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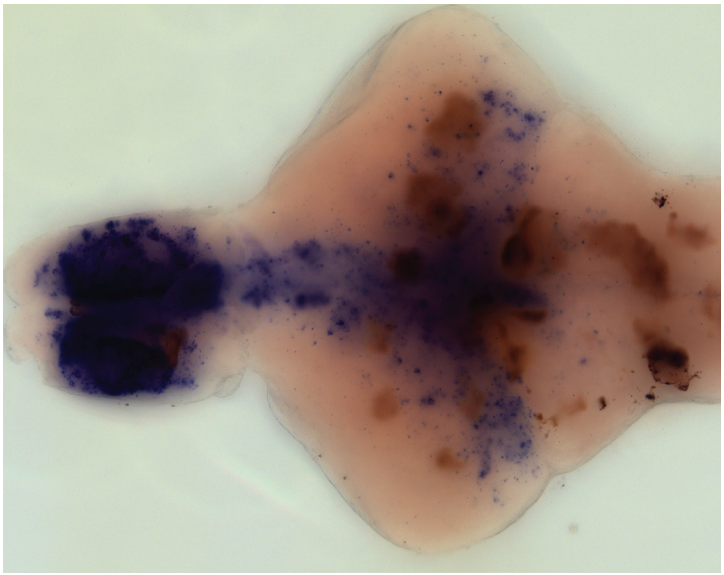
HENRI A. J. PUTTONEN

Neuropharmacological Properties of the Histaminergic System in the Zebrafish



DEPARTMENT OF ANATOMY
FACULTY OF MEDICINE AND
NEUROSCIENCE CENTER
DOCTORAL PROGRAMME IN BIOMEDICINE
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Neuropharmacological Properties of the Histaminergic System in the Zebrafish

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Academic dissertation

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Original publications

I

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Puttonen HAJ, Sundvik M, Rozov S, Chen YC, Panula P.

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II

Storage of neural histamine and histaminergic neurotransmission is VMAT2 dependent in the zebrafish

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III

Knockout of histamine receptor H3 alters adaptation to sudden darkness and monoamine levels in the zebrafish

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In addition, some unpublished results are displayed. No publications have been included in other dissertations. In the text, the original publications are referred to by roman numerals (I–III). Publications I and II are reproduced in accordance with the Creative Commons Attribution License. Publication III is reproduced with permission from John Wiley and Sons (license number 4225010132956).

Index of abbreviations

5-HT 5-hydroxytryptamine, serotonin
AADC aromatic L-amino acid decarboxylase
AMP adenosine monophosphate
ANOVA analysis of variance
BSA bovine serum albumin
CaMKII calcium-calmodulin dependent protein kinase II
cAMP cyclic adenosine monophosphate
COMT catechol-*O*-methyltransferase
DRD1 dopamine receptor D1
DRD2 dopamine receptor D2
DAO diamine oxidase
DAT dopamine transporter
DBH dopamine beta-hydroxylase
L-DOPA L-3,4-dihydroxyphenylalanine
DOPAC dihydroxyphenylacetic acid
dpf days post fertilization
ENU N-ethyl-N-nitrosurea
FAM 5-(and-6)-carboxyfluorescein
GABA gamma-aminobutyric acid
GPCR G-protein coupled receptor
HRH1 histamine receptor H1
HRH2 histamine receptor H2
HRH3 histamine receptor H3
HRH4 histamine receptor H4
HB hybridization buffer
HDC histidine decarboxylase
HNMT histamine N-methyltransferase
hpf hours post fertilization

HPLC high-performance liquid chromatography

HVA homovanillic acid

LC locus coeruleus

MAO monoamine oxidase

NET norepinephrine (=noradrenaline) transporter

NGS normal goat serum

NSS normal sheep serum

OCT2 organic cation transporter 2

OCT3 organic cation transporter 3

PBS phosphate buffered saline

PBSTw phosphate buffered saline with 0.1% Tween-20

PBSTx phosphate buffered saline with 0.3% Triton X-100

PFA paraformaldehyde

PKA protein kinase A

PMAT plasma membrane monoamine transporter

RT room temperature

SERT serotonin transporter

SSC sodium salt citrate

TAMRA 5-(and-6)-carboxytetramethylrhodamine

TH tyrosine hydroxylase

TPH tryptophan hydroxylase

TSA tyramide signal amplification

VMAT1 vesicular monoamine transporter 1

VMAT2 vesicular monoamine transporter 2

VTA ventral tegmental area

Abstract

Biogenic amine neurotransmitters are important modulators of the most basic functions of the central nervous system. The histaminergic system is one of the last biogenic amine systems to be discovered. Its neurons are located in a single nucleus of the hypothalamus, from where they send projections to almost all regions of the brain. Studies on the roles of the histaminergic system have revealed that it regulates many brain functions, such as motor functions, cognition, sleep, and brain plasticity and development. It has also been implicated to be involved in many neurological and psychiatric conditions, such as Parkinson's disease, narcolepsy and alcoholism. Modulation of histamine signalling may thus provide an option for the treatment of these and other conditions. However, due to its relatively recent discovery, many unanswered questions about the histaminergic system remain. In this thesis, I studied the neuropharmacology of the histaminergic system using the zebrafish as a model of the vertebrate brain.

In the first study, we analysed the effect of acute ethanol on the histaminergic and dopaminergic systems. The mRNAs of enzymes synthesizing dopamine and histamine were rapidly upregulated by a short ethanol exposure in a dose-dependent manner. Further immunohistochemical and *in situ* hybridization revealed that the number and localization of histaminergic and dopaminergic neurons was unaltered by ethanol treatment, suggesting that the transcriptional changes observed were localized to the cells that produce histamine and dopamine under normal conditions. When analysing neurotransmitter levels by HPLC, dopamine levels were decreased after a short treatment with ethanol but recovered to baseline levels during longer treatments, suggesting that the transcriptional changes observed represent a compensatory response. Interestingly, no significant changes were seen in histamine levels following ethanol treatment. Ethanol treatment resulted in a rapid and dose-dependent increase in locomotion of larvae, which declined over time with higher treatment doses. As the behavioural and transcriptional changes occurred within a similar time frame, our results suggest that histamine and dopamine may play a role in the locomotor effects of ethanol.

In the second study, we showed that pharmacological inhibition of the vesicular monoamine transporter 2 (VMAT2) using reserpine depleted neural histamine from the brain, resulting in an almost total loss of histamine immunoreactivity in histaminergic fibres and a decreased number of histamine immunoreactive neurons. These results were supported by decreased levels of histamine and other amine neurotransmitters. Interestingly, depletion of histamine did not result in the upregulation of its synthesis, although enzymes synthesizing the other aminergic transmitters were significantly upregulated after VMAT2 inhibition. Behaviourally, VMAT2 inhibition resulted in a decreased dark-induced flash response of zebrafish larvae, a brief spike in locomotor activity during the first second of sudden onset of darkness, which has been described as histamine dependent. However, aside from the dark-flash response, reserpine-treated

larvae showed an increased hypermotor response to sudden darkness, which suggests that monoaminergic transmission regulates darkness adaptation of zebrafish.

