compared for differential splicing using a beta regression for proportions with the model PSI ~ Test|1, assuming constant precision with the *betareg* R-package (v3.2.1). For both differential expression and splicing, p-values were adjusted with a false discovery rate (FDR). Events were considered differentially spliced if FDR adjusted p-value was below 0.1 and absolute deltaPSI (*rbfox1*^{sa15940/sa15940} - wildtype) was above 15³⁶.

Enrichment analyses of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were performed on the differentially expressed and spliced, using the WEB-based GENE Set Analysis Toolkit (WebGestalt) 2024³⁷. For the GO analysis the category “geneontology - Biological Process noRedundant” was chosen, and for all analyses the reference set “genome protein-coding” was selected. The redundancy removal method “weighted set cover” was used, and the multiple test adjustment was set to Benjamini Hochberg (BH) with an FDR cut-off of 0.05 (5 % FDR).

3 RESULTS

3.1 Serotonin, dopamine, GABA, and glutamate levels are reduced in forebrain regions of *rbfox1* mutant zebrafish

We measured the levels of the neurotransmitters serotonin, dopamine, GABA and glutamate, as well as the precursors tryptophan and tyrosine, in the telencephalon, diencephalon, and hindbrain of adult wildtype, *rbfox1*^{sa15940/+}, and *rbfox1*^{sa15940/sa15940} zebrafish (Figure 39, Supplementary Figure 1). We observed decreased levels of all measured neurotransmitters in the telencephalon and diencephalon of *rbfox1*^{sa15940/+} and/or *rbfox1*^{sa15940/sa15940} zebrafish compared to wildtype (Figure 39, Supplementary Table 1). In contrast, no significant differences were found in the hindbrain.

Among the analysed neurotransmitters, serotonin levels showed the most substantial decrease in *rbfox1* mutant zebrafish compared to wildtype, particularly in the telencephalon (FC = -12.35, $p_{\text{adj}} = 2.3\text{e-}05$ in *rbfox1*^{sa15940/+} and FC = -6.46, $p_{\text{adj}} = 9.9\text{e-}04$ in *rbfox1*^{sa15940/sa15940}). Dopamine levels were also notably reduced in this region (FC = -3.93, $p_{\text{adj}} = 3.3\text{e-}04$ in *rbfox1*^{sa15940/+} and FC = -2.70, $p_{\text{adj}} = 4.12\text{e-}02$ in *rbfox1*^{sa15940/sa15940}), followed by GABA (FC = -2.13, $p_{\text{adj}} = 5.5\text{e-}04$ in *rbfox1*^{sa15940/+}) and glutamate (FC = -2.0, $p_{\text{adj}} = 3.0\text{e-}04$ in *rbfox1*^{sa15940/+} and FC = -1.78, $p_{\text{adj}} = 1.17\text{e-}02$ in *rbfox1*^{sa15940/sa15940}) (Figure 39, Supplementary Table 1).

In the diencephalon, serotonin (FC = -4.2, $p_{\text{adj}} = 4.37\text{e-}02$), dopamine (FC = -4.2, $p_{\text{adj}} = 1.02\text{e-}02$), and glutamate (FC = -2.4, $p_{\text{adj}} = 1.25\text{e-}02$) levels were significantly reduced only in *rbfox1*^{sa15940/+} zebrafish compared to wildtype. In contrast, only *rbfox1*^{sa15940/sa15940} zebrafish showed a significant decrease in GABA levels in this region (FC = -2.96, $p_{\text{adj}} = 2.0\text{e-}03$) (Figure 39, Supplementary Table 1).

Additionally, levels of tryptophan, the precursor of serotonin, were significantly lower in the telencephalon of both *rbfox1*^{sa15940/+} and *rbfox1*^{sa15940/sa15940} fish, as well as in the diencephalon of *rbfox1*^{sa15940/+} zebrafish, compared to wildtype (Supplementary Figure 1A; Supplementary Table 2). No significant changes were observed in the levels of the dopamine precursor tyrosine across the tested groups (Supplementary Figure 1B, Supplementary Table 2).

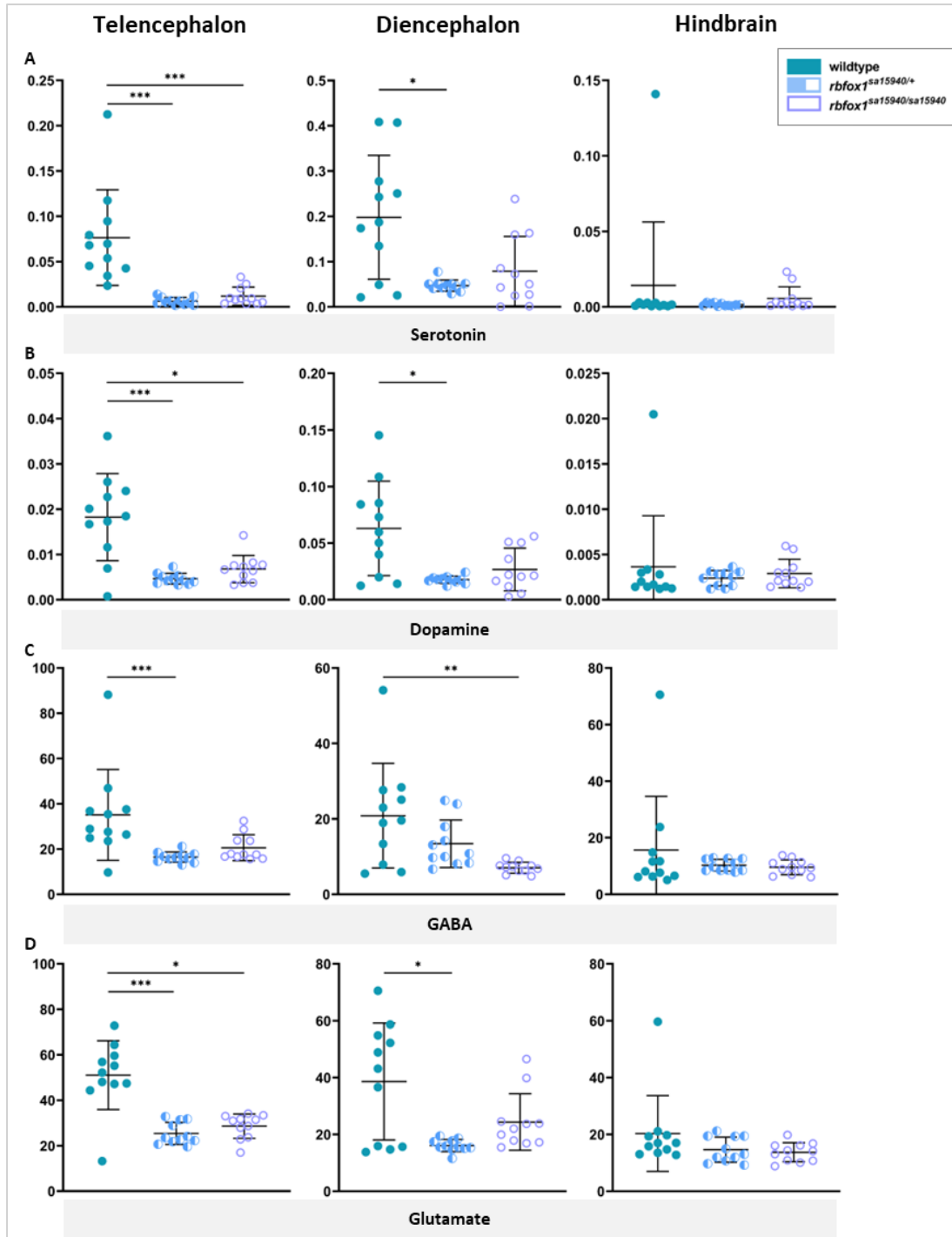


Figure 39: Alterations in neurotransmitter levels in telencephalon and diencephalon of *rbfox1* mutant zebrafish. Levels of A serotonin, B dopamine, C GABA, and D glutamate measured via ultra-high-performance liquid chromatography-tandem mass spectrometry and normalised to the phenylalanine concentration of the respective sample. $N = 11$ per group; Kruskal-Wallis test with Dunn's correction for multiple testing; mean $\pm$ SD; * $p_{adj} < 0.05$, ** $p_{adj} \leq 0.01$, *** $p_{adj} \leq 0.001$

3.2 Increased neuronal activity in brain regions of *rbfox1* mutant zebrafish

To investigate the effect of the *rbfox1*^{sa15940} mutation on neuronal activity, we performed *in vivo* whole brain imaging in 6 dpf old larvae. We observed an increased number of active neurons in

the midbrain of *rbfox1*^{sa15940/+} larvae ($p = 0.03$) and the medulla oblongata of *rbfox1*^{sa15940/sa15940} larvae ($p = 0.04$) compared to wildtype (**Figure 40**). Additionally, the fraction of single cells spikes occurring in bursts was significantly higher in the cerebellum of *rbfox1*^{sa15940/sa15940} compared to both *rbfox1*^{sa15940/+} and wildtype larvae ($p = 8.62\text{e-}03$ and $p = 0.03$, respectively) (**Figure 40**), while the absolute number of single cells spikes did not differ significantly between the genotypes (Supplementary Figure 2).

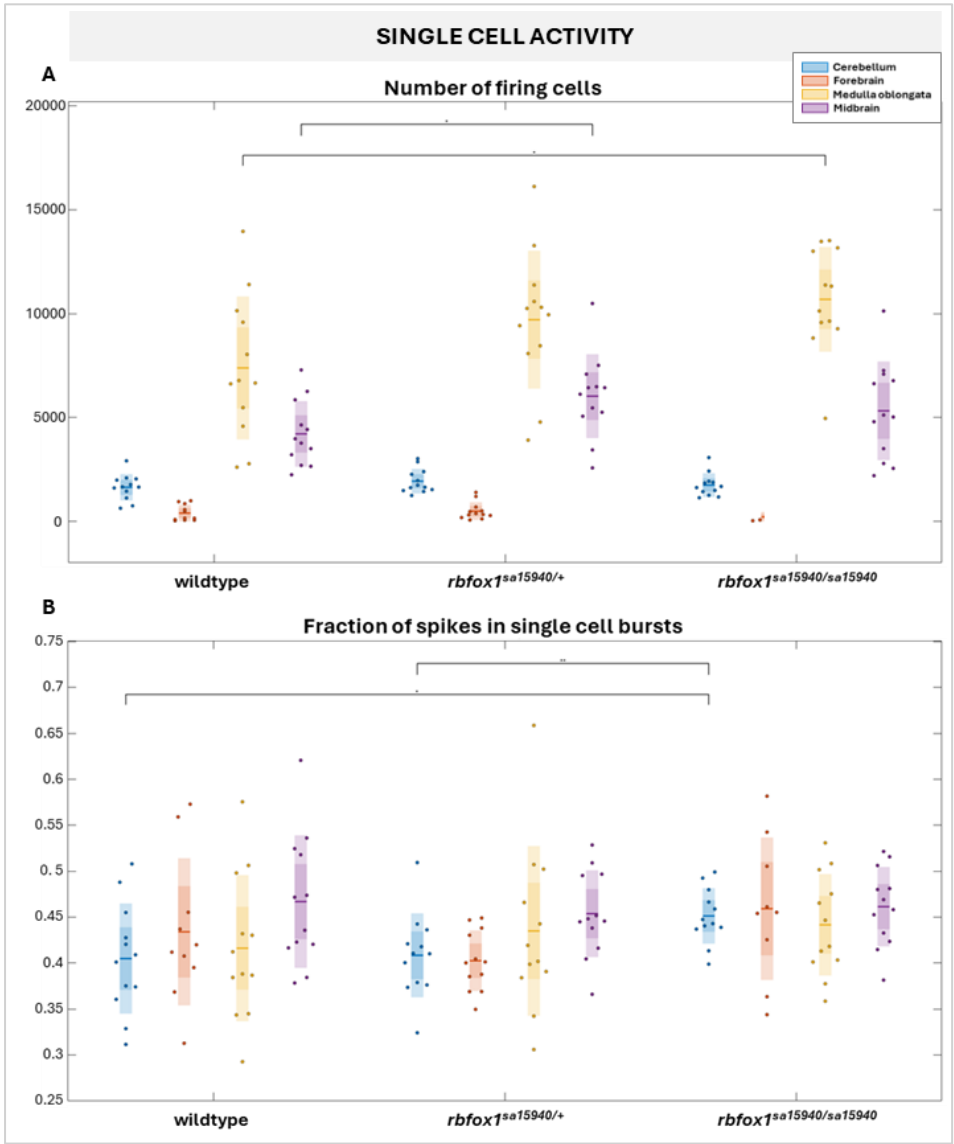


Figure 40: Single cell activity in wildtype, *rbfox1*^{sa15940/+}, and *rbfox1*^{sa15940/sa15940} zebrafish larvae. A total number of firing neurons and B fraction of spikes in single cell bursts. The number of active neurons was significantly increased in the midbrain of *rbfox1*^{sa15940/+} larvae and in the medulla oblongata of *rbfox1*^{sa15940/sa15940} larvae compared to wildtypes. Additionally, the fraction of single-cell spikes contained in bursts was significantly higher in the cerebellum of *rbfox1*^{sa15940/sa15940} larvae compared to *rbfox1*^{sa15940/+} and wildtype larvae. * $p_{adj} < 0.05$, ** $p_{adj} < 0.01$

When activity was analysed at the population level, we observed a higher collective bursting rate in the cerebellum and midbrain of *rbfox1*^{sa15940/+} compared to wildtype larvae ($p = 0.04$ and

$p = 0.03$, respectively), as well as in the medulla oblongata of *rbfox1*^{sa15940/sa15940} compared to wildtype ($p = 8.5e-03$) (**Figure 41**). Furthermore, the duration of the collective bursts was significantly shorter in the cerebellum and forebrain of the *rbfox1*^{sa15940/sa15940} compared to wildtype larvae ($p = 0.04$ and $p = 3.0e-03$, respectively) (**Figure 41**).

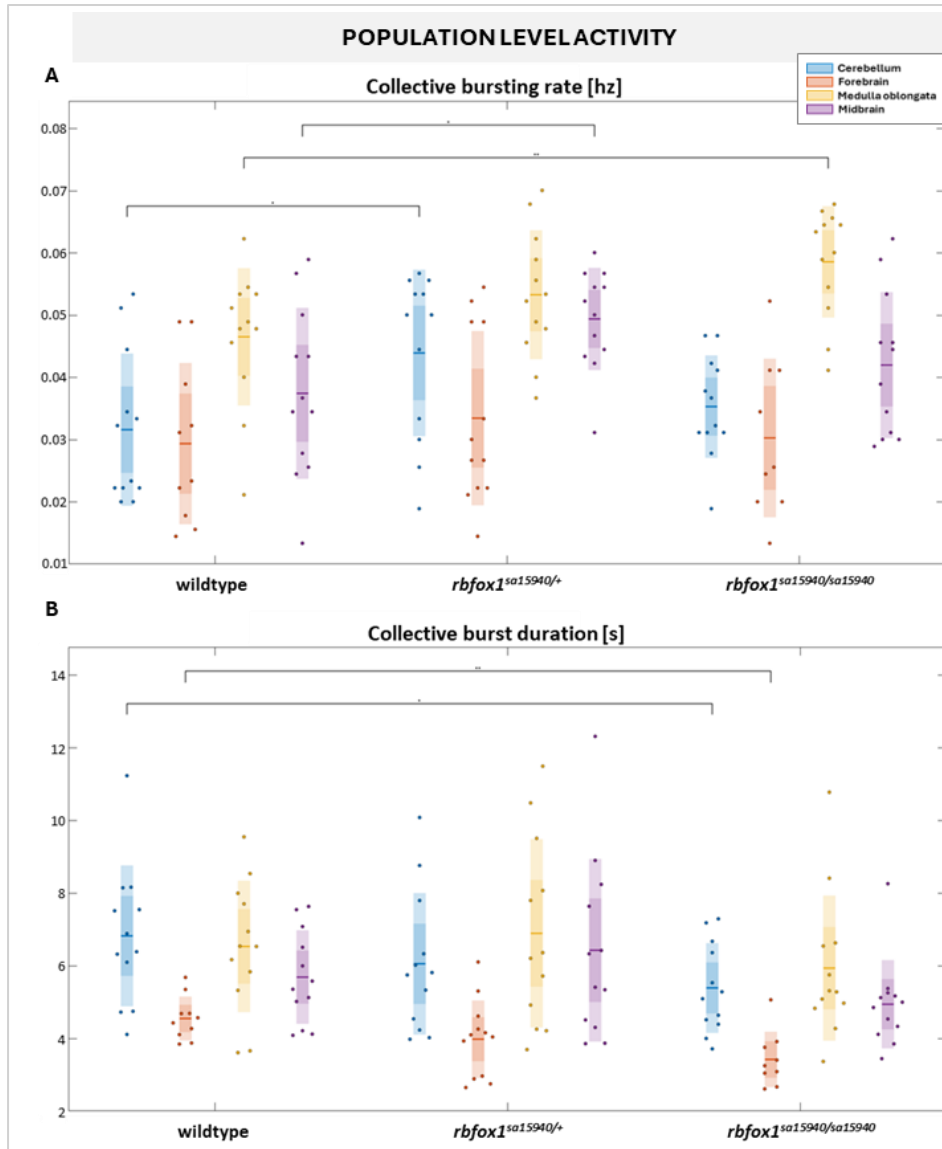


Figure 41: Collective burst activity in wildtype, *rbfox1*^{sa15940/+}, and *rbfox1*^{sa15940/sa15940} zebrafish larvae. A collective bursting rate and B collective burst duration. The collective bursting rate was significantly higher in the cerebellum and midbrain of *rbfox1*^{sa15940/+} larvae and in the medulla oblongata of *rbfox1*^{sa15940/sa15940} larvae compared to wildtype controls. Additionally, the duration of collective bursts was significantly shorter in the cerebellum and forebrain of *rbfox1*^{sa15940/sa15940} larvae compared to wildtype larvae. * $p_{adj} < 0.05$, ** $p_{adj} < 0.01$

3.3 Transcriptomic changes in the brain of *rbfox1* mutant zebrafish

To investigate transcriptomic changes in the brain of adult *rbfox1*^{sa15940/sa15940} zebrafish compared to wildtype we performed differential expression and splicing analyses. We observed significant alterations in gene expression and splicing, with the most pronounced changes in the

telencephalon and hindbrain. Genes differentially expressed in the telencephalon were enriched in genes involved in cell development.

A total of 1062 differentially expressed genes were found across all brain regions in *rbfox1*^{sa15940/sa15940} zebrafish compared to wildtype, with the most differentially expressed genes observed in the telencephalon and hindbrain (Figure 42A, Supplementary Tables S3-6). Of these genes, 59 were differentially expressed in all areas, including in the analyses of the whole brain (Figure 42A, Fehler! Verweisquelle konnte nicht gefunden werden., Supplementary Tables S7).

Regarding splicing alterations, we identified 331 differentially spliced genes across all regions, with 44 genes shared between all of them (Figure 42B, Supplementary Tables S8-11). Notably, 29 of these genes were found to be both differentially expressed and spliced (Supplementary Tables S12).

Table 1: Differentially expressed genes in *rbfox1^{sa15940/sa15940}* zebrafish overlapping in all brain areas and the whole-brain analysis. A $p_{adj} < 0.1$ and $abs(log2FC) > 2.5$ threshold was applied.

