BRAIN AMINERGIC SYSTEMS AND AUTISM-ASSOCIATED RISK FACTORS IN ZEBRAFISH

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1 LIST OF ORIGINAL PUBLICATIONS

I
Embryonic exposure to valproic acid affects the histaminergic system and the social behaviour of adult zebrafish (Danio rerio).
Baronio D*, Puttonen HAJ*, Sundvik M, Semenova S, Lehtonen E, Panula P
Br J Pharmacol. 2018 Mar; 175:797-809. doi: 10.1111/bph.14124.

II
Vesicular monoamine transporter 2 (SLC18A2) regulates monoamine turnover and brain development in zebrafish.
Baronio D, Chen YC, Decker AR, Enckell L, Fernández-López B, Semenova S, Puttonen HAJ, Cornell RA, Panula P
Acta Physiol (Oxf). 2022 Jan; 234:e13725. doi: 10.1111/apha.13725.

III
Abnormal brain development of monoamine oxidase mutant zebrafish and impaired social interaction of heterozygous fish.
Baronio D, Chen YC and Panula P
Dis Model Mech. 2022 Mar; 15:dmm049133. doi: 10.1242/dmm.049133.

*Shared first authorship. No publications have been included in other dissertations. In the text, the original publications are referred to by roman numerals (**I-III**).

Candidate's contributions in the original publications:

I: The candidate performed the drug treatment, behavioral analysis of larval and adult zebrafish, reverse transcription-quantitative PCR (RT-qPCR) and immunohistochemistry. The candidate also participated in the analysis of the results and writing of the manuscript.

II: The candidate performed all the experiments involving *in situ* hybridization, RT-qPCR and behavioural analysis of this manuscript. Additionally, the candidate also performed the serotonin and tyrosine hydroxylase immunostaining. The candidate contributed with the analysis of the results and writing of the manuscript.

III: All the behavioural, *in situ* hybridization and RT-qPCR data were acquired by the candidate. Additionally, the candidate also performed the monoamine oxidase activity assay. Serotonin immunostaining and cell counting were performed by the candidate. The candidate contributed with the analysis of the results and writing of the manuscript.

2 LIST OF ABBREVIATIONS

3-MT - 3-methoxytyramine

5-HIAA - 5-hydroyindoleacetic acid

5-HTP - 5-hydroytryptophan

AADC - aromatic amino acid decarboxylase

AChE - acetylcholine esterase

ADHD - attention deficit hyperactivity disorder

APOEB - apolipoprotein Eb

ASD - autism spectrum disorder

BBB - blood brain barrier

cAMP - cyclic adenosine monophosphate

CMA - chromosomal microarray

CNS - central nervous system

CNVs - copy number variants

COMT - catechol-*Oxi*-methyltransferase

DAT - dopamine transporter

DBH - dopamine beta hydroxylase

DIG - digoxigenin

dpf - days post-fertilization

EG - eminentia granularis

ERZC - european resource zebrafish center

HDAC - histone deacetylases

HDC - histidine decarboxylase

HNMT - histamine-n-methyltransferase

hpf - hour post-fertilization

HPLC - high-performance liquid chromatography

HRH1 - histamine receptor h1

HRH2 - histamine receptor h2

HRH3 - histamine receptor h3

HRH4 - histamine receptor h4

HVA - homovanillic acid

ISI - inter-stimulus-interval

MANF - mesencephalic astrocyte-derived neurotrophic factor

MAO - monoamine oxidase

MB - metabisulfite

MECP2 - methyl cpg binding protein 2

mpf - months post-fertilization

NECs - neuroepithelial cells

NGS - next generation sequencing

NPCs - neural progenitor cells

OCT3 - organic cation transporter 3

PCD - programmed cell death

PCNA - proliferating cell nuclear antigen

PCPA - Trans-2-phenylcyclopropylamine

PEA - β-phenylethylamine

PET - Positron emission tomography

PLC-β - phospholipase Cβ

PMAT - plasma membrane monoamine transporter

PVOa - Paraventricular organ anterior part

PVOi - Paraventricular organ intermediate part

PVOp - paraventricular organ posterior part

Rpl13a - ribosomal protein large subunit 13a

RT-qPCR - reverse transcription -quantitative pcr

SERT - serotonin transporter

SFARI - simons foundation autism research initiative

Shank3b - sh3 and multiple ankyrin repeat domains 3

SNPs - single nucleotide polymorphisms

SNVs - single nucleotide variants

SSRIs - selective erotonin reuptake inhibitors

TH - tyrosine hydroxylase

t-mHA - tele-methylhistamine

TPH - tryptophan hydroxylase

uVNTRs - upstream variable number of tandem repeats

VMAT2 - vesicular monoamine transporter 2

VPA - valproic acid

VTA - ventral tegmental area

3 ABSTRACT

Brain amines are neurotransmitters that modulate important functions in the central nervous system, including behavior and brain development. Several brain disorders are characterized by impairments in aminergic systems, including autism spectrum disorder. Autism spectrum disorder is characterized by impaired social behavior, difficulty in communication and stereotypies. The etiology of autism is poorly understood, but both environmental and genetic risk factors are known to play a role on it. Valproic acid, a drug commonly used to treat bipolar disorder, and mutations in the monoamine oxidase a gene are both environmental and genetic risk factors. Animal models are useful tools to better understand the mechanisms and outcomes involved in aminergic-associated neurodevelopmental disorders and for testing new drugs that could attenuate the neurobehavioral outcomes associated with these conditions.

Zebrafish shares relevant neurochemical aspects with humans and display a wide range of behaviors, which contribute to its appreciation as a model organism in neuroscience. Some functional aspects of aminergic neurotransmission in the zebrafish brain, such as vesicular transport and metabolism, have been studied by pharmacological manipulation and there are no reports of *vesicular monoamine transporter 2* (*vmat2*, also known as *slc18a2*) and *monoamine oxidase* (*mao*) genetic ablation in zebrafish so far. Characterizing such mutants could contribute with the existing pharmacological data and yield insights about the role of aminergic neurotransmission on embryonic development, as well as larval and adult behavior and brain function. Additionally, because zebrafish is becoming a popular tool in neuroscience, a well-characterized set of neurobiological mechanisms will strength its value as a model organism.

This study characterized the phenotypes of zebrafish with pharmacologically and genetically manipulated ASD risk factors. We were particularly interested in the behavioral outcomes and the aminergic systems of these animals. The aminergic populations, transport and metabolism of animals that lacked *vmat2* and *mao,* relevant genes for aminergic neurotransmission, were also studied. Methods such as immunohistochemistry, Quantitative PCR, *in situ* hybridization, high-performance liquid chromatography and behavioral analysis of larval and adult zebrafish were used in this thesis.

In the first publication of this thesis, larval zebrafish embryonically exposed to valproic acid showed a significant reduction in the number of histaminergic neurons and in the levels of histamine when compared to control animals. Besides the reduction in the number of histaminergic cells and histamine levels, the histaminergic system was affected by a downregulation of *histidine*

decarboxylase and histamine receptors (*hrh1*, *hrh2*, and *hrh3*) when compared with control larvae. Some of these abnormalities persisted until adulthood, and valproic acid-exposed adult animals displayed reduced levels of *histidine decarboxylase* and *hrh3* along with impaired social behavior. Valproic acid-exposed zebrafish have the potential to be useful tools in ASD research, however, this model needs further validation. Additionally, this study brings more attention to a possible involvement of the histaminergic system in the outcomes related to autism.

In the second article, *vmat2* mutants, dopamine, noradrenaline, serotonin and histamine levels were decreased, whereas levels of dopamine and serotonin metabolites were increased, indicating elevated amine turnover. There were fewer histamine, serotonin and dopamine immunoreactive cells. Despite reduced levels of dopamine and histamine, mutants presented upregulated synthesizing enzymes. Further, in mutants *notch1a* and *pax2a* were downregulated in brain proliferative zones. This mutant line may be used in the investigation of how amines transport affects brain development and function, and for use in high-throughput and drug screening.

The *mao*$^{-/-}$ larvae showed a hyperserotonergic phenotype that was characterized by extracellular serotonin immunoreactivity and increased density of serotonin-immunoreactive fibers. However, this exacerbated extracellular serotonin was associated with damage in aminergic systems. They also showed weaker responses to visual and acoustic stimuli, abnormal expression of developmental markers and died within 20 days post-fertilization. *mao*$^{+/-}$ fish were viable, grew until adulthood, and demonstrated anxiety-like behavior and impaired social interactions compared with adult *mao*$^{+/+}$ siblings. In conclusion, the present work contributes to previous pharmacological data concerning the behavioral and neurochemical consequences of Mao inactivation in zebrafish. *mao*$^{-/-}$ zebrafish could be used in investigations aiming to assess the roles of MAOA/B and amines during in brain development. *mao*$^{+/-}$ zebrafish is a promising model to study the developmental and behavioral outcomes of interaction between environmental factors and MAOA/B genotype.

Collectively, the results of the present thesis support zebrafish as a tool to investigate mechanisms underlying autism spectrum disorder. Additionally, it presented two new models to study important aspects of aminergic neurotransmission in zebrafish and its role in brain function and behavior.

4 INTRODUCTION

There are five major amine neurotransmitters: the three catecholamines - dopamine, norepinephrine (noradrenaline), and epinephrine (adrenaline) - and histamine and serotonin. These molecules are implicated in a wide range of behaviors (from central homeostatic functions to cognitive processes). Dysfunctions in aminergic systems can lead to various brain disorders, such as Parkinson's disease, depression, schizophrenia and Autism Spectrum Disorder (ASD).

ASD is a developmental disorder featured by impairments in social behavior, communication and flexibility of thought and behavior. An interaction between genetic components and environmental factors play a role in the pathogenesis of this disorder. Among the genes associated with ASD are *Monoamine oxidases,* which encode the enzymes responsible for amines metabolism. Prenatal exposure to valproic acid, a drug commonly used to treat bipolar disorder and epilepsy, is considered an environmental risk factor. Despite numerous abnormalities described in patients with ASD, including neuroanatomical, neurochemical and molecular alterations, the etiology of this disorder is not fully understood.

Although the translation of results to humans can be challenging, animal models are valuable tools to study neurobiological mechanisms of ASD and develop new therapies to treat specific symptoms of this disorder. Zebrafish is a popular model organism in neuroscience because of its high genetic homology with humans and the availability of genome editing tools, such as the CRISPR/Cas9 system. Additionally, it shares relevant neurochemical aspects with humans. All the main neurotransmitters and their systems' components, such as receptors and enzymes, are present in the zebrafish brain. It also displays a robust behavioral repertoire that can be evaluated through various tests from early stage of development to adulthood. Because of that, zebrafish is among the organisms currently used to model ASD-like features.

In this this thesis we performed pharmacological and genetic interventions relevant to the pathophysiology of ASD, and other brain disorders, using zebrafish as a model organism and to study behavioral outcomes and the aminergic systems. Increasing the knowledge about important aspects of the aminergic systems in zebrafish, such as development, transport and metabolism, was necessary.

5 REVIEW OF THE LITERATURE

5.1 Autism spectrum disorder (ASD)

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental condition diagnosed in childhood the symptoms which persist throughout the patient's life. ASD is characterized by impairments in communication and social interaction (Lord et al., 2020). Individuals diagnosed with ASD may also present a restricted repertoire of activities and stereotypies. Sleep disturbances, gastrointestinal problems and immune alterations are among common ASD comorbidities (Accardo and Malow, 2015; Edmiston et al., 2017; Hsiao, 2014). ASD is estimated to affect ~1–1.5% of the global population and it represents with comorbidities a financial burden to public health (Buescher et al., 2014). Males have a four times higher risk of developing the disorder than females (Fombonne, 2009). Despite a variety of neuroanatomical, neurochemical and molecular alterations described in patients with ASD, research has identified no specific biomarkers to diagnose the disorder (Pardo and Eberhart, 2007). The etiology of ASD is not fully understood, but environmental and genetic factors are known to have a role in the development of this disorder.

5.1.1 Environmental factors associated with ASD

The developing central nervous system (CNS) is vulnerable to external insults and exposure to different prenatal environmental agents have a major impact on the risk of ASD. Additionally, the maternal health condition might also play an important role on the development of ASD in the offspring. Alterations in the maternal immune system during pregnancy have been considered risk factors, as maternal viral and bacterial infections (Jiang et al., 2016), and autoimmune diseases (Chen et al., 2016b) have been linked with increased risk of ASD in offspring. The increased levels of proinflammatory factors and autoantibodies in these conditions might have a role in the pathophysiology of ASD (Gottfried et al., 2015).

Toxic xenobiotics are potential environmental risk factors for ASD. Exposure to both heavy metals and organophosphates are associated with a 60% increase in the risk of ASD (Shelton et al., 2014; Yoshimasu et al., 2014). Exposure to valproic acid (VPA), a drug used to treat epilepsy and psychiatric disorders, has been associated with a 5-fold increase in ASD risk in general population (Christensen et al., 2013). ASD patients exposed prenatally to VPA can present craniofacial and limb malformations, which indicate that the window of exposure to this teratogen is open during early

embryogenesis (Ardinger et al., 1988). This is true for thalidomide, another teratogen associated with ASD. After analyzing the Swedish thalidomide registry, Strömland et al. found several patients with ASD and all of them have been exposed to thalidomide between gestational days 20 and 24 (Strömland et al., 1994). These findings led to the development of a rodent model of ASD based on prenatal exposure to VPA (Rodier et al., 1996). Later, this approach was used in other model organisms, such as fish (Zimmermann et al., 2015), chicks (Zachar et al., 2019) and non-human primates (Zhao et al., 2019).

5.1.2 Genetic factors associated with ASD

The genetic component in the pathophysiology of ASD is rather heterogeneous. Cytogenetic studies were the first to observe genetic abnormalities associated with ASD (Gillberg and Wahlström, 1985). However, the low resolution of karyotyping analysis did not allow an association between a specific gene and ASD. Rare chromosomal abnormalities, such as 15q11–q13 duplication (of the maternal allele) of the Prader-Willi/Angelman syndrome region also, affect approximately 2% of individuals diagnosed with ASD (Baker et al., 1994).

The development of new technologies, such as Comparative Genomic Hybridization and single nucleotide polymorphisms (SNPs) arrays, allowed a more precise evaluation of microscopic and submicroscopic deletions and duplications in different genomic regions (Pinto et al., 2011). They have indicated that multiple copy number variants (CNVs) are risk factors for ASD (Sebat et al., 2007). These CNVs can be inherited or *de novo*, a genetic variation that occurs spontaneously in the parental germline. Many of these *de novo* CNVs are proposed to affect several genes that are often associated with synaptic function, such as *NRXN1* (Kim et al., 2008), *NLGN3* (Quartier et al., 2019), *SHANK3* (Durand et al., 2007), *SHANK2* (Berkel et al., 2012), and *SYNGAP1* (Hamdan et al., 2011). The identification of these candidate genes is important because it enhances clinical definition and diagnosis. Using Whole Exome/Genome sequencing, studies can also investigate the contribution of inherited and *de novo* Single Nucleotide Variants (SNVs) in ASD (An et al., 2014).

ASD heritability is mostly associated with common genetic variants called SNPs observed in the general population, with only a small contribution from inherited rare variants (Gaugler et al., 2014). The common variants are estimated to contribute with approximately 50% of ASD liability (Gaugler et al., 2014). However, most of SNPs have a small effect alone and most likely many common genetic variants collectively contribute to ASD (Devlin et al., 2011).

ASD can also be part of different monogenic syndromes, which include Fragile X syndrome (Belmonte and Bourgeron, 2006), tuberous sclerosis (Sundberg and Sahin, 2015), Rett syndrome (Richards et al., 2015) and neurofibromatosis (Garg et al., 2015). Mitochondrial dysfunction has also been implicated in ASD, as patients with untreated phenylketonuria may present ASD features (Baieli et al., 2003).

5.1.3 Animal models of ASD

Different rodent models to study ASD have been developed based on environmental risk factors, such as maternal infection and maternal VPA administration (Bambini-Junior et al., 2014a; Patterson, 2011a), and genetic variants associated with ASD or on syndromic disorders with autistic features (Bortolato et al., 2013; Hori et al., 2020).

One of the well-established rodent models of ASD based on exposure to an environmental risk factor is the VPA model. VPA is a non-selective inhibitor of histone deacetylases (HDACs), enzymes that regulate chromatin structure and function through the removal of acetyl groups from lysine residues of core histones, facilitating a closed chromatin state and transcriptional repression (Dozawa et al., 2014). Inhibition of class I HDACs can lead to increases in synapse numbers and excitatory synapse maturation (Akhtar et al., 2009). Additionally, it has been reported that VPA increases neurite growth and promotes neural proliferation (Go et al., 2012). However, exposure to VPA during early developmental stages may also cause DNA damage, activation of apoptotic mechanisms and impair neural tube closure (Tung and Winn, 2011).

VPA has the ability to inhibit neuronal excitability through effects on the GABAergic and glutamatergic systems. Increased GABA levels in the brains of rodents and CSF of humans have been reported after VPA treatment, most likely due to effects on enzymes that may modulate GABA levels, such as GABA transaminase and glutamic acid decarboxylase. These effects on the GABAergic system are consistent with the known anticonvulsant activity of VPA on seizures induced by GABA antagonists (Badawy et al., 2020; Nau and Löscher, 1982). Decreased levels of aspartate have been reported in whole brains of rodents after treatment with 200–400 mg/kg VPA (Johannessen, 2000). Additionally, aspartate release *in vitro* is decreased after VPA exposure (Crowder and Bradford, 1987).

Prenatal exposure to VPA in rodents leads to several behavioral and morphological abnormalities similar with those exhibited by individuals with ASD. Disruption in the monoaminergic systems, misbalanced excitation/inhibition, alterations in the nociceptive threshold, anxious-like

behavior, impaired social behavior and aberrant exploratory behavior are all noted in this model (Baronio et al., 2015; Narita et al., 2002; Schneider et al., 2006; Tsujino et al., 2007). Beneficial effects of environmental enrichment on the behavior of VPA-exposed rodents resembles the outcomes of early behavioral intervention and sensorimotor enrichment in patients with ASD (Favre et al., 2015; Schneider et al., 2006), suggesting that the VPA rodent model has predictive validity. Additionally, this model has been used for the investigation of new therapies for ASD. Recently, the interest in studying the histaminergic system in ASD pathophysiology increased. The use of histamine receptor H3 (H3R) inverse agonists in the VPA model led to behavioral improvements and reduction of brain inflammation (Baronio et al., 2015; Eissa et al., 2018).

Among the several genes thought to be involved in the pathophysiological processes of ASD, many encode important components for the serotonergic system, such as serotonin transporter (*SERT,* also known as *SLC6A4*) and monoamine oxidase a (*MAOA*) (Cohen et al., 2003; Veenstra-VanderWeele et al., 2012). *Sert* KO and *MaoA* KO mice recapitulate some of the symptoms displayed by patients with ASD and can be used to investigate the role of serotonin in brain development, ASD-like behavior, and abnormalities related with serotonin levels (Bortolato et al., 2013; Ellenbroek et al., 2016). Additionally, based on the hundreds of genes known to have an association with ASD risk, several other genetic models were generated to investigate various biological processes that might play a role on ASD pathophysiology, such as synaptic function and neuronal activity, postsynaptic density protein metabolism and neuronal cell adhesion (Li et al., 2021).