In the third study, we characterized the expression of the zebrafish histamine receptor *hrh3* and generated a *hrh3* knockout strain using the CRISPR-Cas9 method of targeted genome editing. Expression of *hrh3* was most prominent in the pallium of the telencephalon, the region containing higher centers of zebrafish brain function, functionally analogous to the mammalian cortex, amygdala and hippocampus. Double fluorescent *in situ* hybridization revealed that the majority of *hrh3* expressing neurons in the telencephalon were glutamatergic, suggesting that *hrh3* regulates glutamatergic neurotransmission in the zebrafish. This is some of the only direct molecular evidence of the transmitter phenotype of *hrh3*-expressing cells. In our knockout model, we saw that *hrh3* knockout larval zebrafish adapted faster to sudden onset of darkness, indicating that *hrh3* can regulate adaptation to sudden changes in the environment of the zebrafish. Although basic behavioural parameters of *hrh3* knockout larvae were unaltered, adult *hrh3* knockout fish showed decreased locomotor activity, suggesting that *hrh3* signalling may have different functional roles at different ages. Neurochemically, zebrafish larvae had decreased levels of dopamine and serotonin, supporting previous findings that *hrh3* is involved in the regulation of these systems. We saw no changes in the expression of genes relevant to aminergic neurotransmission or the organisation of the aminergic networks, suggesting that the altered neurotransmitter levels reflect functional changes in otherwise intact networks.

In total, these results provide some new answers to open questions in the field of histamine neuropharmacology, specifically concerning the effects of alcohol on the histaminergic system and the mechanisms of vesicular storage of histamine. Additionally, we provide the first thorough characterization of a histamine receptor knockout model in a diurnal vertebrate, the zebrafish. In concordance with previous studies in rodents, we found that *hrh3* knockout results in relatively modest phenotypes and might be relevant in the adaptation to sudden changes in the environment. Also, we provided further evidence of the role of *hrh3* in the regulation of other aminergic networks, i.e. the serotonergic and dopaminergic systems. We also noted that *hrh3* is particularly strongly expressed in glutamatergic cells of the telencephalon, suggesting that the role of *hrh3* in the modulation of glutamatergic transmission and its functional significance should be more thoroughly studied.

1. Introduction

The vertebrate brain is the most complex organ, consisting of a complex network of neurons that are connected to each other by synapses. It forms the basis of our consciousness, being responsible for interpretation of sensory information from our surroundings and for execution of behaviour. The complex and robust signalling between neurons required for these functions is made possible by neurotransmitters, small endogenous molecules that are released by neurons in synapses. Released neurotransmitters bind to receptors on the postsynaptic neuron, thus altering its membrane potential, excitability or firing rate. Given the importance of neurotransmitters, identification of the function of specific transmitter molecules and their receptors in the healthy and pathological brain has provided the foundation for modern neuropharmacology.

Of the different neurotransmitter classes, the biogenic amine transmitters dopamine, serotonin and noradrenaline have attracted particular neuropharmacological interest for their importance in many neurological and psychiatric conditions. This has been the result of intensive research into the structural organisation and receptor properties of these transmitter networks both in experimental animals and clinical settings, which has led to successful drug development. For instance, drugs that potentiate dopaminergic transmission form the cornerstone of pharmacological treatment of motor disorders such as Parkinson's disease, and serotonin and noradrenaline reuptake inhibitors are widely used in the treatment of depression.

Histamine is one of the last biogenic amine neurotransmitters to be discovered. It has been implicated to be of importance in the regulation of motor functions, the neurobiology of alcoholism, many psychiatric disorders and cognitive functions, for instance. In many of the previous studies, histamine has been characterized as having primarily a modulatory role, adjusting transmitter release and signalling of other networks. However, the neurobiology of histamine has attracted considerably less research attention than other neurotransmitter systems, and many key questions about fundamental properties of the histamine neurons and histamine receptors still remain to be properly investigated. Further research into the histaminergic system is thus warranted, and a better understanding of the pharmacology of histamine in the brain could potentially lead to the development of new treatment options for neurological and psychiatric diseases.

In this thesis, I have studied the pharmacology, neuroanatomy and molecular properties of histamine and its receptor Hrh3 using the zebrafish as a model of the vertebrate brain, in comparison to the other aminergic neurotransmitter networks. I present novel findings concerning the effects of alcohol on the histaminergic and dopaminergic systems, the mechanism of vesicular storage of histamine and the molecular and behavioural changes following monoamine depletion. Additionally, I describe the phenotype of a zebrafish Hrh3 knockout animal. Taken

together, these results contribute one piece to the puzzle of the neuropharmacology of histamine.

2. Review of the Literature

2.1 The histaminergic neurotransmitter system in the vertebrate brain

2.1.1 Histaminergic neurons and their projections

The physiological properties of histamine were first described in 1910 by Dale and Laidlaw, who initially characterized histamine as a strong vasodilator and constrictor of bronchial and other smooth muscle tissue [1]. Histamine was soon thereafter identified as a mediator of inflammation [2,3] and gastric acid secretion [4]. The research and pharmacological interest in histamine was for a long time concerned merely with these peripheral effects [5].

Kwiatkowski, who in 1943 discovered histamine in homogenates of brain and nerves of different mammalian species, was first to suggest that histamine acts as a neurotransmitter [6]. Further studies revealed that histamine from brain extracts was localized in the same subcellular fraction as synaptosomes containing noradrenaline and serotonin, suggesting that brain histamine might be localized in neurons [7]. Mammalian histamine and histidine decarboxylase (HDC) -containing neuronal cell bodies were first identified in the rat, located exclusively in the tuberomammillary nucleus of the posterior hypothalamus [8,9]. Although no histamine or HDC containing neuronal soma were seen in other parts of the brain, these studies found extensive histamine and HDC containing fibres in many brain regions, such as the cortex, hippocampus, amygdala, basal ganglia, hypothalamus and many brainstem regions. This organization is similar to that observed in other aminergic neurotransmitter systems, such as the noradrenergic and dopaminergic systems – the aminergic neuronal cell bodies are localized in one or a few distinct regions, but they project extensively to many different brain regions [10-14]. Later studies showed that the organization of the histaminergic network is similar in humans [15], other mammalian models [16-18], amphibians [19] and fish [20], indicating that this neurotransmitter system is well conserved in vertebrates. The wide distribution in the brain suggested a modulatory role of histamine in brain functions. After its discovery, histamine has been implicated in many key functions of the brain, as shall be described later in this review.