Gene ID	Gene name	Whole brain			Telencephalon			Diencephalon			Hindbrain		
		log2 FC	p	p _{adj}	log2 FC	p	p _{adj}	log2 FC	p	p _{adj}	log2 FC	p	p _{adj}
ENSDARG000000061585	cyp4v2b	4.61	8.76E-24	1.37E-20	4.08	9.95E-08	2.92E-05	4.84	8.26E-10	8.62E-07	4.32	2.83E-08	1.11E-05
ENSDARG000000099926	si:dkey-26m3.1	4.09	1.53E-19	2.08E-16	3.55	2.23E-06	3.91E-04	4.33	3.55E-08	2.22E-05	3.57	2.19E-06	4.04E-04
ENSDARG00000108432		3.51	2.37E-39	8.90E-36	2.96	5.54E-11	3.63E-08	3.35	2.03E-13	4.77E-10	3.80	1.47E-16	1.65E-13
ENSDARG00000107760		3.27	7.69E-39	2.41E-35	2.73	5.51E-12	5.75E-09	3.02	3.62E-14	9.71E-11	3.66	1.31E-19	3.07E-16
ENSDARG00000116065		2.93	8.20E-36	2.20E-32	2.40	1.25E-10	7.32E-08	2.67	1.30E-12	2.44E-09	3.43	3.75E-19	7.05E-16
ENSDARG00000038072	ptx4	2.74	7.24E-19	9.08E-16	3.24	4.20E-17	8.77E-14	2.47	2.39E-11	3.46E-08	1.97	3.93E-06	6.41E-04
ENSDARG00000057984	eps8l1a	2.66	1.62E-09	3.88E-07	2.99	7.49E-14	9.39E-11	2.01	2.06E-08	1.45E-05	2.81	3.96E-17	4.96E-14
ENSDARG000000096217	si:dkey-51d8.9	2.39	1.01E-12	4.30E-10	2.36	2.41E-05	2.50E-03	1.94	1.89E-04	2.52E-02	1.77	4.92E-04	1.89E-02
ENSDARG00000110192		2.31	6.15E-28	1.28E-24	2.11	4.08E-11	3.03E-08	1.74	2.38E-08	1.54E-05	2.74	3.80E-17	4.96E-14
ENSDARG000000092106	si:ch211-214p13.8	2.10	1.02E-19	1.47E-16	2.03	6.50E-12	6.43E-09	1.57	5.30E-08	2.93E-05	2.40	8.43E-16	8.34E-13
ENSDARG000000094557	nupr1b	2.00	2.33E-16	1.82E-13	1.31	1.94E-06	3.51E-04	1.79	2.50E-10	2.94E-07	2.54	3.68E-18	6.29E-15
ENSDARG000000097456	coa3b	1.86	5.84E-14	2.95E-11	1.54	4.74E-05	4.12E-03	1.48	8.25E-05	1.32E-02	2.14	1.44E-07	4.29E-05
ENSDARG000000069961	il21r.1	1.75	1.19E-13	6.06E-11	1.42	4.99E-05	4.27E-03	1.59	1.15E-05	3.19E-03	1.92	3.67E-07	9.57E-05
ENSDARG00000111899		1.69	1.35E-12	5.53E-10	1.75	2.34E-06	4.00E-04	2.07	3.21E-07	1.47E-04	0.70	5.18E-03	7.06E-02
ENSDARG000000086853	dpyda.3	1.49	3.10E-07	3.62E-05	1.44	3.57E-06	5.68E-04	1.75	5.50E-08	2.95E-05	0.75	2.49E-03	4.70E-02
ENSDARG00000017579	si:ch211-195b15.7	1.39	2.41E-05	1.35E-03	1.84	4.82E-11	3.36E-08	0.91	2.73E-04	3.23E-02	1.00	3.32E-04	1.47E-02
ENSDARG00000043480	rbbp8	1.39	3.47E-16	2.56E-13	0.66	7.87E-04	2.87E-02	1.02	5.91E-06	1.94E-03	2.10	1.25E-17	1.96E-14
ENSDARG000000037954	tnnt1	1.36	1.26E-14	7.38E-12	1.49	9.16E-11	5.74E-08	1.03	3.09E-06	1.12E-03	1.44	1.18E-10	7.13E-08
ENSDARG000000090033	si:ch211-162i8.8	1.31	7.96E-09	1.51E-06	1.23	3.16E-04	1.52E-02	1.06	9.72E-04	7.91E-02	0.84	3.28E-03	5.40E-02

ENSARG00000051798	brd1b	0.84	3.31E-10	9.15E-08	0.58	2.74E-04	1.38E-02	0.61	1.53E-04	2.13E-02	1.08	3.43E-09	1.61E-06
ENSARG0000013839	aldh3b1	0.81	5.01E-06	3.69E-04	0.90	4.88E-10	2.70E-07	0.83	5.81E-08	3.04E-05	0.73	4.42E-06	6.98E-04
ENSARG0000027357	cd2bp2	0.81	8.36E-27	1.57E-23	0.90	3.12E-14	4.19E-11	0.68	5.15E-09	4.03E-06	0.78	2.27E-11	1.58E-08
ENSARG0000007370	dysf	0.77	2.66E-10	7.46E-08	0.60	6.29E-04	2.45E-02	0.68	1.87E-04	2.51E-02	0.75	6.92E-05	5.45E-03
ENSARG0000037955	plh1d1	0.74	1.20E-11	4.33E-09	0.82	2.01E-15	3.44E-12	0.62	1.15E-09	1.08E-06	0.78	1.20E-14	1.13E-11
ENSARG0000026048	tsc1a	0.67	6.15E-08	8.90E-06	0.70	4.01E-05	3.63E-03	0.51	7.71E-04	6.59E-02	0.61	2.17E-04	1.14E-02
ENSARG0000052734	hmgcr	0.66	1.11E-05	7.28E-04	0.51	6.42E-04	2.50E-02	0.64	6.35E-05	1.11E-02	0.81	1.96E-06	3.73E-04
ENSARG0000054570	smg1	-0.59	1.32E-17	1.38E-14	-0.64	1.53E-19	4.10E-16	-0.64	1.75E-19	6.58E-16	-0.50	1.08E-13	9.19E-11
ENSARG0000062138	ranbp10	-0.67	1.42E-15	9.22E-13	-0.58	6.94E-06	9.67E-04	-0.67	6.79E-07	2.71E-04	-0.65	8.78E-07	1.92E-04
ENSARG00000102512	kdm8	-0.70	1.46E-12	5.82E-10	-0.60	6.36E-05	4.96E-03	-0.68	1.50E-05	4.03E-03	-0.64	2.52E-05	2.68E-03
ENSARG0000059902	vps16	-0.72	5.60E-09	1.12E-06	-0.59	9.79E-04	3.30E-02	-0.57	1.19E-03	8.90E-02	-0.61	8.45E-04	2.59E-02
ENSARG0000099023	CABZ01034698.3	-0.75	1.71E-04	5.87E-03	-0.84	3.76E-04	1.74E-02	-0.88	3.33E-05	7.36E-03	-0.54	3.15E-03	5.30E-02
ENSARG0000058020	fbxw9	-0.78	3.57E-14	1.92E-11	-0.77	5.24E-07	1.19E-04	-0.62	2.76E-05	6.39E-03	-0.80	2.53E-07	7.21E-05
ENSARG0000003270	dhps	-0.79	3.54E-16	2.56E-13	-0.76	1.46E-06	2.74E-04	-0.65	1.84E-05	4.71E-03	-0.80	5.54E-07	1.35E-04
ENSARG0000039661	slc36a4	-0.91	1.71E-10	5.01E-08	-0.76	2.18E-04	1.19E-02	-0.84	5.99E-05	1.07E-02	-0.77	2.02E-04	1.10E-02
ENSARG00000103248	CABZ01064771.1	-1.02	1.35E-07	1.75E-05	-0.84	3.56E-04	1.68E-02	-1.06	2.79E-05	6.39E-03	-0.89	1.97E-04	1.09E-02
ENSARG0000099867	si:dkey-16m19.6	-1.07	5.90E-11	1.91E-08	-1.10	7.73E-06	1.04E-03	-0.98	5.56E-05	1.05E-02	-0.84	2.40E-04	1.21E-02
ENSARG0000027749	hrip3	-1.08	5.92E-10	1.59E-07	-0.69	9.49E-04	3.23E-02	-1.26	4.55E-07	1.99E-04	-0.99	3.23E-05	3.17E-03
ENSARG00000115857		-1.08	3.28E-14	1.82E-11	-0.69	5.25E-04	2.18E-02	-1.13	2.19E-06	8.25E-04	-1.22	2.98E-07	8.13E-05
ENSARG00000113971	si:cabz01069016.2	-1.14	9.52E-11	3.03E-08	-1.32	1.01E-06	2.03E-04	-1.00	7.68E-05	1.28E-02	-0.74	1.16E-03	3.06E-02
ENSARG0000055731	si:dkeyp-69e1.8	-1.14	1.05E-16	8.95E-14	-0.96	1.67E-06	3.07E-04	-1.29	9.66E-10	9.56E-07	-0.99	1.79E-06	3.44E-04

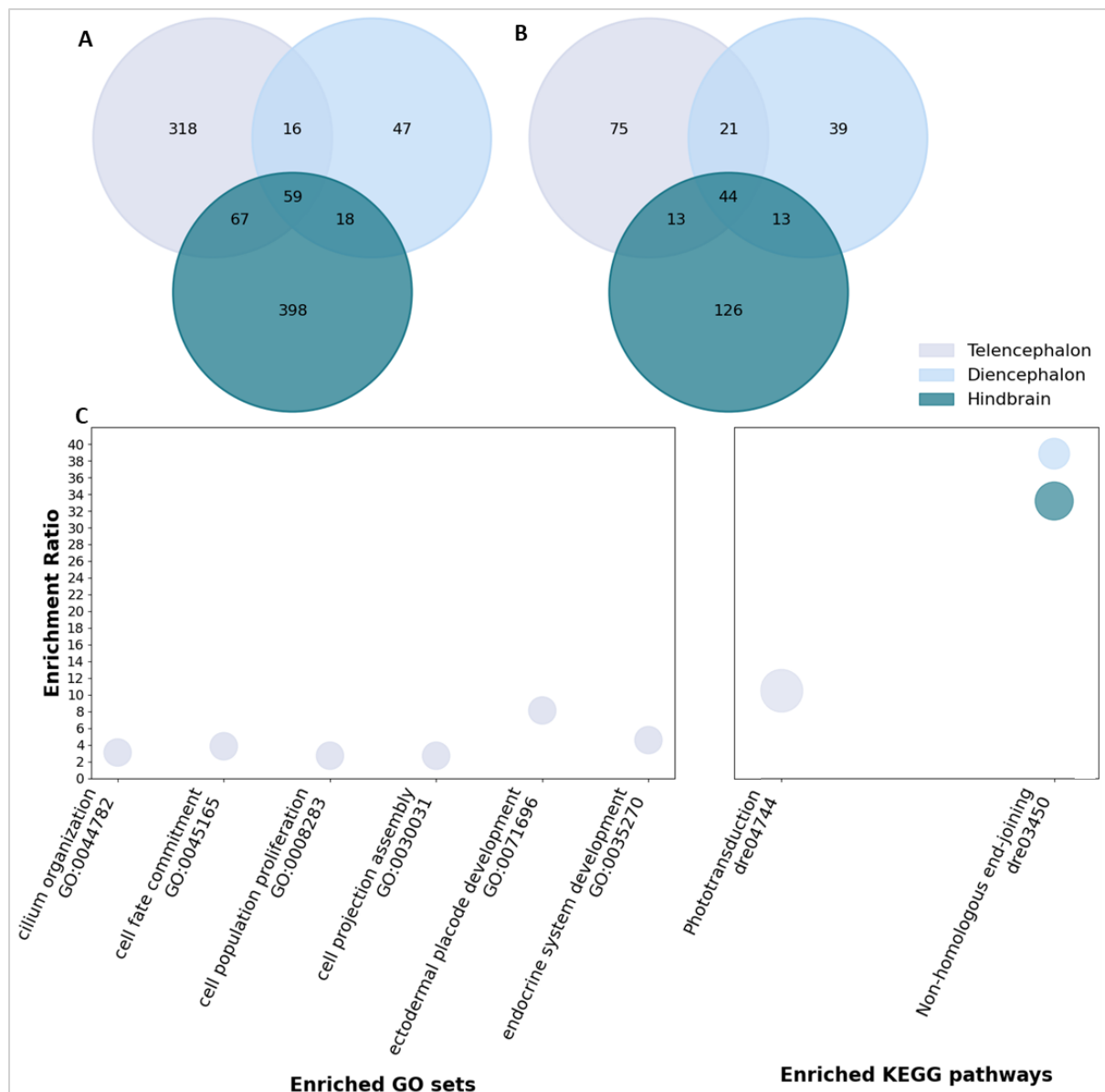


Figure 42: Transcriptomic changes in adult *rbfox1*^{sa15940/sa15940} zebrafish. A Differentially expressed and B differentially spliced genes and in the telencephalon, diencephalon, and hindbrain of adult *rbfox1*^{sa15940/sa15940} compared to wildtype fish overcoming a $p_{adj} < 0.1$ and $abs(log2FC) > 0.5$ threshold. C Enriched biological processes from GO and KEGG pathways. Marker size represents the $-\log_{10}$ of the FDR corrected value

To further investigate the biological implications of the *rbfox1*^{sa15940/sa15940} mutation, we performed functional enrichment analyses of GO biological processes and KEGG pathways. Our analyses revealed that differentially expressed genes were most strongly associated with cell development, while differentially spliced genes were related to DNA repair mechanisms.

In the telencephalon, differentially expressed genes were enriched in biological functions related to cell development, such as “cilium organization” (GO:0044782), “cell fate commitment” (GO:0045165), “cell population proliferation” (GO:0008283), and “cell projection assembly” (GO:0030031) (Figure 42C, Supplementary Table S13). Additionally, the KEGG pathway

“Phototransduction” (dre04744) was enriched in differentially expressed genes in the telencephalon as well as across the whole brain (**Figure 42C**, Supplementary Table S14-15). Notably, no enriched GO biological functions or KEGG pathways were found in the differentially expressed in diencephalon and hindbrain.

Enrichment analysis of the differentially spliced genes revealed that the KEGG pathway “Non-homologous end-joining” (dre03450) was significant in both the diencephalon and hindbrain (**Figure 42C**, Supplementary Table S16-17), with no further enriched GO biological functions or KEGG pathways identified in any brain regions.

Interestingly, joint enrichment analyses of differentially expressed and spliced together revealed similar enriched biological processes and pathways. In the telencephalon, “cilium organization” (GO:0044782), “cell projection assembly” (GO:0030031), and “Phototransduction” (dre04744) were enriched (Supplementary Tables S18-19). Both in the diencephalon and hindbrain, “Non-homologous end-joining” (dre03450) was enriched (Supplementary Tables S20-21).

Comparison of our results with published transcriptomic data revealed that 100 out of 1062 differentially expressed genes across all brain regions had previously been identified to be regulated by *Rbfox1* in mice^{8,12} (Supplementary Tables S12). Out of the 331 differentially spliced genes, 42 had previously been identified as *Rbfox1*-regulated, although they were not previously known to be splicing targets. Three genes, namely *GAA*, *OTUD6B*, and *PAX6*, were both differentially expressed and spliced, and were also identified in previous studies (Supplementary Tables S12)^{8,12}.

4 DISCUSSION

In this study, we investigated the role of *rbfox1* in neurotransmission, neural activity, and transcriptomic alterations in a zebrafish model, that could lead to alterations in behaviour previously described. We included multiple life stages, from larval stages, where fundamental functions are established, to adult stages with increased complexity and specialization of brain structures. By doing this, we aimed to gain insight into the potentially distinct effects of *rbfox1* throughout development, as well as the long-term effects of alterations during early life stages. This approach revealed complex interactions of changes in neurotransmission, neural activity, and gene expression and splicing in *rbfox1* mutant zebrafish. In this study we observed reduced levels of serotonin, dopamine, GABA, and glutamate in the forebrain, and altered neuronal activity in the midbrain and hindbrain. Furthermore, we identified differential expression and splicing of genes across the entire brain, primarily associated with cell developmental processes. While *RBFOX1* has previously been implicated in various psychiatric disorders, our findings offer

novel insights into the underlying mechanisms by which this gene contributes to these conditions by influencing neurotransmission, neuronal activity, and transcriptomic changes.

The observed reduced levels of serotonin, dopamine, GABA, and glutamate in the forebrain of *rbfox1*^{sa15940} mutant zebrafish parallel neurotransmitter disruptions in patients with major psychiatric disorders, such as ADHD, ASD, bipolar disorder, MDD, and schizophrenia^{38–44}. Altered monoaminergic, GABAergic, and glutamatergic signalling are known to contribute to the development and treatment of these conditions, and often manifest in symptoms such as anhedonia, anxiety, hyperactivity, stereotyped behaviours, and social impairments^{38–44}. Interestingly, these behavioural changes were also partially observed in *rbfox1* mutant fish^{18,19}. This demonstrates the potential clinical relevance of our zebrafish model, where alterations in neurotransmitter systems may provide insights into the underlying biological mechanisms of psychiatric phenotypes.

Our findings suggest that *RBFOX1* may play a significant role in mediating the complex interactions between disrupted neurotransmitter systems and resulting behavioural changes, such as pronounced hyperactivity, increased aggression, and impaired social behaviour, which are common symptoms of major psychiatric disorders^{18,19}. In zebrafish, the serotonergic system is known to regulate fear, anxiety, and stress-related behaviours, and it is also involved in aggression and locomotion; however, findings vary regarding the role of serotonin in these behaviours^{45–47}. Similarly, dopaminergic signalling is implicated in regulating learning, locomotion, reward processing, social behaviour, and stereotypy^{48–53}. GABA and glutamate are the primary inhibitory and excitatory neurotransmitters in vertebrates, respectively^{54,55}. The GABAergic system in zebrafish is involved in modulating anxiety and motor control, while glutamatergic signalling is crucial for developmental processes, environmental adaptation, memory, and learning^{56–60}. Additionally, glutamatergic neurons are involved in the regulation of neuronal migration in the cerebellum, with disruptions in this process potentially affecting behaviour⁶¹. Given these functions, the alterations in neurotransmission in *rbfox1* mutants likely contribute to the behavioural changes previously reported in these zebrafish models, as well as human patients, including hyperactivity, aggression, and social impairments.

Interestingly, we found reduced neurotransmitter levels in the forebrain but not hindbrain of *rbfox1*^{sa15940} mutant fish. One explanation for this could be that these neurons are mostly located in or project to forebrain regions. In zebrafish, serotonergic nuclei are primarily located in the raphe nuclei of the hindbrain as well as in the medulla oblongata, from where they project mainly to the forebrain and midbrain⁴⁵. Further populations of serotonergic neurons are present in the telencephalon, specifically in the forebrain and olfactory bulb, as well as in the diencephalon, specifically in the hypothalamus, pineal gland, pretectal area and posterior tuberculum^{45,62}. Dopaminergic neurons in zebrafish are primarily located in the diencephalon, particularly in the

ventral diencephalon, which is considered analogous to the substantia nigra in mammals, as well as in the olfactory bulb and pretectum⁶³. Additionally, there are dopaminergic neurons in parts of the telencephalon, as well as potentially in parts of the hindbrain⁶³. In zebrafish, GABAergic neurons are present in the olfactory bulb, the telencephalon, tectum stratum, and in the diencephalon, including the hypothalamus, thalamus, preoptic region, and posterior tuberculum, as well as in the parts of the hindbrain, including in the cerebellum and medulla oblongata^{64–67}. Glutamatergic neurons in zebrafish are also present in the telencephalon, specifically in the forebrain, the optic tectum, and in the posterior tuberculum of the diencephalon, and, to a lesser extent, in the hindbrain⁶⁸. Taken together, this could explain why reduced levels of these neurotransmitters were observed in the telencephalon and diencephalon, where the projections of these neurons are located, but not the in the hindbrain.

Our findings of *in vivo* whole brain imaging in zebrafish provide insight into how *RBFOX1* affects psychiatric phenotypes, not only through neurotransmitter imbalances but also through alterations in brain activity. With preclinical studies modelling alterations in neuronal activity and hyperactivity due to *Rbfox1*, it is thought to contribute to the disruptions in neuronal activity observed in conditions such as depression, epilepsy, ASD, and neuropathic pain^{9,10,17,69,70}. Our study further supports this connection, as *rbfox1*^{sa15940} mutant zebrafish exhibited increased neuronal activity and synchronicity, particularly in midbrain and hindbrain regions. These findings align with structural and functional alterations of the cerebellum in psychiatric disorders such as ADHD, ASD, MDD, and schizophrenia⁷¹. These alterations may partially explain certain cognitive symptoms and mood dysregulation in these disorders, as the cerebellum also plays a critical role in cognition and emotional processing beyond its role in locomotion⁷¹. Moreover, hyperexcitability of the lateral habenula, which inhibits a monoaminergic centre in the midbrain, has been implicated in MDD, and is associated with depressive- and anxiety-like behaviours in preclinical studies⁷². This suggests *RBFOX1* as a key regulators factor in cognition and mood⁴⁰.

Notably, our findings do not model other prominent brain regions typically associated with depression in humans, such as cortical and limbic regions, which would be more homologous to forebrain regions in zebrafish, as they lack a neocortex^{40,69}. This suggests that *RBFOX1* mutations may influence distinct neural circuits that are not traditionally linked to depression but could still contribute to mood regulation and stress vulnerability. However, changes in neuronal activity do not necessarily reflect alterations in connectivity. Thus, comparing our findings of increased neuronal activity and synchronicity with functional connectivity changes in human patients should be done with caution. Functional connectivity analyses could provide deeper insights into the role of *rbfox1* in brain connectivity.