5.2 Zebrafish as a model organism

Zebrafish is a small teleost that is currently widely used as a model organism in scientific research. These animals are cost-effective because they are easy to breed and can be kept in large numbers in relatively small spaces. A pair of zebrafish is able to spawn 100-300 eggs per week. Thus, even a small zebrafish unit is able to produce a large number of embryos. The eggs and developing embryos are transparent, making it possible to observe the structures of the zebrafish organs during development (Kalueff et al., 2014). They achieve the early larval stage from 2 to 3 days post fertilization (dpf). Zebrafish progress through the early larval, late larval and juvenile stages before reaching adulthood and sexual maturity at around 3 months post-fertilization (mpf). It is a relevant model for high-throughput screening and drug testing because its capacity of generating a large number of offspring and its transparent body at early stages of development allow the visualization of effects of chemical exposure on different organs (Gupta et al., 2014; Nishimura et al., 2016).

Zebrafish is an attractive tool in neuroscience research because it shares relevant neurochemical aspects with humans. All the main neurotransmitters are present in the zebrafish brain. Their systems' components, such as receptors and enzymes, can be easily studied and visualized through whole-mount *in situ* hybridization and immunofluorescence at early stage of development since zebrafish brains can be dissected already at 2 dpf (Chen et al., 2016a; Kaslin and Panula, 2001; Panula et al., 2006; Panula et al., 2021). Zebrafish also possess a blood brain barrier (BBB), which start to develop on 3 dpf (Fleming et al., 2013). Similarly with mammals, the adult zebrafish brain is divided in forebrain, midbrain and hindbrain. The forebrain is composed of the telencephalon and diencephalon. In the telencephalon, regions called subpallium and pallium are the zebrafish homologues of the mammalian basal ganglia and hippocampus and amygdala, respectively (Gerlach and Wullimann, 2021). It is unknown if zebrafish possess a region that would be functionally equivalent to the mammalian neocortex. The diencephalon contains major five parts: epithalamus, dorsal thalamus, ventral thalamus, posterior tuberculum and hypothalamus (Wullimann et al., 1996). The habenulae (right and left) are important epithalamic nuclei that are involved in the regulation of fear, anxiety and social behavior (Agetsuma et al., 2010). The midbrain is the area between the forebrain and hindbrain, divided into optic tectum, torus semicircularis, torus longitudinalis, and midbrain tegmentum (Folgueira et al., 2020). The hindbrain is composed of the cerebellum and medulla oblongata (Wullimann et al., 1996). Figure 1 provides a more detailed representation of the zebrafish neuroanatomy.

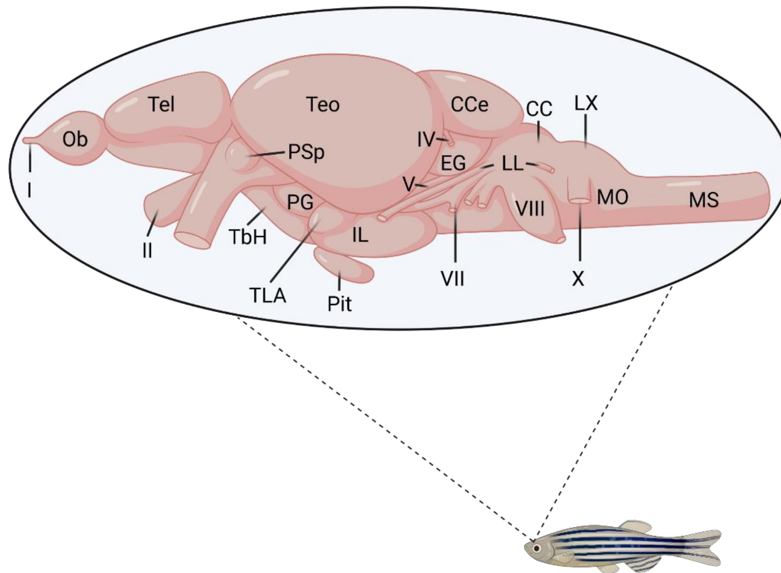

Figure 1- Lateral view of the zebrafish adult brain. Telencephalon is formed by the dorsal and ventral telencephalic area as well as the olfactory bulb, which is entered rostrally by the olfactory nerve. The diencephalon is covered by the optic tectum and externally visible parts are the optic nerve, the preglomerular area, the torus lateralis and the hypothalamus. The cerebellum (corpus cereberelli and granular eminence) covers the brain stem, which comprises most of the cranial nerves, except for the optic and olfactory nerves, and, more caudally, the crista cerebellaris. The medulla oblongata is the most posterior part of the brainstem and merges with the spinal cord. **CC**: crista cerebellaris, **CCe**: corpus cerebelli, **EG**: eminentia granularis, **IL**: inferior lobe of hypothalamus, **LL**: lateral line nerves, **LX**: vagal lobe, **MO**: medulla oblongata, **MS**: medulla spinalis, **OB**: olfactory bulb, **PG**: preglomerular area, **Pit**: pituitary, **PSp**: parvocellular superficial pretectal nucleus, **Tel**: telencephalon, **TeO**: tectum opticum, **TH**: tuberal hypothalamus, **TLa**: torus lateralis, **I**: olfactory nerve, **II**: optic nerve, **IV**: trochlear nerve, **V**: trigeminal nerve, **VII**: facial nerve, **VIII**: octaval nerve, **X**: vagal nerve. Modified from Wullimann et al., 1996. Figure created with BioRender.com.

The wide range of behaviors displayed by zebrafish also contribute to its appreciation in neurobiology research. Behavioral aspects can be monitored already at early stages of development. Zebrafish tails begin to bend spontaneously at 18 h post fertilization (hpf), due to large calcium depolarizations flowing from head to tail, and touch evoked evasive movements are noted at 28 hpf (Brustein et al., 2003). Different apparatuses available in the market allow the simultaneous tracking of up to 96 zebrafish larvae placed in multi-well plates, which make them suitable for high-throughput testing. These observation chambers make it possible to evaluate larval basic locomotor activity, response to acoustic and visual stimuli, mimicking day/night rhythm and locomotor activity response to pharmacological treatments (Puttonen et al., 2017; Puttonen et al., 2018; Van Den Bos et al., 2017). The Mauthner cells modulate the escape response in zebrafish after acoustic/vibrational stimuli (Burgess and Granato, 2007a). After such stimuli, larvae will display a short latency response with

an increase in locomotor activity and velocity. This phenotype is thought to be a form predation avoidance. A hyperactive phenotype is also noted upon abrupt decrements in illumination, a behavior often referred as dark-flash response. Initially, it was hypothesized that this was also an escape response to predation. However, it was proved that this behavior is not mediated by the Mauthner cells and is more likely to be navigational rather than defensive behavior. In this particular situation, larvae execute a maneuver called *O-bend*, characterized by a large angle body bend into an “O” shape. The darkness induced hyperactivity and the 180° turn (o-bend) are hypothesized to facilitate navigation back to well-lit environments (Burgess and Granato, 2007b).

As they grow older, zebrafish start to develop a more complex behavioral repertoire relevant for the study of brain disorders. Social behavior is of particular interest because it is affected in numerous brain disorders. Zebrafish are social animals that are visually attracted to conspecifics and have the tendency to form tight groups of individuals, a behavior called *shoaling*. This behavior serves different purposes, including breeding, avoiding predation and foraging (Pitcher, 1986). It is possible to evaluate the shoaling behavior in the laboratory by placing a group of fish (shoal) in a tank and recording their movements during a trial. Parameters, such as the mean of the interfish distance and duration that individuals are in proximity, can be analyzed with video tracking software that tracks and analyzes the behavior, movement, and activity of any animal (Chen et al., 2020). The fact that zebrafish is visually attracted to conspecifics also allowed the establishment of a paradigm where an isolated social stimulus (a shoal) is presented to a testing fish in an arena. This approach is based on the well-established three-chamber sociability test used in rodent studies (Baronio et al., 2015). Additionally, the use of robotic fish and virtual shoals displayed in a monitor screen have been used to study zebrafish social behavior (Abaid et al., 2012; Gerlai, 2017).

Anxiety-like behavior is also easily assessed with zebrafish by different tests. Thigmotaxis is one of the most commonly used behaviors assessed in rodent models of brain disorders and it is a well-established index of anxiety. This behavior is also assessed in studies with larval and adult zebrafish (Blaser et al., 2010; Schnörr et al., 2012). Zebrafish has the tendency to initially seek protection in an unfamiliar environment before start exploring it. This phenomenon can be mimicked and studied in the laboratory through the *novel tank diving test* (Stewart et al., 2013). The test consists on transferring a zebrafish from its home tank to an unfamiliar tank. The fish initially prefers to explore only the bottom of the tank often displays erratic movements and freezing behavior. Eventually the tested fish becomes bolder and explores the rest of the tank. Dividing the tank in virtual zones with the appropriate software while recording this behavior allows the researcher to evaluate the degree of anxiety-like behavior by the fish, as we can quantify the time spent in each zone, the

latency to reach other zones of the tank and velocity (Chen et al., 2020). This behavior can be pharmacologically manipulated by the previous exposure of the fish to anxiolytic or anxiogenic substances (Egan et al., 2009).

5.2.1 Zebrafish as a model organism to study ASD

Because of its high genetic homology (approximately 70% of all human disease genes have functional homologs in zebrafish) (Santoriello and Zon, 2012) with humans and the availability of genome editing tools, such as the CRISPR/Cas9 system, zebrafish is also used to develop different models of brain disorders trough the generation of mutants. It is important to highlight that, as many teleost species, zebrafish went through a genome duplication event (Postlethwait et al., 1998). This means that the zebrafish genome has two or more isoforms of many genes that have only one copy in mammals. That is the case for a few genes relevant for aminergic transmission. However, the isoforms often have complementary expression patterns in the zebrafish brain or are restricted to a certain developmental stage, which allows the investigation of loss of function of a gene in a certain region or stretch of time. For instance, three different *tryptophan hydroxylase (tph)* genes (*tph1a*, *tph1b* and *tph2*) are present in the zebrafish genome and show distinct patterns of expression, which will be discussed in the following sections of this thesis (Gaspar and Lillesaar, 2012).

Zebrafish models of ASD risk genes and genes linked to ASD-associated syndromes, such as Fragile X syndrome (Constantin et al., 2020), tuberous sclerosis complex (Kim et al., 2011) and Rett syndrome (Cortelazzo et al., 2017), have been developed and helped to shed light on relevant neurobiological mechanisms. These mutants recapitulate important behavioral and molecular aspects of ASD. Additionally, zebrafish can be easily exposed to environmental factors known to be associated with the development of brain disorders, such as drugs and toxins, by injection or immersing these compounds in the fish water (Kalueff et al., 2014).

VPA exposure has been used to produce ASD-like features in zebrafish. This approach is based on reports of high incidence of ASD-symptoms in children with fetal valproate syndrome and it was already used in studies with rodent (Bambini-Junior et al., 2014b; Baronio et al., 2015; Christensen et al., 2013; Eissa et al., 2018). Zimmermann et al. reported that zebrafish exposed to VPA during the first 48 h of life exhibit deficits in social interaction, anxiety, and hyperactivity at different periods of development (Zimmermann et al., 2015). In the report by Chen et al. VPA exposure began at 8 hpf and ended at 4.5 dpf and zebrafish displayed macrocephaly, which could be explained by increases in cell proliferation, the proportion of mature newborn neurons, and neural stem cell proliferation

(Chen et al., 2018). Continuous 4.2-day exposure to 10 μM VPA leads to an early and persistent ASD-like phenotype along with hyperactivity and anxiety, in larvae and adult zebrafish. Additionally, it increases acetylcholine esterase (AChE) activity and reduced Akt–mTOR signaling in the brain (Joseph et al., 2021). The use of VPA-exposed zebrafish to model behavioral and molecular abnormalities associated with ASD could be advantageous. However, the number of published studies on the topic are insufficient to make a conclusion. Additionally, there are differences in methods between the few published articles regarding periods of exposure and concentrations of VPA used to generate an ASD-like phenotype in zebrafish. Thus, more studies and further validation of the already published models are necessary to confirm if this is a valid approach. More examples of ASD-like phenotypes in experimentally manipulated (genetically and environmentally) zebrafish are displayed in Table 1.

Table 1 - Phenotypical observations in zebrafish ASD models generated through genetic and environmental manipulation.

Manipulation	Risk factors associated with ASD	Zebrafish model	Main phenotypical observations in zebrafish	References
GENETIC	*SHANK3* mutation	*shank3ab* $^{-/-}$	Impaired social preference, repetitive swimming behaviors, hypoactivity	(Durand et al., 2007; Liu et al., 2021)
	NRXN2 mutation	*nrxn2aa* $^{-/-}$	Axon pathfinding defects and increased anxiety	(Gauthier et al., 2011; Koh et al., 2021)
	NR3C2 mutation	*nr3c2* $^{-/-}$	Social behavioral deficit. Increased sleep latency, longer wake bouts and shorter sleep bouts.	(Ruzzo et al., 2019)
	CNTNAP2 mutation	*cntnap2ab* $^{-/-}$	GABAergic deficits and sensitivity to drug-induced seizures	(Hoffman et al., 2016; Strauss et al., 2006)
	DYRK1A mutation	*dyrk1aa* $^{-/-}$	Microcephaly and social behavior deficit	(Bronicki et al., 2015; Kim et al., 2017)
ENVIRONMENTAL	Fetal valproate syndrome	Larval exposure to VPA	Social interaction impairment and hyperactivity	(Bescoby-Chambers et al., 2001; Robea et al., 2021)
	Prenatal pollutant exposures	Maternal exposure to water soluble fraction of crude oil and lead (Pb)	Altered expression levels of multiple genes relevant for ASD pathophysiology	(Dutheil et al., 2021; Wang et al., 2016b)

5.3 Aminergic systems

Dopamine, noradrenaline, adrenaline, serotonin and histamine are aminergic neurotransmitters that share an amine group ($-NH_2$) with a catechol, indolamine or imidazole backbone and exert numerous physiological functions in the CNS. Dopamine, noradrenaline and adrenaline are considered catecholamines because the catechol moiety that they share, whereas histamine is a diamine with imidazole backbone (Bindoli et al., 1992; Parsons and Ganellin, 2006). Serotonin belongs to another class of amines, called indolamines. Synthesis, transport and storage are similar between these neurotransmitters because they are chemically related. The availability of the precursor amino acids in the brain and the amount of active synthesizing enzymes are considered rate-limiting steps in the biosynthesis. After synthesis, these neurotransmitters are packed and stored in intracellular vesicles by vesicular monoamine transporter 2 (VMAT2, also known as SLC18A2), which is present in all aminergic neurons (Wimalasena, 2011). Neurotransmitters are removed from the synaptic cleft by cell membrane transporters and can be recycled or metabolized to inactive

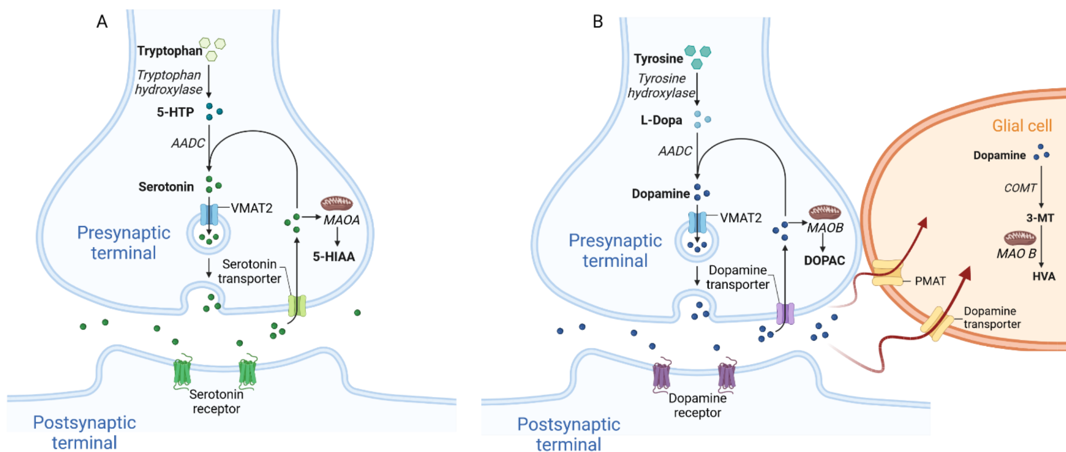

Figure 2- A simplified overview of the basic mechanisms involving the synthesis and metabolism of serotonin (A) and dopamine (B). Tryptophan hydroxylase converts tryptophan into 5-HTP, which is turned into serotonin by AADC. Tyrosine is converted into L-Dopa by tyrosine hydroxylase, which is turned into dopamine by aromatic aminoacid decarboxylase (AADC). Neurotransmitters are packed into transport vesicles by VMAT2. Neurotransmitters are released from the vesicle by exocytosis into the extracellular space. Some of the released neurotransmitters bind to their specific targets, the receptors expressed in the postsynaptic neuron. Plasma membrane transporters for dopamine and serotonin expressed in the presynaptic neurons take up some of the released neurotransmitters, which are degraded by catabolic enzymes (MAOA or MAOB). Glial cells also play a role in the metabolism of aminergic neurotransmitters. As an example, panel B shows dopamine transporter and plasma membrane monoamine transporter (PMAT) expressed in glial cells taking up extraneuronal dopamine. Once dopamine is transported into glial cells, catechol-*Oxi*-methyltransferase (COMT) converts it into 3-methoxytyramine (3-MT), which is transformed into homovanillic acid (HVA) by MAO B. Figure created with BioRender.com.

metabolites by specific enzymes. Figure 2 provides an overview of the synthesis of serotonin and dopamine. In the following sections the dopaminergic, serotonergic and histaminergic systems and their roles in the pathophysiology of ASD are discussed.

5.3.1 The dopaminergic system

Dopamine and other cathecolamines are synthesized from a common precursor, the nonessential amino acid tyrosine. The conversion of tyrosine to L-DOPA by tyrosine hydroxylase (TH) is the rate-limiting step in the synthesis of cathecolamines (Nagatsu et al., 1964). Aromatic amino acid decarboxylase (AADC) is responsible to convert L-DOPA into dopamine (Lovenberg et al., 1962). In addition to TH, noradrenergic neurons express dopamine beta hydroxylase (DBH), which converts dopamine into noradrenaline (Joh and Hwang, 1987).

Similarly with other biogenic amines, dopamine is stored (or transported into) in vesicles by VMAT2 and released into the synaptic cleft after the arrival of an action potential (Weihe and Eiden, 2000). After activation of postsynaptic dopamine receptors, dopamine is removed from the synaptic cleft by the dopamine transporter (DAT), which is a transmembrane protein expressed in the presynaptic neuron and glial cells (Iversen, 1971; Segura-Aguilar et al., 2022). The dopamine that is taken up by the presynaptic neuron can be either recycled and stored again in vesicles or degraded. Dopamine is degraded to DOPAC or 3-methoxytyramine (3-MT) by monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT), respectively. DOPAC and 3-MT are degraded to homovanillic acid (HVA), by COMT and MAO, respectively (Napolitano et al., 1995). It is noteworthy that the presence of COMT in presynaptic neurons is controversial, with reports of this protein expressed in postsynaptic neurons and non-neuronal cells such as ependymal cells of the cerebral ventricles and glial cells (Karhunen et al., 1994; Karhunen et al., 1995; Männistö and Kaakkola, 1999).

Dopaminergic neurons in mammals can be divided into different groups: A8–A10 (diencephalon-midbrain), A11–A15 (diencephalon), A16 (olfactory bulb), and A17 (retinal) groups (Hökfelt et al., 1973). A8 neurons are located in the retrorubral field, a midbrain region situated dorsal and posterior to the substantia nigra. A9 population is located in the substantia nigra pars compacta, whereas A10 forms the ventral tegmental area. In the diencephalic group, A11 is located in the caudal diencephalon and gives rise to diencephalo-spinal projections. Dopaminergic cells located in the arcuate nucleus of the hypothalamus belong to A12 population and project to the pituitary. A13 and A14 are located in the dorsal hypothalamus and in the rostral periventricular hypothalamus,

respectively. Together they form the incerto-hypothalamic neuronal system. A15 is located in the rostral hypothalamus and preoptic area. In the forebrain, TH-positive periglomerular cells form the A16 population. A17 cells are in the retina and are amacrine cells (Nguyen-Legros et al., 1982).