2.1.2 The biosynthesis of neural histamine and its regulation

Histamine in vertebrates is synthesized from the essential amino-acid L-histidine in a single step decarboxylation reaction catalysed by HDC [21]. Neural uptake of L-histidine does not seem to be an important point of regulation in histamine synthesis, as histidine uptake into neurons is independent of changes in the neuronal membrane potential [22,23]. However, it should be noted that substrate availability seems to be essential in the synthesis of histamine in the brain, as the activity of HDC is not saturated with physiological concentrations of L-histidine in the mammalian brain, although the

relevance of this mechanism is poorly understood [24]. The synthesis of histamine is also regulated through the activity of HDC. Phosphorylation by the cyclic adenosine monophosphate (cAMP) dependent protein kinase A (PKA) increases histamine synthesis, suggesting that HDC is positively regulated by phosphorylation [25]. Another phosphorylation pathway, mediated by the calcium-calmodulin dependent protein kinase II (CaMKII) also stimulates histamine synthesis and increases HDC activity [26]. As would be expected by these results, protein phosphatases regulate HDC activity negatively [27]. Post-translational modifications thus play a key role in the regulation of HDC activity. However, the role of HDC synthesis in the regulation of histamine metabolism is poorly studied and understood. In the gut, gastrin and the pituitary adenylate cyclase-activating peptide (PACAP) are known to increase HDC mRNA synthesis [28], but this effect has not yet been described in the brain. Several regulatory elements were identified in proximity to the gene coding for human HDC [29]. This suggests that regulation of histamine synthesis by HDC could also occur at a transcriptional level, but it is currently unknown what signalling pathways or biological conditions would cause this in the brain.

2.1.3 Storage of histamine in neurons

Immunohistochemical data support the conclusion that histamine is stored in vesicle-like structures, comparable to other aminergic transmitters [30]. Histamine, like the catecholamines and serotonin, is a substrate of VMAT2 [31], which is also expressed in histaminergic neurons [31]. However, the role of VMAT2 in histamine metabolism in the brain is partially unclear. When the function of VMAT2 is inhibited by the indole alkaloid reserpine, the storage of catecholamines and serotonin is inhibited, leading to the subsequent metabolism of these transmitters and a dramatic decrease of their measured concentrations in the brain [32]. Results with histamine have been unclear and contradictory, with some studies indicating that reserpine lowers brain histamine levels [33,34], while other studies point to unchanged histamine levels following reserpine treatment [35,36]. Interpretation of many of these studies is further complicated by the presence of histamine in mast cells in the brain [8,9]. Further studies are thus needed to conclusively demonstrate the relationship between neural histamine and VMAT2.

2.1.4 Reuptake and metabolism of histamine in the brain

All the other monoamines in the brain – dopamine, serotonin and noradrenaline – have specific reuptake transporters that terminate the action of the transmitter in the synaptic cleft after release and that are expressed on neurons or glia [37]. In the case of histamine, no such transporters have been described. Instead, histamine has been shown to be transported by several nonspecific cell membrane transporters – the organic cation transporters (OCT) 2 and 3 [38,39] and the plasma membrane monoamine transporter (PMAT) [40]. OCT3 and PMAT are expressed in astrocytes and transport histamine *in vitro*, with PMAT being the major transporter and OCT3 playing a more minor role [41].

This transport is likely to be functionally relevant, as toxic inhibition of astrocyte function increases histamine levels [42]. Astrocytes also express histamine *n*-methyltransferase (HNMT), the cytosolic enzyme responsible for metabolizing histamine into *n*-methylhistamine [41]. It is thus likely that extracellular histamine (i.e. from the synaptic cleft after transmitter release) is taken into astrocytes for enzymatic degradation. However, there is also evidence of neuronal reuptake of histamine [43] and neural expression of HNMT, suggesting that neurons might also partake in the reuptake and metabolism of histamine [44,45]. It should be noted that although histamine can also be metabolized by diamine oxidase (DAO), activity of this enzyme is minimal in the brain [46].

2.1.5 Histamine receptors

In vertebrates, four different types of histamine receptors have been described (HRH1, HRH2, HRH3 and HRH4) [47]. All of these are metabotropic GPCRs (G-protein-coupled receptors) – there is also evidence of histamine action on certain types of $GABA_A$ channels, but the *in vivo* relevance of this remains unknown [48]. Additionally, histamine also seems to potentiate the current of N-methyl-D-aspartate receptors by binding to the polyamine binding site on this receptor, thus reinforcing the action of glutamate at this receptor [49,50]. Here, the basic signalling properties and brain locations of the different histamine receptors are briefly described; their role in specific brain functions will be discussed later.

2.1.5.1 Receptor abbreviation nomenclature

In the literature, several different abbreviations and symbols have been used to refer to the histaminergic receptors. In this thesis, the abbreviations and symbols recommended by the International Union of Basic and Clinical Pharmacology are used [51].

2.1.5.2 Histamine receptor HRH1

HRH1 is linked to the $G_{q/11}$ class of G-proteins [52]. Activation of the receptor thus leads to the activation of phospholipase C, which produces the secondary messengers diacyl glycerol (DAG) and inositol trisphosphate (IP_3) , leading to the activation of protein kinase C and release of calcium from the endoplasmic reticulum, respectively [53]. HRH1 is also linked to several other signalling pathways through stimulation of the production of arachidonic acid and nitric oxide [54].

HRH1 is expressed widely in the central nervous system. As well as in neurons, it is also found in astrocytes [55], brain endothelium [56] and cells of the immune system circulating in the brain [57]. Thus, not all HRH1 mediated effects on brain function are caused by neurotransmission, but might also result from modulation of glial, vascular and inflammatory functions in the brain. Neural HRH1 is present postsynaptically in many brain regions, such as the cerebral cortex, the limbic system, thalamus,

hippocampus, basal ganglia and cerebellum. In most of these areas, HRH1 signalling is excitatory, although inhibitory signalling has been reported in the hippocampus [54,58,59]. HRH1 also displays constitutive activity – active signalling even in the absence of a ligand, a property occasionally seen in GPCRs [60,61].

2.1.5.3 Histamine receptor HRH2

HRH2 is linked to the mainly excitatory G_s class of G-proteins, and acts through stimulation of adenylate cyclase and production of cAMP, which further leads to the activation of protein kinase A and the transcriptional regulator CREB [54]. This signalling pathway is implicated especially in neural plasticity and development, and has also been suggested as being linked to the pathogenesis of neurodegenerative diseases [62,63]. Like HRH1, HRH2 displays constitutive signalling activity [64,65], suggesting that HRH2 might be important in maintaining a baseline activity of protein kinase A and CREB in neurons, although the relevance of this has not been studied. HRH2 has a widespread expression in the brain, being present in neurons, astrocytes, endothelium and leukocytes [54,56,57]. Although HRH2 and HRH1 are expressed in the same brain regions (such as the cortex and limbic system), their expression patterns within these regions differ, suggesting that the receptors have different target cells [54,66,67].