Furthermore, *RBFOX1* may have different roles in higher brain regions in zebrafish than in humans. This idea is supported by different expression patterns of *RBFOX1* across brain areas

and developmental stages in humans and zebrafish. In humans, *RBFOX1* is prominently expressed in the neocortex, among other regions, especially during prenatal stages and throughout adolescence and adulthood⁷³. In zebrafish larvae, *rbfox1* is predominantly expressed in midbrain and hindbrain regions, with more prominent expression in telencephalic and diencephalic regions in adult zebrafish¹⁸. These findings suggest that the timing and regional distribution of *rbfox1* expression may play a crucial role in the observed effects on brain activity at different stages of development, and may differ from *RBFOX1* expression patterns in humans.

Along with clinical findings in psychiatric disorders, our observations of increased neuronal activity and synchronicity in the zebrafish larvae also align with previous studies implicating *RBFOX1* in the regulation of neuronal excitation^{13–17}. For instance, Gehman et al. (2011) reported heightened neuronal excitability in the dentate gyrus of *Rbfox1* knockout mice, which also exhibited an increased susceptibility to spontaneous and induced seizures, likely due to impaired neuronal inhibition¹⁷. In line with these findings, we detected an increased number of firing neurons in the midbrain and hindbrain of the *rbfox1*^{sa15940} mutant zebrafish, which were more likely to fire in single cell bursts, indicating higher neuronal activation and probably higher excitability. Additionally, we found a higher collective burst rate in those brain regions, suggesting a higher synchronicity of neuronal activity across populations of neurons²².

Interestingly, we did not observe increased spontaneous seizures in *rbfox1*^{sa15940} mutant zebrafish. This may be due to the distinct nature of the *rbfox1* mutation in our study. Unlike the *Rbfox1* knockdown used in the study by Gehman et al. (2011), the *rbfox1*^{sa15940} mutation results in an exon skipping event, leading to a shorter, aberrant mRNA that may retain some function, potentially protecting against seizures¹⁷. Furthermore, the absence of seizures in our model could be related to differences in brain morphology or compensatory mechanisms in zebrafish that prevent seizure onset. However, other factors are also likely to contribute to seizure susceptibility, as studies in other *Rbfox1* models have also failed to show an increased risk of seizures. For instance, studies of *rbfox1* mutant zebrafish with a full knockout, such as the *rbfox1*^{del19} line, found no increased incidence of seizures¹⁸. Additionally, a 2022 study by O’Leary et al. did not report increased seizures in *Rbfox1* knockout mice, suggesting that both the genetic background and the specific nature of the *Rbfox1* mutation may influence susceptibility to seizures². Nevertheless, further research is needed to clarify the precise role of *RBFOX1* in seizure susceptibility and to understand the mechanisms underlying the observed differences.

During brain development, spontaneous bursting activity is essential for the correct development of neural circuits, as it synchronizes local and large-scale cortical networks^{74,75}. The observed increased neuronal activity in *rbfox1*^{sa15940} mutant zebrafish could indicate early disruptions in neural circuit development, which could affect the establishment of balanced excitatory and inhibitory signalling and monoaminergic circuit formation in adulthood, resulting in the observed

neurotransmitter alterations. Notably, we detected the alterations in larval brain activity and synchronicity in the midbrain and hindbrain, i.e., cerebellum and medulla oblongata, areas in which no alterations of the neurotransmitter levels were observed in adult zebrafish. This opens several lines of interpretations. One possibility is that the lower levels of the neurotransmitters we observed could be a long-term consequence of the early increased neuronal activity. Neuronal hyperactivity of midbrain and hindbrain regions during the brain development could lead to compensatory downregulation of different neurotransmitters in the forebrain to stabilize network activity. Alternatively, if neurotransmitter alterations are already present in larval zebrafish, the increased activity in the other brain regions may be the result of compensatory mechanism of the brain to maintain homeostasis in response to reduced neurotransmitter levels in the forebrain. Also, lower GABA levels could lead to reduced inhibitory GABAergic signalling, further increasing hyperactivity of different brain regions.

It is important to note that, while basic functions of the different brain regions are conserved among larval and adult zebrafish, neurotransmitter systems and neuronal activity become increasingly complex during the development. Therefore, our findings of the brain activity in larvae and the neurotransmitter levels in adult fish and may not be directly comparable. Additional experiments measuring neurotransmitter levels in larval zebrafish are needed to better understand the causal relationship between alterations in the brain activity and neurotransmission.

Transcriptomic analyses in adult *rbfox1* mutant zebrafish revealed numerous differentially expressed and spliced genes, which may underlie the observed neurotransmitter imbalances and alterations in brain activity. Disturbances in cell development processes in the telencephalon could impact the formation and maintenance of balanced excitatory and inhibitory, as well as monoaminergic neurotransmission, potentially leading to the observed neurotransmitter imbalances and alterations in neuronal activity. These findings align with previous studies relating *rbfox1* with developmental processes essential for neuronal growth and maintenance, including cell proliferation, signalling, cytoskeletal organization, and neuronal differentiation^{13–17}. The alterations in neurotransmission in the forebrain could furthermore have an indirect impact on brain activity in other regions, possibly through compensatory mechanisms aimed at maintaining overall neural homeostasis, as discussed above. However, given that *rbfox1* expression is more prominent in the midbrain and hindbrain of zebrafish larvae, it is possible that the transcriptomic changes in these regions could be driving the increased neuronal activity and synchronicity observed in early developmental stages, which could then result in decreased neurotransmission in the forebrain¹⁸. As *rbfox1* is known to play a crucial role during neurodevelopment, we will perform transcriptomic analyses in zebrafish larvae to explore the specific effects of *rbfox1* at different developmental stages. This would help to better understand

the causal relationship between altered brain activity, neurotransmission, and gene expression and splicing throughout the development.

Another benefit of investigating transcriptomic alterations during larval stages is the opportunity to assess whether the *rbfox1*^{sa15940} mutation leads to differential expression of *rbfox1* itself during early development. In adults, we found no significant differential expression of *rbfox1*, but we did observe significant alternative splicing across all brain regions, which could be attributed to the exon skipping event that results in a shorter and aberrant mRNA sequence¹⁸. If there are changes in *rbfox1* expression patterns during early development, these could potentially have different effects on neurotransmission compared to those observed in adults. By exploring this in larvae, we aim to better understand how *rbfox1* expression influences brain development and contributes to the alterations in brain activity and neurotransmission observed in our study.

Interestingly, several of the differentially spliced and expressed genes in *rbfox1*^{sa15940} mutant zebrafish were previously identified as *Rbfox1*-regulated in two studies by Lee et al. (2016)¹² and Wamsely et al. (2018)⁸. These findings, along with the overlapping behavioural alterations and changes in neuronal activity observed in different *Rbfox1* animal models, suggest that the regulatory role of *RBFOX1* is partially evolutionarily conserved across vertebrates.

In conclusion, *RBFOX1* plays an important role in regulating the intricate interplay between neurotransmission, neuronal activity, and transcriptomic changes implicated in neurodevelopmental disruptions. All these alterations in brain activity could explain the behavioural alterations observed in mutant *rbfox1* animal models. Together, these findings help to elucidate the underlying mechanisms by which *RBFOX1* contributes to different psychiatric disorders.

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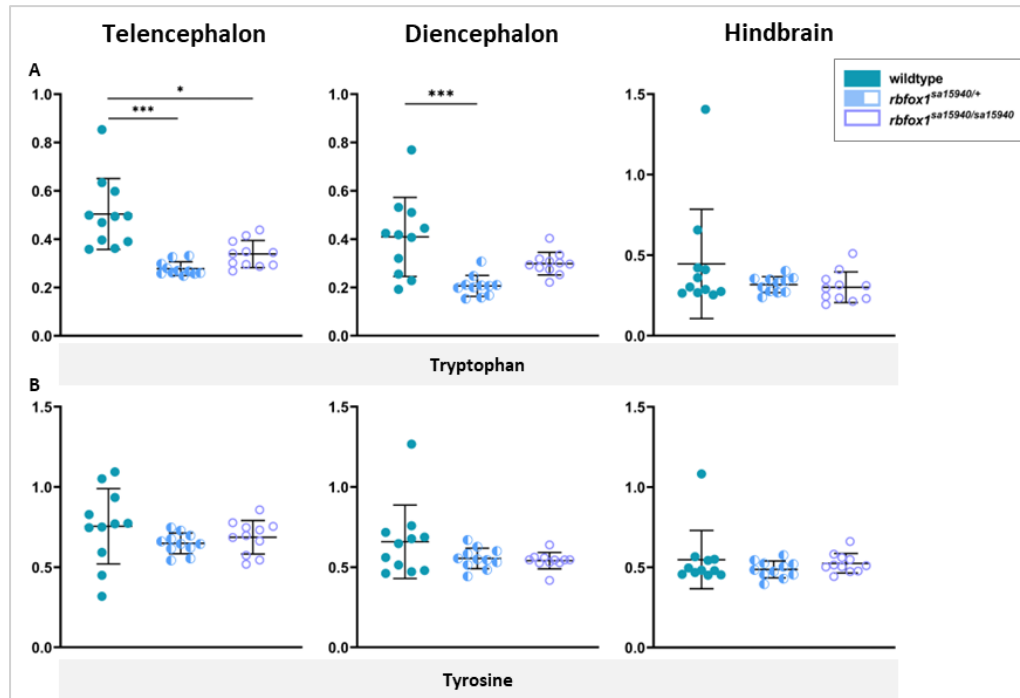
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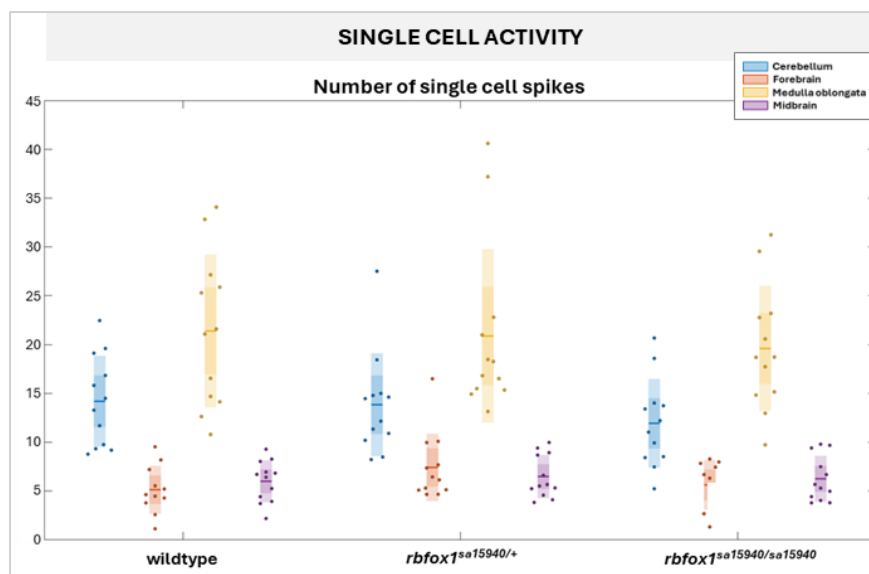
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6 SUPPLEMENTARY MATERIAL

6.1 Supplementary Figures



Supplementary Figure 2: Basal neurotransmitter precursor levels in telencephalon, diencephalon, and hindbrain of adult wildtype, $rbfox1^{sa15940/+}$, and $rbfox1^{sa15940/sa15940}$ zebrafish. Levels of A tryptophan and B tyrosine were measured via ultra-high-performance liquid chromatography-tandem mass spectrometry and normalised to the phenylalanine concentration of the respective sample. $N = 11$ per group; Kruskal-Wallis test with Dunn's correction for multiple testing; mean $\pm$ SD; * $p_{adj} < 0.05$, *** $p_{adj} \leq 0.001$



Supplementary Figure 3: Number of single cell spikes in wildtype, $rbfox1^{sa15940/+}$, and $rbfox1^{sa15940/sa15940}$ zebrafish larvae. No significant differences were observed in the absolute number of single-cell spikes between the genotypes

6.2 Supplementary Tables

The following supplementary tables in **bold** were included as summaries of the results in this thesis.

Supplementary Table 1: Basal neurotransmitter levels in the telencephalon (Tel), diencephalon (Die), and hindbrain (Hind) of adult wildtype (WT), *rbfox1*^{sa15940/+} (HZ), and *rbfox1*^{sa15940/sa15940} (HOM) zebrafish.

Supplementary Table 2: Basal levels of the serotonin and dopamine precursors tryptophan and tyrosine in the telencephalon (Tel), diencephalon (Die), and hindbrain (Hind) of adult wildtype (WT), *rbfox1*^{sa15940/+} (HZ), and *rbfox1*^{sa15940/sa15940}.

Supplementary Table 3: Differentially expressed genes in adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish across the whole brain

Supplementary Table 4: Differentially expressed genes in adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish in the telencephalon.

Supplementary Table 5: Differentially expressed genes in adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish in the diencephalon.

Supplementary Table 6: Differentially expressed genes in adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish in the hindbrain.

Supplementary Table 7: Overview of the differentially expressed genes in adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish in all brain regions.

Supplementary Table 8: Differential splicing events in adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish in the telencephalon.

Supplementary Table 9: Differential splicing events in adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish in the diencephalon.

Supplementary Table 10: Differential splicing events in adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish in the hindbrain.

Supplementary Table 11: Overview of the differentially spliced genes in adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish in all brain regions.

Supplementary Table 12: Overlap of all differentially expressed and spliced genes in adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish across all brain regions with previously identified RBFOX1 regulated genes.

Supplementary Table 13: Biological Processes from GO enriched in differentially expressed genes in the telencephalon of adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish.

Supplementary Table 14: Enriched KEGG pathways in differentially expressed genes in the telencephalon of adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish.

Supplementary Table 15: Enriched KEGG pathways in differentially expressed genes across the whole brain of adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish.

Supplementary Table 16: Enriched KEGG pathways in differentially spliced genes in the diencephalon of adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish.

Supplementary Table 17: Enriched KEGG pathways in differentially spliced genes in the hindbrain of adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish.

Supplementary Table 18: Biological Processes from GO enriched in differentially expressed and spliced genes in the telencephalon of adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish.

Supplementary Table 19: Enriched KEGG pathways in differentially expressed and spliced genes in the telencephalon of adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish.

Supplementary Table 20: Enriched KEGG pathways in differentially expressed and spliced genes in the diencephalon of adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish.

Supplementary Table 21: Enriched KEGG pathways in differentially expressed and spliced genes in the hindbrain of adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish.

Supplementary Table 1: Basal neurotransmitter levels in the telencephalon (Tel), diencephalon (Die), and hindbrain (Hind) of adult wildtype (WT), *rbfox1*^{sa15940/+} (HZ), and *rbfox1*^{sa15940/sa15940} (HOM) zebrafish. Mean ± standard deviation.

		Serotonin	Dopamine	GABA	Glutamate
WT	Tel	0.07640 ± 0.05280	0.01824 ± 0.009589	35.07 ± 20.05	51.01 ± 15.13
	Die	0.19780 ± 0.1368	0.06302 ± 0.04171	20.84 ± 13.86	38.62 ± 20.56
	Hind	0.01415 ± 0.04205	0.00364 ± 0.005635	15.64 ± 18.99	20.33 ± 13.34
HZ	Tel	0.006184 ± 0.004181	0.004645 ± 0.001192	16.43 ± 2.262	25.38 ± 4.843
	Die	0.04702 ± 0.01246	0.01772 ± 0.003201	13.41 ± 6.297	16.07 ± 2.174
	Hind	0.001463 ± 0.0009370	0.002375 ± 0.0008480	10.28 ± 2.080	14.61 ± 4.436
HOM	Tel	0.01183 ± 0.009990	0.006757 ± 0.003032	20.57 ± 5.746	28.60 ± 5.375
	Die	0.07866 ± 0.07679	0.02674 ± 0.01886	7.031 ± 1.450	24.34 ± 9.929
	Hind	0.005494 ± 0.007825	0.002892 ± 0.002892	9.546 ± 2.621	13.71 ± 3.358

Supplementary Table 2: Basal levels of the serotonin and dopamine precursors tryptophan and tyrosine in the telencephalon (Tel), diencephalon (Die), and hindbrain (Hind) of adult wildtype (WT), *rbfox1*^{sa15940/+} (HZ), and *rbfox1*^{sa15940/sa15940} (HOM) zebrafish. Mean ± standard deviation.

		Tryptophan	Tyrosine
WT	Tel	0.5045 ± 0.1467	0.7550 ± 0.2349
	Die	0.4090 ± 0.1636	0.6579 ± 0.2285
	Hind	0.4455 ± 0.3392	0.5477 ± 0.1819
HZ	Tel	0.2777 ± 0.02841	0.6481 ± 0.06438
	Die	0.2059 ± 0.04320	0.5544 ± 0.06413
	Hind	0.3170 ± 0.04967	0.4871 ± 0.05278
HOM	Tel	0.3384 ± 0.05618	0.6861 ± 0.1048
	Die	0.2985 ± 0.04660	0.5400 ± 0.05130
	Hind	0.3007 ± 0.09509	0.5248 ± 0.06049

Supplementary Table 12: Overlap of all differentially expressed and spliced genes in adult *rbfox1*^{sa15940/sa15940} vs wildtype zebrafish across all brain regions with previously identified *RBFOX1* regulated genes.