There are five families of dopamine receptors (D_1, D_2, D_3, D_4 and D_5) that can be categorized according to their functional properties (Lachowicz and Sibley, 1997). D_1-like (D_1, D_5) couple to the G_s family of G proteins (G_s and G_{olf}), which increases cyclic adenosine monophosphate (cAMP) production and are expressed postsynaptically (Beaulieu and Gainetdinov, 2011). D_2-like receptors (D_2, D_3 and D_4) couple primarily to the $G_{i/o}$ family, which leads to a decreased cAMP production, and can be expressed both postsynaptically on dopamine target cells and in presynaptic dopaminergic neurons (Usiello et al., 2000). Dopamine receptors are involved in important processes, such as movement control, cognition and reward.

5.3.1.1 The dopaminergic system of zebrafish

As a consequence of genome duplication which occurred within the teleost lineage, many genes from the zebrafish genome present two or more isoforms that are only in one copy in mammals (Postlethwait et al., 1998). That is the case for *TH*, which has two isoforms in zebrafish (*th1* and *th2*) (Chen et al., 2009). Groups of Th1-immunoreactive cells have been described and categorized in the zebrafish brain through a numbering system (1-17). Some of these groups can be detected already at 24 hpf and all populations present in the adult brain are detected at 72 hpf. This numbering system (Sallinen et al., 2009a) was created after analysis of the development of catecholaminergic populations using 3D confocal microscopy and is different from the previous nomenclature that was established based on 2D data and an investigation mainly focusing on diencephalic populations (Rink and Wullimann, 2002).

In the forebrain, 13 groups are of dopaminergic and one is of noradrenergic cells (group 14, locus coeruleus). Groups 1 and 2 are located in the olfactory bulb and telencephalon, respectively. Cells of group 3 and 4 are in the preoptic area, where group 4 is ventrally positioned and projects to the pituitary. Groups 5 (postoptic), 6 (thalamic) and 11 (posterior tuberculum) can be detected from 48 hpf onwards, but are more easily separable in the adult than in the larval brain. The pretectal population (group 7) project laterally to the optic tectum. Group 8 is located in the anterior part of the paraventricular organ, whereas group 9 and group 10 are in the intermediate and posterior parts, respectively. Group 12 is located in the paraventricular organ and posterior tuberculum; group 13 is part of the periventricular hypothalamus and posterior tuberculum. In the hindbrain, groups 15, 16

and 17 are locates in the internal reticular formation, caudal lobe commissural nucleus of Cajal, respectively (Sallinen et al., 2009a). Th2-immunoreactive cells are found in 4 additional groups that were assigned the same numbers as the neighboring Th1-immunoreactive cells with the added letter "b" (3b, 8b, 9b, 10b) (Chen et al., 2009).

As mentioned in the previous section, dopamine is metabolized by MAO and COMT in mammals. Zebrafish treated with selegiline (l-deprenyl), which inhibits the Mao activity in these animals, presented decreased levels of DOPAC, a dopamine metabolite. However, no alterations in the immunoreactivity of Th cells and in the levels of dopamine were detected, suggesting that Mao does not metabolize dopamine efficiently in zebrafish (Sallinen et al., 2009b). Two *comt* genes (*comta* and *comtb*) are found in zebrafish and the encoded enzymes are thought to play part in the metabolism of dopamine and other cathecolamines, as the product of dopamine methylation by COMT, 3-MT is present in zebrafish (Semenova et al., 2017).

In zebrafish, eight dopamine receptor subtypes have been reported so far, *drd1*, *drd2a*, *drd2b*, *drd2c*, *drd3*, *drd4a*, *drd4b* and *drd4C*. Based on the amino acid sequence similarity and phylogenetic analysis, drd1 is the homolog of the mammalian D1-like receptor type, and the rest belong to the mammalian D2-like receptor type. Zebrafish larvae exposed to D_1 and D_2 antagonists display marked hypoactivity while agonism of either receptor subtype leads to hyperactivity (Giacomini et al., 2006; Irons et al., 2013). Adults acutely treated with dopamine receptor antagonist improved spatial learning and memory, while fish dosed with dopamine receptor agonists showed impaired performance (Naderi et al., 2016).

5.3.1.2 The dopaminergic system in ASD

Since dopaminergic projections innervate and modulate different brain structures that are associated with ASD, it has been hypothesized that dysfunctions in different dopaminergic pathways could be responsible to specific behavioral alterations commonly detected in patients with ASD. The mesolimbic pathway, for instance, which connects dopaminergic neurons from the ventral tegmental area (VTA) with the frontal cortex, if disrupted, could reduce reward value linked to social stimuli. As a consequence, reduced social interaction and poor development of verbal skills could manifest as symptoms. Supporting this hypothesis, positron emission tomography (PET) studies have shown that presynaptic dopamine levels are reduced in the prefrontal cortex of children with ASD (Ernst et al., 1997). During a behavioral task decreased phasic dopamine release to rewards was detected in

several striatal clusters, including the putamen and caudate nucleus, of patients with ASD (Zürcher et al., 2021).

The use of atypical antipsychotic drugs, such as risperidone and aripiprazole, seem to have improving effects on the behavioral traits displayed by individuals with ASD. These dopamine receptor D2 subtype ligands are able to reduce irritability, aggression and hyperactivity of patients (McCracken et al., 2002; Scott and Dhillon, 2007). Additionally, these drugs also block serotonin receptors. However, there is no evidence that these pharmacological interventions have beneficial effects on core symptoms of ASD (Marrus et al., 2014).

Mutations in genes encoding proteins that are modulators of dopaminergic neurotransmission have been associated with ASD. A possible risk haplotype was identified in the *DRD1* gene which was over-transmitted from mothers to sons with ASD (Hettinger et al., 2008). This haplotype was associated with more severe core symptoms of ASD. A significant association between ASD and the SNP rs167771 located in the *DRD3* gene was found in British and Dutch samples of ASD (de Krom et al., 2009). Whole-exome sequencing of ASD families has identified a novel *de novo* missense mutation in the DAT, which is predicted to alter extracellular dopamine levels by anomalous dopamine efflux (Hamilton et al., 2013).

5.3.2 The serotonergic system

Serotonin is synthesized from the amino acid tryptophan in two steps. Firstly, the rate limiting enzyme TPH converts tryptophan into 5-hydroytryptophan (5-HTP) (Lovenberg et al., 1967). In the periphery the isoform responsible for serotonin synthesis is called TPH1, while in the CNS the enzymatic reaction is mediated by TPH2 (Walther et al., 2003). In the second step serotonin is produced by the decarboxylation of 5-HTP by AADC (Lovenberg et al., 1962). The product of serotonin degradation by MAOA is the metabolite 5-hydroyindoleacetic acid (5-HIAA). Before its release in the synaptic cleft, serotonin is stored in vesicles by VMAT2.

There are nine serotonergic populations (B1-B9), which form the raphe nuclei in the mammalian brain. The more caudal raphe nuclei (B1-B5) send projections to the peripheral nervous system, while the rostral populations (B6-B9) project to forebrain structures (Dahlström and Fuxe, 1964). In mammals, there are 14 receptors for serotonin that have distinct temporal and spatial patterns of expression and regulate important physiological processes and behaviors (Barnes et al., 2021). They are divided into seven families (5-HT_1 - 5-HT_7) largely on the basis of their structural and operational characteristics and are part of the G-protein-coupled receptor family, except for 5-

HT_3 receptors, which are ligand-gated ion channels. These receptors have been linked to diverse processes, such as the pathophysiology of different brain disorders (i.e., anxiety, depression and schizophrenia) and regulation of sleep, spatial memory and learning (Hannon and Hoyer, 2008).

After stimulation of its receptors, serotonin is removed from the synaptic cleft by SERT, which is expressed in the presynaptic neurons. The serotonin removed by SERT can be recycled or degraded by MAOA. SERT is an important pharmacological target, as the selective serotonin reuptake inhibitors (SSRIs) bind to SERT and prevent serotonin reuptake, in disorders such as depression and anxiety (Vahid-Ansari and Albert, 2021).

The serotonergic system has important roles during development and serotonin functions as a trophic factor that regulates neuronal growth, differentiation, migration and survival before synapse formation (Brüning et al., 1997; Riccio et al., 2011). Thus, a disrupted serotonergic system during development can lead to impairments in brain function and behavior. In fact, alterations in the developing serotonergic system have been linked to the pathophysiology of different brain disorders, including ASD. These alterations can be consequences of genetic (i.e., *SERT* or *MAOA* allelic variants) or environmental (prenatal exposure to SSRIs) factors (Cohen et al., 2011; Man et al., 2015; Sutcliffe et al., 2005).

5.3.2.1 The serotonergic system in ASD

Hyperserotonemia was first reported in patients with ASD in 1961. It was estimated in a meta-analysis that approximately 25% of the ASD population presents this alteration (Gabriele et al., 2014). This phenomenon has been associated with the severity of specific symptoms of the disorder, such as self-injurious behavior. However, hyperserotonemia cannot be reliably used as a biomarker for ASD because the results presented by different studies are inconsistent. It has been hypothesized that increased levels of serotonin in the blood of patients with ASD could be a consequence of different mechanisms, including increased production by enterochromaffin cells in the gut, increased uptake into the platelet, decreased metabolic rate, or altered platelet release. Hyperserotonemia can be problematic during early development since serotonin can enter the developing brain and lead to an impaired serotonergic innervation due to a negative feedback (Whitaker-Azmitia, 2005). This could consequently generate ASD symptoms later in life.

Evidence also points to an altered serotonergic system in the CNS of ASD patients. Several neuroimaging and postmortem studies reported decreased serotonin receptors binding and density in the brains of ASD patients (Goldberg et al., 2009; Murphy et al., 2006; Oblak et al., 2013). Increased

SERT expression in ASD has been associated with increased stereotypies (Mulder et al., 2005). In contrast, decreased SERT expression has been linked to impaired social behavior (Brune et al., 2006). However, in a PET study of SERT binding in adults with Asperger's disorder, no alterations were found (Girgis et al., 2011). *Sert* KO mice display increased stress reactivity, macrocephaly and decreased sociability (Brune et al., 2006; Mulder et al., 2005). However, knock-in of the SERT Ala56 variant, which is associated with compulsive behavior and sensory aversion in individuals with ASD (Sutcliffe et al., 2005), in mice displayed alterations in social function, communication, and repetitive behavior (Veenstra-VanderWeele et al., 2012). Serotonin receptors also might play a role in this disorder, as there are reports of reduced binding potentials of serotonin receptors 5-HT_{1A} and 5-HT_{2A} in the thalamus, the posterior cingulate cortex, and the fusiform gyrus (Beversdorf et al., 2012; Oblak et al., 2013). The role of MAOA in the pathophysiology of ASD will be discussed in other section of this thesis.

5.3.2.2 The serotonergic system of zebrafish

In zebrafish, the serotonergic cells can be detected at 1 dpf in the brain and the serotonergic system is developed by 5 dpf, with serotonin immunoreactive cells detected in raphe nuclei (Sallinen et al., 2009b). In addition, and contrary to mammals, zebrafish also display serotonergic clusters in in the diencephalon and hypothalamus. In the diencephalon, pretectal serotonergic neurons (population 1) are thought to be involved in visual processing, as they innervate the optic tectum. The hypothalamus contains the highest number of serotonin immunoreactive neurons, which can be divided into three distinct populations according to their location: paraventricular organ anterior part (PVOa, population 2), paraventricular organ intermediate part (PVOi, population 3) and paraventricular organ posterior part (PVOp, population 4) (Sallinen et al., 2009b). In the hindbrain, the zebrafish raphe serotonergic populations display similarities to the mammalian raphe serotonergic system. Raphe populations in the zebrafish hindbrain can be divided in rostral raphe complex (populations 5-7), caudal raphe complex (population 8) and area postrema complex (population 9) (Panula et al., 2010; Sallinen et al., 2009b).

Three isoforms of *tph* (*tph1a*, *tph1b* and *tph2*) and two *sert* genes (*serta* and *sertb*) are expressed in the zebrafish brain. *tph1a* and *sertb* are expressed in cells of the embryonic and adult posterior tuberculum and hypothalamus (Lillesaar et al., 2007; Norton et al., 2008). Additionally, *tph1a* is transiently expressed in cells along the floor plate of the spinal cord. *tph1b* is expressed transiently in neuroepithelial cells during early development (Pan et al., 2021). Neurons of the raphe nuclei and in

the pretectal area of the diencephalon from embryos and adult zebrafish express *tph2* and *serta* (Lillesaar et al., 2007; Norton et al., 2008; Teraoka et al., 2004). All three *tph* paralogs have been described in the pineal gland, where serotonin serves as a precursor of melatonin. Serotonin is metabolized by Mao in zebrafish, an enzyme that shares similarities with both mammalian MAOA and MAOB (Anichtchik et al., 2006). Only a few serotonergic receptors have been cloned from fish, with the pattern of expression of three receptors of the type 1 family more well characterized in zebrafish (Norton et al., 2008). It has been demonstrated that serotonin receptors promote antiepileptic activity in zebrafish, since treatment with agonists of 5-HT_{2b} receptors exerts a powerful suppression of convulsive swim behavior and electrographic seizure activity in a zebrafish model of Dravet syndrome (Griffin et al., 2019).

5.3.3 The histaminergic system

Neuronal histamine is produced from the amino acid L-histidine, which is taken up by neurons and converted to histamine by the enzyme histidine decarboxylase (HDC) (Martres et al., 1975). Histamine is then packed in vesicles by the VMAT2 (Merickel and Edwards, 1995). Tele-methylhistamine (t-mHA) is the product of histamine degradation by histamine-N-methyltransferase (HNMT) in the central nervous system (Brown et al., 1959). However, the molecular mechanism of histamine clearance is not fully understood. HNMT has been reported to be expressed in primary human astrocytes and the astrocytes of human brain specimens. Histamine uptake by these cells is dependent on the plasma membrane monoamine transporter (PMAT) with a minor contribution of organic cation transporter 3 (OCT3) (Yoshikawa et al., 2013). Figure 3 provides an overview of synthesis and metabolism of histamine. In the mammalian brain, histaminergic neurons are located in the tuberomamillary nucleus and send projections to different areas of the brain through two ascending and one descending histaminergic pathways (Haas and Panula, 2003; Panula et al., 1990). The ventral part of the brain, including the hypothalamus and the septum, receive projections from the ventral ascending pathway. The thalamus, amygdala, hippocampus and rostral forebrain structures are innervated by projections from the dorsal ascending pathway. Cerebellum, brain stem and spinal cord are innervated by projections from the descending pathway. The organization of the histaminergic network is similar in humans, other mammals, amphibians and fish (Airaksinen and Panula, 1990; Airaksinen et al., 1992; Eriksson et al., 1998).

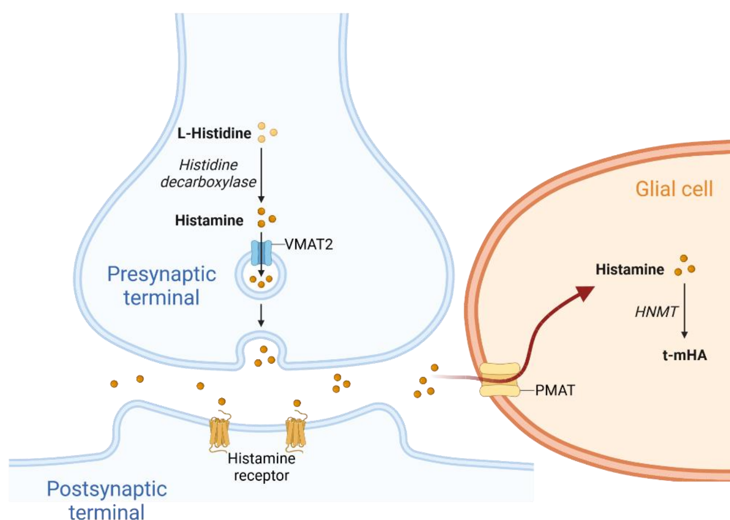

Figure 3 - A simplified overview of the basic mechanisms involving the synthesis and metabolism of histamine. L-histidine is converted into histamine by histidine decarboxylase. Histamine is packed into transport vesicles by VMAT2 and released from the vesicle by exocytosis into the extracellular space. Some of the released neurotransmitters bind to their specific targets, the receptors expressed in the postsynaptic neuron. Plasma membrane monoamine transporter (PMAT) is expressed in astrocytes and play a role in the regulation of extraneuronal histamine. Once histamine is transported into astrocytes, Histamine N-methyltransferase (HNMT) converts it into tele-methylhistamine (t-mHA). Figure created with BioRender.com.

There are four histamine receptors (H1R, H2R, H3R3 and H4R) that belong to the GPCR family and present constitutive activity. H1R is expressed different cell types of the central nervous, including neurons, astrocytes and cells of the immune system circulating in the brain (Jurič et al., 2016; Karlstedt et al., 2013; Passani and Blandina, 2011). Regions where this receptor is expressed includes hypothalamus, thalamus, cortex and brainstem. The signal transduction of H1R includes activation of phospholipase C, which promotes the inositol triphosphate-dependent release of $Ca2^{+}$ from intracellular stores and diacylglycerol-sensitive activation of protein kinase C (Smit et al., 1999). Activation of H2R stimulates adenylate cyclase and cAMP production, which leads to the activation of protein kinase A and CREB. In the brain, H2R is expressed in areas such as the cortex, hippocampus, basal ganglia and amygdala. A role for H2R in learning and memory has been suggested (Schneider et al., 2014). H3R is widely expressed in the brain and works as a presynaptic auto- or hetero-receptors that regulates the synthesis and release of histamine and other neurotransmitters. Activation of H3R inhibits cAMP synthesis and activates MAP kinases and the AKT/GSK3β axis (Mariottini et al., 2009). A role for H3R in motivation and aversion, locomotor

activity and social behavior has been suggested (Baronio et al., 2015; Nuutinen et al., 2011; Vanhanen et al., 2013). The presence of H4R in the CNS is still debated, although there are reports of the presence of this receptor in brain endothelial cells (Karlstedt et al., 2013). Activation of H4R inhibits the formation of cAMP and is involved in the activation of phospholipase Cβ (PLC-β), which will lead to inositol triphosphate-dependent release of $Ca2^{+}$ from intracellular stores (Leurs et al., 2009).

5.3.3.1 The histaminergic system in ASD

Alterations in different neurotransmitter systems, such as serotonergic, dopaminergic, GABAergic and glutamatergic, have been studied and reported in ASD (Pardo and Eberhart, 2007). Surprisingly, only few studies examined the histaminergic system in ASD. Histamine modulates several behaviours and physiological functions, and its deficiency is associated with neuropsychiatric disorders (Nuutinen and Panula, 2010). The expression of histamine receptors and other histamine signaling genes was performed in a *post mortem* dorsolateral prefrontal cortex samples of ASD patients and none of the analyzed genes were individually identified to be differentially expressed. In contrast to the individual expression analyses, a significant overexpression was detected in ASD samples when the genes were analyzed as a gene set (Wright et al., 2017). However, *HRH3* downregulation was reported in a set of patients with Fragile X syndrome (Rosales-Reynoso et al., 2010), a condition that is associated with ASD (Moss and Howlin, 2009).