2.1.5.4 Histamine receptor HRH3

Signalling by HRH3 is highly complex. It is linked to the inhibitory $G_{i/o}$ class of G-proteins, inhibiting adenylate cyclase function and thus decreasing production of adenylate cyclase [68]. $G_{i/o}$ also directly inhibits voltage-gated calcium channels [69]. Furthermore, HRH3 activation is positively coupled to the mitogen-activated protein kinase (MAPK) [70] and Akt signalling pathways [71], both of which are relevant in brain plasticity and development. Action of HRH3 on neurons is mainly inhibitory, although the multiple downstream signalling pathways of the receptor make this distinction unclear [72]. HRH3 also displays an unusual multitude of functional isoforms, resulting from alternative splicing processes, with different isoforms displaying differences in signalling properties and ligand responses [70,73,74]. Although both HRH1 and HRH2 display constitutive signalling activity, HRH3 has an exceptionally high constitutive activity [75], with most HRH3 antagonists actually acting as inverse agonists, decreasing basal signalling [58]. The high constitutive activity enables HRH3 to have functional significance regardless of the presence of histamine. For instance, HRH3 antagonists have been shown to have neuroprotective effects even in animals lacking histamine [76].

In contrast to the other histamine receptors, HRH3 was long believed to be expressed primarily in histaminergic neurons in the brain, where HRH3 was first identified as an autoreceptor on histaminergic neurons regulating histamine release [77]. Later, HRH3 was shown to act as a presynaptic receptor regulating the release of many other neurotransmitters [78-81], supported by HRH3 ligand binding and mRNA expression often being localized at different sites [82]. Similarly as HRH1 and HRH2, HRH3 expression is

found in many key regions of the brain [54]. HRH3 was also relatively recently described as a postsynaptic receptor in the basal ganglia, where it forms heterodimers with dopamine receptors DRD1 and DRD2, thus modulating dopaminergic signalling [83]. However, the exact transmitter phenotype of neurons expressing HRH3 remains unknown in most brain regions.

Although initially believed to be restricted to neurons, HRH3 expression was recently also found in astrocytes [55]. As other histamine receptors, HRH3 is expressed in the brain endothelium [84]. However, it has so far not been found in brain leukocytes [58].

2.1.5.5 Histamine receptor HRH4

Like HRH3, HRH4 is also linked to $G_{i/o}$, acting through inhibition of the adenylate cyclase – cAMP pathway [85]. Currently, involvement in other signalling pathways has not been described. So far, there is insufficient proof of HRH4 expression in the neurons of the central nervous system, although HRH4 has been detected in peripheral neurons [86]. HRH4 is, however, expressed in leukocytes [57] and endothelial [84] cells in the brain, and HRH4 signalling might be relevant in brain function through this mechanism.

2.1.6 Cotransmitters and non-histamine mediated effects of histaminergic neurons

Aminergic neurons often co-release other neurotransmitters, such as the small molecule transmitters glutamate or GABA [87,88]. Histaminergic neurons in mammals also coexpress several other transmitters, most notably GABA, which has been found in a majority of histaminergic neurons [89]. The role of these cotransmitters in the function of the histaminergic network is poorly understood. A recent study identified that inhibition of GABA release in histaminergic neurons of mice increased their activity, suggesting that GABA limits and modulates the effects of released histamine [90]. Thus, not all functions of the histaminergic neurons involve histamine, which should be taken into account when studying the role of histamine in the brain.

2.2 Overview of the other biogenic amine neurotransmitter systems in the brain

Modulation of other biogenic amine systems in the brain seems to be a key role of the histaminergic system, as shall be reviewed in chapter 2.3. Thus, it is necessary to briefly present the organization of these systems in the brain. The biogenic amine neurotransmitter systems were first described by Dahlström and Fuxe in 1964 [13], and have since been extensively studied in different species. Figure 1 provides an overview of the synthesis of aminergic transmitters.

2.2.1 The dopaminergic system

Dopamine, previously also known as 3-hydroxytyramine, was initially believed to be merely an intermediate product in the synthesis of noradrenaline. It was first suggested as acting as a neurotransmitter in 1957, when Dr. A. Carlsson and colleagues described substantial amounts of dopamine in the striatum [91]. Dopamine is synthesized in a two-step reaction from the amino acid L-tyrosine. First, L-tyrosine is converted into L-DOPA (3,4-dihydroxyphenylalanine) by the enzyme tyrosine hydroxylase (TH) [92]. This is the rate-limiting step in dopamine biosynthesis, and both transcriptional and phosphorylation-based post-translational mechanisms for the regulation of dopamine biosynthesis have been described at this step [93,94]. L-DOPA is further converted into dopamine by aromatic L-amino acid decarboxylase (AADC) [95]. In the brain, synthesized dopamine is loaded into vesicles by VMAT2 [96]. Dopamine released into the synaptic cleft is cleared by a high-affinity reuptake mechanism through the dopamine transporter (DAT), although in some brain regions, dopamine can also be removed by the noradrenaline transporter (NET) [97]. Dopamine is metabolized by two main pathways – through oxidative deamination by the enzyme monoamine oxidase (MAO) into dihydroxyphenylacetic acid (DOPAC), and through *O*-methylation by catechol-*O*-methyltransferase (COMT) into 3-methoxytyramine (3-MT) [98]. In general, MAO is more abundantly present in neurons, while COMT is more prominent in peripheral organs and in glial cells [98]. The action of dopamine is mediated by at least five different types of metabotropic GPCRs, all of which are expressed in the brain [99].

In mammals and other vertebrates, dopaminergic neurons in the brain are found in populations A8-A17 of catecholaminergic cells, which are located in the midbrain (A8-A10), diencephalon (A11-A15), olfactory bulb (A16) and retina (A17), although individual dopaminergic cells have also been described in other regions [100]. Dopamine is involved in many key functions of the brain. For instance, the nigrostriatal dopaminergic system (consisting of projections from population A9) is pivotal in the regulation of voluntary motion and other motor functions [101] and the mesolimbic pathway (A10) is involved in the perception of rewarding stimuli and the pathogenesis of addiction and abuse of intoxicating substances such as ethanol [102,103].

2.2.2 The noradrenergic system

Noradrenaline was one of the first neurotransmitters to be discovered, initially described as the transmitter of the sympathetic nervous system [104]. Noradrenaline is synthesized from dopamine in a single step through beta-hydroxylation by dopamine beta-hydroxylase (DBH)[105]. In contrast to other monoamine transmitters, noradrenaline is synthesized inside synaptic vesicles. Noradrenergic transmission can thus be considered to be VMAT2 dependent, as functional VMAT2 is required for dopamine to be available in the vesicles for synthesis. Released noradrenaline is transported back into the presynaptic terminal by the noradrenaline transporter (NET) [37], and neural noradrenaline is degraded primarily through the action of MAO [106]. Noradrenaline acts through α and β adrenergic receptors, both of which are expressed in the brain [107].