Zebrafish Ensembl ID	Zebrafish gene name	Human homolog gene name	Differentially expressed	Splicing target	Prev. identified ¹
ENSDARG00000090714	<i>gaa</i>	GAA	y	y	y
ENSDARG00000102705	<i>otud6b</i>	OTUD6B	y	y	y
ENSDARG00000103379	<i>pax6a</i>	PAX6	y	y	y
ENSDARG00000001733	<i>gulp1a</i>	GULP1	y		y
ENSDARG000000001953	<i>pfkfb3</i>	PFKFB3	y		y
ENSDARG00000002241	<i>KCNA2</i>	KCNA2	y		y
ENSDARG00000002847	<i>fndc1</i>	FNDC1	y		y
ENSDARG00000003303	<i>stc1</i>	STC1	y		y
ENSDARG00000003822	<i>ankrd16</i>	ANKRD16	y		y
ENSDARG00000004150	<i>grm2a</i>	GRM2	y		y
ENSDARG00000005274	<i>rad52</i>	RAD52	y		y
ENSDARG00000006031	<i>abat</i>	ABAT	y		y
ENSDARG00000006896	<i>lhx6a</i>	LHX6	y		y
ENSDARG00000007788	<i>atp2b1b</i>	ATP2B1	y		y
ENSDARG00000008858	<i>cyp7b1</i>	CYP7B1	y		y
ENSDARG00000010420	<i>ndrg1b</i>	NDRG1	y		y
ENSDARG00000012968	<i>rhoub</i>	RHOU	y		y
ENSDARG00000014246	<i>jag2a</i>	JAG2	y		y
ENSDARG00000016412	<i>agt</i>	AGT	y		y
ENSDARG00000019341	<i>gpc1a</i>	GPC1	y		y
ENSDARG00000020395	<i>mical2b</i>	MICAL2	y		y
ENSDARG00000020898	<i>cyb5r4</i>	CYB5R4	y		y
ENSDARG00000021948	<i>tncb</i>	TNC	y		y
ENSDARG00000022841	<i>ptk2ba</i>	PTK2B	y		y
ENSDARG00000023217	<i>crema</i>	CREM	y		y
ENSDARG00000027495	<i>elovl4b</i>	ELOVL4	y		y
ENSDARG00000029936	<i>grtp1a</i>	GRTP1	y		y
ENSDARG00000030417	<i>pkd1a</i>	PKD1	y		y
ENSDARG00000031817	<i>trim2a</i>	TRIM2	y		y
ENSDARG00000033498	<i>rorb</i>	RORB	y		y
ENSDARG00000035018	<i>thy1</i>	THY1	y		y
ENSDARG00000035253	<i>npr3</i>	NPR3	y		y
ENSDARG00000037429	<i>tll1</i>	TLL1	y		y
ENSDARG00000037794	<i>nptx2a</i>	NPTX2	y		y
ENSDARG00000037916	<i>cdk5r1a</i>	CDK5R1	y		y
ENSDARG00000039517	<i>c8b</i>	C8B	y		y
ENSDARG00000039880	<i>arpp19b</i>	ARPP19	y		y
ENSDARG00000042296	<i>dlx5a</i>	DLX5	y		y
ENSDARG00000042409	<i>ralgds</i>	RALGDS	y		y
ENSDARG00000043986	<i>setd6</i>	SETD6	y		y
ENSDARG00000044175	<i>oxtrb</i>	OXTR	y		y
ENSDARG00000044278	<i>synprb</i>	SYNPR	y		y

ENSDARG00000045644	CA12	CA12	y	y
ENSDARG00000045799	acanb	ACAN	y	y
ENSDARG00000052638	fam210b	FAM210B	y	y
ENSDARG00000052642	shisa9b	SHISA9	y	y
ENSDARG00000054619	fras1	FRAS1	y	y
ENSDARG00000055855	kcnc3a	KCNC3	y	y
ENSDARG00000056625	trpc6a	TRPC6	y	y
ENSDARG00000056764	hydin	HYDIN	y	y
ENSDARG00000057098	htr1ab	HTR1A	y	y
ENSDARG00000057239	tph2	TPH2	y	y
ENSDARG00000059054	pdk2b	PDK2	y	y
ENSDARG00000060116	camk1a	CAMK1	y	y
ENSDARG00000060192	rpud1	RPUSD1	y	y
ENSDARG00000060348	map3k3	MAP3K3	y	y
ENSDARG00000060374	ttl11	TTL11	y	y
ENSDARG00000060638	clstn2b	CLSTN2	y	y
ENSDARG00000061240	cbln4	CBLN4	y	y
ENSDARG00000061379	cmya5	CMYA5	y	y
ENSDARG00000061636	kcnj9	KCNJ9	y	y
ENSDARG00000062138	ranbp10	RANBP10	y	y
ENSDARG00000062350	synm	SYNM	y	y
ENSDARG00000062821	slc6a15	SLC6A15	y	y
ENSDARG00000062915	polr3b	POLR3B	y	y
ENSDARG00000063264	pcdh9	PCDH9	y	y
ENSDARG00000063475	abcg1	ABCG1	y	y
ENSDARG00000063634	hpse	HPSE	y	y
ENSDARG00000067673	snapc4	SNAPC4	y	y
ENSDARG00000069560	kcnh3	KCNH3	y	y
ENSDARG00000069669	adra2c	ADRA2C	y	y
ENSDARG00000070683	dkk3b	DKK3	y	y
ENSDARG00000070831	rftn1a	RFTN1	y	y
ENSDARG00000075110	dab2ipb	DAB2IP	y	y
ENSDARG00000075831	slc7a8a	SLC7A8	y	y
ENSDARG00000076650	pde9ac	PDE9A	y	y
ENSDARG00000077167	nmbb	NMB	y	y
ENSDARG00000077178	sun1a	SUN1	y	y
ENSDARG00000077569	LRRN3	LRRN3	y	y
ENSDARG00000078707	sema7a	SEMA7A	y	y
ENSDARG00000078736	doc2a	DOC2A	y	y
ENSDARG00000078915	mmp17a	MMP17	y	y
ENSDARG00000079241	wdr90	WDR90	y	y
ENSDARG00000080675	otoa	OTOA	y	y
ENSDARG00000087013	cubn	CUBN	y	y
ENSDARG00000093286	ankrd34c	ANKRD34C	y	y
ENSDARG00000093677	ppp1r17	PPP1R17	y	y
ENSDARG00000093864	wee1	WEE1	y	y

ENSDARG00000095180	abr	ABR	y	y
ENSDARG00000098280	rbm5	RBM5	y	y
ENSDARG00000100821	scrt1a	SCRT1	y	y
ENSDARG00000101322	tfr1a	TFRC	y	y
ENSDARG00000101512	tmx4	TMX4	y	y
ENSDARG00000102483	numa1	NUMA1	y	y
ENSDARG00000102899	cremb	CREM	y	y
ENSDARG00000104291	gpr151	GPR151	y	y
ENSDARG00000104922	sstr1a	SSTR1	y	y
ENSDARG00000108939	pde9ab	PDE9A	y	y
ENSDARG00000111188	cdaa	CDA	y	y
ENSDARG00000116493	GAA	GAA	y	y
ENSDARG00000001162	matr3l1.1	MATR3	y	y
ENSDARG00000001939	b3gat3	B3GAT3	y	y
ENSDARG00000003751	lats1	LATS1	y	y
ENSDARG00000004930	lmo7a	LMO7	y	y
ENSDARG00000004988	ppp3ca	PPP3CA	y	y
ENSDARG00000005775	scn8aa	SCN8A	y	y
ENSDARG00000006878	phf21aa	PHF21A	y	y
ENSDARG00000014746	rbfox1	RBFOX1	y	y
ENSDARG00000019874	hsph1	HSPH1	y	y
ENSDARG00000026796	grm1a	GRM1	y	y
ENSDARG00000032737	gria3a	GRIA3	y	y
ENSDARG00000036239	gatm	GATM	y	y
ENSDARG00000036695	calcoco1a	CALCOCO1	y	y
ENSDARG00000037905	cacna1aa	CACNA1A	y	y
ENSDARG00000040396	bcl7bb	BCL7B	y	y
ENSDARG00000045437	slc35b3	SLC35B3	y	y
ENSDARG00000052348	smarca5	SMARCA5	y	y
ENSDARG00000054076	TATDN3	TATDN3	y	y
ENSDARG00000055108	gde1	GDE1	y	y
ENSDARG00000059474	mlxip	MLXIP	y	y
ENSDARG00000059553	sympk	SYMPK	y	y
ENSDARG00000060093	dapk1	DAPK1	y	y
ENSDARG00000071107	wnt7bb	WNT7B	y	y
ENSDARG00000074531	spag9a	SPAG9	y	y
ENSDARG00000074604	phc3	PHC3	y	y
ENSDARG00000075327	dock10	DOCK10	y	y
ENSDARG00000077047	ptprnb	PTPRN	y	y
ENSDARG00000077060	rbm6	RBM6	y	y
ENSDARG00000077582	ank3b	ANK3	y	y
ENSDARG00000077810	otud4	OTUD4	y	y
ENSDARG00000078006	wscd1b	WSCD1	y	y
ENSDARG00000087921	plce1	PLCE1	y	y
ENSDARG00000089213	adam15	ADAM15	y	y
ENSDARG00000098670	map4k4	MAP4K4	y	y

ENSDARG00000098802	map7d1a	MAP7D1	y	y
ENSDARG00000099841	prkcab	PRKCA	y	y
ENSDARG00000101120	kif3b	KIF3B	y	y
ENSDARG00000103109	chl1b	CHL1	y	y

¹*identified to be regulated by Rbfox1 in Lee et al. (2016), Supplementary Table S1 and/or Wamsley et al. (2018), Supplementary Table S5*

DISCUSSION

MDD is a complex disorder with biological predispositions, including a highly pleiotropic genetic basis, and environmental factors are known to contributing to its aetiology^{1,2}. It commonly co-occurs with other psychiatric phenotypes, with largely shared genetic factors. One key factor in the genetic architecture of MDD and other psychiatric and neurodevelopmental disorders is the gene *RBFOX1*, which was highlighted in several GWAS and case studies³. However, the exact mechanisms through which *RBFOX1* contributes to the aetiology of MDD and related traits remained largely unknown.

This thesis aimed at unravelling the mechanisms through which *RBFOX1* contributes to these psychiatric phenotypes. To obtain a comprehensive overview over the different *RBFOX1* functions, we examined the role of *RBFOX1* and its regulated genes in MDD, anxiety, irritability, and neuroticism using genomic data from patients, and complemented these findings with studies in zebrafish by transcriptomic analyses, neurotransmitter measures, *in vivo* whole-brain imaging, and behavioural analyses, including pharmacological rescue experiments in *rbfox1* mutant zebrafish. These different approaches revealed that the *RBFOX1* regulatory network substantially contributes to the shared genetic basis of MDD, anxiety, irritability, and neuroticism, and is mainly involved in biological processes related to neuron development, synapse function, and neurotransmission. We discovered a complex interaction of differentially spliced and expressed genes involved in cell development in *rbfox1* mutant zebrafish, which could contribute to the reduced levels of major neurotransmitters and increased neuronal activity and synchronicity we observed. This suggests that *RBFOX1* is implicated in dysfunctions in signal transduction and neuronal communication related to psychiatric disorders. The behavioural consequences of these disruptions in *rbfox1* mutant zebrafish paralleled common symptoms seen in psychiatric disorders, such as hyperactivity and altered social behaviour, and could partially be rescued by the SSRI fluoxetine.

This discussion will integrate the findings obtained in the different chapters of this thesis on the role of *RBFOX1* at different levels. Starting from gene regulation to then neurotransmission, brain activity, behaviour, and finally pharmacological interventions. First, we will examine the role of *RBFOX1* and its regulatory network, as well as affected biological processes and pathways at the gene level, and how they contribute to the genetic basis of MDD and its comorbidities. Then, the influence of *RBFOX1* on neurotransmission will be discussed, along with how this affects neuronal activity. These effects will be explored in terms of how they translate into behavioural alterations and the partial rescue by medication targeting different neurotransmitter systems.

The findings presented in this thesis, based on GWAS summary statistics, transcriptomic data from mice and zebrafish, as well as neurotransmitter levels, brain activity, and behavioural experiments in zebrafish, compared to clinical observations in patients, provide novel insights into how *RBFOX1* may contribute to the pathophysiology of MDD and other psychiatric

phenotypes, which will be explored in a hypothesis of a working mechanism at the end of this chapter.

1 THE EFFECTS OF *RBFOX1* ON GENE REGULATION

Our *RBFOX1* regulatory network analysis, in which we integrated transcriptomic data with GWAS summary statistics, revealed that *RBFOX1* and its regulated genes contribute to MDD, anxiety, irritability, and neuroticism mainly through pathways related to neuronal development and neurotransmission. Notably, we also found that *RBFOX1*-regulated genes contribute to rare disorders with symptoms of depression and anxiety, suggesting that *RBFOX1* may influence these symptoms through similar mechanisms across various conditions. Our transcriptomic analyses in adult *rbfox1*^{sa15940/sa15940} zebrafish revealed numerous differentially spliced and expressed genes, mainly involved in cell development and DNA repair mechanisms, particularly in the telencephalon and hindbrain. These findings align with studies implicating *RBFOX1* in brain developmental processes, which could partially explain its involvement in neurodevelopmental disorders like ASD. Our results suggest that certain functions of *RBFOX1* are evolutionarily conserved among vertebrates and play a crucial role in the regulation of various essential processes across different life stages, which may contribute to neurodevelopmental and psychiatric disorders when disrupted. Together, our findings provide new insights into the role of *RBFOX1* in the shared genetic architecture of these conditions.

1.1 Differential splicing and expression in zebrafish

Our transcriptomic analyses identified numerous differentially spliced and expressed genes in adult *rbfox1*^{sa15940/sa15940} zebrafish, most prominently in the telencephalon and hindbrain. These genes were primarily associated with cell development and DNA-repair mechanisms. Interestingly, the differentially expressed genes in the *rbfox1* mutant zebrafish partially overlap with those identified in mouse models of *Rbfox1*, which were enriched in neuronal and cortical development, and associated with ASD^{4,5}.

The overlap of the differentially expressed genes involved in developmental processes in the zebrafish brain with findings from studies in mouse models and human patients suggests that some of the functions of *RBFOX1* may be evolutionarily conserved among vertebrates^{6–10}. These studies have shown that *RBFOX1*-regulated genes are involved in essential neurodevelopmental processes such as neuronal differentiation, neuronal migration, and maintenance^{6–10}. Therefore, the regulatory role of *RBFOX1* in neurodevelopmental processes is likely conserved across vertebrates, at least partially^{6,11,12}.

Our discovery that *rbfox1* is involved in cell development and DNA repair during adulthood show that its effects extend beyond the neurodevelopmental period. This aligns with findings from human studies. While *RBFOX1* is known to be highly active and expressed during neurodevelopment, gene expression studies of human *post mortem* brain tissues show that

RBFOX1 is prominently expressed during different life stages, especially during prenatal stages and throughout adolescence and adulthood, particularly in the neocortex (**Figure**)¹³.

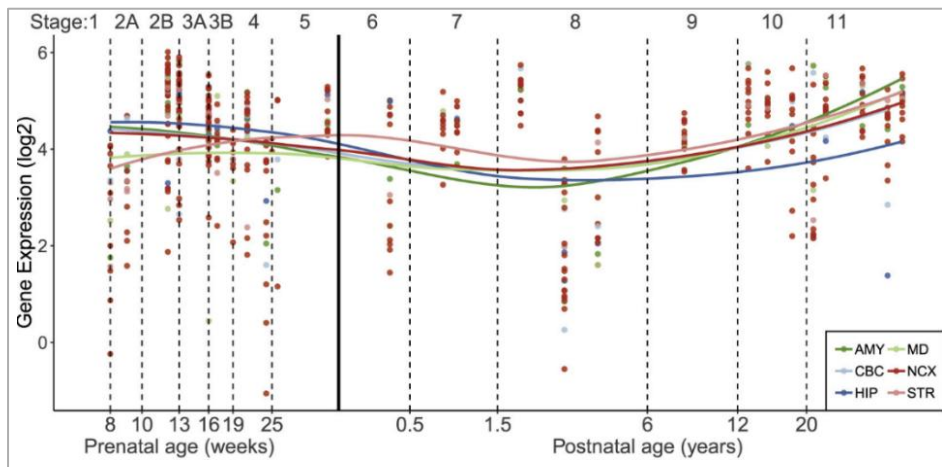


Figure 31: Gene expression trajectory of *RBFOX1* from a transcriptome atlas of human post-mortem brain tissue across development. AMY amygdala, MD mediodorsal nucleus of the thalamus, CBC cerebellar cortex, NCX neocortex, HIP hippocampus, STR striatum. Lee et al. (2019)¹³

Alterations in *rbfox1* activity may have distinct effects during different life stages. Therefore, to gain a comprehensive understanding of the role of *RBFOX1* in neurodevelopmental and psychiatric disorders, it is essential to examine its activity across all stages of life. In future studies we aim to conduct additional transcriptomic analyses in larval zebrafish to compare its role during development and adulthood.

1.2 The *RBFOX1* regulatory network in psychiatric disorders

To investigate the role of *RBFOX1* in MDD, anxiety, irritability, and neuroticism, we used transcriptomic data of *RBFOX1*-regulated genes and GWAS summary statistics on MDD, anxiety, irritability, neuroticism, and suicide attempt. We found *RBFOX1* to be associated with MDD, anxiety, and neuroticism, but not irritability or suicide attempt. Our analysis revealed enrichment of *RBFOX1*-regulated genes in all phenotypes except suicide attempt and showed that these genes are mainly involved in biological processes and pathways belonging to neurodevelopment and neurotransmission. Additionally, we found that *RBFOX1*-regulated genes contribute to rare disorders with symptoms of depression and anxiety, suggesting that *RBFOX1* may contribute to depressive and anxious symptoms through similar mechanisms across different disorders. Together, these findings offer new insights into the role of *RBFOX1* and its regulatory network in the genetic architecture of psychiatric and rare disorders with related symptomatology.

While *RBFOX1* has been previously been investigated mainly in terms of its involvement with individual disorders, over the past years the focus has shifted towards its pleiotropic effects on

multiple psychiatric disorders^{3,14–19}. In a 2019 study by the cross-disorder group of the PGC, *RBFOX1* was highlighted as the second most highly pleiotropic *locus*, as it was associated with seven out of the eight tested disorders (**Figure 32**)¹³. A 2022 study investigating the role of *RBFOX1* in different psychiatric phenotypes also found *RBFOX1* associated with eight different psychiatric conditions, including MDD, and furthermore associated it with a cross-disorder phenotype²⁰.

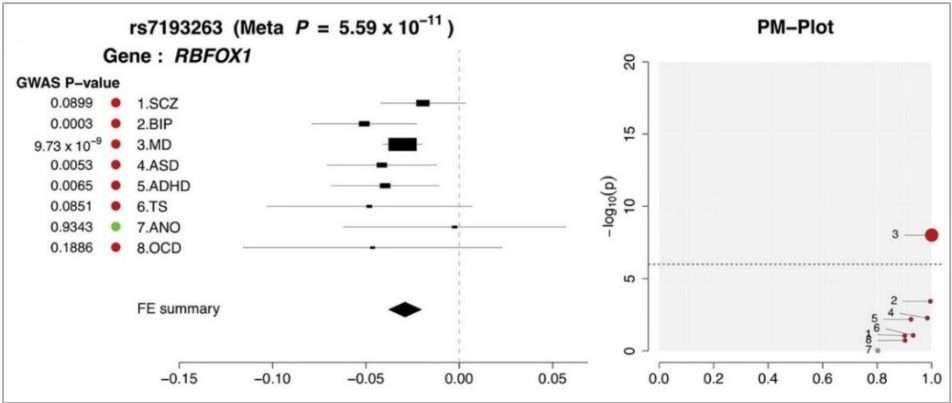


Figure 32: Associations of an *RBFOX1* locus with seven out of eight psychiatric disorders. Disorder-specific effects of the index SNP are in the forest plot in the first panel, displaying disorder-specific association *p* value, log odds ratios (ORs), and standard errors of the SNP. The meta-analysis *p* value and the corresponding summary statistic are displayed on the top and the bottom of the forest plot, respectively. The second panel is the PM-plot in which *x* axis represents the *m*-value, the posterior probability that the effect exists in each disorder, and the *y* axis represents the disorder-specific association *p* value as $-\log_{10}(p \text{ value})$. Disorders are depicted as a dot whose size represents the sample size of individual GWAS. Disorders with estimated *m*-values of at least 0.9 are in red, while those with *m*-values less than 0.9 are green. Lee et al. (2019)¹³

The pleiotropic effects of *RBFOX1* on different psychiatric disorders suggest that *RBFOX1* may contribute to these disorders through similar working mechanisms. Thus, if *RBFOX1* plays a key role, genes regulated by *RBFOX1* may also contribute psychiatric disorders. To investigate this theory, we explored the *RBFOX1* regulatory network, i.e. genes whose splicing and differential expression is influenced by *RBFOX1*, to elucidate their contribution to the genetic architecture of MDD and comorbid traits. For this we used previously described *RBFOX1*-regulated genes from two transcriptomic studies in mice by Lee et al. (2016) and Wamsley et al. (2018)^{4,5}. We chose to include all *RBFOX1*-regulated genes, not just splicing targets, as *RBFOX1* may have important downstream effects beyond its role in gene splicing. As a member of the *Rbfox* proteins family, it likely influences other essential processes, including transcriptional regulation, miRNA biogenesis, and mRNA stability and translation (**Figure 33**)²¹. We therefore consider it crucial to include genes regulated through other mechanisms to comprehensively understand the role of *RBFOX1* in psychiatric disorders.