Histamine receptors play important roles in the CNS making them attractive targets to develop new therapeutical approaches for different brain disorders. Currently, the H3R receptor antagonist pitolisant is used to treat excessive daytime sleepiness and cataplexy in patients with narcolepsy (Schwartz, 2011; Syed, 2016). Recently, pitolisant was tested and improved many symptoms in a 15-year-old female with Prader-Willi syndrome and ASD (Pennington et al., 2021). Ligands of H3R have also been tested in ASD models and significant behavioural improvements regarding sociability and stereotypies were reported (Baronio et al., 2015; Eissa et al., 2018). A few studies proposed and tested the use of an H2R antagonist in patients (Linday, 1997; Linday et al., 2001). Symptoms like irritability, hyperactivity, and atypical pattern of eye contact were attenuated after treatment with famotidine. Thus, further investigation should be made to characterize this system in ASD. Animal models that recapitulate the core symptoms of the disorder are tools that can shed some light on possible roles of the histaminergic system in ASD pathophysiology.

5.3.3.2 The histaminergic system of zebrafish

The histaminergic system of zebrafish consists of neuronal cell bodies, which can start to be visualized at 85 hpf, and are grouped in the posterior hypothalamus sending projections throughout the brain, similarly to other vertebrates (Eriksson et al., 1998). Histamine is synthesized by Hdc, which is only detected in the brain and with the same anatomical position as the histamine-immunoreactive neurons (Eriksson et al., 1998). *Hnmt* is expressed in the raphe, preoptic area of the hypothalamus and periventricular nucleus of the hypothalamus of the zebrafish brain and is responsible for histamine metabolism (Norton et al., 2011). Three histamine receptors (Hrh1, Hrh2 and Hrh3) are present in the zebrafish brain (Peitsaro et al., 2007). Hrh1 is found in the dorsal telencephalon in an area that corresponds to the mammalian cortex, amygdala, hippocampus, and in habenula. There are two copies of Hrh2-like receptor in the zebrafish, in the brain the binding sites are mainly found in the optic tectum and central diencephalon. As an autoreceptor, Hrh3 is prominently expressed in the hypothalamus, where histaminergic neuronal cell bodies are locates. It is also present in other areas innervated by histaminergic projections, such as the telencephalon, thalamus and optic tectum (Panula et al., 2021).

5.4 Monoamine oxidases

MAOA and MAOB are mitochondrial membrane proteins that are responsible for the metabolism of aminergic neurotransmitters. They catalyze the oxidative deamination of endogenous and exogenic amines avoiding their accumulation and potentially toxic effects. However, the products from this reaction (aldehydes, ammonia and hydrogen peroxide) are toxic and are also metabolized by aldehyde dehydrogenase, glutamine synthetase and catalase, respectively (Bortolato et al., 2008).

Despite sharing similar structural features and intracellular location, mammalian MAOA and MAOB differ by their substrate affinity, anatomical distribution and responsiveness to inhibitors. MAOA has a higher affinity for serotonin and noradrenaline, whereas MAOB oxidizes trace amines like β-phenylethylamine (PEA). Both isoenzymes metabolize dopamine, but the level of affinity depends on the species. It has been suggested that dopamine is mainly metabolized by MAOA in rodents and MAOB in humans (Glover et al., 1977). Clorgyline at low doses is known to inhibit MAOA, whereas MAOB is inhibited by deprenyl. MAOA is mainly expressed in catecholaminergic neurons and MAOB in serotonergic and histaminergic neurons. Interestingly, as mentioned before, MAOB has lower affinity for serotonin than MAOA. The reasons of this mismatch are not fully understood, but it has been suggested that serotonin is metabolized by MAOA outside serotonergic

neurons, most likely by glial cells. Alternatively, it has been suggested that serotonin could be taken up by PMAT, which transports many types of neurotransmitters, including serotonin and dopamine, and metabolized by non-serotonergic neurons (Daws, 2009; Zhou et al., 2007).

MAO inhibitors were found to be effective in the treatment of depressive symptoms. However, hypertensive crises with cerebral hemorrhages was a side effect reported by patients treated with MAO inhibitors following the intake of foods rich in tyramine and sympathomimetic amines (Anderson et al., 1993). Because of inhibition of MAOB activity in the intestines, these amines are absorbed and induce increased norepinephrine release and increased blood pressure. Additionally, MAO inhibitors, along with other proserotonergic drugs, like SSRIs, may cause serotonin syndrome. The highly increased levels of serotonin in serotonin syndrome can lead to various complications, such as altered mental state, autonomic hyperactivity and neuromuscular abnormalities, and in some cases, death (Francescangeli et al., 2019).

MAO deficiency in humans was originally described in a few patients with atypical Norrie disease, a condition where large deletions in the X chromosome encompassed the *ND*, *MAOA* and *MAOB* genes. These patients showed developmental delay, sleep disturbance, and autistic-like symptoms (Sims et al., 1989). MAOA congenital deficiency was reported by Brunner in a group of Dutch patients that presented aggressive and antisocial behavior, maladaptive responses to environmental triggers and stereotypies. They also had reduced levels of the serotonin metabolite 5-HIAA and increase in serotonin in their urine (Brunner et al., 1993). *MaoA* KO mice are characterized by high levels of serotonin and noradrenaline, followed by aggressive behavior and reduced exploratory behavior. Additionally, these mice recapitulate the core deficits observed in ASD, including social and communication impairments (as assessed by a lower number of ultrasonic vocalization in response to maternal separation) (Bortolato et al., 2013). Clinical findings that point to a role for MAOA in the pathophysiology of ASD will be discussed in the next section of this thesis. Low activity of MAOB has been reported in platelets of individuals that often display features associated to impulsive behavior (Oreland and Hallman, 1995). *MaoB* KO mice showed high levels of PEA in their brains and a behavior that resembles the one displayed by patients with low MAOB activity in platelets, characterized by lower neophobia and risk-taking behavior (Bortolato et al., 2009). A spontaneous mutation in *MaoA* gene was detected in a *MaoB* KO colony of mice, leading to the establishment of the *MaoA/B* KO mice line (Chen et al., 2004). The increase in monoamines levels in the brains of these animals is more significant than hose observed in either *MaoA* or *MaoB* KO mice, which supports the hypothesis that one enzyme can partially compensate the absence of the other.

Besides the aforementioned disorders, pathophysiological roles of MAO have been established in other several conditions. MAOA has been linked to different forms of depression, where increased MAOA levels were detected in different brain areas of patients with postpartum depression and high activity of MAOA has been considered a risk factor for major depression (Sacher et al., 2010; Schulze et al., 2000). A role for MAOB in neurodegeneration diseases is suggested, as it is upregulated in Parkinson's disease and Alzheimer's disease where it is involved in amyloid β peptide formation (Damier et al., 1996; Schedin-Weiss et al., 2017).

5.4.1 MAOA and ASD

Foundation Autism Risk Initiative (SFARI) database lists over a thousand genes associated with ASD (WWW.SFARI.ORG). Among them is *MAOA*, which has also been associated with disorders that share symptomatology with ASD, such as attention deficit hyperactivity disorder (ADHD). Among common variations of *MAOA*, an upstream variable number of tandem repeats (uVNTRs) at the *MAOA* promoter is associated with variations in enzymatic activity. *In vitro* data indicated that the low-repeat variant (i.e., two or three copies) was associated with significantly lower enzymatic activity compared to the high-repeat variant (i.e., four or five copies) (Sabol et al., 1998). In a Korean study with 151 complete trios, comprising patients with ASD and their biological parents, and unrelated controls evaluated MAOA uVNTR and SNPs, demonstrating a significant difference in haplotype frequencies between ASD patients and controls. A three-repeat allele of a MAOA-uVNTR marker was preferentially transmitted in ASD individuals. The results suggested a potential association between *MAOA* and ASD (Yoo et al., 2009). Patients with the low activity three-repeat *MAOA* allele present more severe behavioral impairments, such as aggression and deficits in social communication (Cohen et al., 2011). Additionally, the low-activity MAOA allele was associated with larger cortical volumes in males with ASD (Davis et al., 2008). A similar finding was reported when patients with the low activity allele of a functional MAOA promoter polymorphism and fragile X syndrome, a condition related to ASD, were examined and showed increased cerebral cortical gray and white matter volumes (Wassink et al., 2014). A significant impairment in cerebellar MAOA activity was detected in postmortem samples of children with ASD compared with age-matched controls has been reported. In the frontal cortex, MAOA activity in children with ASD was also reduced by 30% compared with controls (Gu et al., 2017). Lower MAOA activity leads to increased levels of monoaminergic neurotransmitters, including serotonin, which have been reported to be abnormal in a subset of ASD patients (Anderson et al., 1990; Veenstra-VanderWeele et al., 2012).

5.4.2 The zebrafish Mao

Only one *mao* gene, located in chromosome 9, is found in the zebrafish genome (Anichtchik et al., 2006). The existence of only form of Mao is also commonly reported in other fish species. In zebrafish Mao has 67–69% nucleotide and protein identity with human MAOA and MAOB. The active site in human MAOA and MAOB contains 12 amino acid residues. Eight of these residues are identical in MAOA, MAOB and zebrafish Mao. From the other four amino acid residues in the zebrafish Mao substrate binding domain, two are the same as in human MAOA and two are unique to zebrafish Mao. The enzyme has high specificity for serotonin, PEA and tyramine, whereas it shows a modest one for dopamine. Mao inhibition is known to increase only serotonin levels in zebrafish (Sallinen et al., 2009b), which could indicate that dopamine metabolism is regulated by other enzymes, such as COMT. This substrate specificity and the structural properties of the binding site of the enzyme could indicate that it is more related with the human MAOA. However, zebrafish Mao can be inhibited by the MAOB specific inhibitor, deprenyl (Anichtchik et al., 2006; Sallinen et al., 2009b). The highest expression of *mao* is in the brain, followed by the gut, liver heart and gills. In the brain, it is widely expressed and can be detected in noradrenergic cells in the locus coeruleus and in the histaminergic cells of posterior hypothalamus. Regarding serotonergic cells, Mao activity was detected in some cell populations and adjacent to others in the neuropil. In the habenula, rostral raphe and internal reticular formation, serotonergic cells express *mao*. Serotonergic cells of the hypothalamus did not show signs of Mao activity, however, strong Mao activity was detected between the intermediate and posterior part of the paraventricular organ and it could be from glial nature (Finberg, 2014).

5.5 Vesicular monoamine transporter 2

Vesicular monoamine transporters (VMAT1 and VMAT2) are responsible for the uptake of cytosolic monoamines into synaptic vesicles in monoaminergic neurons. Two closely related VMATs with distinct pharmacological properties and tissue distributions have been characterized. VMAT1 is preferentially expressed in neuroendocrine cells and VMAT2 is primarily expressed in the CNS (Wimalasena, 2011).

VMAT2 is a transmembrane protein located in all aminergic neurons and responsible for transport of aminergic neurotransmitters from the cytosol to vesicles. It is an H^+-ATPase antiporter, which uses the vesicular electrochemical gradient to drive the packaging of cytosolic transmitter into vesicles (Liu et al., 1992). This process is fundamental for neurotransmission, but it also prevents

cytosolic amine accumulation and its neurotoxic effects, in the case of dopamine and serotonin. It has been hypothesized that VMAT2 is also involved in the storage of trace amines, such as tyramine and PEA (Eiden and Weihe, 2011).

VMAT2 inhibitors have been used in the treatment of disease. Reserpine was previously used in the treatment of hypertension based on reduction of sympathetic tone and, in turn, reduced blood pressure, caused by VMAT2 inhibition in the sympathetic nervous system. However, side effects including depression, Parkinsonism and gastric symptoms, were detected and reserpine is no longer commonly prescribed (Bernstein et al., 2014). Tetrabenazine has been used for the treatment of Huntington's disease, but it also leads to similar side effects as reserpine use. It also showed some efficacy in the treatment of disorders characterized by a hyperdopaminergic phenotype, such as Gilles de la Tourette syndrome (Kaur et al., 2016).

Mice homozygous for loss-of-function alleles of *Vmat2* are unable to feed properly and die a few days after birth. In the neonatal brain, mutants relative to wild types display a 94%-99% reduction of monoamines (dopamine, noradrenaline and serotonin) levels. Heterozygous *Vmat2* mutants show a reduction in dopamine (42%), noradrenaline (23%) and serotonin (34%) levels, but are able to survive until adulthood. They display a depressive-like phenotype and increased sensitivity to parkinsonian toxins (Fon et al., 1997).

Many brain disorders can be linked to dysfunction of monoaminergic systems, including Parkinson's disease, ADHD, dystonia, schizophrenia, addiction, and depression. A role for VMAT2 in the pathophysiology of some of these disorders is discussed in the next session.

5.5.1 Pathophysiological roles of VMAT2

Considering that it is one of the important modulators of monoaminergic neurotransmission, VMAT2 have been linked to a variety of disorders. In Parkinson's disease, impaired packaging of dopamine into vesicles has been proposed as one of the key players in the disease process (Lohr and Miller, 2014). This is supported by a mutation in the gene encoding VMAT2 that compromises transport of biogenic amines into synaptic vesicles, resulting in impaired neurotransmission without affecting the amount of dopamine, but causing parkinsonian condition with profound motor and cognitive impairments (Rilstone et al., 2013). Additionally, it has been suggested that VMAT2 overexpression might reduce the risk of Parkinson's disease (Brighina et al., 2013). The platelets of patients with depression showed increased density of VMAT2, which could reflect a response to lower monoaminergic turnover in these patients (Zucker et al., 2002). As mentioned before, reserpine,

a VMAT2 inhibitor, was commonly used to treat hypertension, but the reports of patients displaying depressive symptoms contributed to its popularity decline (Baumeister et al., 2003). This reinforces a likely role for this transporter in the pathophysiology of depression. Genetic variants of *VMAT2* have been linked to substance dependence (Fehr et al., 2013; Randesi et al., 2019). Increased VMAT2 concentration has been detected in the ventral brainstem of patients with schizophrenia and bipolar disorder (Zubieta et al., 2001). In this region are located the raphe nuclei, from where serotonergic projections originate and innervate different areas of the CNS (Berger et al., 2009).

Well-established rodent models that lack *Vmat2* have been used to investigate neurobehavioral impairments caused by VMAT2 deficit (Fon et al., 1997; Fukui et al., 2007; Isingrini et al., 2016). Some of these impairments are also displayed by patients with brain diseases where this transporter plays a role, making these models important tools to understand pathophysiological processes. Mice homozygous for loss-of-function alleles of *Vmat2* die at early age. Their brains, when compared to wild type siblings, show a 94%-99% reduction of dopamine, noradrenaline and serotonin levels (Fon et al., 1997). Additionally, they are hypoactive and unable to feed properly. Heterozygous *Vmat2* mutants present a less drastic reduction in brain amines and are able to grow into adulthood (Fon et al., 1997). They display a depressive-like phenotype and increased sensitivity to parkinsonian toxins (Fukui et al., 2007). In this thesis, we present a zebrafish model that could contribute with future studies with the same aim and that has the advantage of being a convenient tool for high throughput screening and drug discovery.

5.5.2 The zebrafish Vmat2

The *vmat2* gene is located on chromosome 17, contains 16 exons and phylogenetic analysis indicates that this gene is closely related to the *VMAT2* genes in human and mouse (Wen et al., 2008). In zebrafish, *vmat2* is primarily expressed in aminergic populations. *In situ* hybridization shows that it is firstly detected at 22 hpf in the diencephalon. At 48 hpf the distribution and number of *vmat2*- and *th*-positive ventral diencephalic neurons are similar. Also, at 48 hpf *vmat2* is expressed in the raphe nuclei and other serotonergic clusters, such as the anterior, intermediate and posterior part of the paraventricular organ. A weak expression can be also noticed in the locus coeruleus (Wen et al., 2008). The majority of *hdc*-positive neurons in the hypothalamus also express *vmat2*, as demonstrated by double fluorescent *in situ* hybridization (Puttonen et al., 2017). The mechanism of storage and release of histamine in the small group of neurons that do not express *vmat2* is still uncertain. *Vmat1*

was investigated in the zebrafish brain to verify if this other transport could participate in this process, but no expression of this gene was detected in the zebrafish brain (Puttonen et al., 2017).

VMAT2 inhibitors have been used in zebrafish and the neurochemical and behavioral outcomes are similar to what have been reported in studies with rodents, including depletion of aminergic neurotransmitters, hypoactivity and depressive-like state (Puttonen et al., 2017; Wang et al., 2019). CRISPR/Cas9 technology has been recently applied to modify *vmat2* in zebrafish. In this study, only heterozygous animals were evaluated and they displayed anxiety-like phonotype and significantly decreased dopamine, serotonin and noradrenaline when compared to their littermates (Wang et al., 2016a). In this thesis we characterize for the first time a *vmat2*$^{-/-}$ zebrafish.

6 AIMS

The aim of this thesis was to perform pharmacological and genetic interventions relevant to the pathophysiology of ASD and other brain disorders using zebrafish as a model organism and to study behavioral outcomes and the aminergic systems. Increasing the knowledge about important aspects of the aminergic systems in zebrafish, such as development, transport and metabolism, was necessary. Thus, we characterized the aminergic populations, transport and metabolism of animals that lacked *vesicular monoamine transporter 2* (*vmat2*) and *monoamine oxidase (mao)*.

The specific aims of the study are:

1. To expose zebrafish embryos to VPA, an ASD-associated drug, and evaluate their histaminergic system and behavior at different stages of development (I).
2. To characterize the effects of *vmat2*-loss of function on the brain development, aminergic neurotransmitter systems and behavior (II).
3. To characterize the effects of *mao*-loss of function on the brain development, aminergic neurotransmitter systems and behavior (III).
4. To characterize the phenotype of *mao* mutant zebrafish, particularly aspects relevant for basic mechanisms of ASD (III).

7 MATERIALS AND METHODS

Experimental animals (I-III)

Zebrafish embryos were collected after spawning and placed onto a Petri dish containing 1xE3 medium (5mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$). Larvae were raised on 14:10 (light/dark, lights on at 8:00 A.M.) cycles at 28°C and fed daily once with dry food and two times with live artemia. Adult fish were raised in continuously cycling Aquatic Habitats Systems with complete exchange of water in each tank every 6-10 min. Circulating water was UV-sterilized, and filtered with foam filters and activated charcoal. Water quality, including temperature (28 ± 0.5°C), pH value (7.4 ± 0.2), and conductivity (450 ± 10 mS), was monitored continuously.

In the first article included in this thesis we used zebrafish from the wild-type Turku strain. This line has been maintained in our laboratory for several years and has been used in different publications (Kaslin and Panula, 2001; Puttonen et al., 2013; Sundvik and Panula, 2012). In the second publication, *vmat2* mutant was generated at the University of Iowa kindly given to us by Dr. Robert Cornell. CRISPR/Cas9 system was used to a 5-base-pair (5-bp), frameshift-inducing deletion in exon 3 of *slc18a2* (*vmat2*). In the third publication, *mao* mutant (sa^{31732}) was generated by the Sanger Institute Zebrafish Mutation Project and was purchased by us from the European Resource Zebrafish Center (ERZC). It contains an A/T nonsense mutation at nucleotide 685 of the open-reading frame, which is predicted to generate a 229-amino acid protein compared with the 522-amino acid WT protein.

Mutant larvae were genotyped at 3 dpf according to a protocol previously described in our group's publications (Chen et al., 2020; Puttonen et al., 2018). Briefly, when genomic DNA was lysed after individual tail clippings were incubated in 50 µL lysis buffer (10mM Tris-HCl pH8.3, 50mM KCl, 0.3% Tween-20 and 0.3% NP-40) at 98°C for 10 min, followed by incubation on ice for 2 min. 1 µL of Proteinase K (20 mg/ml) was added to remove protein, and the mixture was incubated at 55°C for at least 4 h. To inactivate Proteinase K, samples were incubated at 98°C for 10 min and quenched on ice. Genotyping primers (Table 2) flanking the mutation site were designed using Primer-BLAST. Mutations were detected after HRM curve acquisition and analysis. We utilized a LightCycler® 480 instrument (Roche) and curves were analyzed using the LightCycler® 480 gene-scanning software (version 1.5) according to the manufacturer's instructions (Roche Diagnostics Ltd., Switzerland).