The majority of noradrenergic neurons in vertebrates are located in the sympathetic ganglia of the autonomous nervous system, where noradrenaline is an essential regulator of cardiovascular tone and other functions in peripheral organs [108,109]. In the brain, noradrenergic neurons are relatively few in number and concentrated primarily in the locus coeruleus (LC) in the pons, projecting widely to different regions of the brain, where it acts as an important modulator of cognitive functions, being an important target for the treatment of various psychiatric disorders [100,110,111].

2.2.3 The serotonergic system

Like noradrenaline, serotonin (also known as 5-hydroxytryptamine, 5-HT) was also initially identified due to its properties in peripheral organs, being first described as a vasoconstrictor by M. Rapport *et al.* in 1948 [112]. Later, serotonin was also found in the brain and shown to act as a neurotransmitter [113,114]. Serotonin is synthesized from L-tryptophan in two steps by the enzymes tryptophan hydroxylase (TPH) and AADC, with TPH acting as the rate-limiting enzyme [95,115]. In mammals, two isoforms of TPH have been characterized – TPH1, expressed primarily in peripheral organs, and TPH2, expressed primarily in the central nervous system [116,117]. Like dopamine, serotonin is packed into vesicles by VMAT2 for release [96]. Released serotonin can be removed from the synaptic cleft both by high-affinity transport by the serotonin transporter (SERT) and by a recently discovered low-affinity transport mechanism, mediated by PMAT [118]. Degradation of serotonin in the brain is mostly mediated by isoform B of MAO [119]. The serotonin receptors are highly heterogenous – seven different groups of receptors are known, with many of these displaying several subtypes [120,121].

The vertebrate serotonergic neurons are located along the brainstem in several groups known as the Raphe nuclei, and send extensive projections to many brain regions [122]. While involved in many brain functions, the serotonergic system has been of particular interest due to its involvement in depression, anxiety and other mood disorders, and substances modulating serotonergic neurotransmission have revolutionized treatment of these common psychiatric conditions [123-125].

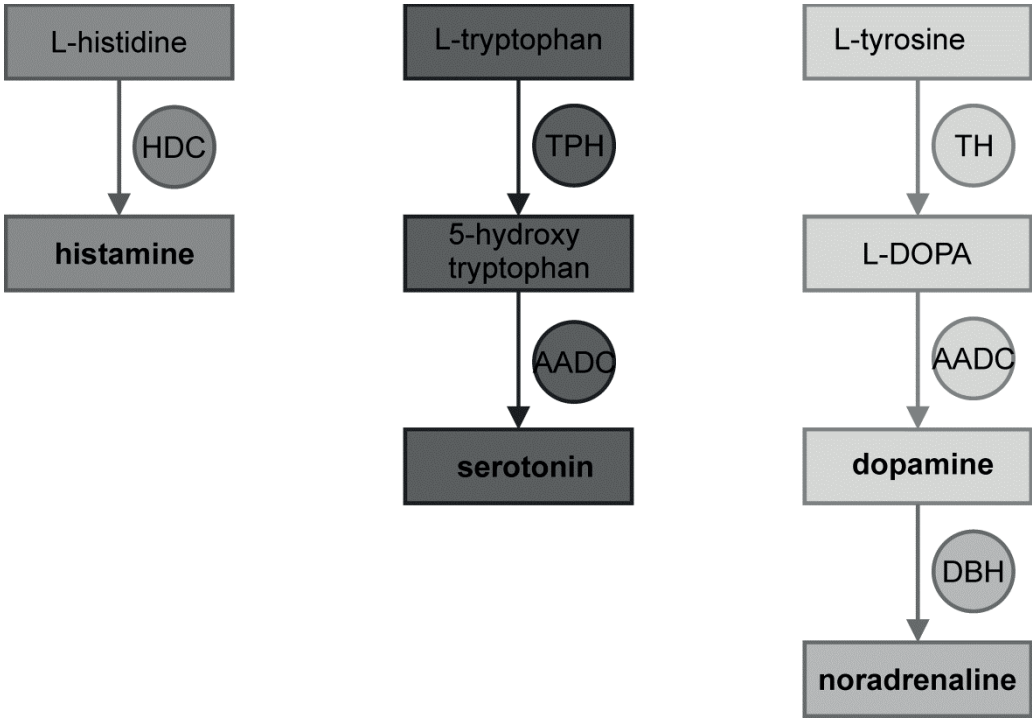

Figure 1. Overview of the synthesis of amine neurotransmitters.

2.3 Histamine in the healthy and pathological brain

In this chapter, key functions of the histaminergic system addressed and studied in this dissertation are reviewed.

2.3.1 Histamine in the developing brain

Although the functional relevance of histamine in brain development still remains poorly understood, histamine has been implicated as a regulator of neural differentiation. Histamine receptors HRH1, HRH2 and HRH3 are present in cultured cortical neural stem cells (NSCs) of the rat, and the expression of receptor mRNA is detected in many areas of the developing brain [126-129]. Histamine was also found to stimulate proliferation, survival and differentiation of these NSCs, with HRH2 signalling stimulating neural proliferation and HRH1 signalling steering the differentiation of NSCs into a neural direction [126]. The functional relevance of this effect *in vivo* is not well understood. Pharmacological inhibition of HRH1 signalling during rat development results in a smaller population of FOXP2-positive neurons in the developing cerebral cortex. The effect of this change on the morphology of the

adult brain was, however, not studied [130]. Mice lacking histamine or histamine receptors have shown no gross morphological changes in the brain or phenotypes indicating severe neural dysfunction, suggesting that these effects on proliferation can be compensated by other signalling mechanisms [131,132]. However, studies on the anatomy of different neural circuits in these mutant models are yet to be done. In the zebrafish, knock-down of histamine synthesis and pharmacologic inhibition of Hrh1 signalling during development results in impaired development of hypocretin-expressing neurons [133]. In the mice, HRH1 signalling was shown to inhibit development of dopaminergic neurons of the midbrain [134]. These results further suggest that histamine might have relevance for the development of different neuronal populations *in vivo*, especially through HRH1 signalling. Elucidation of the role of the other histamine receptors in brain development would still merit further studies.