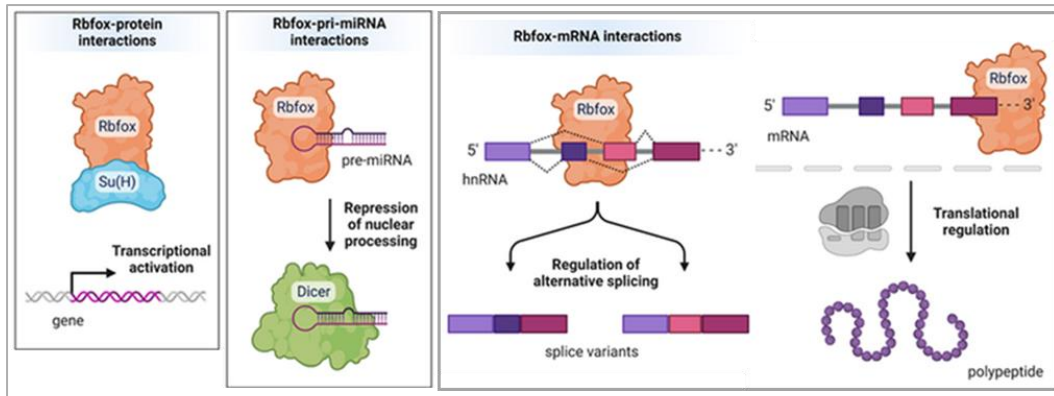


Figure 33: The different roles of Rbfox proteins beyond splicing regulation. Rbfox proteins play a role in transcriptional regulation, miRNA biogenesis, and mRNA stability and translation. Through these diverse functions, Rbfox1 controls the tissue-specific expression of critical developmental and disease-associated genes. Adapted from Mukherjee and Amartya (2024)²¹

Our approach including all RBFOX1-regulated genes is supported by studies demonstrating that along with direct splicing targets, downstream RBFOX1-regulated genes play a role in pathways implicated in ASD and other neurodevelopmental disorders⁶. RBFOX1 target genes form large alternative splicing networks, and alterations in these networks can lead to neurodevelopmental disruptions and contribute to various psychiatric phenotypes^{6–10,14,22}. RBFOX1 targets include other splicing and transcription factors, as well as RNA-binding proteins, which influence the downstream differential expression of other genes^{6,7}. Similar to the alternatively spliced targets, this extended network of RBFOX1-regulated genes is involved in pathways implicated in ASD and other neurodevelopmental diseases, as evidenced by a study by Fogel et al. using RNASeq in primary human neural stem cells during differentiation⁶. By generating an *RBFOX1* knockdown in these cells at a similar level to the haploinsufficiency observed in human ASD patients, they found that differentially expressed and spliced genes were enriched in similar biological pathways⁶. These pathways were found to contribute to neurodevelopmental disorders like ASD⁶. Interestingly, while the differentially spliced genes represented no more than 1–2% of the genes within the disease gene sets, differentially expressed genes were three times more enriched⁶. Additionally, they used weighted gene co-expression network analysis (WGCNA), to identify differences between *RBFOX1* knockdown and controls⁶. They found that the two modules with the largest differences included known risk genes for ASD such as the transcription factor *FOXP2* and the gene *DLG1*, which modulates cytoskeletal interactions to establish cell polarity⁶.

Our gene-based analysis showed that *RBFOX1* itself was associated with MDD, anxiety, and neuroticism, but not with irritability. Importantly, RBFOX1-regulated genes were associated with all four phenotypes. This shows that similar mechanisms in this *RBFOX1* regulatory network are underlying all these highly comorbid traits, but the link with irritability might be not directly related to *RBFOX1*.

In this line, we further discovered that RBFOX1-regulated genes contribute to these phenotypes through largely overlapping biological processes. Previous studies have established the involvement of *RBFOX1* in multiple disorders individually, including MDD, anxiety, and neuroticism. Our findings provide novel insights into the mechanisms through which *RBFOX1* affects these phenotypes at genetic and molecular levels. Within the RBFOX1-regulated genes we found associated with all these phenotypes, the top enriched biological processes shared across all of them involve neuron projection and synaptic processes. These processes had been related to *RBFOX1* before, however, the contribution of *RBFOX1* to MDD and related traits through these pathways was not previously described. Notably, RBFOX1-regulated genes in MDD and neuroticism, but not anxiety or irritability, share substantial biological pathways, primarily involving neurotransmitter systems and neuroplasticity. The monoaminergic system as well as in the balance of excitatory and inhibitory neurotransmission are key factors in the aetiology as well as treatment of depression^{2,23–25}. Antidepressants targeting these systems are thought to partially act through enhancing neuroplasticity^{26–29}. This highlights the importance of RBFOX1-regulated genes as significant contributors to the overlapping genetic basis of these phenotypes, with a major role in neurodevelopment and neurotransmission.

To confirm that the downstream effect of *RBFOX1* on MDD and the related traits is not driven by one or several of its targets, we constructed a network including both directly and indirectly regulated genes involved in these disorders. In this network, *RBFOX1* is one of the most central and well-connected nodes, and it is essential for network stability. This taken together with the somewhat low network heterogeneity confirms a broad regulatory role of *RBFOX1* on direct and downstream targets, without being driven by one of the target genes. These findings show that *RBFOX1* affects a wide range of transcripts contributing to diverse biological processes across the network.

We found six transcription factors, namely *CAMTA1*, *PAX6*, *SOX6*, *SP4*, *TCF4*, and *TEF*, among the RBFOX1-regulated genes associated with MDD and related traits. Similar results were obtained by Fogel et al. (2012) in their study on *RBFOX1* in neurodevelopmental disorders, where they also discovered several transcription factors among RBFOX1-regulated genes. While the effect of the *RBFOX1* regulatory network on MDD, anxiety, irritability, and neuroticism are not caused by a single or small group of downstream targets, *PAX6* stood out as highly pleiotropic and a potential candidate for future studies. Our study revealed a strong association of *PAX6* with MDD, anxiety, irritability, and neuroticism, and it was one of the most essential nodes in the *RBFOX1* regulatory network. *PAX6* encodes a transcription factor crucial for the development of neuronal tissue and the eye, and it is predominantly expressed in the brain, specifically in the cerebellar hemisphere and cerebellum³⁰. *PAX6* was also identified in the above-mentioned study by Fogel et al. (2012)⁶, and was previously identified as a RBFOX1 splicing target by Lee et al. (2016)⁴. Our transcriptomic

study confirmed the *PAX6* zebrafish homolog *pax6a* as a splicing target of *rbfox1* in zebrafish. We furthermore found it to be upregulated in the telencephalon of adult *rbfox1*^{sa15940} mutant zebrafish. This falls in line with a 2021 study on depression in over 1.2 million individuals, which found *PAX6* upregulated in the *caudate nucleus*³¹. Considering these findings, we suggest *PAX6* as a target for future studies of MDD, as well as potentially other psychiatric phenotypes.

The connection between *RBFOX1* and depressive and anxious phenotypes is supported by our analysis of genes causal for rare disorders. We examined genes causal for rare disorders with symptoms of depression, anxiety, irritability, and neuroticism and identified *RBFOX1*-regulated genes among them. While *RBFOX1* itself is not directly associated with any rare disorder exhibiting these symptoms, *RBFOX1*-regulated genes are enriched in genes causal for rare disorders with symptoms of depression and/or anxiety. Notably, several *RBFOX1*-regulated genes overlapped between rare disorders with symptoms of depression, anxiety and/or irritability. This provides novel insight into the pleiotropic influence of *RBFOX1* on similar symptoms in common psychiatric and rare disorders through potentially shared pathways. One of the shared genes between rare disorders with symptoms of depression and anxiety is *TCF4*. Plasma levels of *RBFOX1* and *TCF4* have previously been proposed as potential biomarkers for depression³². Our findings indicate that these genes might contribute through similar pathways to the symptomatology of depression and anxiety in rare and psychiatric disorders. Future studies investigating plasma levels of *RBFOX1* and *TCF4* in patients with the associated rare disorders could provide further insights into the potentially shared underlying working mechanisms.

1.3 Limitations of GWAS studies

Since the first GWAS was published in 2005, these studies have become increasingly popular to identify genotype-phenotype associations^{33,34}. In just over a decade alone, 50000 genome-wide significant associations of genetic variants with common diseases and traits had been reported in over 3700 GWASs, and the numbers constantly increase^{33,35}. These studies have provided valuable insights into the architecture of different diseases and uncovered novel disease-risk genes and mechanisms^{33,36}. They have also contributed to advancements in clinical treatment, including the discovery of new drug targets and disease biomarkers, as well as in personalized medicine, like improving risk prediction and optimizing therapies³³.

However, GWAS studies also have limitations. On one hand, the identified single-nucleotide variants (SNVs) only explain a small fraction of the heritability of complex traits as they typically have very small effect sizes^{33,37}. Rare and ultra-rare variants are particularly challenging to detect because of limited statistical power and sample size³⁸. As they occur with a very low frequency, detected associations bear a higher risk of being a false positive³⁸. Additionally, identified genetic variants may represent spurious associations, for example due to confounding factors or

sampling bias^{33,38,39}. This false detection can also happen when underlying genetic structures are not considered, for example, when genomic-window based tests do not account for heterogeneous SNP density in the genome⁴⁰. Another challenge when interpreting findings from GWAS studies is that not all identified SNVs are causal^{33,36}. Other factors such as cellular contexts and complex genetic interactions can make it difficult to elucidate the biological significance of GWAS signals^{33,36}.

One important consideration when analysing GWAS studies in complex disorders like depression is the great heterogeneity and complexity in the ways in which these disorders present. For instance, there are 227 possible combinations to combine the symptoms of MDD to meet the criteria for a diagnosis with MDD⁴¹. In practice, not all of these symptom combinations occur, but still 170 different ways of meeting the criteria for MDD were observed⁴¹. This can potentially reduce the ability to detect true genetic associations⁴¹. Additionally, differences in the developmental timing of the symptoms, the presence of other concurrent disorders, and the unique environmental factors affecting each person or study group further contribute to the diversity of the manifestations of MDD^{42,43}. This diagnostic heterogeneity is typically not fully accounted for in GWAS studies, as these studies usually only compare healthy controls with patients with a diagnosis, obtained with any kind of symptom combination.

The ways in which information about a diagnosis can be obtained, i.e., through self-reported clinical diagnosis, electronic health records codes, or structured diagnostic interviews, can also potentially add further noise⁴⁴. The GWAS data on anxiety, irritability, and neuroticism we obtained from the UK Biobank measured these phenotypes as continuous traits with scores based on self-reports. This might not be as reliable as a clinical diagnosis, as it was the case in the GWAS on MDD.

Using data from the UK Biobank can furthermore introduce a selection bias. The UK Biobank recruits participants through a volunteer-based strategy, and participants are, on average, healthier, wealthier and more educated than the general population^{45,46}.

Another limitation of GWAS analyses is the challenge of obtaining large enough sample sizes to accurately analyse traits. To increase the sample size for MDD, we utilized data from a meta-analysis of three large GWASs on MDD⁴⁷. However, for analysing suicide attempts, no GWAS on this trait with enough statistical power is available. The GWAS summary statistics that we used included fewer than 30 000 cases, and this relatively small sample size may have masked potential associations and effects that we were unable to detect. Future studies including larger sample sizes might provide more insight into potential effects of *RBFOX1* on suicide attempt or discard its contribution.

Moreover, in our analyses we used GWAS data including only individuals of European ancestry, which might not be translatable to other populations. Another key limitation is that the GWAS data used in this study were not stratified by sex, which could potentially obscure sex-specific genetic effects. This could be a strong limitation, as complex disorders like MDD may present differently in men and women and may have somewhat distinct genetic foundations. Future studies including GWAS separated by sex, as well as including other ancestries, could provide a better insight into the potentially different effects of *RBFOX1*.

In summary, our analyses were limited by the moderate sample sizes of the GWAS studies, especially of suicide attempt. Using only data of individuals with European ancestry further limited us from discovering potentially distinct effects of the *RBFOX1* regulatory network in different ancestries. Similarly, as the used GWAS were not separated by sex, we could not investigate potential sex differences in the effects of *RBFOX1*. These GWAS could also not account for the heterogeneity of symptoms with which the investigated psychiatric phenotypes present. If *RBFOX1* only affects a subset of symptoms, some of these effects might have been hidden. Future studies including bigger sample sizes of different ancestries and stratified by sex might provide more detailed insights into the effects of *RBFOX1* on different cohorts.

1.4 Limitations of transcriptomic studies

Our study of the transcriptomic alterations in *rbfox1*^{sa15940/sa15940} zebrafish was done in form of a bulk RNASeq. This allowed us to obtain a comprehensive overview of the gene expression and splicing differences. One advantage of performing bulk RNASeq is that it can detect low-expressed RNAs and non-coding RNAs by averaging gene expression across all cells in the sample. However, this approach also has certain limitations. For example, signals from specific cell types that are crucial for biological processes may be missed, as the averaging effect masks cell-type-specific responses. This could obscure important insights into the cellular mechanisms that drive the observed transcriptomic changes. Moreover, the averaging effect could also lead to false positives, where genes are identified as differentially expressed but do not truly reflect biological significance⁴⁸. Future studies performing single-cell RNASeq could obtain more high-resolution insights into individual cell types. This could potentially help to uncover more nuanced mechanisms underlying the functions of *RBFOX1*.

Moreover, as our study was conducted in zebrafish, statistically, only about 70% of the detected genes are likely to have a human ortholog. Therefore, comparing expression data across different models is essential to enhance our understanding of the transcriptomic changes caused by *RBFOX1*. We therefore compared our results with published studies on *Rbfox1*-regulated genes in mice. This revealed evidence of evolutionarily conserved effects on cell development, and supported the validity of our findings.

1.5 Limitations of translatability to human studies

An important limitation of our study is the translatability of our findings from the zebrafish model to human psychiatric disorders. In humans, *RBFOX1* itself undergoes complex regulatory processes, including autoregulation, with multiple initiation sites for transcription and translation^{11,12}. This results in a range of structurally distinct, tissue-specific isoforms^{11,12}. Our *rbfox1* zebrafish model cannot capture the full spectrum of *RBFOX1* isoforms and therefore not all functions of *RBFOX1* that may be isoform specific. It is likely that our findings reflect the more evolutionarily conserved and essential functions of *RBFOX1*. To broaden the scope of our findings, we compared transcriptomic data from *rbfox1*^{sa15940} zebrafish with data from *Rbfox1* mouse models, however, further research is needed to compare our findings with additional models to better understand the other aspects of the functions of *RBFOX1*.

Additionally, different kinds of *RBFOX1* genetic variants were found in different psychiatric disorders. For instance, partial or complete CNVs in *RBFOX1*, including both deletions and duplications, are observed in patients with ASD but also with schizophrenia, while common genetic variants with small effects are associated with MDD. The two zebrafish lines used in our studies have high impact *rbfox1* mutations, leading to an exon skipping event and a full knockout, respectively. These mutations allow for the functional characterization of *rbfox1*, however, they do not necessarily model what is observed in patients. The effect of other genetic variants, which might be partially influenced by *RBFOX1*, play an important role in MDD and other disorders. To take this into account, we performed an *RBFOX1* regulatory network, allowing us to observe the downstream effects of *RBFOX1*.

Another limitation in all our studies with zebrafish is that the results of male and female zebrafish were pooled. We did not investigate both sexes separately as we cannot easily ascertain sex in this species. The sex of the zebrafish can only be presumed morphologically and this is prone to errors due to the visual similarity of the male and female fish at the tested ages (6 dpf to 6 months). Separating the results by the morphologically determined sex of the fish might therefore introduce more errors and false positive or negative findings. In future studies, this could be addressed by having a veterinarian separate the fish by sex prior to the studies. However, as zebrafish are social animals living in shoals of mixed sexes, separating them can cause stress and affect the study results. This should be taken into account when specifically testing for sex differences in zebrafish.

In summary, the *rbfox1* zebrafish models used in this study allow for the functional characterization of the gene. However, they might not be exactly comparable to the genetic variations observed in psychiatric disorders. Therefore, we compared our findings with data from human studies, ensuring the validity of our conclusions.

2 THE EFFECTS OF *RBFOX1* ON NEUROTRANSMISSION

Our findings of reduced basal levels of serotonin, dopamine, GABA, and glutamate in the forebrain of adult *rbfox1*^{sa15940} mutant zebrafish align with observations in patients with major psychiatric disorders. Monoaminergic, GABAergic, and glutamatergic neurotransmission are key factors in the development and treatment of ADHD, ASD, bipolar disorder, depression, OCD, and schizophrenia, among others^{2,23–25,49–51}. These findings suggest a potentially causal link between impaired *rbfox1* functioning and disruptions in neurotransmission, which could underlie some of the findings in psychiatric patients.

2.1 Altered serotonin in *rbfox1*^{sa15940} zebrafish and psychiatric disorders

Several lines of evidence implicate serotonin in the aetiology of depression, and medications targeting the serotonergic system are the most commonly used antidepressants, although the underlying mechanisms are not fully understood^{52,53}. The reduced basal levels of serotonin and its precursor tryptophan in the forebrain of *rbfox1*^{sa15940} mutant zebrafish align with our findings in genomic data from patients. We found that *RBFOX1*-regulated genes in MDD are enriched in biological processes related to the serotonergic synapse. This implicates *RBFOX1* in the complex relationship between serotonin and MDD. While there is no conclusive evidence for a causal role of lowered serotonin in depression, it may contribute to this disease through other indirect effects, partially mediated by *RBFOX1*⁵².

Early research suggested a causal relationship of low serotonin levels with depression, however, more recent studies have provided a more nuanced understanding^{52,53}. Although lowered serotonin levels are not directly linked to MDD, serotonin still seems to be a factor in its aetiology, as medications targeting the serotonergic system are among the most prescribed antidepressant drugs. Moreover, serotonin is highly involved in the regulation of mood, aggression, anxiety, among other behaviours and physiological functions, which are commonly dysregulated in depression²³. On a genetic level, serotonin is also linked to MDD, among other psychiatric disorders, as evidenced by a recent study by Cabana-Domínguez et al. (2022)⁵⁰. In this study, the involvement of serotonergic as well as dopaminergic gene sets was investigated in eight different psychiatric disorders, namely ADHD, anorexia, ASD, bipolar disorder, MDD, OCD, schizophrenia, and Tourette's syndrome⁵⁰. While most genes from both genes sets were not enriched in any of the investigated disorders, a gene-set enrichment analysis revealed significant associations of the serotonergic gene set with bipolar disorder and MDD⁵⁰.

Interestingly, several genes within the serotonergic gene set are regulated by *RBFOX1*, namely *AKT3*, *MAPK1*, *MAPK3*, and *GRIN2A*^{4,5,50}. These genes have pleiotropic effects, particularly on bipolar disorder, and schizophrenia⁵⁰. The involvement of these genes in multiple psychiatric

disorders suggests they may act through similar mechanisms and pathways across these conditions. Interestingly, these genes also partially overlap with the dopaminergic gene set, highlighting the interconnectedness of these systems⁵⁰.

One recent theory relates serotonin, and by extension the effect of SSRIs, to depression through its neuroplasticity modulating properties^{52,53}. This could also partially explain the delayed onset of antidepressant medication targeting the serotonergic system^{52,53}. Our study of enriched biological pathways in depression and related traits implicates *RBFOX1* in this mechanism: Along with biological processes related to the serotonergic system, we found long-term depression and long-term potentiation enriched in *RBFOX1*-regulated genes in MDD and neuroticism. This indicates that *RBFOX1* may affect neuroplasticity in depression, potentially through the modulation of serotonin. However, based on our analyses it is also possible that *RBFOX1* may influence serotonergic transmission and neuroplasticity independently of each other. Either of these hypotheses would be supported by our findings of lowered serotonin levels in the forebrain of *rbfox1*^{sa15940} mutant zebrafish. However, as the complex role of serotonin and neuroplasticity in depression is not fully understood, further research into the role of *RBFOX1* in these mechanisms is necessary⁵⁴.

Interestingly, we found lower levels of serotonin in the forebrain of adult *rbfox1*^{sa15940} mutant zebrafish, whereas reduced serotonin levels in MDD patients are not consistently reported. This suggests that *rbfox1* may influence serotonergic neurotransmission in zebrafish differentially than in humans, at least in part. One possible reason for this could be that, unlike mammals, zebrafish retain the ability to undergo neurogenesis throughout adulthood^{55,56}. One key modulator of this process is serotonin, which can both promote and suppress neurogenesis depending on the physiological or pathological context^{55,56}. In addition to its role in regulating serotonergic transmission and plasticity, *rbfox1* may also influence neurogenesis in adult zebrafish, possibly through serotonergic modulation. Our findings of differentially expressed genes related to cell development in the forebrain of adult *rbfox1*^{sa15940} mutant fish support this idea. As humans are not capable of neurogenesis throughout all life stages in the same way as zebrafish, this role of *rbfox1* might not be conserved. Another possibility is that humans could possess compensatory mechanisms that prevent serotonin levels from dropping in the same way as in zebrafish. Taken together, these findings make the role of *rbfox1* in the interaction between serotonin and neuroplasticity an interesting area for future research.

Together, these findings implicate *RBFOX1* as a modulatory element of the complex effects of serotonergic neurotransmission in psychiatric disorders. While the role of the serotonergic system in MDD is more complex than originally proposed in the monoamine hypothesis, serotonin remains an important factor in the aetiology of MDD and other psychiatric disorders. Our findings suggest that these processes may be partially modulated by *RBFOX1*.

2.2 Altered dopamine in *rbfox1*^{sa15940} zebrafish and psychiatric disorders

Alterations in dopaminergic transmission are implicated in several major psychiatric disorders, including ADHD, ASD, bipolar disorder, MDD, post-traumatic stress disorder (PTSD), OCD and schizophrenia^{50,51}. Reduced dopamine levels in *rbfox1*^{sa15940} mutant zebrafish, along with the enrichment of dopaminergic pathways in RBFOX1-regulated genes in MDD, suggest a potentially causal role of *RBFOX1* in the dysregulation of dopaminergic system.