Larvae and adult animals were killed in ice-cold water for all the experiments where tissues needed to be collected. All animal care and experimental procedures complied with the ethical

guidelines of the European convention (ESAVI/6100/04.10.07/2015) and the permits for all experiments were obtained from the Office of the Regional Government of Southern Finland, in agreement with the ethical guidelines of the European convention.

Table 2 - Primers used for qPCR and genotyping.

PURPOSE	GENE	FORWARD PRIMER	REVERSE PRIMER
qPCR	*hdc*	TTCATGCGTCCTCTCCTGC	CCCCAGGCATGATGATGTTC
	th1	GACGGAAGATGATCGGAGACA	CCGCCATGTTCCGATTTCT
	th2	CTCCAGAAGAGAATGCCACATG	ACGTTCACTCTCCAGCTGAGTG
	dbh	TGCAACCAGTCCACAGCGCA	GCTGTCCGCTCGCACCTCTG
	rpl13a	AGAGAAAGCGCATGGTTGTCC	GCCTGGTACTTCCAGCCAACTT
	hrh3	CGCCACCGTCCTTGGGAACG	GGGGATGCAAAACCCGCCGA
	hrh2	GGCCACTAGGGGCGCACTTC	AGCGGAGCAGTGACCGCAAA
	hrh1	TCCTGATCCCGTCCGCACCA	CCCGACGGTATGCAGCGTCC
	pcna	ACGCCTTGGCACTGGTCTT	CTCTGGAATGCCAAGCTGCT
	vmat2	TGCCTATTATCCCAAGTTACCTGT	TGAGGGCTCACAAAAGTAGGA
	comta	TCTGGCACGATGTGGTCCAT	TCAAGATGCGCTGTGGTCGT
	mao	TGGTGGAGGTCAGGACGGTGA	GCTGGTTCCTCAGAGGCGGC
	notch1a	AGAGCCGGATTCAGCGGTC	TTACAGGGACGTGGAGAACAAG
	manf	AGATGGAGAGTGTGAAGTCTGTGTG	CAATTGAGTCGCTGTCAAACTTG
	gfap	GAAGCAGGAGGCCAATGACTATC	GGACTCATTAGACCCACGGAGAG
	neurod1	ACACACCCTAGAGTTCCGAC	GTCCACGTCTCGTTCGTCTT
	apoeb	AACGCCTGAACAAGGACACA	GTATGGCTGGAAACGGTCCT
	mecp2	ACGTCTACCTTATCAACCCAGA	CCTTCCACGTCCAGAGGG
	shank3b	GGTTTTATTAGGGTTGTGAGGCCG	CCCAGGAGAGGTCCGAATACTGTC
	serta	ACAACCGATGGAACACTCCC	CAACACCTGCCGGACATAAA
Genotyping	*mao*	AATGACAGGAGCGCAAGTTT	GTAAACCTCCTCATTCACCGTC
	vmat2	TCTCTCTAGTGCCTATTATCCCAAG	AGGTGCTCGATGGAGAAAGA

Valproic acid exposure (I)

A stock solution (1 mM) of VPA (Sigma P4543, Darmstadt, Germany) was made after diluting the chemical in 1xE3 medium. Zebrafish embryos at 10 hpf were placed in 6-well plates, each well containing 30 embryos and 3 mL of 1xE3 medium or 25 µM of VPA diluted in E3. This dose was chosen based on pilot studies performed in our laboratory and was the tested dose that caused the lowest death rate or embryo exclusion due to obvious malformations (27%). At 24 hpf VPA was removed from the wells, embryos were washed several times with E3 medium and raised until larval stage or adulthood. The time window of exposure to VPA was selected based on previous publications that showed that VPA exposure during the first trimester of pregnancy is associated with higher incidence of ASD. During this time window, the neural tube closure takes place (Bayer et al., 1993). Based on that, a rodent model of ASD was developed with prenatal exposure to VPA during the

neural tube closure (Rodier et al., 1996). We exposed zebrafish embryos to VPA during a period that corresponds to the end of gastrulation to neural tube formation (Compagnon and Heisenberg, 2013). Figure 4 shows the timeline of valproic exposure and experiments.

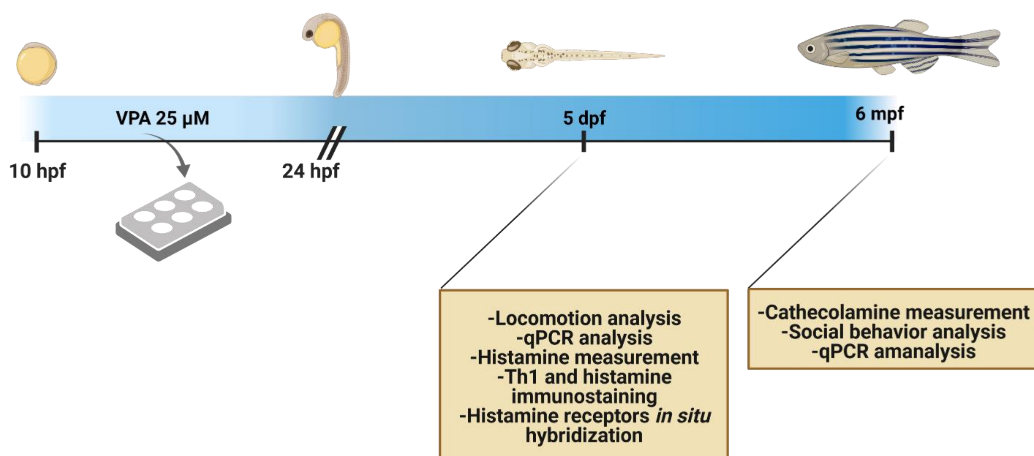

Figure 4 - Timeline of the experiments and VPA exposure. Exposure to 25 µM of VPA was applied from 10 to 24 hpf. Behavioral tests and the study of histaminergic and catecholaminergic systems of zebrafish were performed at 5 dpf and 6 mpf. Figure created with BioRender.com.

Behavioral tests (I-III)

24h locomotion tracking (II, III)

We tracked larvae for 24 h with the light conditions following the regular light/dark cycle of the larvae. The trial was started at 12:00 noon. Day and night activity was analyzed in 60 min bins by calculating the total distance moved. Larvae were individually tracked using the DanioVision (Noldus Information Technology, Wageningen, The Netherlands) system and EthoVision 13 software DanioVision (Noldus Information Technology, Wageningen, The Netherlands) in 24 or 48 well-plates.

Dark-flash response (I-III)

This behavioral test was carried out according to a previous publication (Puttonen et al., 2018). Briefly, larvae were individually placed in wells of multiwell plates filled with 1 mL E3 medium and, after an initial assessment of basic locomotor activity tracking, larvae were exposed to alternating 2 min periods of darkness and light, and with three periods of darkness in total. The locomotor activity was analyzed in 1 min, 30 s and 1 s bins using the DanioVision system and EthoVision 13 software.

Larval acoustic/vibrational startle (III)

This behavioral protocol has been described previously (Van Den Bos et al., 2017). Briefly, larvae were placed into 48-well plates filled with 1 ml E3 medium. After 10 min of acclimation, ten acoustic/vibrational stimuli (DanioVision intensity setting 6) with a 20 s inter-stimulus-interval (ISI) were given to the larvae. The variable of interest to show the startle response was maximum velocity (mm/s) with 1 s intervals, because the startle response is a short burst of activity best captured by this parameter. When subjects did not show a clear response to the first stimulus (values lower than 15 mm/s), they were discarded from the analysis. Larvae were individually tracked using the DanioVision system and EthoVision 13 software.

Adult locomotor activity and thigmotaxis (I, III)

The basic locomotor activity of adult fish was tracked in cylindrical observation tanks (inner diameter 22 cm and water depth 8 cm). Single fish were acclimated for 10 min in the tank before the tracking started. The total distance moved and velocity were automatically evaluated for 10 min using a digital video camera connected to a standard PC computer system running the EthoVision 13 software.

Juvenile social interaction (III)

This assay was carried out using a glass tank (9 cm length×5 cm height×7 cm width). Juvenile fish were separated into pairs of the same genotype and placed into glass tanks with 150 ml water (water depth, 3 cm). Each individual of the pairs was taken from different home tanks. The pairs were tracked for 6 min, and parameters analyzed included the total duration in proximity (with proximity defined as when the distance between two larvae was ≤0.8 cm). Each group had 16 fishes for the data analysis using EthoVision 13 software.

Social preference (I,III)

Zebrafish have the innate tendency to aggregate into shoals. We used this innate behavior to develop a visual choice test to evaluate the social preference a fish. Our apparatus to test social preference was developed based on previous publications with the rodent three-chamber sociability test (Baronio et al., 2015) and similar approaches that have been utilized with zebrafish (Zimmermann et al., 2015). An acrylic apparatus (29 cm length × 19 cm height × 29 cm width) was divided by a transparent wall into two chambers, one of which was subdivided in two smaller compartments. A shoal of eight fish, serving as a visual social cue, was placed in one of the compartments, and the other compartment was filled with stones and plant imitations. The other chamber was digitally divided in 3 zones (distal, social and non-social) and the time spent by the tested adult fish in each zone during a 10 min trial was measured with EthoVision 13 software.

Novel tank test (III)

Zebrafish has the natural tendency to seek protection in an unfamiliar environment before developing confidence to explore it. In the laboratory, a similar phenomenon is observed when a fish is placed in a novel tank and it initially dives to the bottom and stays there before exploring the rest of the tank. The assessment of this behavior can provide information regarding the exploratory activity and the status of anxiety-like behavior of the fish. During our test, the novel tank (24 cm × 14.5 cm × 5 cm) with 1 L of system water rested on a level, stable surface and was virtually divided into three equal portions (bottom, middle, and top of the tank). A single fish was transferred from its home tank to the novel tank and immediately tracked for 6 min using EthoVision 13 software. The time spent in each zone of the tank was measured and the degree of 'bottom dwelling' can be interpreted as an index of anxiety-like. Figure 5 displays the set up for the novel tank diving test.

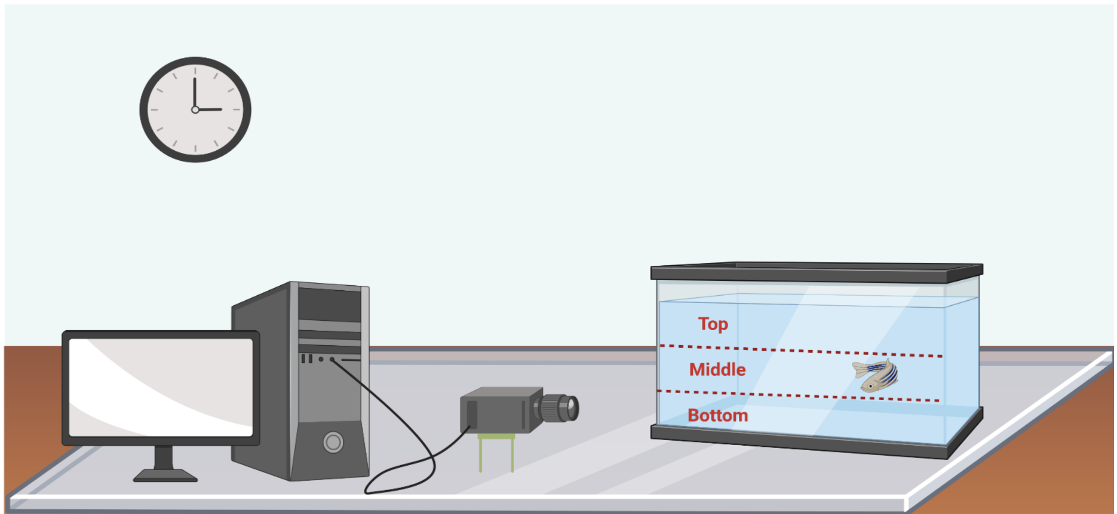

Figure 5 - Scheme of the novel tank test with three digital zones during a 6 min recording period. A single fish is transferred from its home tank to the novel tank and immediately recorded by a Basler acA1300-60gm industrial CCD video camera connected to a computer. The time spent in each zone was quantified using EthoVision 13 software. Figure created with BioRender.com.

Shoaling behavior (III)

The establishment of tight social groups (shoals) by zebrafish serves different purposes in their natural habitat, including foraging, avoiding predation and mating (Pitcher, 1986). This is a form of social interaction and can be studied in the laboratory by placing a group of zebrafish in observational tanks and evaluating parameters such as distance between subjects of a shoal. We utilized cohorts of 4-5 fish in a round white polyethylene plastic flat-bottomed container for 10 min. For 40 dpf fish the setting was: (12 cm height, 12 cm diameter) with 350 mL of fish system water (5 cm depth). The setting for adult fish was (23 cm height, 23 cm diameter) with 2 L of fish system water (5 cm depth. All videos were analyzed with EthoVision 13 software, using the default setting (the center-point detection of the unmarked animals). The mean of the interfish distance (defined as distance between the body center of every member of the shoal) was quantified from the average data from all trials (n = 4 trials per genotype). The proximity duration (s) was defined as cumulative duration of time a fish stayed close to the shoal fish (within 1 cm). Figure 6 displays the set up for the shoaling behavior.

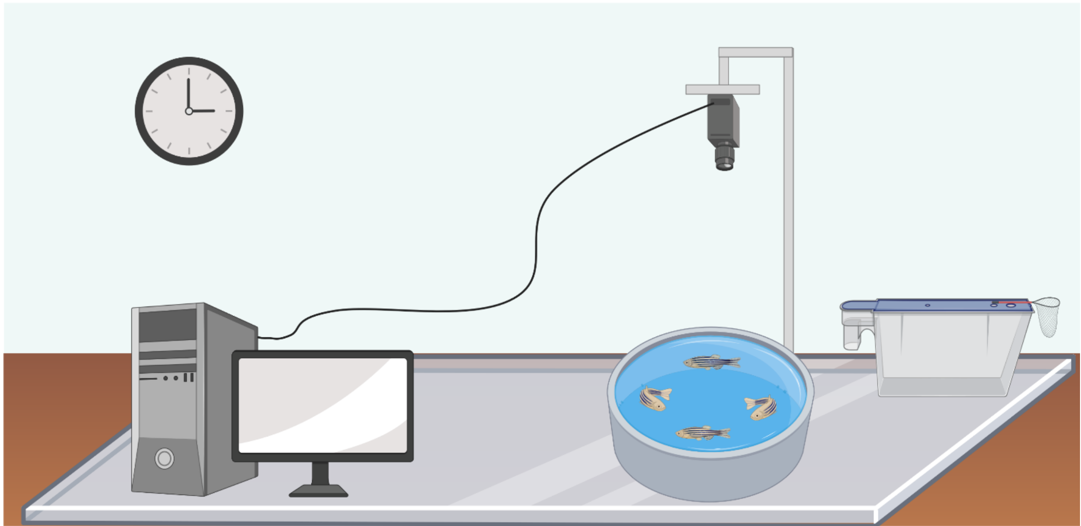

Figure 6 - Shoaling behavior test. The test consists in placing a group of conspecific fish into a round polyethlene plastic flat-bottomed container and quantifying social cohesion in the group of fish, which can be measured by the average mean distance among individuals. Fish are recorded during a 10 min trial by a Basler acA1300-60gm industrial CCD video camera connected to a computer. Parameters of interest are quantified using EthoVision 13 software. Figure created with BioRender.com.

Heart rate measurement (II)

After anesthesia with 0.02% tricaine, zebrafish larvae were placed on a glass slide. Larvae were observed under 10 × objective of the Leica inverted microscope DMi1. The heart rate measurement was done by counting the contractions of either of the two chambers for 20 s. Videos were recorded using a Leica digital HDMI camera attached to the back of the microscope.

Reverse transcription -quantitative PCR (RT-qPCR) (I-III)

Larval zebrafish were sacrificed in ice-cold water prior to RNA extraction, and total RNA was extracted using the RNeasy mini Kit (Qiagen, Hilden, Germany). Larval samples (10 pooled larvae per sample) were collected and 1.0 µg of total RNA was reverse-transcribed using SuperScriptTM III reverse transcriptase (Invitrogen, Carlsbad, USA). RT-qPCR was done with the LightCycler 480 realtime PCR system and the LightCycler 480 SYBR green I master kit (Roche Applied Science,

Mannheim, Germany). Primers for amplification were designed with Primer-BLAST (NCBI), and sequences are shown in Table 1. Cycling parameters were as follows: 95°C for 30 s and 45 cycles of the following, 95°C for 10 s and 62°C for 45 s. Fluorescence changes were monitored with SYBR Green after every cycle. Dissociation curve analysis was performed (0.2°C per s increase from 60 to 95°C with continuous fluorescence readings) at the end of cycles to ensure that only a single amplicon was obtained. All reactions were performed in duplicate. Results were evaluated with the LightCycler 480 Software version 1.5. Quantification was done by Ct value comparison, using the Ct value of *ribosomal protein large subunit 13a* (*rpl13a*) as the reference control.

High-performance liquid chromatography (HPLC) (I, II)

Groups of 10 whole larvae were killed in ice-cold water and homogenized by sonication in 2% perchloric acid for each sample, centrifuged for 30min at 15 000 g at 4°C, and filtered through a 0.45-μm PVDF filter (Pall Life Sciences, Ann Arbor, USA) before loading onto the HPLC system. All analyses were done in duplicate to ensure the reliability of values. The detection details have been described earlier (Puttonen et al., 2013; Rozov et al., 2014; Yamatodani et al., 1985). In order to normalize the results from HPLC measurement, we utilized the bicinchoninic acid assay kit (Thermo Fisher Scientific, Waltham, USA) to measure protein concentration.

In situ hybridization (I-III)

4% PFA-fixed dissected larval brains were used as samples and experiments were carried out according the protocol described by Thisse & Thisse with slight modifications (Thisse and Thisse, 2008). The digoxigenin (DIG) RNA labelling kit (Roche Diagnostics, Germany) was used to produce antisense DIG-labelled RNA probes. The specificity of the probes and clones have been described in previous publications (Chen et al., 2009; Chen et al., 2016a; Kaslin et al., 2004; Peitsaro et al., 2007; Sundvik et al., 2011; Wang et al., 2006). Sheep anti-digoxigenin-AP Fab fragments (1:5000; Roche Diagnostics, Germany) were used to detect the *in situ* hybridization signals. Staining was performed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate and samples were incubated at room temperature in the dark. After staining, samples were immersed in 80% glycerol, placed between two cover glasses and visualized under brightfield optics using a Leica DM IRB inverted microscope.

Fluorescent in situ hybridization (I)

Larvae killed in ice-cold water were collected and fixed overnight in 4% PFA diluted in PBS at 4°C. Fixed larvae were washed in PBS and brains were dissected. Brains were dehydrated in methanol. *In situ* hybridization was carried out as described previously (Lauter et al., 2014), with minor modifications as described before (Chen et al., 2016a; Sundvik et al., 2011). Digoxigenin- and fluorescein-labelled riboprobes against *hrh1* and *hrh3* specified earlier were used (Sundvik et al., 2011). DY-647P1 (Dyomics 647P1-01, Jena, Germany) and 5(6)-TAMRA (Thermo Fisher Scientific C1171, Waltham, USA) conjugated tyramides were synthesized as described (Speel et al., 1998). The tyramides were diluted 1:250 in amplification buffer, and amplification was carried out for 15 min. Samples were mounted in 75% glycerol diluted with PBS for imaging.