2.3.2 Functional interactions between the histaminergic and other aminergic systems

Studies on the histaminergic system have revealed that histamine regulates the function of all monoaminergic systems, acting largely through histamine receptor HRH3. Firstly, it was shown that histamine receptor HRH3 is located on the presynaptic terminals of monoaminergic neurons, with HRH3 signalling inhibiting the release of serotonin, noradrenaline and dopamine in the target areas of these neurons in different brain regions in rats and mice [78-81,135-137]. Only the interactions with HRH3 and dopamine have been extensively studied further. A recent study questioned this mechanism with regard to dopamine. In rat and mouse synaptosomes from nucleus accumbens, a part of the ventral striatum, it was discovered that HRH3 signalling inhibited dopamine synthesis through inhibition of cAMP synthesis, but HRH3 signalling did not affect dopamine release [138]. In the striatum, HRH3 also has extensive postsynaptic interactions with dopaminergic circuits. GABAergic striatal medium spiny neurons, the main neuronal subtype in the striatum, express HRH3 receptors that form dimers with dopamine receptors DRD1 and DRD2 and thus modulate their signalling [139,140]. Activation of HRH3 counteracts both DRD1 and DRD2 mediated increases in locomotor activity in mice, while inhibition of HRH3 has the opposite effect [139]. Functional HRH3 expression seems to also be necessary for dopaminergic signalling in the striatum, as mice lacking HRH3 have lower levels of *DRD1* mRNA and show no phosphorylation of the extracellular signal-regulated kinase 1/2, one of the secondary messengers of dopaminergic signalling [141]. HRH3 also modulates striatal dopaminergic circuits indirectly, through inhibition of glutamatergic excitatory input from the cortex [142]. Taken together, it is evident that HRH3 is an important mediator of monoaminergic, notably dopaminergic, signalling. There are also some studies implicating other histamine receptors in the regulation of dopaminergic circuits. HRH2 excites striatal medium spiny neurons, and HRH1 positively regulates calcium levels in dopaminergic cells of the retina [142,143].

2.3.3 Regulation of motor circuits by histamine

Locomotion and other motor processes constitute one of the main functions of the central nervous system and make it possible for the organism to react to environmental cues and interact with the environment – thus, motor functions can be seen as the main expressive result of brain function. Histamine has been implicated in motor control in several brain levels. The vestibular nuclei in the brain stem, important centres in the control of posture and muscle tone, receive histaminergic innervation, and HRH2 signalling facilitates motor functions of the lateral vestibular nucleus [144-147]. The HRH1 agonist / HRH3 antagonist betahistine has also been clinically used in the treatment of vestibular disorders [148,149]. Histaminergic innervation is also prominent in the cerebellum, forming excitatory synapses with neurons in the cerebellar cortex and deep cerebellar nuclei [150]. Injection of histamine into deep cerebellar nuclei enhances the performance of rats in several motor tasks [151,152]. Additionally, both *HRH1* and *HRH3* mRNAs are widely expressed in a majority of the nuclei of the thalamus, an important centre in the regulation of voluntary movement, although the precise role of histamine in the motor functions of the thalamus remains unknown [66,153].

Studies in mutant mice also support a role of histamine in the regulation of motor functions. Mice lacking HDC, HRH1 or HRH3 receptor show reduced locomotor activity, and HRH1 knockout mice have reduced exploratory behaviour [154-158]. However, it should be noted that histamine also strongly promotes wakefulness, and therefore these phenotypes may simply reflect a lower activity state of animals with genetically disturbed histamine signalling [90,159]. HDC or histamine receptor knockout mice models have not shown impaired motor coordination [131,132].

2.3.4 Histamine and the neurobiology of ethanol

Ethanol is the most common substance of abuse, and ethanol addiction and overuse causes substantial morbidity and social issues worldwide. There is thus a great need to understand the neural circuitry and mechanisms underlying the neural effects of ethanol. The strong involvement of the dopaminergic network in the behavioural and reinforcing effects of ethanol administration has been well documented [160]. Ethanol activates the neurons of the mesolimbic pathway, which leads to an increased release of dopamine in the nucleus accumbens, a key area in stimulant-induced locomotor activation [161-163]. Dopamine receptor antagonists are able to inhibit ethanol-induced hyperactivity, further suggesting that dopaminergic pathways are essential in this response [164,165]. The mesolimbic pathway, originating from neurons in the ventral tegmental area (VTA), is also essential in the development of substance addiction [166]. It is thus evident that dopaminergic signalling is of pivotal relevance in the neurobiology of ethanol. However, the effects of ethanol in the brain are highly non-specific and not confined to one system.

In animal models, addiction mechanisms are often studied using conditioned place preference (CPP), a setup in which an animal develops a preference for the

compartment in which it receives the addictive or rewarding substance [167]. Several studies have shown that mice lacking histamine demonstrate stronger CPP by morphine and ethanol, which suggests that histamine would be relevant in the pathogenesis of addiction [168-170]. Further studies into the mechanism behind this effect revealed that HRH3 antagonists inhibited ethanol-induced CPP and increased the release of histamine in the nucleus accumbens, suggesting that the mesolimbic dopaminergic pathway could be modulated by histamine at this site [171,172]. The mechanism for this modulation is likely to be at least partially attributable to the dimerization of HRH3 with dopamine receptors in the striatum, as knockout of HRH3 in mice results in impaired downstream signalling of DRD1 and DRD2 receptors in the striatum [141]. Histamine has also been implicated as a mediator of the acute effects of ethanol, as mice lacking histamine show a decreased locomotor activation following ethanol administration [169]. The mechanism by which histamine modulates acute effects of ethanol is still not completely understood. The HRH3 dimerization is likely to also modulate the dopamine signalling following the increased release of dopamine after an acute dose of ethanol. It is, however, important to also consider that the histaminergic system itself is affected by ethanol. In rats, acute ethanol treatment has been shown to increase histamine content and HDC activity in the hypothalamus, although the underlying mechanism has not been studied further [173]. Thus, further studies into how the histaminergic system itself is affected by ethanol are warranted.

2.3.5 Histaminergic signalling in the pathology of psychiatric conditions

2.3.5.1 Tourette's syndrome and autism spectrum disorders

The pathogenetic processes behind psychiatric disorders are poorly and incompletely understood and generally involve changes in a multitude of neurotransmitter systems. However, even partial understanding of the involvement of individual neurotransmitters and neurotransmitter receptors in these conditions is of importance, as this could open up new avenues for development of better pharmacological therapies. Histamine has been implicated in several psychiatric diseases [174]. Its involvement has probably best been described for Gilles de la Tourette's syndrome, a condition characterized by involuntary repetitive motor behaviours called tics, after the discovery of a dominant mutant loss of function allele of HDC in a family with an autosomal dominant inheritance pattern of Tourette's syndrome [175]. A tic-like phenotype of repetitive behaviour was later reproduced in HDC knockout mice, which was reversible by injection of histamine into the brain, providing further evidence of the relevance of histamine signalling in Tourette's syndrome [176]. There is, however, no understanding of the mechanism by which histamine deficiency triggers Tourette's syndrome. As the association between histamine and Tourette's syndrome has so far been discovered in only one family, it is unlikely to explain most cases of Tourette's syndrome. Interestingly, Tourette's syndrome has substantial comorbidity with diseases of the autism spectrum disorder [177]. These conditions are characterized by

impairments of social behaviour, communication deficits and repetitive, stereotypic behaviour [178]. Some evidence suggests that histamine might also be relevant in autism spectrum disorders. Treatment with the HRH3 antagonist ciproxifan reversed the social behaviour impairment in a mouse model of autism, and a recent study found that patients with autism spectrum disorder had altered expression of genes in the histaminergic system, although this study was unable to identify which individual genes were affected in detail [179]. These pioneer studies imply that histamine could regulate social behaviour, although further studies are necessary in order to understand the mechanism behind this.