Dopamine is primarily implicated in depression through its role in anhedonia, which is a core symptom. A crucial part of the development of anhedonia is the disruption of the reward system, which is modulated primarily by dopamine, and may be partially regulated by *RBFOX1*²⁴. Evidence from several studies points to downregulation of the dopaminergic system in MDD patients: Lower levels of dopamine transporter binding and increased binding of the dopamine receptor D₂ were found in striatal regions of MDD patients, which might be compensatory mechanisms due to downregulation of the dopaminergic system⁴⁹. However, the afferent circuits regulating these mechanisms are not yet fully understood⁴⁹. Our neurotransmitter analyses implicate *rbfox1* in the downregulation of dopamine, as lower levels were found in the forebrain of *rbfox1*^{sa15940} mutant zebrafish compared to wildtypes. This suggests that variants in *RBFOX1* may be involved in the disruptions of the reward system in MDD patients. Our findings of enriched dopaminergic processes in RBFOX1-regulated genes in MDD and neuroticism align with these findings, further implicating *RBFOX1* in the intricate mechanisms of dopaminergic homeostasis and reward signalling. However, the exact working mechanisms through which *RBFOX1* might influence dopaminergic neurotransmission are not yet understood.

RBFOX1 was also linked to the genetic basis of the dopaminergic system in other psychiatric disorders⁵⁰: The genes *AKT3*, *MAPK10*, and *GRIN2A*, which are regulated by RBFOX1, belong to the dopaminergic gene set identified in the aforementioned study by Cabana-Domínguez et al. (2022), and show pleiotropic influences on bipolar disorder and schizophrenia^{4,5,50}.

Taken together, these findings suggest that *RBFOX1* may play a causal role in the dysregulation of dopaminergic transmission in depression, bipolar disorder, and schizophrenia. This might occur through shared pathways, potentially related to the reward system, as anhedonia is a common symptom across these disorders^{50,57}. However, the absence of shared genes in the dopaminergic gene set among all three disorders suggests that the interaction between *RBFOX1* and dopaminergic dysregulation may vary between conditions. *RBFOX1* itself could be affected in distinct ways in each disorder, either through genetic variations or distinct alternative splicing, leading to different downstream effects on dopaminergic neurotransmission. Alternatively, the same *RBFOX1* variants or isoforms, in combination with varying genetic backgrounds, could influence dopaminergic neurotransmission differently, resulting in distinct dopaminergic

alterations and symptoms. Further research is needed to investigate the pathways through which *RBFOX1* affects the dopaminergic system and the resulting phenotypic consequences of disruptions of these processes.

2.3 Altered GABA and glutamate in *rbfox1*^{sa15940} zebrafish and psychiatric disorders

Disruptions in GABA- and glutamatergic neurotransmission are implicated in various psychiatric disorders, including MDD, bipolar disorder, and schizophrenia^{26,58–60}. Reduced levels of these neurotransmitters in *rbfox1*^{sa15940} mutant zebrafish, along with the enrichment of GABA- and glutamatergic processes in *RBFOX1*-regulated genes in MDD, suggest that *RBFOX1* contributes to the disruptions and imbalances of these systems in depression.

GABA levels are consistently reported to be reduced in patients with MDD, particularly in cortical regions, and they have been shown to normalize with remission^{26,59,60}. Furthermore, post-mortem brain samples of patients with depression show reduced expression of glutamic acid decarboxylase (GAD), the primary enzyme for GABA synthesis, and reduced peptide markers for subsets of GABAergic interneurons^{61,62}. GABA deficits impair cortical inhibition, a key process in depression⁶³. Interestingly, similar deficits have been observed in patients with OCD and schizophrenia, suggesting common biological pathways across these disorders⁶⁴. Similarly, reduced levels of glutamate metabolites and synaptic markers have been reported in cortical regions and the ACC in MDD patients^{26,58,65}. Imaging studies also show that lower glutamate in the subgenual ACC is associated with decreased connectivity to other brain areas, such as the insula, and a reduced response to emotional stimuli in MDD patients²⁶.

Our findings suggest that *RBFOX1* is involved in the regulation of GABA and glutamate systems. We identified biological pathways related to both GABA- and glutamatergic systems in *RBFOX1*-regulated genes in MDD, and neurotransmitter analyses revealed reduced levels of both GABA and glutamate in the forebrain of *rbfox1*^{sa15940} mutant zebrafish, a region homologous to the human cortex. This suggests that alterations in *RBFOX1* may contribute to disrupted inhibitory and excitatory signalling.

Notably, chronic stress, a commonly used depression model in preclinical research, can lead to a shift in GABA and glutamate functioning and reduced GABA markers²⁶. As *RBFOX1* was implicated in stress response processes, it might be implicated in GABA- and glutamatergic dysfunctions in stress and depression^{14,66–68}. Both clinical and preclinical studies showed that chronic stress and depression can cause deficits in GABA and glutamate systems. These findings include reduced volume in the PFC and hippocampus, lower levels of synaptic markers, fewer somatostatin (SST) interneurons, and decreased GAD levels (**Figure 34**)⁵⁸. Furthermore, chronic stress in mice resulted in decreased glutamate cycling in the PFC, atrophy of dendrites of

pyramidal neurons and fewer synapses, reduced levels of synaptic proteins, and lower vesicular glutamate transporter (VGLUT) levels (**Figure 34**)^{26,58}. Also observed are reductions in GABA neuronal markers⁵⁸. These disruptions in the GABA and glutamate systems lead to reduced signal-to-noise ratio and degradation of signal integrity⁵⁸. The fact that ketamine, an NMDA receptor antagonist, can rapidly increase GABA and glutamate systems and reverse deficits caused by chronic stress exposure supports the link between stress, alterations in GABA and glutamate systems, and depressive symptoms as potentially causal (**Figure 34**)²⁶.

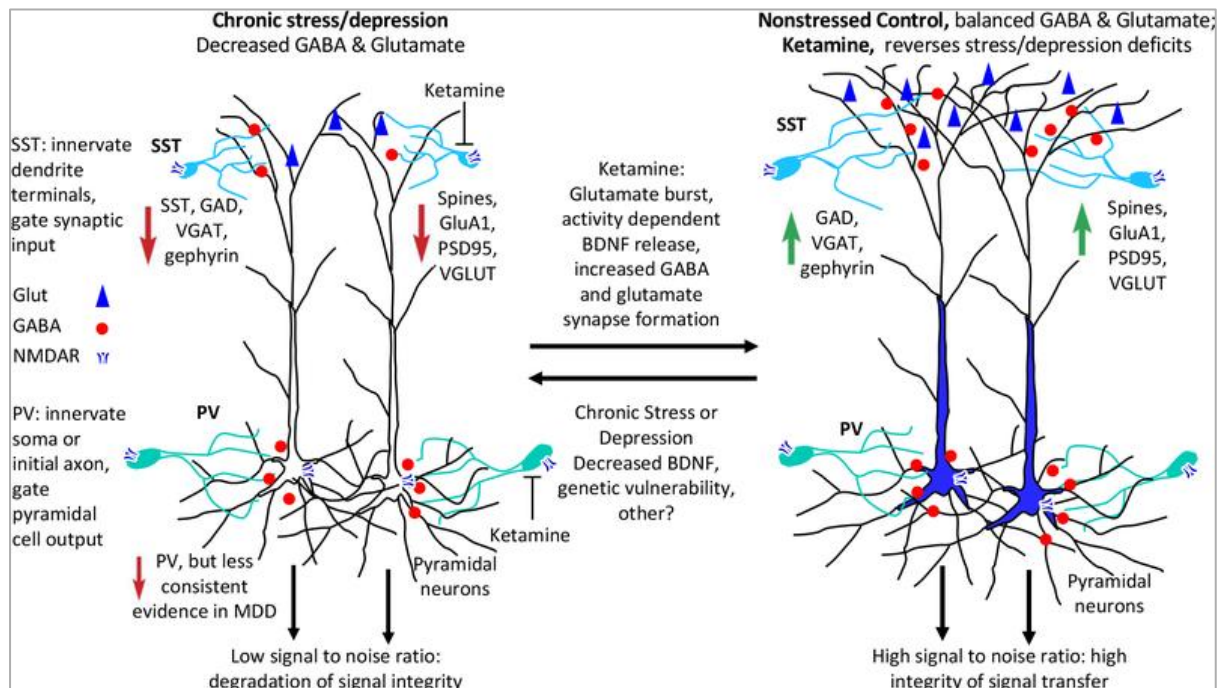


Figure 34: Stress and depression decrease both glutamate and GABA neurotransmitter systems in cortical and limbic brain regions, which can be reversed by ketamine. SST interneurons innervated dendrites of principle neurons and gate synaptic input while PV interneuron subtypes innervate the soma (basket cells) and initial axon (chandelier cells) and gate output. Under normal, non-stressed conditions, GABA and glutamate transmission is balanced and provides high signal to noise ratio and integrity of signal transfer. Depression and chronic stress have been shown to cause deficits of both GABA and glutamate systems, and reduce the volume of prefrontal cortex and hippocampus, reduce synaptic markers, decrease SST interneuron markers, and decrease levels of glutamic acid decarboxylase (GAD). There is less consistent evidence for deficits of PV interneurons. Preclinical studies demonstrate atrophy of pyramidal neuron dendrites in PFC and hippocampus, reductions of synapse number and proteins (PSD95, GluA1), and decreased levels of the vesicular glutamate transporter (VGLUT) in response to chronic stress. Chronic stress furthermore causes reductions of GABA neuronal markers, notably SST, the vesicular GABA transporter (VGAT), GAD, and gephyrin. Reductions in GABA and glutamate neurotransmitter systems decrease signal to noise ratio and result in degradation of signal integrity in these regions. Ketamine rapidly increases both GABA and glutamate systems and reverses the deficits caused by chronic stress exposure. It bursts glutamate via blockade of NMDARs on GABA interneurons, causing activity dependent release of BDNF and increased synapse number and function in layer 5 pyramidal neurons in the medial prefrontal cortex (mPFC), as well as increased synaptic markers (PSD95, GluA1, VGLUT). GABA interneurons are more sensitive to ketamine because their tonic firing removes Mg²⁺ from the channel, allowing ketamine to enter and block the channel. Ketamine rapidly increases GABA function in the mPFC including increased levels the VGAT, GAD, and gephyrin. Up-regulation of GABA and glutamate neurotransmitter systems

reverses the effects of chronic stress exposure, and thereby increases signal to noise and enhances integrity of signalling in these regions. PV parvalbumin, SST somatostatin. Duman et al. (2019)⁵⁸

RBFOX1 has been linked to stress response mechanisms, which in turn is associated with lowered GABA and glutamate^{14,66–68}. Genetic variants in *RBFOX1* could therefore hinder adaptive mechanisms to chronic stress, leading to the disruptions in GABA and glutamate neurotransmission observed in depression. Interestingly, we observed reduced basal GABA and glutamate levels in unstressed animals. This could suggest that *RBFOX1* may influence GABA- and glutamatergic neurotransmission also independently of stress. However, the similarity of the observed GABA and glutamate alterations in unstressed *rbfox1* zebrafish to neurochemical changes observed in chronic stress and depression could also indicate another mechanism: *RBFOX1* dysfunction may predispose individuals to stress-related disorders by creating a neurochemical environment that resembles a chronically stressed state. Such a dysregulation of the stress response system could lead to an exaggerated or prolonged stress response, even in the absence of stressors, and increase vulnerability to psychiatric conditions. However, further research into the effects of *RBFOX1* on GABA and glutamate under stress and normal conditions is needed to confirm this hypothesis.

Together, our findings implicate *RBFOX1* in the homeostatic regulation of the excitation and inhibition, with disturbances potentially leading to stress-like states and psychiatric phenotypes.

2.4 Limitations of modelling neurotransmitter systems in zebrafish

To ensure the clinical relevance of our data, we compared our findings of zebrafish neurotransmitter levels with studies in human patients. However, this bears certain limitations. For one, while vertebrate neurotransmitter systems are highly conserved, animal models like mice and zebrafish may not fully capture the complexity of the human brain⁶⁹. Zebrafish, unlike mammals, do not possess a neocortex; therefore, the zebrafish brain lacks higher-order cortical structures critical for complex cognitive processes such as abstract reasoning and language. Functions like learning, memory, social interactions, and decision-making are instead carried out by the telencephalon^{70–72}. This limits the comparability between human and zebrafish studies, as the anatomy and functions of these regions differ to some extent. Performing neurotransmitter analyses in a mammalian model, e.g. in *Rbfox1* deficient mice, could provide further insight into how *Rbfox1* affects neurotransmitter levels in cortical structures.

Another limitation of our study is that, due to the small size of the zebrafish brain, we had to sample relatively large regions to be able to perform neurotransmitter analyses. Therefore, we had to compare wider brain regions in zebrafish with more specific regions of the human brain. For instance, we had to compare the whole zebrafish telencephalon with the human striatum. As

a result, comparing neurotransmitter alterations in specific brain areas in zebrafish is less precise than studies conducted in humans. This could potentially be addressed by dissecting the zebrafish brain into smaller regions and then pooling the samples. This approach could offer more detailed insights into neurotransmitter alterations between smaller brain structures. It should be considered however that this strategy does not allow for addressing inter-individual differences of the fish and might therefore introduce variability.

Additionally, future research on the impact of early life and/or chronic stress on neurotransmitter changes in the *rbfox1*^{sa15940} mutant zebrafish would offer valuable insights into how *RBFOX1* influences the stress response in depression, given that stress plays a key role in depression².

3 THE EFFECTS OF *RBFOX1* ON BRAIN ACTIVITY

Alterations in brain activity and connectivity are consistently reported in major psychiatric disorders, including ASD, bipolar disorder, epilepsy, MDD, and schizophrenia^{10,73–76}. Our findings of increased neuronal activity and synchronicity in *rbfox1*^{sa15940} mutant zebrafish implicates *RBFOX1* in these alterations, in line with findings from different preclinical and clinical studies^{10,73–75}.

3.1 Altered neuronal activity in *rbfox1*^{sa15940} zebrafish and psychiatric disorders

Altered neuronal activity and excitability is associated with disorders such as depression, epilepsy, ASD, and neuropathic pain, and has been modelled in preclinical studies^{10,73–75,77}. Our study of *in vivo* brain activity of *rbfox1*^{sa15940} mutant and wildtype zebrafish are in line with these studies, as neurons in *rbfox1*^{sa15940} mutant zebrafish were more active and showed more activity in collective bursts, confirming the role of this gene in psychiatric disorders.

The interaction between neuronal excitability and connectivity has been shown to play a role in disorders like ASD, where factors such as the homogeneity of network excitability and the strength of functional connectivity can significantly impact information processing and cognitive outcomes⁷⁸. Given that *RBFOX1* is a known risk gene for ASD, our findings suggest that the link between *RBFOX1* and the disorder may, in part, be mediated by alterations in neuronal activity. The consistent findings across zebrafish, mice, and humans regarding the role of *RBFOX1* in neuronal activity indicate that this function may be evolutionarily conserved among vertebrates. For instance, mice with a neuron-specific deletion of *Rbfox1* exhibited increased neuronal excitability¹⁰. Interestingly, this mutation also heightened the susceptibility of these mice to both induced and spontaneous seizures¹⁰. We did not observe increased spontaneous seizures in either the heterozygous or the homozygous *rbfox1*^{sa15940} mutant zebrafish. This could be due to the distinct natures of the *rbfox1* mutation: While the aforementioned study used an *Rbfox1* knockout, the *rbfox1*^{sa15940} mutation in our study causes an exon skipping event, leading to a shorter, aberrant mRNA¹⁰. This may preserve some functions and protect against seizures. However, in a second line of *rbfox1* mutant zebrafish with a full knockout, namely *rbfox1*^{del19}, we did not observe any seizures either. This suggests that the absence of seizures may result from differences in brain morphology or compensatory or protective mechanisms in zebrafish that prevent seizure onset. Nevertheless, more research is needed to clarify the precise role of *RBFOX1* in seizures and understand the underlying mechanisms contributing to these differences.

In summary, our study confirms the role of *RBFOX1* in altered neuronal activity in psychiatric phenotypes. Our findings also provide a more nuanced perspective on the link between *Rbfox1*

deficiency and susceptibility to seizures, highlighting the importance of considering the contribution of the genetic background.

3.2 Altered neuronal activity in *rbfox1*^{sa15940} zebrafish and psychiatric disorders

Numerous connectivity studies offer strong evidence of altered brain activity and connectivity in patients with psychiatric disorders such as depression, bipolar disorder, and schizophrenia^{23,76}. Interestingly, in our study, we observed changes in brain activity in *rbfox1*^{sa15940} mutant zebrafish, primarily in the midbrain and hindbrain, rather than in brain regions typically associated with depression, such as cortical and limbic regions, which would be homologous to forebrain regions in zebrafish⁷⁷.

In humans, depression is frequently associated with upregulation and increased connectivity of the affective and the default mode networks, along with downregulation and decreased connectivity of the cognitive control and reward networks (Figure 6)²³. In their 2015 meta-analysis, Palmer et al. reviewed the overlap and divergence in regions with altered activity in adults diagnosed with depression and healthy controls under different conditions⁷⁷. They report several divergent clusters of activity in emotionally valenced and cognitively demanding tasks. In depression, no significant overlap was found between brain regions altered during emotional tasks and cognitive tasks, whereas in healthy controls, integrated activation patterns are typically observed across these tasks⁷⁷. During cognitively demanding tasks, the right medial frontal and insula regions were recruited in patients with depression, possibly as a compensatory mechanism⁷⁷. During emotional tasks, bilateral amygdala activity was significantly altered⁷⁷. However, activity overlap was present in the left amygdala and the right subcallosal cingulate between emotion and resting state in patients with depression⁷⁷. This activity overlap was not observed in healthy controls, suggesting alterations in baseline activity potentially contributing to emotional dysregulation⁷⁷. These areas are more homologous to forebrain regions in zebrafish. Interestingly, our *in vivo* whole brain imaging study revealed increased neuronal activity and increased synchronicity in regions of the midbrain and hindbrain in *rbfox1*^{sa15940} mutant zebrafish. This could suggest that *RBFOX1* affects neuronal activity and connectivity differently in humans and zebrafish. Our findings could also indicate that *RBFOX1* may influence distinct neural circuits that are not conventionally linked to depression but still contribute to mood regulation and stress vulnerability. However, it is important to note that changes in neuronal activity do not necessarily reflect alterations in connectivity, and these findings should therefore be interpreted with caution. Further research is needed to elucidate the relationship between the observed changes in neuronal activity in *rbfox1* mutant zebrafish and potential connectivity alterations in these circuits.

3.3 Limitations of modelling brain activity in zebrafish

One major limitation of our brain imaging study in zebrafish larvae is the lower levels of complexity of the zebrafish brain compared to humans, as discussed in chapter 2.4. Neural circuits in zebrafish are less intricate and contain fewer neurons than those of the human brain⁷⁹. This limits the interpretability of our results. Our study only provides insights into how the *rbfox1* mutation affects activity in the described regions of the zebrafish brain. To obtain clearer insights into how it affects cortical activity, and how this translates into depressive symptoms or other symptoms in psychiatric disorders, a mammalian model, for example *Rbfox1* deficient mice, could be more suitable. However, *in vivo* whole brain imaging is only possible in transparent brains, as is the case in zebrafish larvae, but not in mammals.

Another important limitation is that our study was conducted in zebrafish larvae at an early developmental stage, when the brain is still maturing both structurally and functionally. In contrast, most fMRI studies on depression in humans are performed in fully developed adult brains and assess functional connectivity within fully developed networks. Similarly to the functions of the maturing brain regions, the functions of *rbfox1* and by extensions the consequences of its mutation may manifest differently at the different life stages.

It is also important to consider the fact that in our study on brain imaging we only modelled resting-state activity. Comparisons with task-dependent activity and connectivity changes should be done with great care, as task-dependent activity often involves specific circuits that may not be engaged at rest. These limitations should be addressed in future studies. For instance, while *in vivo* brain activity can not be measured in zebrafish, the *c-fos* proto-oncogene could be utilized as a brain activity marker⁸⁰. This way, neuronal activity could also be compared after testing the zebrafish in different paradigms, which might be more comparable with task-dependent fMRI studies in humans. This could also provide better insights into which neuronal circuits influence different behaviours.