Immunohistochemistry (I-III)

Larvae were sacrificed in ice-cold water and collected for overnight fixation in 4% 1-ethyl-3,3 (dimethyl-aminopropyl) carbodiimide (EDAC; Carbosynth, Berkshire, UK) or 2% PFA. The detailed protocol for immunohistochemistry and specificity of the antibodies have been described previously. Primary and secondary antibodies information is described in Table 3.

For the dopamine staining, zebrafish larvae (6 dpf; vmat2$^{+/+}$ and vmat2$^{-/-)}$ were fixed by immersion in 5% glutaraldehyde and 1% sodium metabisulfite (MB) in 1X phosphate-buffered saline (PBS; pH 7.4) for 20 h at 4 °C. Brains were dissected out and washed in PBS with 0.3% Triton X-100 and 1% MB (PBST-MB), O/N at 4 ºC. To increase antibody penetration, we incubated the samples in 0.5 mg/ml collagenase in the following buffer (100 mM Tris base; 1 mM CaCl2; 0.1% Triton X-100; pH 7.4) for 30 min at 37 ºC, in gentle agitation. After washing in PBST-MB, the samples were pre-treated with 0.5% NaBH4 in deionized water for 1 hour to quench auto fluorescence. Following washes in PBST-MB, brains were incubated in blocking solution (4% NGS, 1% DMSO in PBST-MB), O/N at 4 ºC. We then incubated the samples with a rabbit polyclonal anti-dopamine antibody (1:750) in blocking solution for 3 days at 4 °C. Then, samples were incubated for 1 hour at room temperature with Alexa-conjugated antibodies (Alexa Anti-mouse 568, Invitrogen, Carlsbad, USA) diluted 1:1000 in blocking solution with PBST. Samples were rinsed in PBST and PBS, incubated in increasing glycerol concentrations and mounted in glycerol.

For double immunofluorescence WT Turku zebrafish larvae (6 dpf) were fixed by immersion in a mix containing 2.5% glutaraldehyde + 2% PFA and 1% sodium MB in 1X phosphate-buffered

saline (PBS; pH 7.4) for 20 h at 4 °C. Brain dissection and washes were done as described above. Samples were then incubated with a rabbit polyclonal anti-dopamine antibody (Dr. Steinbusch, Maastricht University; 1:750), and a mouse monoclonal anti-TH antibody (1:1000; Product No 22941, Immunostar, Hudson, WI, USA) in blocking solution for 3 days at 4 °C. Then, the samples were rinsed of primary antibodies and subsequently incubated for 1 hour at room temperature with Alexa-conjugated antibodies (Alexa Anti-rabbit 488 and Anti-mouse 568, Invitrogen, Carlsbad, USA) diluted 1:1000 in blocking solution with PBST. Samples were rinsed in PBST and PBS, incubated in increasing glycerol concentrations and mounted in glycerol between two glass coverslips with spacers.

Table 3 - Primary and secondary antibodies used for immunohistochemistry.

ANTIBODY	DILUTION	SUPPLIER	PRODUCT NUMBER	REFERENCE
Rabbit anti-histamine	1:10000	Prof. Panula	19C	(Panula et al., 1990)
Mouse anti-proliferating cell nuclear antigen	1:1000	Abcam	GR201287	(Thummel et al., 2008)
Mouse anti-tyrosine hydroxylase	1:1000	Immunostar	907001	(Semenova et al., 2014)
Rabbit anti-serotonin	1:1000	Sigma	S5545	(Kaslin and Panula, 2001)
Mouse anti-HuC/D	1:1000	Invitrogen	A21271	(Chen et al., 2012)
Rabbit anti-dopamine	1:750	Dr. Steinbusch, Maastricht University	-	(Steinbusch et al., 1991)
Alexa goat anti-rabbit 488	1:1000	Invitrogen	A 11034	(Sundvik and Panula, 2012)
Alexa goat anti-mouse 568	1:1000	Invitrogen	A 11031	(Sundvik and Panula, 2012)
Alexa goat anti-rabbit 568	1:1000	Invitrogen	A 11036	(Sundvik and Panula, 2012)

Mao activity histochemistry (III)

We performed Mao activity histochemistry assay according to a previous publication (Anichtchik et al., 2006). Briefly, 4% EDAC fixed 40 dpf brain samples were washed 2 x 10 min in PBS and then incubated in 0.05 M Tris-HCl buffer containing 0.08 g/l DAB, 1 g/l tyramine, 1 g/l peroxidase, 6 g/l NiSO4 for 2h at RT.

Microscopy and imaging (I-III)

Leica DM IRB inverted microscope with a DFC 480 charge-coupled device camera was used to take brightfield images. Z-stacks were processed with Leica Application Suite software. Immunofluorescence samples were analysed with a Leica TCS SP2 AOBS confocal microscope. The Alexa 488- and 568-labelled secondary antibodies were detected using a 488 nm argon laser and a 568 nm diode laser respectively. Emission was detected at 500–550 nm and 560–620 nm, respectively. Stacks of images taken at 1.0 µm intervals were compiled, and the maximum intensity projection algorithm was used to produce final images with Leica Confocal software. Cell numbers were counted in each 1.0 µm optical slice using ImageJ 1.52b software (National Institutes of Health, Bethesda, USA). All cell counts were performed by an investigator blinded to the sample type.

Data and statistical analysis (I-III)

Data shown are representative of a minimum of two independent biological replicates. Data were analysed by unpaired, two-tailed Student's t-test, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test or two-way multiple comparisons ANOVA followed by Tukey's post hoc test, and $P<0.05$ was considered statistically significant. Statistical analysis was performed by Prism version 7 (GraphPad Software, San Diego, CA, USA).

8 RESULTS

The main findings regarding the development behaviour and aminergic neurotransmitter systems of VPA exposed zebrafish, *mao* and *vmat2* mutants are summarized in Table 4. Findings are reported in detail in the following sections of this thesis.

Table 4 - Summary of VPA exposed, *mao* and *vmat2* mutants phenotypes.

<table>
<tr><th rowspan="2">Phenotype</th><th colspan="4">Model</th></tr>
<tr><th>VPA exposed</th><th>mao $^{-/-}$</th><th>mao $^{+/-}$</th><th>vmat2 $^{-/-}$</th></tr>
<tr><td>Development</td><td>Hypoactive at larval stage.</td><td>Hypoactive and die within 20 days. Downregulation of developmental genes.</td><td>No alterations found.</td><td>Hypoactive and die within 20 days. Downregulation of developmental genes.</td></tr>
<tr><td>Adult Behavior</td><td>Impaired social behavior.</td><td>-</td><td>Anxious-like and impaired social behavior</td><td>-</td></tr>
<tr><td>Dopaminergic system</td><td>Reduced number of Th1-immunoreactive cells in the preoptic area and downregulation of Th1.</td><td>Reduced number of Th1-immunoreactive cells.</td><td rowspan="3">No main alterations in aminergic systems at a larval stage. Upregulated vmat2 in adult brains.</td><td>Virtually no immunoreactivity to dopamine. Reduced levels of the neurotransmitter. th1 and th2 were upregulated.</td></tr>
<tr><td>Serotonergic system</td><td>No alterations found.</td><td>Increased extracellular serotonin immunoreactivity and increased density of serotonin-immunoreactive fibers.</td><td>Virtually no immunoreactivity to serotonin. Reduced levels of the neurotransmitter. tph1a was upregulated.</td></tr>
<tr><td>Histaminergic system</td><td>Reduced number of histaminergic cells and histamine levels in larvae. Downregulation of histamine receptors and hdc in both larvae and adults.</td><td>Reduced number of histaminergic cells.</td><td>Reduced number of histaminergic cells and histamine levels. Increased number of hdc-positive cells.</td></tr>
</table>

8.1 Embryonic exposure to valproic acid affects the aminergic systems of zebrafish (I)

After embryonic exposure to VPA, zebrafish were raised until 5 dpf and 6 mpf to be collected for the evaluation of their aminergic systems. At 5 dpf, we started by analysing the histaminergic system by fluorescence *in situ* hybridization, which revealed weaker *hrh3* and *hrh1* signals in the dorsal telencephalon of VPA-exposed larvae than in wild-type larvae. This result was supported by qPCR analysis that indicated a significant downregulation of *hrh1* and *hrh3*, as well as of *hrh2* and *hdc*, in the VPA-exposed group. Additionally, a reduced number of histamine-immunoreactive cells and lower levels of the neurotransmitter histamine were also detected at larval stage in VPA-exposed animals. These larvae also presented some alterations regarding catecholaminergic systems, including downregulation of *th1* and *dbh* and reduced number of Th1-immunoreactive cells in the preoptic area. Other TH-positive populations, such as the diencephalic and the hypothalamic ones, were also evaluated but no differences in cell number were detected. VPA-exposed larval brains did not show any evident alterations when immune staining with proliferating cell nuclear antigen (PCNA) antibody was performed and no significant difference in *pcna* levels were detected when VPA and control groups were compared.

At 6 mpf, the brains of animals that were embryonically exposed to VPA presented a downregulation of *hdc*, *hrh3*, *th1* and *dbh*. Interestingly, the histamine levels of VPA-exposed and control brains were not significantly different. However, noradrenaline and DOPAC were significantly reduced in the VPA brains. We also quantified the levels of serotonin and its metabolites, such as 5-HIAA and HVA, but no differences between groups were detected.

8.2 VPA-exposed larvae are hypoactive and present altered dark-flash response (I)

At 5 dpf, VPA-exposed larvae showed a hypoactive phenotype when compared to control larvae during a basic 15 min locomotor activity assessment. After the locomotor activity evaluation, the dark-flash response of larvae was tested. Larvae were exposed to alternating 2 min periods of sudden darkness and light, with three periods of darkness in total. When the light was switched off for the first time both control and VPA-exposed larvae became hyperactive, with VPA-exposed larvae moving significantly more than control animals during the 2 min period. In the following periods of darkness, no differences between. Interestingly, when we analysed the light-dark transitions at a 1s resolution, the dark-flash response in the VPA-exposed group was stronger than the control in the first transition, but significantly weaker in the second and third transitions.

8.3 Impaired transport into synaptic vesicles leads to amine depletion in zebrafish (II)

Zebrafish larvae lacking *vmat2* are unable to package and store into intracellular vesicles aminergic neurotransmitters. The failure to package amines into synaptic vesicles increases the time spent in the cytoplasm and hence the accessibility to enzymes such as monoamine oxidase. The lack of vmat2 is not compatible with life and zebrafish die within 2 weeks post-fertilizations. A similar phenotype has been described when *Vmat2* KO mice were analyzed and died a few days after birth.

When we compared *vmat2*$^{-/-}$ larvae with *vmat2*$^{+/+}$ siblings, the mutants had a strong reduction in the levels of dopamine and noradrenaline and dopamine immunoreactivity was absent in their brains. DOPAC, a dopamine metabolite was significantly increased in *vmat2*$^{-/-}$ larvae, which indicates an accelerated dopamine turnover. Immunostaining did not reveal alterations in the number of th1-positive cells in *vmat2*$^{-/-}$ larval brains. However, RT-qPCR analysis of whole embryos lysates indicated an upregulation of *th1* and *th2* in comparison with their *vmat2*$^{+/+}$ siblings. In situ hybridization demonstrated th2 signal was particularly stronger that the neuron groups 9b and 10b of *vmat2*$^{-/-}$ larval brains.

Similarly with the reduction in dopamine immunoreactive cells, the number of serotonin immunoreactive cells was significantly reduced in *vmat2*$^{-/-}$ larval brains relative to *vmat2*$^{+/+}$ larval brains. *In situ* hybridization showed that *tph1a* signal, encoding tryptophan hydroxylase 1a, the enzyme responsible for serotonin synthesis, was clearly upregulated in *vmat2*$^{-/-}$ larval brains relative to *vmat2*$^{+/+}$ larval brains. Additionally, the levels of serotonin were significantly decreased in *vmat2*$^{-/-}$ larvae, whereas the metabolites 5-HIAA as increased. Interestingly, RT-qPCR analysis of whole embryo lysates did not show a difference in *mao* and *comta* expression in *vmat2*$^{-/-}$ larvae when compared to *vmat2*$^{+/+}$ siblings.

We also found alterations in the histaminergic system, where *vmat2*$^{-/-}$ larvae showed a reduction in the number of histamine immunoreactive cell bodies and fibers in the ascending, descending and commissural fiber network in the telencephalon. HPLC analysis revealed that histamine levels were significantly reduced *vmat2*$^{-/-}$ larvae when compared to *vmat2*$^{+/+}$ siblings. Similarly to *th1*, *th2* and *tph1a*, the number of *hdc* cells (encoding histidine decarboxylase) mRNA-positive was significantly increased in *vmat2*$^{-/-}$ larval brains relative to *vmat2*$^{+/+}$ larval brains. That could indicate the existence of a feedback mechanism meant to maintain histamine levels. Interestingly, despite the reduction of histamine levels in *vmat2*$^{-/-}$ larvae, the expression of the histamine receptor *hrh1* was unaltered in these mutants. *Vmat2*$^{-/-}$ larvae died before reaching 20 dpf.

8.4 Absence of monoamine breakdown is damaging to zebrafish neurotransmitters systems (III)

Mao-loss of function in zebrafish leads to early death with fish not being able to reach the juvenile stage of development. This is most likely caused by a toxic effect caused by exacerbated levels of serotonin, one of the main substrates for Mao in the zebrafish, which affected other aminergic neurotransmitter systems. A reduction of 50% in *mao* expression in heterozygous did not cause major alterations in aminergic systems and fish were able to develop until adulthood.

We verified that both *mao*$^{-/-}$ and *mao*$^{+/-}$ larvae displayed a stronger serotonin immunoreactivity in their brains when compared with *mao*$^{+/+}$ siblings. This result possibly reflects increased levels of serotonin in these genotypes, a hypothesis supported by previous reports of zebrafish treated with MAO inhibitors displaying increased levels of serotonin after HPLC analysis. The immunostaining revealed that the stronger serotonin immunoreactivity in *mao*$^{-/-}$ fish brains is against extracellular serotonin and fibers. When we assessed the numbers of serotonergic cells in different subpopulations (PVOa, PVOi and PVOp), *mao*$^{-/-}$ brains showed a significant reduction in the number of these cells when compared with both *mao*$^{+/+}$ and *mao*$^{+/-}$ brains. The excess of serotonin in the extracellular space seemed to be taken up by cells which are unlikely to be serotonergic. *Mao*$^{+/-}$ brains had increased number of serotonergic cells in the PVOi when compared to *mao*$^{+/+}$ and *mao*$^{-/-}$ brains, but no differences between *mao*$^{+/+}$ and *mao*$^{+/-}$ brains were detected when PVOa and PVOp were evaluated. *Mao*$^{-/-}$ larvae also presented an upregulation of *tph1a* when compared to *mao*$^{+/+}$ and *mao*$^{+/-}$ siblings.

The dopaminergic and histaminergic systems of *mao*$^{-/-}$ larvae were also affected, as we detected significant reduction in the number of Th1 immunoreactive cells in cell group 10 (Sallinen et al., 2009a) of posterior hypothalamus and histamine immunoreactive cells in the brains of these mutants when compared to their *mao*$^{+/+}$ and *mao*$^{+/-}$ siblings. However, no differences between *mao*$^{+/+}$, *mao*$^{+/-}$ and *mao*$^{-/-}$ genotypes were detected when *th1* and *hdc* expression was analyzed in whole larvae lysates by RT-qPCR. *Mao*$^{-/-}$ larvae died before reaching 20 dpf.

We evaluated the expression of some essential genes relevant for aminergic neurotransmission in samples of adult *mao*$^{+/+}$ and *mao*$^{+/-}$ fish. A significant increase in *vmat2* expression was detected in the brains of *mao*$^{+/-}$ fish by RT-qPCR. Since a hyperserotonergic phenotype is expected in these animals we also evaluated the expression of *serta*, but both RT-qPCR and *in situ* hybridization showed no differences between genotypes.

8.5 Disrupted aminergic neurotransmission affects brain development in zebrafish (II, III)

We have verified that genes relevant for brain development are altered in zebrafish mutants that have impaired aminergic neurotransmission due to either compromised transport of biogenic amines into synaptic vesicles or to absence of the enzyme that catalyzes the oxidation of monoamines. After *in situ* hybridization we verified that brains of both *vmat2*$^{-/-}$ and *mao*$^{-/-}$ larvae presented a clearly weaker expression of the early neuronal marker *notch1a* in comparison with their wild-type siblings. RT-qPCR analysis confirmed that *notch1a* expression was significantly downregulated in both *vmat2*$^{-/-}$ and mao$^{-/-}$ larvae. *Mao*$^{-/-}$ larvae also presented downregulation of *neurod1*, a transcription factor that is critical for survival and maturation of newborn neurons. *Vmat2*$^{-/-}$ larvae expressed higher levels of *mesencephalic astrocyte-derived neurotrophic factor* (*manf*), which encodes a protein that has neuroprotective properties in apoptotic conditions, whereas the microglial marker *apolipoprotein Eb* (*apoeb*) was upregulated in *mao*$^{-/-}$ larvae, raising the possibility of increased apoptosis in both mutants. Both mutants died before reaching 20 dpf.

8.6 Vmat2 loss-of-function increases the heart rate and disrupts zebrafish behavior (II)

The significant decrease in amine levels detected in *vmat2*$^{-/-}$ larvae is likely to have affected peripheral organs, since *vmat2*$^{-/-}$ larvae displayed an increase in the heart rate when compared with *vmat2*$^{+/+}$ siblings. It has been reported that zebrafish *vmat2* is present in arch-associated neurons rostral to the heart.

We tracked the locomotor activity of *vmat2*$^{+/+}$ and *vmat2*$^{-/-}$ larvae at 6 dpf for 24 h and we verified that *vmat2*$^{-/-}$ larvae were hypoactive relative to their *vmat2*$^{+/+}$ siblings during day time. The dark-flash response was also evaluated in a trial that consisted of 10 min of basic locomotor activity evaluation followed by alternating 2 min periods of sudden darkness and light, with three periods of darkness in total. *Vmat2*$^{-/-}$ larvae showed a greater increase in locomotion activity after sudden darkness when compared to *vmat2*$^{+/+}$ siblings.

8.7 Mao deficiency leads to anxiety-like traits in adult zebrafish (III)

We evaluated the locomotor activity of *mao*$^{+/+}$ and *mao*$^{+/-}$ adult fish that were placed individually in a cylindrical tank for 10 min. The total distance moved during the trial did not significantly differ between genotypes (n=7 for each genotype; $p > 0.05$). Thigmotaxis was also assessed, as we virtually divided the arena in zones (border and center) and quantified the time spent

in each zone of the arena. No differences between genotypes was detected and both *mao*$^{+/+}$ and *mao*$^{+/-}$ preferred to stay longer in the zone corresponding to the border of the arena (n=7 for each genotype; $p < 0.01$).

The novel tank test is used to assess traits of anxiety in zebrafish. The innate tendency of the zebrafish to swim to the bottom of a novel environment is usually interpreted as a precautionary antipredator response, which is gradually replaced by a more exploratory behavior after alleviation of anxiety. We digitally divided the novel tank diving area into three zones: bottom, middle and top of the tank. Compared with their *mao*$^{+/+}$ siblings, *mao*$^{+/-}$ adult fish spent similar time exploring the top zone (n=8 for each genotype; $p > 0.05$). However, they spent less time in the middle zone (n=8 for each genotype; $p < 0.05$) and longer time in the bottom of the tank (n=8 for each genotype; $p < 0.05$).