2.3.5.2 Anxiety disorders

Early pharmacological studies demonstrated that HRH1 agonists increase, while HRH1 antagonists reduce, anxiety-like light-avoidance behaviour in mice, suggesting that histamine acts as a modulator of anxiety [180,181]. By contrast, HRH3 antagonists had an anxiogenic effect, which was attributed to inhibition of HRH3 autoreceptors on histaminergic terminals, resulting in increased histamine release and increased HRH1 activation [181]. Supporting this view, HRH3 agonists show anxiolytic effects in both rats and guinea pigs [182]. In rats, the anxiogenic effect of histamine is at least in part mediated through histaminergic modulation of the amygdala, as histamine injected into the amygdala is anxiogenic, acting through a mechanism involving HRH1 but also DRD1 and DRD2 [183,184].

However, more recent studies in mutant mice have challenged the simplistic notion that histamine would be anxiogenic. Although HRH1 knockout mice display decreased anxiety-like behaviour in the elevated plus maze test, they do not show altered light-avoidance behaviour in comparison to controls [185,186]. HDC knockout mice that lack histamine completely show an increase in anxiety-like behaviour in several studies and test paradigms [158,187,188]. An anxiogenic phenotype has also been observed after lowering brain histamine levels through limiting the availability of histidine in the food of mice [189]. Knockout of HRH3 seems to have a complex effect on anxiety, increasing startle-induced anxiety responses but decreasing anxiety behaviour in the elevated plus maze [190]. The discrepancy between results in mutant mice and pharmacological studies could imply that chronic inhibition of histamine signalling (i.e. mutant models) and acute inhibition (the current pharmacological studies) affect anxiety-like behaviour differently.

2.3.5.3 Other psychiatric conditions

Histamine has also been linked to several other psychiatric conditions. Patients with schizophrenia display decreased HRH1 and increased HRH3 radioligand binding in the frontal cortex [191,192]. In a British study, a one amino acid substitution in the gene of HRH2 was found to be enriched in patients with schizophrenia, but this discovery was

not reproducible in other populations, thus casting doubts on its overall relevance [193,194]. Interestingly, HRH2 antagonists have shown some promise in the treatment of negative symptoms in schizophrenia. However, their beneficial effect may be transient due to the development of tolerance [195,196]. Due to their pro-cognitive effects in preclinical trials, attempts have been made to use HRH3 antagonists for treatment of psychotic conditions [197]. However, so far, clinical trials with HRH3 antagonists have not demonstrated efficacy in the treatment of schizophrenia [198].

Patients suffering from depression have been shown to have lower levels of HRH1 binding in the prefrontal cortex and gyrus cinguli in comparison to healthy controls, with the magnitude of this effect correlating with the severity of the symptoms [199]. The HRH3 antagonist clobenpropit demonstrated antidepressant activity in a rat model of depression, and another HRH3 antagonist, pitolisant, was able to reverse depressive-like symptoms caused by olanzapine in mice [200,201]. However, the precise role of histamine in depression still remains unknown.

2.4 The zebrafish as a model of the vertebrate brain

2.4.1 Introduction of the zebrafish as a model organism

The use of the zebrafish (*Danio rerio*) as a model organism was pioneered by Dr. George Streisinger at the University of Oregon almost 50 years ago. Streisinger's goal was to find a vertebrate animal that could be used as a model for study of the genetics of the developing nervous system and be simple to maintain in a laboratory setting [202]. The zebrafish had several properties making it a suitable candidate [202]. Zebrafish breed readily all year round in a laboratory setting and several hundred embryos can be obtained from a single breeding, enabling experiments requiring a large number of individuals, such as drug screening setups [203]. Zebrafish, like many other species of fish, are ovuliparous, and thus both the gametes and developing embryos are easily accessible to the researcher. This enables the use of several methods of genetic manipulation – indeed, the first major zebrafish paper from 1981 described the generation of homozygous diploid zebrafish embryos through conversion of haploid zebrafish eggs [204]. In addition, zebrafish embryos are transparent until the end of organogenesis (achieved at 24 hours post fertilization, hpf), and the transparency can be retained even longer by treating the embryos with 1-phenyl 2-thiourea or by using zebrafish strains that are unable to produce pigment [205,206]. This has allowed for the development of non-invasive, light-based and high-resolution *in vivo* imaging techniques of different organ systems, which are virtually impossible to develop in other established vertebrate models in life sciences [207-209]. Zebrafish embryos and larvae can easily be grown on a Petri dish and adult fish require considerably less space than a similar number of rodents [210]. The easy accessibility of the developing zebrafish embryo has also facilitated the development of many different tools for genetic manipulation and gene function regulation [211-214]. The zebrafish genome has also been

fully sequenced, which has made it possible to reliably identify the zebrafish orthologues of human genes [215]. Taken together, these fundamental advantages of the zebrafish attracted greater interest and, eventually, led to general acceptance of this animal model in life sciences.

2.4.2 Similarities and differences between the zebrafish and the mammalian brain

Several fundamental principles of neurotransmission, such as the mechanism behind the action potential, have originally been discovered in organisms with quite simple neural networks [216]. However, in order to model the function of neurotransmitter networks relevant to the mammal brain, these networks must be present in the model organism. At first glance, the morphology of the zebrafish brain appears quite different from its mammalian counterparts. The telencephalon in mammals contains many different regions that can be identified macroscopically, while the zebrafish telencephalon is much simpler and encompasses a much smaller fraction of the brain mass in comparison to mammals [217]. However, although small in size, the zebrafish telencephalon contains regions homologous to mammalian structures, such as the amygdala, cortex and hippocampus [218,219]. The fish also contain all major small molecule and amine neurotransmitter systems that have been described in mammals, and many of the neuropeptides are conserved as well [220]. Additionally, important genes in brain development, such as the different classes of neurotrophic factors, have all been described in the zebrafish, suggesting that these developmental mechanisms are present and conserved also in the zebrafish [221-224]. Although many of the systems are conserved, they still have some essential functional differences that will shortly be reviewed here for the systems addressed in this dissertation.