Neuronal excitability can modulate functional connectivity within and between brain areas, and vice versa, as changes in network connectivity can influence its excitability^{81,82}. However, our findings of altered neuronal activity and synchronicity do not necessarily reflect the connectivity in the zebrafish brain^{81,82}. Therefore, future studies investigating connectivity alterations in *rbfox1*^{sa15940} mutant zebrafish or using mammalian animal models are necessary to better characterize how *rbfox1* affects neuronal activity and connectivity.

4 THE EFFECTS OF *RBFOX1* ON BEHAVIOUR

Major psychiatric disorders such as MDD, ADHD and ASD are associated with behavioural changes like hyperactivity or restlessness, stereotyped behaviours, and social impairments. Similar behavioural alterations, including hyperactivity and changes in social behaviour and aggression, are observed in two different lines of *rbfox1* mutant zebrafish, as well as *Rbfox1* mutant mice, indicating a potential causal role of *RBFOX1* in these behaviours.

There is a great variety of inherited as well as *de novo* *RBFOX1* variants observed in patients with psychiatric disorders, along with potential genetic heterogeneity²⁰. These different variants in combinations with distinct genetic backgrounds may have different effects on psychiatric phenotypes. We therefore aimed to investigate how different *rbfox1* mutations and genetic backgrounds can influence behaviour. To address this, we performed behavioural tests in two zebrafish lines from different strains and with different *rbfox1* mutations. The first line, *rbfox1*^{sa15940}, was the line we also used for the transcriptomic, neurotransmitter, and neuronal activity experiments. This line has a Tübingen longfin (TL) background and a point mutation leading to an exon-skipping event, resulting in a short, aberrant mRNA that may be translated to a shorter protein. The second line, *rbfox1*^{del19}, has a Tübingen (TU) background and a frameshift mutation leading to a premature stop codon in the second exon, thus not encoding functional protein. This second line was obtained from a group from Queen Mary University of London, UK, which had generated the *rbfox1*^{del19} line independently. As comparing the behavioural alterations observed in the *rbfox1*^{sa15940} line with this second line provides additional insights into the effects of the different backgrounds and mutations, we decided to repeat the same behavioural test in the *rbfox1*^{del19} line. This approach led to several interesting discoveries of consistent but also specific behavioural alterations. Both zebrafish lines displayed hyperactivity and thigmotaxis, as well as alterations in social behaviours. While hyperactivity and thigmotaxis were more pronounced in the *rbfox1*^{sa15940} line, the *rbfox1*^{del19} line displayed stronger alterations in social tests (Table 3).

Table 3: Summary of behavioural alterations in *rbfox1*^{sa15940} and *rbfox1*^{del19} mutant zebrafish and *Rbfox1* depleted mice²⁰.

Behavioural alterations	Zebrafish TL (♀, ♂)	Zebrafish TU (♀, ♂)	Mice (♂)
Hyperactivity	Total distance and swimming speed increased in HZ and HOM fish in the Open Field test Total distance and swimming speed increased in HOM fish in the VMSP test Number of crossings increased in HOM fish in the Black and White test	Total distance and swimming speed increased in HOM fish in the VMSP test Number of crossings increased in HZ and HOM fish in the Black and White test	Total distance increased in HOM mice in the Open field test with and without the presence of a novel object in the centre Total distance increased in HOM mice in the Light-dark box test Total distance increased in HOM mice in the Marble-burying test
Increased thigmotaxis behaviour	Thigmotaxis increased in HZ and HOM fish in the shoaling and Open Field test	Thigmotaxis increased in HZ fish in the Open Field test Thigmotaxis increased in HZ and HOM fish in the Shoaling test	Thigmotaxis increased in HOM mice in the Open Field test Thigmotaxis decreased in HOM mice in the Novel Object Exploration paradigm
Decreased freezing behaviour	Decreased freezing in HZ and HOM fish in the Open Field	No differences found between the genotypes	Decreased freezing in HOM mice in the fear conditioning test in all stages Decreased freezing in HZ mice in the fear conditioning test in extinction phase
Decreased social interest	Increased interindividual distance in HZ and HOM fish in the Shoaling test	Increased interindividual distance in HZ and HOM fish in the Shoaling test Increased nearest neighbour distance in HOM fish in the Shoaling test HOM fish spend less time near the 1st strangers in first step of the VMSP paradigm	Less social interest in social interaction test in the HOM mice
Altered aggressive behaviour	No difference between mutant and control fish in aggressive behaviour in the mirror test, but aggression is increased in HOM compared to HZ fish	No differences found between the genotypes	Lack of aggressive behaviour in HOM mice in the escalated aggression test

TL *rbfox1*^{sa15940}, TU *rbfox1*^{del19}, HOM homozygous individuals for the respective *rbfox1* mutation, HZ heterozygous individuals for the respective *rbfox1* mutation, VMSP visually-mediated social preference test, WT wild-type individuals, ♀ female individuals, ♂ male individuals. Adapted from Antón-Galindo et al. (2024)⁸³

4.1 Hyperactivity in *rbfox1* mutant zebrafish and psychiatric disorders

Both the *rbfox1*^{sa15940} and the *rbfox1*^{del19} mutant zebrafish displayed hyperactivity, and, remarkably, more pronounced in the *rbfox1*^{sa15940} line. This aligns with finding from a 2022 study by O’Leary et al. which detected hyperactivity in a *Rbfox1* knockout mouse line (Table), as well as clinical findings^{20,57}.

Hyperactivity is a core symptom of ADHD, and patients with ASD can also exhibit hyperactivity^{20,57}. Furthermore, psychomotor agitation or restlessness can be a symptom of MDD⁵⁷. A proposed pathophysiological mechanism that has long been associated with ADHD is the dysregulation of monoaminergic neurotransmission systems, particularly the dopaminergic and noradrenergic systems⁸⁴. Findings of reduced dopamine levels in the synaptic cleft as well as the efficacy of medication targeting the dopaminergic system in patients with ADHD support this theory⁸⁴. In our *rbfox1*^{sa15940} zebrafish model, we observed reduced dopamine in forebrain regions, which are homologous to the regions where dopamine dysregulation was observed in patients with ADHD, namely the midbrain, nucleus accumbens, caudate nucleus, and prefrontal cortex^{85,86}. Considering that we also found significant enrichment of dopaminergic processes in RBFOX1-regulated genes, it is plausible to suggest a causal role of *RBFOX1* variants in the dopamine-mediated hyperactivity. Neurotransmitter analyses in the *rbfox1*^{del19} line would provide further insight into this theory.

4.2 Thigmotaxis and anxiety in *rbfox1* mutant zebrafish and psychiatric disorders

Both the *rbfox1*^{sa15940} and the *rbfox1*^{del19} mutant zebrafish also displayed increased thigmotaxis, i.e. “wall-hugging” behaviour, which is a potential sign of anxiety in zebrafish (Table). While thigmotaxis can be related to anxiety, it can also indicate stereotyped behaviour typically observed in patients with ASD. Like the hyperactivity, this behaviour was more pronounced in the *rbfox1*^{sa15940} line.

Anxiety is a common symptom in major psychiatric disorders, including in anxiety disorders, OCD, mood disorders, psychotic disorders like schizophrenia, and neurodevelopmental disorders like ASD⁵⁷. Prominent brain regions involved in the modulation of anxiety are the amygdala, the ACC, the hippocampus, the hypothalamus, the NACC, the PFC, and the thalamus^{87,88}. These regions modulate anxiety primarily through the GABAergic and serotonergic systems, however, other neurotransmitters might also be involved⁸⁸. We found lowered levels of both GABA and serotonin in the telencephalon and diencephalon in *rbfox1*^{sa15940} mutant zebrafish, which are homologous to the regions involved in anxiety in humans. If the thigmotaxis displayed by the *rbfox1* mutant zebrafish was a sign of anxiety, it could be assumed that the lowered levels of GABA and serotonin are a potential explanation. However, it is important to consider that in a

test specific for anxiety, namely the black- and white test, we did not find increased anxiety in either line of the zebrafish, nor in *Rbfox1* knockout mice²⁰. Interestingly, thigmotaxis was also observed in *Rbfox1* knockout mice in the open field test²⁰. However, when an object was placed in the arena, the thigmotaxis was not detected any more, suggesting, that it is not exclusively driven by anxiety (Table)²⁰.

Considering the lack of anxiety displayed in the black- and white test along with the findings in *Rbfox1* knockout mice, the thigmotaxis displayed by the *rbfox1* mutant zebrafish likely indicates stereotyped behaviour. Additional experiments including other paradigms like the novel tank diving test might clarify whether the thigmotaxis displayed by the *rbfox1* mutant zebrafish is a sign of anxiety.

4.3 Social impairments in *rbfox1* mutant zebrafish and psychiatric disorders

Different impairments in social behaviours, including social withdrawal and reduced social interest, are key symptoms for several psychiatric disorders, including anxiety disorders, ASD, mood disorders, personality disorders, and schizophrenia, among others⁵⁷. Mirroring these changes in human patients, we observe alterations in social behaviours in both lines in two different paradigms, namely in the social novelty preference test and the shoaling test. Here, the *rbfox1*^{del19} line displayed more pronounced alterations (Table).

Our version of the social novelty preference test consists of two steps. In the first step, the tested fish is presented with a group of fish, and interaction time is measured. In the second step, another group of fish is added, and time spent next to the first and second group is measured. We found that in both lines, the *rbfox1* mutant and wildtype fish showed a clear preference for the first group of strangers versus an empty compartment. However, during the second step, the *rbfox1* mutant fish of both lines hardly interacted with the new group of strangers, whereas their wildtype counterparts spent equal amounts of time with both groups. This reduced social novelty interest parallels alterations in social behaviours observed in *Rbfox1* knockout mice: *Rbfox1* deficient mice were also found to display less interest in the social interaction test (Table)²⁰. However, this test does not measure social novelty interest. A specific test for social novelty interest in *Rbfox1* knockout mice would shed further light on the role of *Rbfox1* in different social paradigms in mice.

Additionally, when testing shoaling behaviour in groups of fish that were housed together, the *rbfox1* mutant fish of both lines did not swim as closely together as the wildtype fish, suggesting further social impairments. Like in the social novelty preference tests, alterations in the shoaling behaviour were more pronounced in the *rbfox1*^{del19} line.

Importantly, different social behaviours are modulated by different brain regions and circuits⁵⁷. For instance, in humans, social exploration is mainly regulated by connections between the mPFC, hippocampus, and amygdala⁸⁹. Disruptions in these circuits can lead to reduced social exploration and interaction, which correlates with social interaction alterations observed in individuals with ASD⁸⁹. Main neurotransmitters mediating this behaviour are dopamine, through the mesolimbic pathways, and GABA⁸⁹. The social novelty preference test models social exploration, while shoaling test measures social cohesion. The reduced exploration and group cohesion of the *rbfox1* mutant fish mirrors some aspect of the alterations in social interactions observed in ASD patients. As the *rbfox1*^{sa15940} mutant fish were found to have lowered levels of both dopamine and GABA, an evolutionarily conserved relationship of *RBFOX1* and the dopaminergic and GABAergic modulation of social behaviour could be suggested. However, this theory should be verified in future experiments, including neurotransmitter analyses in the *rbfox1*^{del19} line.

Our findings of alterations in social in both *rbfox1* mutant zebrafish line, which align with findings of reduced social interest in *Rbfox1* deficient mice, implicate *RBFOX1* in the social impairments frequently observed in patients with neurodevelopmental and psychiatric disorders. These changes in social behaviour are likely partially caused by alterations in monoaminergic and GABAergic neurotransmission, as observed in *rbfox1* mutant fish.

4.4 Aggression in *rbfox1* mutant zebrafish and psychiatric disorders

Aggressive behaviour and irritability are commonly comorbid with different psychiatric disorders, such as ASD, ADHD, and MDD, and can present in a variety of different forms.

One key difference we found between the two tested zebrafish lines was that only the *rbfox1*^{sa15940} mutant zebrafish displayed increased aggression, not the *rbfox1*^{del19} fish. Interestingly, in the aforementioned study by O'Leary et al., the aggressive behaviour displayed by *Rbfox1* knockout mice was not present, unlike in their wildtype counterparts (Table)²⁰. It is possible that the brain regions and biological processes that modulate aggression differ between zebrafish and mice. Therefore, mutations in *Rbfox1* could have distinct effects in the different species. However, another potential reason could be the difference in the kinds of aggression tests that were used. In humans, aggressive behaviour can be classified into proactive and reactive⁹⁰. The zebrafish were tested in the mirror test, where aggressive behaviour toward a perceived stranger in a novel environment is assessed. The *Rbfox1* knockout mice were tested in a resident-intruder test. In this test aggressive behaviour is displayed in terms of territoriality, so the underlying motivation for the aggressive behaviour might be different²⁰. In the resident intruder test, aggressive behaviour is displayed in terms of territoriality.

Aggressive behaviour is modulated by brain regions such as the amygdala and hypothalamus, where disruptions in neurotransmission and circuitry can influence aggression⁹¹. These regions modulate aggression primarily through neurotransmitters like serotonin, dopamine, and GABA⁹¹. In the *rbfox1*^{sa15940} mutant zebrafish, these neurotransmitters were lowered in forebrain regions homologous to the amygdala and hypothalamus in humans.

Our findings suggest that genetic variants in *RBFOX1* may contribute to aggressive behaviour by altering neurotransmission in critical regions. While our findings align with previous preclinical and clinical studies relating *RBFOX1* to aggression, further research is necessary to fully understand the relationship between *RBFOX1* and aggression^{92–94}.

4.5 Limitations of behavioural animal models of psychiatric phenotypes

A growing body of preclinical research, including *Rbfox1* animal models, has advanced our understanding of its working mechanisms. These studies include transcriptomic, electrophysiological, and behavioural analyses in animals, specifically mice, with partial or complete *Rbfox1* knockouts. They were able to relate this gene to the regulation of vast differential splicing and expression gene networks, increased neuronal excitation, and behavioural alterations paralleling important aspects of the symptomatology in psychiatric disorders^{4,5,10,20}. By incorporating zebrafish, another vertebrate model, our studies add an important evolutionary dimension to our research, offering valuable insights into the conserved functions of *RBFOX1* across species and further informing its role in psychiatric disorders. The fact that consistent alterations are found across different animal models strengthens the validity of the findings.

However, there are several important limitations to consider when modelling psychiatric disorders in animals. One major challenge is that these disorders often involve human-specific changes in complex cognitive, emotional, and linguistic processes, which cannot be fully replicated in animal models, as discussed in chapter 2.4⁹⁵. Zebrafish do not possess a neocortex, so complex behaviours, including social behaviour, are regulated by the forebrain. This affects, among others, the modelling of social behaviours. Social interactions are highly complex behaviours involving different brain areas and circuits and can differ substantially between zebrafish and humans. Modelling the full range of human social behaviours is not possible in zebrafish, or likely any other animal. Therefore, it is crucial to acknowledge that only certain aspects of the behaviour can be accurately modelled at a time. When interpreting behavioural study results, it is important to specify which particular aspect is being modelled to properly contextualize the findings.

Psychiatric disorders are highly heterogeneous, and modelling the full spectrum of symptoms is not possible. It is therefore not possible to model a whole psychiatric disorder in an animal. Animal studies can only be used to address specific research questions or model behavioural traits related to specific symptoms. For instance, the observed hyperactivity in *rbfox1* mutant fish and *Rbfox1* deficient mice suggests that *RBFOX1* could play a role in the hyperactivity observed in ADHD patients. However, it does not give insights into all the other symptoms of ADHD, nor why hyperactivity might be more pronounced in some patients than others. Moreover, the modelled hyperactivity is not specific to one clinical phenotype, but a symptom that can occur in different disorders.

Another limitation of our studies is that there is no established behavioural test for depressive-like states in zebrafish. Typically, depressive states are modelled through stress, with cortisol as a marker of stress⁹⁶. In future studies we will explore the involvement of stress in the observed behavioural alterations by measuring cortisol levels of the tested fish. This is especially relevant considering the involvement of *RBFOX1* in the stress response^{66–68,97}.

We could not take sex differences into account in our studies, although they are observed in patients with MDD and other psychiatric disorders, both in prevalence and symptomatology. As discussed in chapter 1.4, we pooled the results of male and female zebrafish for all studies. To ensure that this does not hide sex-specific behavioural effects of *rbfox1*, we tested potential sex differences in the *rbfox1*^{sa15940} line. For these experiments, the fish were separated according to their sex before the experiments to ensure the validity of the sex determination. No significant differences were observed between the sexes in the *rbfox1*^{sa15940} mutant fish. It is important to note however, that no hyperactivity or thigmotaxis was found in the *rbfox1*^{sa15940} mutant fish, so it may be valuable to repeat this testing.

The potential lack of reproducibility is a general important limitation in animal models, as well as in our studies. The behaviour of zebrafish can be affected by a variety of factors, including housing conditions, handling by caretakers and experimenters, group dynamics within the tanks, among others⁹⁸. In our studies, all zebrafish were kept in the same facility, however, some of these factors could not be controlled for by us. Additionally, slight variations in the genetic background of the fish can also affect their behaviour. Zebrafish should not be inbred for more than three or four generations, as this might lead to inbreeding depression. Therefore, the fish used in our studies were inbred for a maximum of three generations. After that, wildtype fish with the same genetic background and from another group of the same facility were introduced. This approach may introduce small genetic changes that can affect the reproducibility of the behaviour. Moreover, the different testing batches of the fish may have slight differences in health or metabolic states that could influence their behaviour, which we cannot control for.

In summary, investigating psychiatric phenotypes through animal models bears several important limitations. It is not possible to completely model a psychiatric disorder in animals due to the more human specific aspects, such as abstract thinking and language, and the high heterogeneity in which the symptoms can manifest in different individuals. However, specific behaviours and symptoms, for instance hyperactivity and anxiety, can be modelled, and the contribution of specific genetic factors to these behaviours can be investigated. This can provide important insights into the underlying genetic basis of psychiatric disorders, and potentially help to improve treatment outcomes.

5 THE EFFECTS OF *RBFOX1* IN PHARMACOLOGICAL INTERVENTIONS

Our findings of reduced neurotransmitter levels and behavioural alterations in the *rbfox1*^{sa15940} mutant zebrafish mirror some aspects observed in patients with depression or other comorbid psychiatric disorders. We therefore aimed to test whether medications targeting the serotonergic, dopaminergic, and GABAergic systems could reverse some of these behavioural changes. Acute exposure to the SSRI fluoxetine, a commonly prescribed antidepressant, was able to partially rescue hyperactivity and social impairments in certain tests. However, when testing medications targeting other neurotransmitter systems, we detected no behavioural changes. This suggests that a complex interplay of various neurotransmitters may influence the observed behavioural alterations.

5.1 Fluoxetine in *rbfox1*^{sa15940} zebrafish

We observed that acute exposure to fluoxetine was able to rescue the hyperactivity observed in *rbfox1*^{sa15940/sa15940} zebrafish in the open field test, but not the shoaling test. Interestingly, in the second step of the social novelty preference test, the *rbfox1*^{sa15940/sa15940} zebrafish exposed to fluoxetine even displayed increased activity. These findings indicate that serotonergic neurotransmission might play a role in the hyperactivity displayed in different paradigms.

In the open field test, the displayed hyperactive behaviour of the *rbfox1*^{sa15940} mutant zebrafish seems to be largely modulated through serotonin, as this behaviour could be rescued through fluoxetine. However, in the mirror and shoaling tests, hyperactivity could not be rescued, and in the second step of the social novelty preference test, the fluoxetine-treated *rbfox1*^{sa15940} mutant zebrafish even displayed increased hyperactivity. This suggests that the hyperactivity of these behaviours might be largely modulated by neurotransmitters other than serotonin. It is possible that the underlying reason for the displayed hyperactivity might differ between the paradigms, which may explain why distinct neurotransmitter systems could be involved. In the open field test, the tested fish is exposed to a novel environment, with no visual stimuli provoking a reaction. In the mirror test, the shoaling test, and the social novelty preference test, the tested fish has visual or direct contact with (perceived) other fish. This might lead to agitation of the fish, which could involve other neurotransmitters such as dopamine or GABA⁹⁹.

In the case of social behaviour, the acute exposure to fluoxetine reverted some of the alterations observed. It rescued the lowered inter-individual distance and increased nearest neighbour distance in *rbfox1*^{sa15940/sa15940} zebrafish the shoaling test. However, acute exposure to fluoxetine did not rescue the lowered social novelty preference observed in *rbfox1*^{sa15940/sa15940} zebrafish. While serotonin is known to play a role in modulating social behaviour, and fluoxetine is sometimes prescribed for social anxiety, increasing serotonin levels might not be enough to

rescue these changes^{100,101}. This was also shown in a double-blind, placebo-controlled pilot study on social phobia, where fluoxetine did not outperform the placebo^{100,101}.