8.8 Dark-flash and startle responses are affected in *mao* mutants and heterozygous larval fish (III)

At 6 dpf *mao*$^{-/-}$ larvae displayed a hypoactive phenotype in comparison to their *mao*$^{+/+}$ and *mao*$^{+/-}$ siblings when their locomotor activity was tracked for 24h (n=16 for *mao*$^{+/+}$, n=16 for *mao*$^{+/-}$ and n=15 for *mao*$^{-/-}$, two-way ANOVA, genotype effect $F(2, 1056) = 352.6$, $p <0.01$. Tukey's post hoc test significances indicated in the graph). At the same age, a similar hypoactive phenotype was also displayed by these mutants in comparison to their *mao*$^{+/+}$ and *mao*$^{+/-}$ siblings when we evaluated their dark-flash response (n=15 *mao*$^{+/+}$ and n=16 for *mao*$^{+/-}$ and *mao*$^{-/-}$; two-way ANOVA, genotype effect $F(2, 1716) = 174.3$, $p < 0.01$. Tukey's multiple comparisons test significances indicated in the graph) during a trial that consisted of 10 min of basic locomotor activity evaluation followed by alternating 2 min periods of sudden darkness and light, with three periods of darkness in total. *Mao*$^{+/+}$ and *mao*$^{+/-}$ larvae displayed similar pattern of locomotor activity in an analysis of 30 s bins. When the larval response to sudden darkness was analyzed in 1 s bins, *mao*$^{+/-}$ larvae showed a significantly stronger reaction than *mao*$^{+/+}$ and *mao*$^{-/-}$ siblings in the first dark-flash. Additionally, compared with *mao*$^{+/+}$ larvae, *mao*$^{+/-}$ larvae showed a longer adaptation period and slower return to baseline activity (two-way ANOVA, genotype effect $F(2, 704) = 30.4$, $p < 0.01$. Tukey's post hoc test significances indicated in the graph). During the following stimuli, *mao*$^{+/+}$ and *mao*$^{+/-}$ larvae showed a similar pattern of locomotor activity, with both genotypes responding significantly stronger than *mao*$^{-/-}$ larvae at the onset of darkness in the second (two-way ANOVA, genotype effect $F(2, 704) = 49.89$, $p < 0.01$. Tukey's post hoc test significances indicated in the graph) and third flashes (two-way ANOVA,

genotype effect F(2, 704) = 41.44, p < 0.01. Tukey's post hoc test significances indicated in the graph).

The startle response was evaluated through the acoustic/vibrational test (at 6 dpf) and we verified that the response after the first stimulus did not differ between *mao*$^{+/+}$, *mao*$^{+/-}$ and *mao*$^{-/-}$ larvae (n=15 *mao*$^{+/+}$, n=16 for *mao*$^{+/-}$ and n= 12 for *mao*$^{-/-}$; two-way ANOVA, genotype effect F(2, 400) = 26.96, p < 0.01. Tukey's post hoc test significances indicated in the graph). After the second, third, and fourth stimuli, *mao*$^{+/-}$ larvae showed a significantly increased startle response compared with their siblings, indicating impaired sensory adaptation and habituation.

Overall, it seemed that *mao*$^{-/-}$ larvae showed a faster habituation to repeated exposure of the acoustic/vibrational stimuli at 20 s ISI, while their siblings did less so. However, it should be considered that these mutants showed impairments in different neurotransmitter systems and altered expression of genes relevant to brain development. Thus, they might have displayed a weaker response to stimuli because a general arrested development.

8.9 ASD-associated risk factors affect the social behavior of zebrafish (I, III)

The social preference of adult zebrafish that were embryonically exposed to VPA, a known ASD risk factor for humans, was evaluated in an apparatus where the testing fish had visual access to a compartment containing a group of conspecifics and to a compartment without conspecifics containing only stones and plants imitation. In comparison with control animals, VPA-exposed fish spent less time in the zone closer to the compartment with the conspecifics. In addition, VPA-exposed fish stayed longer time than the control group in the area closer to the compartment without conspecifics.

Based on human and rodent evidence suggesting an association between *MAOA* and ASD (Bortolato et al., 2011; Cohen et al., 2003; Gu et al., 2017), we aimed to verify if *mao*$^{+/-}$ adult zebrafish show autistic-like social deficits. These animals presented decreased mao activity and 50% reduction in the expression of *mao* mRNA when compared to *mao*$^{+/+}$ siblings. We started by assessing the social interaction of 1-month-old *mao*$^{+/+}$ and *mao*$^{+/-}$ fish. A pair of fish of the same genotype was placed in an arena for 6 min and we verified that *mao*$^{+/-}$ fish were less frequently in proximity (at a distance ≤ 0.8 cm) to each other (n=8 pairs for each genotype; $p < 0.05$). However, *mao*$^{+/+}$ and *mao*$^{+/-}$ fish spent similar time in proximity when compared (n=8 pairs for each genotype; $p > 0.05$). Additionally, we evaluated the frequency of direct contact between the fishes in the arena, but no statistically

significant difference was detected when genotypes were compared (n=8 pairs for each genotype; $p > 0.05$).

The shoaling behavior of juvenile *mao*$^{+/+}$ and *mao*$^{+/-}$ fish when a group of 4 fish of the same was placed in a cylindrical plastic container and tracked for 10 min. In shoals formed by *mao*$^{+/-}$ fish the time individuals spent in proximity was significantly shorter when compared with shoals formed by *mao*$^{+/+}$ siblings (n = 4 trials for each genotype; $p < 0.05$). Furthermore, the average interindividual distance was significantly greater in the *mao*$^{+/-}$ fish group than *mao*$^{+/+}$ sibling group (n = 4 trials for each genotype; $p < 0.05$).

Finally, the social preference of adult fish was tested. Compared with their *mao*$^{+/+}$ siblings, *mao*$^{+/-}$ adult fish did not differ statistically in the time spent in the zone closer to the compartment with conspecifics (n=8 for each genotype; $p > 0.05$) and spent less time in the area closer to the compartment without conspecifics (n=8 for each genotype; $p < 0.05$). However, *mao*$^{+/-}$ adult fish preferred to stay longer in the distal area of the apparatus (n=8 for each genotype; $p < 0.05$). When we compared the time spent in the area closer to conspecifics by the *mao*$^{+/+}$ fish with the time spent in both distal area and area closer to a compartment without conspecifics summed, these animals showed a preference for the area closer to the social stimulus detected (n=8 for each genotype; $p < 0.05$), whereas when the same comparison was made with their *mao*$^{+/-}$ siblings, no significant difference was detected.

We studied if *mao*$^{+/-}$ zebrafish presented abnormal expression of some genes that are linked to social behavior and ASD. We measured the expression of *SH3 and multiple ankyrin repeat domains 3* (*shank3b*) and *methyl CpG binding protein 2* (*mecp2*) because of the extensive literature associating these genes with ASD. Additionally, we also assessed the levels of *hrh3* because of its roles in anxiety and cognition, and findings suggesting that this receptor could be a potential therapeutic target for ASD. However, no differences between *mao*$^{+/+}$ and *mao*$^{+/-}$ zebrafish were detected for the expression of these transcripts (n=4 for each genotype; $p > 0.05$). As expected, the *mao*$^{+/-}$ brains displayed a significantly reduced level of *mao* mRNA compared with the brains of *mao*$^{+/+}$ siblings (n=4 for each genotype; $p < 0.01$).

9 Discussion

9.1 Zebrafish as a model organism to study ASD

One of the goals of this thesis was to verify if zebrafish exposed to different ASD-associated risk factors would display ASD-like behavioral and neurobiological phenotypes. The purpose was to provide new tools that, for example, could be used in high-throughput screening and drug testing, allowing the identification of new chemicals (or drugs) that could prevent or restore a specific phenotype associated with the disorder. Using distinct risk factors to induce ASD-like features is relevant because this disorder is phenotypically heterogeneous, making it difficult to determine the exact etiology and pathophysiology underlying the core symptoms, which can be accompanied by comorbidities such as sleep disturbs, epilepsy, and sensorimotor impairments (Accardo and Malow, 2015). Several animal models have been developed over the years to study ASD and, depending on the etiologic condition used to model the disorder, a different set of behavioral and neurobiological phenotypes is observed (Bambini-Junior et al., 2014b; Hori et al., 2020; Patterson, 2011b). Although it is puzzling to unify and determine common mechanisms and pathways displayed by these different models, they might help us to dissect different subgroups of molecular and neurobiological manifestations of ASD.

Our initial approach was to expose zebrafish embryos to VPA, an ASD-associated drug. VPA exposure has been used before to model ASD in several studies with rodents (Bambini-Junior et al., 2011; Bambini-Junior et al., 2014a; Baronio et al., 2015) and a few with zebrafish (Joseph et al., 2021; Zimmermann et al., 2015; Zimmermann et al., 2017), based on clinical data demonstrating that VPA exposure during the first trimester of pregnancy is associated with higher incidence of ASD in the offspring (Christensen et al., 2013). When such approach is used in rodents, a single VPA intraperitoneal injection is most commonly given to pregnant rats and mice in a time window that varies from gestational day 11 to 12.5 (Bambini-Junior et al., 2014a). This time window is chosen because it was reported that several patients with ASD were exposed *in utero* to thalidomide, another ASD risk factor, between gestational days 20 and 24 (Strömland et al., 1994). During this time period in humans the neural tube closes, the corresponding gestational period in rats is day 11.5. In zebrafish the neurulation process happens between 10 and 24 hpf (Compagnon and Heisenberg, 2013) and that was the time window of 25 µM VPA exposure chosen by us. Other groups have chosen to use different doses of VPA and periods of exposure, including VPA exposure during the first 48 h of life and exposure beginning at 8 hpf ending at 4.5 dpf (Chen et al., 2018; Zimmermann et al., 2015). Researchers aiming to model ASD-like symptoms in zebrafish using VPA should attempt to

standardize the exposure protocol because that would facilitate interpretation of the results and allow comparisons.

It is important to mention that the concentration of VPA used to induce ASD-like features in both rodent and fish models is much higher than the blood concentration of VPA in clinical practice. In rodent models, VPA doses ranged between 300 and 800 mg/kg, while most of the studies used the amount of 600 mg/kg. This dose is often used because generates a significant increase in autistic-like behaviors in a battery of tests in comparison with lower doses (160–100 mg/kg) that have been tested in previous studies (Ergaz et al., 2016). In clinical populations the dose of VPA administered is 200–3600 mg/day, equivalent to 3–55 mg/kg for an average female that weight 65 kg (Favre et al., 2013). The 25 µM VPA exposure in zebrafish embryos was chosen by us after pilot studies where we identified this concentration as optimal, considering that it led to low rates of deformities at 5 dpf and mortality after 10 dpf when compared with higher concentrations.

Since impaired social behavior is one of the core symptoms displayed by ASD patients and animal models of the disorder, we raised the VPA-exposed embryos until adulthood to verify if they would present any behavioral impairments. We evaluated their social preference by testing them in an apparatus that resembles the three-chamber social box commonly used in studies with rodents. Similarly with other studies that have exposed zebrafish embryos or larvae, we detected a deficit in sociability on adult zebrafish embryonically exposed to VPA. Differently from mammals, zebrafish development occurs outside an intrauterine environment, not allowing us to fully recapitulate the conditions involving VPA exposure and ASD development. On the other hand, zebrafish and mammals often present similar concordance in developmental toxicity (Nishimura et al., 2016). Additionally, exposing zebrafish to different drugs can be done in more controlled conditions.

The other model studied in this thesis was the zebrafish *mao* mutant line. A lower MAOA activity has been reported in brains of children with ASD when compared with age-matched controls (Gu et al., 2017). The reason for that is not fully understood, but it might be associated with polymorphism of the *MAOA* gene promoter and mutation of the *MAOA* gene. A 30bas-pair repeat polymorphism (uVNTR) within the *MAOA* promoter region is associated with reduced transcription and, therefore, reduced activity of MAOA (Davis et al., 2008). We decided study *mao*$^{+/-}$ juvenile and adult fish, which presented a 50% reduction in *mao* expression accompanied by reduced mao enzymatic activity detected in a histochemical assay. Our *mao*$^{-/-}$ larvae presented a severe phenotype featured by abnormalities in brain development and neurotransmitter systems and died before reaching the age where social behavior could be evaluated. Additionally, a total congenital loss-of-

function of MAO is rare and has been reported in patients with Brunner syndrome, which display violent and aggressive behavior (Brunner et al., 1993).

Differently from mammals, zebrafish possess only one form of MAO, which resembles mammalian MAOA and MAOB but also shows distinct characteristics that differ from both mammalian enzymes (Anichtchik et al., 2006). Therefore, the structure, activity and function of the zebrafish mao should be taken into consideration when using this model system (Anichtchik et al., 2006; Sallinen et al., 2009b). Interestingly, the zebrafish mao has a preference for serotonin as a substrate and does not seem to have a substantial role on dopamine metabolism, making it a potential tool to study the effects of abnormal levels of serotonin during development (Chugani et al., 1999).

Impairments in the serotonergic system have been described in patients with ASD, including hyperserotonemia, decreased MAO activity and reports of decreased and increased expression of the SERT (Brune et al., 2006; Mulder et al., 2005). Additionally, increased levels of serotonin were detected in the brains of rats prenatally exposed to VPA (Narita et al., 2002). It is likely that the reduced expression of *mao* and weaker signal of mao enzymatic activity detected on the brains of *mao*$^{+/-}$ fish will lead to increased levels of serotonin. Additionally, they also presented an upregulation of *vmat2*, which could be a compensatory mechanism caused by increased levels of aminergic neurotransmitters. Similar upregulation of *Vmat2* was detected in the raphe nuclei, the primary location in the brain for the production of serotonin, of rats that were treated with an MAO inhibitor from gestational day 12 until postnatal day 21 (Blažević and Hranilović, 2013). Increased levels of VMAT2 were found in the brains of patients with schizophrenia (Zubieta et al., 2001), a disorder that shares symptomatology with ASD (Trevisan et al., 2020). No differences in the expression of *serta* were detected in the brains of *mao*$^{+/-}$ fish.

The social behavior of *mao*$^{+/-}$ fish was firstly evaluated at the juvenile stage, when we utilized a paradigm that assesses social contact trough different parameters, such as frequency in proximity and time spent in proximity. Differently from *mao*$^{+/+}$ siblings, *mao*$^{+/-}$ fish were less frequently in proximity with a fish of the same genotype during the trial. We also tested the shoaling behavior by placing groups of 4 fish of the same genotype in a round tank. After the groups of 4 fish swum freely in a 10 min trial, we verified that *mao*$^{+/-}$ fish spent less time in proximity and that the average distance between individuals during the trial was higher when compared with trials containing groups of *mao*$^{+/+}$ fish. Using the same apparatus where we tested the social preference of VPA-exposed animals we verified that *mao*$^{+/-}$ did not show a preference for a social stimulus. *MaoA* KO mice also display an impaired social behavior in addition to perseverative and stereotypical responses (Bortolato et al., 2013). However, they also displays a severe phenotype that includes high levels of serotonin and

noradrenaline and marked reactive aggression, resembling the features of patients with Brunner syndrome (Bortolato et al., 2018). Thus, *mao*$^{+/-}$ fish could be considered as a tool to study a more subtle phenotype shown by individuals with low MAOA activity. In addition to impaired sociability, *mao*$^{+/-}$ fish showed an anxious-like behavior in the novel tank diving test, resembling a phenotype displayed by a subset of patients with ASD (Vasa et al., 2014). *MaoA* KO mice and *MaoB* KO mouse do not display anxious- like behavior in the elevated plus maze (Cases et al., 1995; Grimsby et al., 1997). However, mice lacking both *MaoA* and *MaoB* show reduced exploration in the open-field test, and smaller number of entries into both the open and closed arms of the plus maze (Chen et al., 2004).

The two models presented in this thesis have the potential to be useful tools in ASD research, however, they need further validation. The criteria for a well-established animal model includes: (a) face validity, ie, resemblance to the defining characteristics of the human disorder; (b) construct validity, ie, the biological abnormality that leads to the human disorder, such as a gene mutation or exposure to risk factors; and (c) predictive validity, ie, responsiveness to drugs used to treat symptoms in the human disorder (Crawley, 2012). Future studies are necessary to evaluate other behaviors and neurobiological phenotypes of these models that are relevant for ASD, including stereotypies, aggression, attention, synaptic formation, inhibitory/excitatory balance and synaptic proteins defects. Additionally, it should be verified how they respond to drugs such as haloperidol and risperidone, which are used to treat ASD symptoms and have been proved to be effective in treating other zebrafish models of brain disorders (Banono et al., 2021).

It is noteworthy that the study of molecular underpinnings of ASD can benefit from approaches that not always recapitulate the wide range of alterations displayed by patients, but rather allow the investigation of specific associated symptoms and the diverse set of pathophysiological mechanisms known for playing a role in the disorder. For instance, The X-linked *NLGN3* gene, encoding a postsynaptic cell adhesion molecule, is involved in a nonsyndromic monogenic form of ASD (Quartier et al., 2019), but, even though *Nlgn3* KO mice display reduced ultrasound vocalization and a lack of social novelty preference, they do not show alterations in time spent in social interaction, prepulse inhibition, seizure propensity and repetitive behavior (Radyushkin et al., 2009), features detected in other models of the disorder. Induced pluripotent stem cells (iPSC) are now used to study the cellular phenotype of disorders such as ASD, but in vitro systems do not allow the reproduction of global cellular homeostasis and cell orientation and projections within the distinct cortical layers, which are crucial in the context of brain development (Russo et al., 2019). Nevertheless, these are still important tools.

9.2 The histaminergic system is affected in zebrafish embryonically exposed to VPA

We wanted to verify if VPA-exposed animals presented molecular and/or neurochemical abnormalities that could suggest possible mechanisms that led to the behavioral outcome. We were particularly interested in the histaminergic system, which is less explored than other neurotransmitter systems in ASD studies.

Larval zebrafish exposed to VPA showed a significant reduction in the number of histaminergic neurons and in the levels of histamine when compared to control animals. Different effects of VPA have been reported in the rodent model of ASD. A reduced number of neurons has been reported in different brain areas, including the cerebellum and thalamus (Ingram et al., 2000; Mansour et al., 2021). However, neuronal density in hippocampus was increased in adult rats prenatally exposed to VPA (Edalatmanesh et al., 2013). In postmortem samples of patients with ASD there are significantly fewer neurons in areas such as the amygdala, cerebellum and fusiform gyrus (Schumann and Amaral, 2006; van Kooten et al., 2008; Wegiel et al., 2014). However, brain overgrowth and abnormal excess number of neurons in the prefrontal cortex of patients with ASD has been reported (Courchesne et al., 2011).

It is yet to be determined what caused the reduction in the number of histaminergic neurons in zebrafish. Early exposure to VPA has been reported to decrease cell proliferation in zebrafish brains (Lee et al., 2013). In our study, no differences between VPA-exposed and control larvae were detected when we measured the expression levels of *pcna* and we carried out immunostaining with anti-PCNA antibody on larval brains. Prenatal exposure to VPA has also caused apoptosis in the prefrontal cortex, hippocampus area CA1 and cerebellar cortex of rats (Elnahas et al., 2021).

Besides the reduction in the number of histaminergic cells and histamine levels, the histaminergic system was affected by a downregulation of *hdc*, *hrh1*, *hrh2*, and *hrh3* when compared with control larvae. Some of these abnormalities persisted until adulthood, and VPA-exposed adult animals displayed reduced levels of *hdc* and *hrh3*. Interestingly, no difference between groups was detected when histamine levels were measured. Alterations regarding histaminergic genes have been reported in brain postmortem samples of patients ASD. Histamine receptors and other histamine signalling genes were analyzed as a gene set in the dorsal lateral prefrontal cortex and a significant overexpression was detected (Wright et al., 2017). Patients with fragile X syndrome, which frequently are a diagnosed with ASD, presented a downregulation of *HRH3* in a study that used microarray analysis and peripheral blood as sample (Rosales-Reynoso et al., 2010).