The increased aggression displayed by the *rbfox1*^{sa15940/sa15940} fish was also not rescued by the fluoxetine treatment. While aggressive behaviour is linked to serotonin, it is possible that other neurotransmitter systems may also be involved in the modulation of this behaviour. In this case, targeting the serotonergic system would not be sufficient to rescue the altered aggressive behaviour.

In this study, we only tested acute exposure to fluoxetine, so we could not measure the long-term effects of chronic fluoxetine treatment. While acute exposure to fluoxetine rapidly increased serotonin levels, chronic treatment has been linked to changes in neuroplasticity, which is thought to contribute to its antidepressant effects¹⁰². It is therefore possible that chronic exposure to fluoxetine might rescue some of the behavioural alterations of the *rbfox1*^{sa15940/sa15940} zebrafish that could not be reversed through acute exposure.

Social behaviours are complex, and targeting just one neurotransmitter, i.e., serotonin, might not be enough to fully rescue the alterations. Our findings suggest that while it is likely that serotonin largely modulates the hyperactivity in the open field test, other neurotransmitter systems, mainly dopamine and GABA, also contribute to the alterations observed in the other paradigms.

5.2 Quinpirole and diazepam in *rbfox1*^{sa15940} zebrafish

To test to which extent other neurotransmitters contribute to the behavioural alterations observed in the *rbfox1*^{sa15940} mutant zebrafish, we decided to test quinpirole and diazepam. These drugs target the dopaminergic and GABAergic system, respectively. In two rounds of testing with quinpirole and one round of testing with diazepam, we did not observe consistent differences caused by this treatment on the zebrafish behaviour. The lack of observable treatment effects may be due to the hyperactive behaviour of the wildtype fish, or the inconsistent outcomes of quinpirole and diazepam treatment, as reported in the literature.

Quinpirole has been studied in zebrafish to explore the effects of dopamine on behaviour. However, results vary depending on factors like exposure time, dose, and developmental stage. For instance, one study found that acute exposure to quinpirole increased larval activity, while another study showed decreased locomotor activity at lower doses and no anxiolytic effects^{103,104}. The effects also differ across life stages. Early exposure to quinpirole increased anxiety-like behaviours, stereotypic swimming, and memory impairment in later life, but did not affect social or aggressive behaviour^{105,106}. In another study, quinpirole exposure led to hypolocomotion, anxiety-like behaviour, memory impairment, and stereotypic swimming¹⁰⁷. These studies highlight the complexity of the effects of quinpirole on zebrafish behaviour,

suggesting that the influences of the dopaminergic system on locomotion, anxiety, and stereotypy may be context-dependent.

The effects of diazepam on zebrafish, like quinpirole, are not entirely consistent across studies. For example, one study found no anxiolytic effect in zebrafish larvae, but observed a reduction in swimming speed, possibly due to a sedative effect¹⁰⁸. This contrasts findings of other studies, which could be due to genetic differences in zebrafish strains, as well as variations in procedural and environmental factors^{108–110}. In adult zebrafish, some studies report anxiolytic effects in the novel-tank diving and black-white tests, while others do not replicate these findings^{111–113}. In one study, acute exposure to diazepam reduced stress-related behaviours and cortisol levels, but also decreased social interaction^{96,114}. Additionally, sex-specific responses were observed, with only male zebrafish showing anxiolytic effects¹¹¹. Time- and dose-dependent effects were also noted, with changes in locomotion and exploration at different exposure times¹¹¹. These findings emphasize the importance of accounting for factors like dosage, sex, and exposure duration when investigating the effects of diazepam in zebrafish.

Importantly, we observed inconsistent behaviour in the wildtype zebrafish used in these experiments. The typical pronounced freezing behaviour in the open field test was absent, which may be attributed to batch effects among the groups of fish. These limitations of behavioural studies in animal models are discussed in chapter 4.5.

In summary, we did not observe behavioural effects of quinpirole and diazepam on the *rbfox1*^{sa15940/sa15940} and wildtype zebrafish. However, the untreated groups of fish, especially the wildtypes, behaved inconsistently compared to previous studies. This suggests that the lack of treatment effect in our experiments was due to batch effects. Therefore, it would be convenient repeating the experiments with a larger sample size, potentially including different dosages, and separating the fish by sex.

5.3 Limitations of pharmacological testing in zebrafish

Pharmacological testing in zebrafish has proven to be a valuable tool for studying drug effects, however, it also has several limitations. One limitation is the difficulty in accurately controlling drug dosage due to absorption variability through the water. While it is possible to inject the drugs interperitoneally, this comes with other disadvantages such as increased stress for the animal. Zebrafish have been shown to respond to the here tested medications, however, the drug breakdown and efficacy might differ between strains or even batches. As discussed in chapter 2.4, while zebrafish share many conserved neurotransmitter systems with mammals, their simpler brain structure may limit the direct translation of findings to human neurobiology^{115,116}.

This limitation could be addressed by testing these medications in a mammalian *Rbfox1* model, such as mice, and compare the results to the findings in zebrafish.

Another limitation is the lack of clearly established protocols for testing certain drugs, such as fluoxetine, quinpirole, or diazepam. While zebrafish are becoming increasingly popular as a model organism for pharmacological studies, much of this research is conducted in larvae due to their ease of handling and high-throughput capabilities. It is important to consider that drug responses in zebrafish may vary across life stages, and protocols optimized for larval zebrafish may not be directly translatable to adults. To address this, we initially tested two different doses of fluoxetine to determine which one could rescue the behavioural alterations in the *rbfox1*^{sa15940} mutant zebrafish and proceeded with further testing using the more promising dose. This approach should be repeated with quinpirole and diazepam to confirm why we could not observe any effects of treatment.

Another limitation is that due to the housing conditions of the zebrafish, we could only assess acute effects of the medications in zebrafish. As zebrafish tanks in the facility share a water system, a chronic treatment is not possible. Chronic exposure might affect distinct biological mechanisms with different behavioural implications. For instance, in patients with depression, SSRIs typically take several weeks to exhibit their antidepressant effects, likely due to the modulatory impact of serotonin on neuroplasticity²⁸. The partial rescue of the behavioural alterations observed on *rbfox1*^{sa15940} mutant zebrafish through fluoxetine could be therefore a result of its impact on serotonin levels rather than through effects on neuroplasticity. If some of the effects of SSRIs are due to the modulatory effects of serotonin on neuroplasticity, chronic exposure to fluoxetine could have different impacts on zebrafish behaviour over time.

6 A NEW PERSPECTIVE ON *RBFOX1* IN PSYCHIATRIC PHENOTYPES

RBFOX1 is an important, evolutionarily conserved regulator of gene networks essential for the development and function of the brain. The importance of *RBFOX1*-regulated genes in different psychiatric phenotypes and rare disorders with similar symptoms suggests that their working mechanisms are not exclusive to specific disorders, but rather shared. Based on our findings of differentially expressed and spliced genes, along with alterations in neurotransmitter levels, brain activity, and behaviour, I propose the following hypothesis about how genetic variants affecting *RBFOX1* expression or function can lead to psychiatric phenotypes (**Figure 35**). Certain *RBFOX1* genetic variants could affect mechanisms involved in cell development and DNA repair, among other biological processes. These disruptions, potentially through interactions with environmental factors, may contribute to neurodevelopmental disturbances. This could manifest in increased neuronal activity and synchronicity in midbrain and hindbrain regions. Furthermore, it could result in lowered levels of serotonin, dopamine, GABA, and glutamate in the forebrain. It is also possible that *RBFOX1* affects other neurotransmitter systems which we have not been investigated in the *rbfox1*^{sa15940} mutant zebrafish. The alterations in neuronal activity and neurotransmitter levels could be reciprocally and causally linked, potentially through compensatory mechanisms. The alterations in serotonin could increase anxiety, social alterations, aggression, and hyperactivity. Reduced dopamine could also increase hyperactivity. Reduced GABA and glutamate could impair neuroplasticity and contribute to anxiety. Together, *RBFOX1* may lead to disruptions in neurotransmission and neuronal activity, which may contribute to symptoms such as anxiety, hyperactivity, and social impairments, which are commonly observed in human psychiatric conditions.

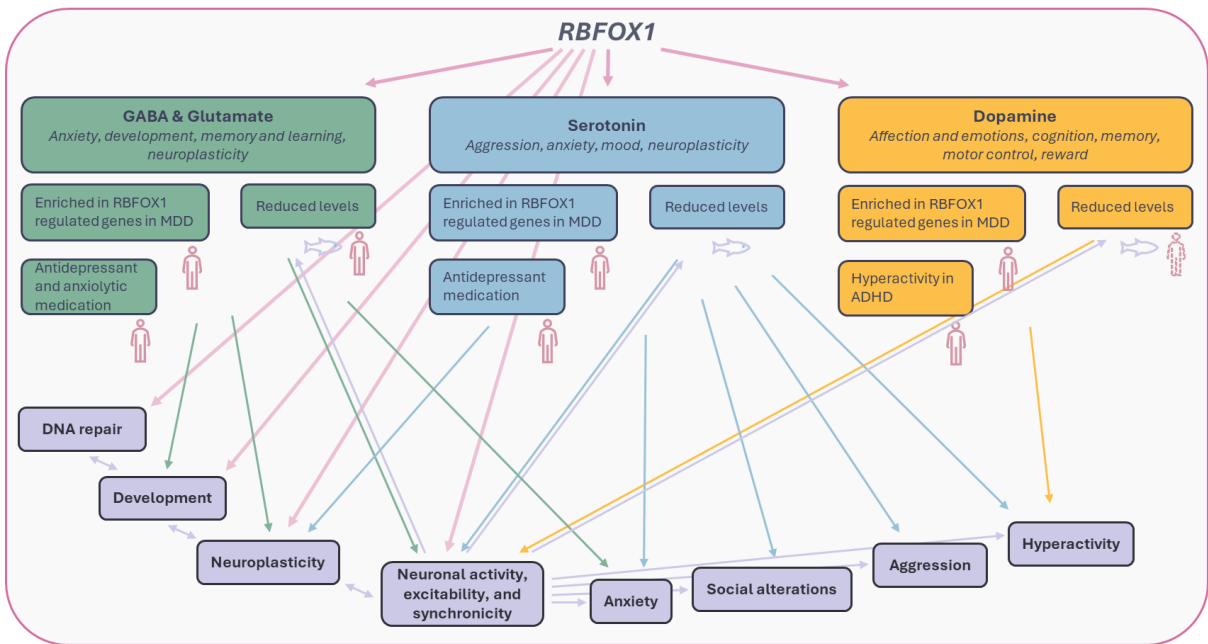


Figure 35: Proposed working mechanism of RBFOX1 in depression and other psychiatric phenotypes. RBFOX1 may affect key neurotransmitter systems implicated in major depression and other psychiatric disorders, including dopamine, serotonin, GABA, and glutamate. This is evidenced by our findings of reduced levels of these neurotransmitters in forebrain regions of *rbfox1*^{sa15940} mutant zebrafish. This could lead to an imbalance between excitatory and inhibitory signals, which may provoke compensatory upregulation of neuronal activity in other brain regions, as observed in the mid- and hindbrain in the zebrafish model. Conversely, neuronal activity may also be directly affected by *rbfox1*. Increased brain activity could then lead to compensatory downregulation of neurotransmitters in other brain regions. Importantly, alterations in neurotransmission are reciprocally and causally linked to changes in neuronal activity and connectivity, which could create a feedback loop impairing neuronal activity and neurotransmission. Reduced serotonin levels could contribute to anxiety, social alterations, aggression, and hyperactivity. Reduced dopamine could also increase hyperactivity. Reduced GABA and glutamate could impair neurodevelopment, neuroplasticity, and contribute to anxiety. Reduced serotonin and glutamate could furthermore impair neuroplasticity mechanisms in the brain. Together, RBFOX1 may lead to disruptions in neurotransmission and neuronal activity, which may contribute to symptoms such as anxiety, hyperactivity, and social impairments, which are commonly observed in human psychiatric conditions.

This hypothesis is backed by findings from our studies, as well as reports in the literature. For instance, we found reduced serotonin levels in the *rbfox1*^{sa15940} mutant zebrafish forebrain. Serotonin is crucial modulator of mood, aggression, and anxiety^{23,52,53}. It has been linked to various psychiatric disorders, including MDD, potentially through its effects on neuroplasticity^{23,50,52,53}. Additionally, monoaminergic neurons were highly enriched in tissue and cell-type enrichment analyses in MDD in a recent study by Adams et al. (2025)¹¹⁷. Interestingly, biological processes related to neuroplasticity were also enriched in RBFOX1-regulated genes associated with MDD in our study. This suggests that RBFOX1 may affect psychiatric phenotypes partially through modulations of serotonin levels, as well as neuroplasticity. It could influence neuroplasticity either independently or through serotonin, or possibly through other factors that affect both processes.

Dopaminergic dysregulation is implicated in the hyperactive symptoms observed in ADHD patients, with commonly prescribed ADHD medications targeting dopaminergic synapses to raise dopamine levels⁸⁴. In our studies, we found reduced dopamine levels in the forebrain of *rbfox1*^{sa15940} mutant zebrafish, which could lead to their hyperactive phenotype. Based on this, I suggest that impaired *RBFOX1* functioning, among other factors, could contribute to hyperactivity in ADHD and restlessness in MDD and other psychiatric disorders associated with *RBFOX1* through disruptions in the dopaminergic system. It is also possible that the observed alterations in dopamine levels could contribute to other symptoms in psychiatric disorders related to the reward system. As we could not test behavioural alterations related to these symptoms in our *rbfox1* zebrafish, future studies in other animal models of *Rbfox1*, for example in mice, could shed light on the contribution of *RBFOX1* on these symptoms.

Furthermore, we observed reduced GABA and glutamate levels in the forebrains of *rbfox1*^{sa15940} mutant zebrafish. In humans, reduced levels of both glutamate and GABA are associated with depressive symptoms and anxiety, and in zebrafish GABA is involved in modulating anxiety and motor control^{118,119}. These alterations are commonly linked to stress in clinical and preclinical studies⁵⁸. Genetic variants affecting *RBFOX1* expression or function may affect GABA and glutamate neurotransmission, potentially predisposing individuals to stress-related disorders by creating a neurochemical environment similar to chronic stress, even in the absence of stressors. Variants in *RBFOX1* could therefore partially explain alterations in GABA and glutamate levels, leading to anxiety and other somatic depressive symptoms. Our findings of enriched biological processes related to glutamate in *RBFOX1*-regulated genes associated with major depression further support the idea that *RBFOX1* contributes to disrupted inhibitory and excitatory signalling via imbalances in GABA and glutamate.

Alterations in neurotransmission, as observed in *rbfox1*^{sa15940} mutant zebrafish, are reciprocally and causally linked to changes in neuronal activity and connectivity^{120,121}. If variants in *RBFOX1* disrupt the balance between excitatory and inhibitory signals during neurodevelopment, it could impair the proper formation of neurotransmitter systems, thereby affecting neuronal activity and connectivity. Conversely, if variants in *RBFOX1* cause lowered neurotransmitter levels during development, as we saw in the *rbfox1*^{sa15940} mutant zebrafish, other brain regions may compensate by upregulating their activity, which we also saw in the *rbfox1*^{sa15940} mutant zebrafish. This reciprocal relationship between *RBFOX1*-driven changes in neurotransmission and neuronal activity may create a feedback loop, further reinforcing the neurotransmission and connectivity disruptions observed in patients with MDD and other psychiatric disorders. The effects of *RBFOX1* variants could vary between developmental stages, which could be relevant in the prevention and treatment of psychiatric disorders.

Importantly, *RBFOX1* alone is unlikely to modulate these phenotypes alone. Our *RBFOX1* network analysis confirms the involvement of large gene networks contributing to the alterations in neurodevelopmental processes and neurotransmission. In conclusion, *RBFOX1* and its regulatory network appear to play a critical role in regulating key neural mechanisms, including neurotransmission pathways, neuroplasticity, and neuronal connectivity, that results in many different behavioural alterations, thus contributing to a wide range of psychiatric phenotypes.

CONCLUSIONS

RBFOX1 exerts its pleiotropic effects on MDD and other psychiatric phenotypes through its regulatory network of genes involved in cell- and neurodevelopment. The underlying mechanisms identified in this thesis involve alterations in different neurotransmitter levels and in neuronal activity in certain brain regions, which could result in behavioural changes such as hyperactivity and social impairments.

The influence of the *RBFOX1* regulatory network on MDD and comorbid traits.

- 1 *RBFOX1* is associated with MDD, anxiety, and neuroticism, but not irritability or suicide attempt.
- 2 Genes associated with MDD, anxiety, irritability, and neuroticism are enriched in *RBFOX1*-regulated genes and largely overlap between these phenotypes.
- 3 *RBFOX1*-regulated genes associated with MDD and with comorbid traits mainly belong to biological processes and pathways related to neurodevelopment and neurotransmission.
- 4 Half of the *RBFOX1*-regulated genes predicted to be differentially expressed in brain of patients were also associated with these phenotypes.
- 5 *RBFOX1* is an essential hub in the network of *RBFOX1*-regulated genes associated with MDD and at least one other traits. The network analysis suggests that *RBFOX1* is crucial for the integration and regulation of a range of different processes and functions.
- 6 Genes causal for rare diseases with symptoms of depression and anxiety are enriched in *RBFOX1*-regulated genes.
- 7 Our integrative analyses highlighted 19 top-ranked *RBFOX1*-regulated genes with a high pleiotropic effect, including *CADM2*, *CTSB*, *MAPT*, *NSF*, *PAX6*, *SCAI*, *SNCA*, *SP4*, and *TCF4*.

The effect of *rbfox1* on behaviour in zebrafish.

- 8 Hyperactivity, thigmotaxis and social impairments were observed in both lines of *rbfox1* mutant zebrafish. These behaviours are consistent with behavioural alterations identified in *Rbfox1*-deficient mice.
- 9 One of the zebrafish lines, *rbfox1*^{sa15940}, which was also used for the transcriptomic, neurotransmitter, and neuronal activity experiments, displayed stronger hyperactivity and thigmotaxis than the other line, *rbfox1*^{del19}. The *rbfox1*^{del19} line displayed stronger

changes in social behaviour. The *rbfox1*^{sa15940} mutant zebrafish displayed increased aggression compared to wildtype, which was not present in the *rbfox1*^{del19} line. *Rbfox1*-deficient mice did not show aggressive behaviour.

- 10 No sex differences were detected in *rbfox1* zebrafish in wildtype or *rbfox1* mutant fish, potentially due to a batch effect.

Pharmacological interventions in *rbfox1* mutant zebrafish.

- 11 Reduced basal levels of serotonin and tryptophan were found in the forebrain of homozygous *rbfox1* mutant zebrafish, which could explain behavioural alterations observed.
- 12 Acute exposure to fluoxetine (SSRI) rescued hyperactivity in the open field test and social alterations in the shoaling test. However, acute exposure to this drug could not rescue hyperactivity in other behavioural tests, nor the increased aggression or reduced social novelty interest, suggesting the involvement of other biological mechanism in these behaviours.
- 13 Acute exposure to quinpirole and diazepam did not alter the behaviour, potentially due to batch effects in these experiments. Wildtype fish displayed inconsistent behaviour, which may also explain the lack of observable effects of these drugs on behaviour.

Central nervous system alterations in *rbfox1* mutant zebrafish.

- 14 Reduced basal levels of serotonin, dopamine, GABA, and glutamate were found in forebrain regions of adult homozygous and heterozygous *rbfox1* mutant zebrafish compared to wildtype.
- 15 Midbrain and hindbrain regions of *rbfox1* mutant zebrafish larvae showed increased neuronal activity. In the hindbrain, the fraction of single-cell spikes occurring in bursts was also significantly higher in *rbfox1* mutant zebrafish compared to wildtype. Midbrain and hindbrain regions in *rbfox1* mutant fish also showed an increase in the collective bursting rate, with these bursts being significantly shorter.
- 16 A total of 1062 genes differentially expressed and 331 differentially spliced were identified in adult brain, most prominently in telencephalon and hindbrain. The *rbfox1*-regulated genes identified were enriched in cell developmental processes in the telencephalon and DNA repair in the diencephalon and hindbrain.

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