The histaminergic system has been linked to the pathophysiology of different brain disorders and some of them share a symptomatology with ASD, including ADHD (Stevenson et al., 2010), schizophrenia (Iwabuchi et al., 2005) and Gilles de la Tourette syndrome (Castellan Baldan et al., 2014). A role for the histaminergic system in ASD pathophysiology would not be surprising, considering that this system is known to modulate important physiological functions such as sleep-wake cycle, energy and endocrine homeostasis, sensory and motor functions, cognition, and attention, which are all often affected in neuropsychiatric disorders (Panula and Nuutinen, 2013). Particularly, H3R antagonists/inverse agonists are known to affect cortical fast rhythms, which are associated with cognitive processes such as attention, alertness, and leaning (Parmentier et al., 2007). This auto and heteroreceptor regulates the release of histamine and other neurotransmitters, which is stimulated by the treatment with these antagonists/inverse agonists (Hatta et al., 1997).

Another mechanism in which histamine could be involved in the pathophysiology of ASD is neuroinflammation. Patients with ASD exhibit ongoing neuroinflammation in various regions of the brain involving microglial activation (Vargas et al., 2005). Histamine plays a role modulating microglia through activation of histamine receptors expressed in these cells (Barata-Antunes et al., 2017). The VPA rodent model of ASD presents high levels of inflammatory cytokines in the brain and increased number of activated microglia. Both are reduced when these animals are treated with an H3R inverse agonist, which is known to induce the release of histamine among other neurotransmitters (Eissa et al., 2019). Additionally, other studies have used H3R ligands to attenuate behavioral impairments in rodent models of ASD (Baronio et al., 2015; Eissa et al., 2018).

In addition to damages in the histaminergic system, VPA-exposed zebrafish also presented abnormalities in catecholaminergic system. At 5 dpf they showed a reduced number of Th-immunoreactive cells in the preoptic area and downregulation of *th1* when compared to controls. Adult animals continued to present lower levels of *th1* and also displayed decreased levels of *dbh,* the enzyme that catalyzes the conversion of dopamine to noradrenaline. Noradrenaline, a neurotransmitter implicated in many cognitive processes, including attention, learning, memory and decision making, was also reduced in VPA-exposed brains. The noradrenergic system has been implicated in the pathophysiology of ADHD, a disorder that shares symptomatology with ASD, and the selective noradrenaline reuptake inhibitor atomoxetine is used in the treatment of ADHD (Del Campo et al., 2011).

The impairments in aminergic system were associated with altered behavior in VPA-exposed larvae. They were hypoactive while the environment was normally illuminated and, when illumination suddenly ceased, displayed a stronger increase in locomotor activity when compared

with control group. A similar phenotype of altered dark-flash response has been described in other studies where zebrafish were exposed to VPA during early stages of development (Cowden et al., 2012; Muhsen et al., 2021). Altered dark-flash response in zebrafish has been previously associated with a functional impairment in histaminergic neurotransmission (Sundvik et al., 2011). Altered motor behavior has also been reported in the VPA rodent model of ASD, where VPA prenatally exposed animals displayed deficits in motor coordination and gait, as well as signs of hypoactivity (Al Sagheer et al., 2018).

9.3 *mao* loss-of-function affects different aminergic populations in the zebrafish brain

In this thesis we also characterized for the first time a *mao*$^{-/-}$ zebrafish. The majority of investigations involving mao functioning in zebrafish were based on a pharmacological approach where MAO inhibitors were used to assess the neurobehavioral outcomes (Bellot et al., 2021; Jaka et al., 2021; Sallinen et al., 2009b). As mentioned before, this is a valid approach but interpretation of the results should be done with caution because different methods and windows of exposure to MAO inhibitors are used, and these drugs may generate effects independent of Mao inhibition. For instance, deprenyl can be metabolized to amphetamine, which could alter dopamine neurotransmission (Karoum et al., 1982). Thus, characterizing a *mao* mutant could contribute to the existent pharmacological data.

Differently from mammals that have two isoforms of MAO (MAOA and MAOB), zebrafish only has one Mao. The zebrafish *mao* shares similarity in exon/intron numbers and structure to *MAOA* and *MAOB* of humans, and the protein shares the properties of both human MAOA and MAOB. Additionally, the overall distribution of zebrafish mao is a combination of MAOA and MAOB distribution in mammals (Anichtchik et al., 2006). The knockout of *mao* in zebrafish led to severe impairments in the aminergic systems and was lethal within 20 dpf.

The zebrafish Mao shows a strong affinity for serotonin and PEA and a weak one for dopamine and noradrenaline. The *mao*$^{-/-}$ larvae showed a hyperserotonergic phenotype that was characterized by extracellular serotonin immunoreactivity and increased density of serotonin-immunoreactive fibers. However, this exacerbated extracellular serotonin was associated with damage in aminergic systems. When we counted the number of cells in different brain serotonergic groups, we found that *mao*$^{-/-}$ larvae had a reduced number of cells in the anterior, intermediate and posterior part of the paraventricular organ when compared to their *mao*$^{+/+}$ and *mao*$^{+/-}$ siblings. These results resemble the reduced serotonin immunoreactivity in the serotonergic cell somata of WT zebrafish treated with

MAO inhibitor (Sallinen et al., 2009b). Additionally, when this comparison was made after quantifying histamine- and Th1-immunoreactive cells, *mao*$^{-/-}$ brains also showed a significant reduction. A similar toxic effect and neuronal death was detected in zebrafish treated with Trans-2-phenylcyclopropylamine (PCPA), a non-selective MAO inhibitor (Jie et al., 2009). It is likely that this general impairment in aminergic systems contributed to the early death of *mao*$^{-/-}$ larvae. The aminergic systems have an important role in feeding control (Wee et al., 2019). Additionally, aminergic inputs innervate the zebrafish swim bladder and mao inhibition affects its control, which could have impaired the larvae mobility and ability to seek for food (Finney et al., 2006; Sallinen et al., 2009a). A hypoactive phenotype was detected when *mao*$^{-/-}$ larvae were evaluated in a 24h locomotor activity evaluation. A similar behavior was reported when WT zebrafish were treated with a Mao inhibitor (Sallinen et al., 2009b). Additionally, weaker reactivity to acoustic/vibrational and visual stimuli was displayed by mao$^{-/-}$ larvae. This could linked with the hypoactive phenotype and impaired development displayed by the mutants, considering that these responses are mediated by independent processes (Burgess and Granato, 2007a; Burgess and Granato, 2007b).

When we analyzed the expression of genes encoding amine-synthesizing enzymes, we did not detect differences in *hdc* and *th1* expression between *mao*$^{-/-}$ larvae and their *mao*$^{+/+}$ and *mao*$^{+/-}$ siblings. However, RT-qPCR showed that *tph1a* was upregulated in *mao*$^{-/-}$ larvae. Interestingly, *in situ* hybridization showed that *tph1a* signal was markedly weaker in the *mao*$^{-/-}$ brains. It is important to highlight that samples for the RT-qPCR analysis were whole larvae lysates and tph1a is expressed in skin and pharyngeal arch neuroepithelial cells (NECs) and nerves innervating NECs (Pan et al., 2021).

9.4 Aminergic signalling and turnover in zebrafish lacking *vmat2*

Several lines of *Vmat2* mutant mice have been used to study the role of this transporter in aminergic signalling, embryonic development and adult physiology (Fon et al., 1997; Fukui et al., 2007; Wang et al., 1997). VMAT2 has been implicated in the pathophysiology of different diseases, such as Parkinson's disease, depression and addiction (Brighina et al., 2013; Fehr et al., 2013; Zalsman et al., 2011). Thus, the availability of different animal models will benefit studies aiming to better understand the molecular underpinnings of these diseases or the development of new therapies.

The use of zebrafish to complement the knowledge obtained with rodent models is attractive because of the several advantages provided by this model organism already discussed in this thesis. Among them are the genetic homology shared with humans, the embryo transparency during

development and the wide range of genetic tools available to generate mutants (Gerlai, 2011). However, similarly to the investigations involving mao, the studies of vmat2 on zebrafish neurobiology have been mainly limited to exposing these animals in different stages of development to different doses of VMAT2 inhibitors, such as reserpine (Puttonen et al., 2017). Thus, we decided to characterize, for the first time, a *vmat2*$^{-/-}$ zebrafish mutant. We expect that this mutant line will contribute to the existing pharmacological data to a better understanding of aminergic neurotransmission in zebrafish. This is relevant because zebrafish is becoming a popular tool in neuroscience and a well-characterized set of neurobiological mechanisms will strength its value as a model organism.

A particular important aspect to be characterized in this mutant was the role played by Vmat2 on the histaminergic system. VMAT2 affinity for histamine and its expression on neuronal and endocrine cells containing HDC have been reported (Merickel and Edwards, 1995; Weihe and Eiden, 2000). Although VMAT2 inhibitor reserpine decreases the levels of histamine on cat and mouse brains, studies with rats showed that histamine levels were unaffected. We showed that zebrafish lacking *vmat2* had reduced histamine immunoreactivity in neuronal cell bodies located in the hypothalamus and their projections to the dorsal telencephalon and habenula. Additionally, we have also verified a decrease in histamine levels by HPLC. Our data is comparable with a previous report where WT zebrafish were exposed to reserpine (Puttonen et al., 2017). The increased number of cells expressing *hdc* mRNA detected in *vmat2*$^{-/-}$ brains imply the existence of a compensatory mechanism to maintain histamine levels. Increase in HDC activity has been reported in the rat brain after treatment with reserpine.

Differently from other systems where the lack of *vmat2* caused a virtual absence of immunoreactivity to aminergic neurotransmitters, in the histaminergic system we found the presence of immunoreactivity to histamine in a small group of neurons. That could indicate the existence of a still uncharacterized mechanism of transport specific for histamine present in a subset of histaminergic neurons. Thus, it would be relevant to determine the extent to which histaminergic neurons are homogenous regarding metabolism and histamine synthesis or consist of distinct subpopulations.

Increased levels of DOPAC, reduced levels of dopamine and the presence of only few scattered dopaminergic neurons indicate an accelerated dopamine turnover in *vmat2*$^{-/-}$ brains. These changes were accompanied by an upregulation of the two tyrosine hydroxylase genes present in the zebrafish (*th1* and *th2*). Similarly, *Vmat2* KO mice show enhanced TH enzymatic activity and increased accumulation of levodopa (Wang et al., 1997). Parkinson's disease patients, which show an impaired

dopamine uptake per VMAT2, also display upregulated *TH* expression in the ventral tegmental area (Tong et al., 2000). It has been proposed that impaired dopamine storage in vesicles by a malfunctioning VMAT2 may be an early critical abnormality promoting degenerative death pathways, through dopamine-derived cytotoxic reactive species, underlying the progressive death of nigrostriatal dopaminergic neurons in this disorder (Pifl et al., 2014).

As expected, the serotonergic system also presented abnormalities. The *vmat2*$^{-/-}$ brains presented virtually no immunoreactivity to serotonin and the levels of this neurotransmitter were significantly reduced on whole larvae lysates. Additionally, the metabolite 5-HIAA was significantly increased. Like the other amine-synthesizing enzymes evaluated in this study, *tph1a* was upregulated. This could indicate an increased rate of amines synthesis, which has been demonstrated in *Vmat2* KO mice (Wang et al., 1997).

The impairments in aminergic systems also affected the behavior of *vmat2*$^{-/-}$, which displayed an abnormally stronger dark-flash response. This behavioral phenotype is comparable to what was detected in VPA-exposed and *mao*$^{-/-}$ larvae, reinforcing the evidence that suggest a role for amines in the regulation of this behavior. Similarly with what happens with *Vmat2* KO mice, *vmat2*$^{-/-}$ fish do not do not survive until adulthood and die within 20 dpf.

This new fish line might be of importance in future investigations of basic mechanisms of aminergic neurotransmission, and to screen potential new drugs or model brain disorders where VMAT2 plays a critical role. Importantly, we brought more information about the role of VMAT2 on histamine packaging and storage, which has received less attention in comparison with the other monoaminergic systems in studies involving this transporter.

9.5 Impaired aminergic neurotransmission affects zebrafish brain development

We verified that zebrafish with impaired aminergic neurotransmission caused by either the lack of capacity to transport into synaptic vesicles or to metabolize aminergic neurotransmitters presented abnormal expression of different genes relevant to brain development. That is not surprising, considering that early brain development is modulated by biogenic amines, and other molecules, such as growth factors and neuropeptides.

The serotonergic system has important roles during development and serotonin functions as a trophic factor that regulates neuronal growth, differentiation, migration and survival before synapse formation (Brüning et al., 1997; Whitaker-Azmitia et al., 1996). Inhibition TPH during development leads to a delay in the onset of differentiation of neurons developing along the serotonergic pathway,

including mesencephalic dopaminergic neurons and serotonergic neurons of the raphe (Whitaker-Azmitia et al., 1996). Excess serotonin reduces migration distance of embryonic interneurons in cortical slice cultures (Riccio et al., 2011). Histamine also has important roles during development. It has been shown to regulate the proliferation and differentiation of neural stem cells *in vitro* through activation of hrh2 and hrh1, respectively (Panula et al., 2014). Prenatal dopamine depletion in the substantia nigra by treatment with 6-hydroxydopamine decreases the number of cortical GABAergic interneurons (Ohira, 2019). Cortical GABAergic circuits are immature at birth and, in adolescence, dopamine is known to modulate the maturation of the GABAergic system, including the expression of GABA synthesizing enzymes and transporters, formation of GABAergic synapses, and the projection pattern of GABAergic axons (Kilb, 2012). Proper functioning of GABAergic inhibitory properties is necessary for the refinement of cortical circuitry. Thus, it is likely that impairments in GABAergic maturation caused by dopamine depletion during development lead to mental illness (Nakazawa et al., 2012). Similar effects are also detected when dopamine is increases during development by exposure to cocaine, which leads to disrupted development of cortical neurons (Crandall et al., 2004).

The release of these amines is VMAT2-dependent; thus, a malfunctioning or inhibition of this transporter is likely to affect brain development. We examined zebrafish *vmat2*$^{-/-}$ larvae, which presented a marked depletion of serotonin, dopamine and histamine. These larvae exhibited downregulation of *notch1a*, a gene known to regulate neuronal differentiation. Notch1 signalling plays a role on the maintenance of neuronal precursor cells in an undifferentiated state, while its suppression induces differentiation of neuronal precursors. Additionally, they also showed a weaker expression of *pax2a*, an early marker of dopaminergic neurons. Dopaminergic neurons are derived from progenitors that express PAX2 and eventually become positive for TH. Interestingly, VMAT2 inhibition stimulates β-cell differentiation *in vitro* (Sakano et al., 2014). The authors proposed that monoamines serve as a brake for differentiation of Pdx1+ pancreatic progenitors into Ngn3+ endocrine precursors and subsequently into Ins+ cells. Once amine release is inhibited by reserpine, the Pdx1+ cells are induced to differentiate into Ngn3+ cells, which quickly turn into Ins+ cells. However, in the brain, VMAT2 is known to have a protective role on neurons and the inhibition or lack of this transporter may cause the accumulation of neurotransmitters and their metabolites in the cytosol which can lead to toxic effects. Zebrafish *vmat2*$^{-/-}$ larvae also showed increased levels of *manf*, which regulates dopaminergic developments and pancreatic differentiation. However, MANF has neuroprotective properties against apoptosis (Mätlik et al., 2018). *Vmat2* KO mice display increased programmed cell death (PCD) in specific cortical areas, whereas a reduced level of amines

caused by synthesis inhibition did not cause visible consequences on PCD. Interestingly, *Vmat2-MaoA* DKO mice, which presented high levels of serotonin, did not show increased cell death as displayed by *Vmat2* KO animals (Stankovski et al., 2007). Increased neurogenesis has been reported in neurodegenerative disorders (Butti et al., 2014; Jin et al., 2004; Shruster et al., 2010). Thus, it is possible to suggest that the loss of Vmat2 leads to neurodegeneration which in turn would stimulate differentiation of neural progenitor cells (NPCs) and hence the reduction in expression of early developmental markers *notch1a* and *pax2a*. However, the fact that the fish examined in this study were still immature makes this possibility questionable. Further investigation is needed to determine if the altered expression of genes relevant for brain development in *vmat2*$^{-/-}$ larvae indicates a general arrested development or if reduced *notch1a* and *pax2a* levels imply that Vmat2-dependent monoaminergic signalling suppresses neuronal differentiation.

Differently from *vmat2* mutants, *mao*$^{-/-}$ larvae are characterized by an accumulation of amines caused by a lack of Mao enzymatic activity. Excess of serotonin, which is the preferred Mao substrate in zebrafish, led to abnormal expression of genes linked to brain development in these mutants. Similarly to *vmat2*$^{-/-}$ larvae, they showed reduced levels of *notch1a*. In this context, we can assume that this may be related to a general impaired brain development because these larvae also showed a downregulation of *neurod1*, a transcription factor that is important for survival and maturation of newborn neurons. They also displayed increased levels of *apoeb* a microglial marker that is commonly associated to apoptosis (Elliott et al., 2007). This result is consistent with the reduced number of histaminergic and Th1-positive neurons detected in these mutants. In fact, the exacerbated levels of serotonin was associated with the loss of serotonin immunoreactive cells in the anterior, intermediate and posterior part of the paraventricular organ. Mao inhibition in zebrafish through PCPA exposure leads to increased neuronal death (Jie et al., 2009).

10 Conclusions

This study characterized behavioral and neurochemical phenotypes of zebrafish with pharmacologically and genetically manipulated with ASD risk factors. Additionally, it characterized the effects of *mao* and *vmat2* loss of function on zebrafish brain development, behavior and aminergic neurotransmitter systems.

Zebrafish embryos exposed to VPA, an ASD-associated drug, displayed molecular and neurochemical alterations that persevered until adulthood and were associated with an impaired social behavior. Some of these alterations were in the histaminergic system, a neurotransmitter system that

have been seldom studied in ASD but is impaired in different brain disorders that share a symptomatology with ASD. Particularly, *hrh3* a promising targets for cognitive disorders, was downregulated in both larvae and adult zebrafish embryonically exposed to VPA. These findings might bring more attention to a possible involvement of the histaminergic system in the pathophysiology of ASD. Furthermore, it supports the use of zebrafish as a useful model system to investigate mechanisms underlying this disorder.

The lack of *vmat2* affected the expression of genes related to brain development, suggesting that this transporter plays a significant role in this processes, most likely through amines stored by this transporter. We provided new essential information regarding the importance of VMAT2 to the histaminergic system, which has received less attention in comparison to the other aminergic systems in studies involving this transporter. Future studies aiming to investigate basic mechanisms involving this monoamine transporter, screen potential new drugs or model brain disorders where VMAT2 plays a critical role might benefit from this model. Additionally, this mutant line contributes with the existing pharmacological data to a better understanding of aminergic neurotransmission in zebrafish. This is relevant because zebrafish is becoming a popular tool in neuroscience and a well-characterized set of neurobiological mechanisms will strength its value as a model organism.

Zebrafish that had a *mao* complete loss of function displayed abnormal expression of relevant genes for brain development, severe impairments in brain aminergic neurotransmitter systems and died at early age. Considering the influence of brain amines on neurogenesis, *mao*$^{-/-}$ fish could be used as a tool to study roles of *MAOA/B* and amines during brain development. Zebrafish with a reduced expression of *mao* survived until adulthood and showed a significant deficit in sociability and displayed anxious-like behavior. It is important to mention that these behavioral impairments were mild, but this model organism has the potential to be used in ASD studies aiming to evaluate neurobehavioral outcomes of interaction between environmental factors and *MAOA/B* genotype.

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