2.4.2.1 The histaminergic system of the zebrafish

The histaminergic neurons are localized in the posterior hypothalamus of the zebrafish and are first detectable by immunohistochemistry after 3 days post fertilization (dpf) [20]. The histaminergic system in the zebrafish is highly similar to that of mammals, with histaminergic fibres projecting to many brain regions [225]. The most notable difference is the absence of histamine fibre projections in the zebrafish cerebellum, while the mammalian cerebellum receives considerable histaminergic innervation (see chapter 2.3.3) [20]. Of the four mammalian histamine receptors, only the first three are found in the zebrafish. All of these are expressed in the brain of both larval and adult fish [226]. Although the exact signalling properties of these receptors have not been published, they display considerable similarity to their mammalian isoforms – for instance, the domain responsible for the high constitutive activity of HRH3 is conserved [226].

The peripheral tissues, including mast cells, are devoid of histamine in the zebrafish [20,227]. Thus, it would seem that biologically relevant histamine in the zebrafish is only synthesized by the hypothalamic neurons. This makes the zebrafish an optimal model

for study of the role of histaminergic neurotransmission, as there is evidence suggesting that mast cell derived histamine would also influence behaviour and interact with neural histamine receptors in mice [228,229].

The metabolism of histamine in the zebrafish has not been thoroughly studied. Although the zebrafish has a homologue of HNMT that is expressed widely in the brain, nothing is known about its properties at the moment [230]. Although low in mammalian brain, expression of DAO has been characterized in the zebrafish habenula, suggesting that oxidative deamination by DAO might partake in the metabolism of neural histamine in the zebrafish [231].

2.4.2.2 The catecholaminergic systems of the zebrafish

The dopaminergic system of the zebrafish is well developed and has been thoroughly characterized in several detailed studies [225,232-234]. All dopaminergic populations that are found in the adult brain are present in the brain at 5 dpf, and the distribution of some of the cell groups are well conserved [225,233]. The most notable difference between the zebrafish and mammals is the absence of dopaminergic neurons in the mesencephalon of the zebrafish, and it has thus been difficult to ascertain which of the populations is homologous with the mammalian substantia nigra [225]. Current evidence implicates the diencephalic posterior tuberal dopaminergic population as the zebrafish equivalent of the substantia nigra [234,235]. Other differences include a prominent population in the telencephalon and a pretectal diencephalic population, both of which are absent in mammals [225].

The zebrafish has two isoforms of TH, named Th1 and Th2, which show a complementary expression pattern in the brain [232]. In the brain, expression of *th2* is confined to the hypothalamus and preoptic region of the diencephalon. Some cell populations express both *th1* and *th2* transcripts; the majority of only *th2*-expressing neurons are located in the posterior hypothalamus [236]. This has allowed for the study of functions specific to the *th2*-expressing subset of dopaminergic neurons; these are currently known to be involved in the regulation of hypothalamic neurogenesis and initiation of locomotion [237,238]. Differences in the transcriptional regulation of these two isoforms are still poorly understood.

The noradrenergic neurons of the zebrafish are mainly located in the locus coeruleus in the hindbrain, with additional noradrenergic neurons being found in the medulla – this is highly similar to the noradrenergic system of mammals [225].

Although poorly understood, notable differences in the metabolism of catecholamines exist between mammals and the zebrafish. The zebrafish has only one isoform of MAO, which seems to be primarily involved in the metabolism of serotonin, as inhibition of MAO has no effect on the concentrations of either dopamine or noradrenaline in the zebrafish [239]. This suggests that catecholamines in the zebrafish are primarily metabolized by COMT, but very little is known about zebrafish COMT at the moment.

2.4.2.3 The serotonergic system of the zebrafish

Like the dopaminergic and histaminergic systems, all serotonergic populations of the zebrafish brain have developed by 5 dpf [233]. The zebrafish has prominent serotonergic populations in the diencephalon, a feature common in other species of fish but not observed in mammals [225,240]. The Raphe nuclei are conserved in the zebrafish and send extensive ascending and descending projections, as is the case in mammals [240].

The zebrafish have three isoforms of *tph* – *tph1a*, *tph1b* and *tph2* – all of which are expressed in the brain [241,242]. Expression of *tph1a* is restricted to the diencephalic populations in the hypothalamus and *tph2* is expressed in the raphe and pretectum [242]. *tph1b* is expressed only transiently in development and its role is not well understood [241]. As mentioned above, serotonin is efficiently metabolized by MAO [239].

2.4.3 Advantages and disadvantages of the zebrafish as a model organism

2.4.3.1 The zebrafish genome duplication event

In early evolution, many teleost species, such as the zebrafish, have undergone a genome duplication event [243]. As a consequence, the zebrafish genome has two or several isoforms of many genes that are present only in one copy in mammals. This is the case for many genes relevant for neurotransmission, as reviewed in the previous chapter. As the isoforms often have an overlapping expression [232,244], it can be difficult to generate knockout models that completely lack the function of this gene. In some cases, however, the expression patterns of the two isoforms are complementary and restricted to specific regions of the organism, thus making it possible to study the effect of regional loss of function of a certain gene. Thus, the genome duplication has created both unique challenges and opportunities for using the zebrafish as a model.

2.4.3.2 Analysing behaviour of the zebrafish

Fruitful study of brain mechanisms usually requires a quantifiable readout. Different forms of behaviour are suitable readouts, as these can be monitored non-invasively and can also be used to model human symptoms, such as motor deficiencies and anxiety [239,245]. Zebrafish display a large repertoire of characteristic behaviours, many of which are already present in early larval stages [246]. Basic locomotion can be easily studied in automated setups, giving high-throughput information about parameters such as locomotion activity and place preference of the fish [226,247]. The small size of larvae allow for tracking of up to 96 individuals in the same trial, which makes it possible to track large numbers of individuals and several experimental groups in the same trial [248]. Additionally, the possibility to combine behavioural trials with non-invasive *in vivo* microscopy techniques has allowed for the detailed identification of cells and circuits

underlying specific forms of behavioural responses [249,250]. These studies provide important information about the organization of neural circuits controlling behaviour at the cellular level, which is not feasibly obtainable in other established vertebrate models.

The zebrafish is a diurnal species, and locomotion of zebrafish larvae is strongly regulated by the level of light and changes in illumination, and larvae display a preference for light [251,252]. As light conditions can easily be controlled during experiments, the sensitivity of larvae to light has been utilized in several behavioural setups. For instance, the preference of light has been used to develop models of fear and anxiety in larvae [253]. Rapid motor responses to sudden changes in illumination have been used to study the neural circuitry regulating alertness of the zebrafish [133]. Several studies have also shown the zebrafish to be a suitable model for studying the mechanisms and pathology of sleep [254-256]. However, it should be noted that sleep in zebrafish can only be defined by behavioural criteria, as it is not feasible to perform electroencephalographic recordings of the fish brain [252].

Juvenile and adult zebrafish display a wide range of social behaviours, such as shoaling, social hierarchy between individuals and mating behaviour [246]. Social behaviour can be analysed quantitatively using video recordings and algorithms that are able to track individual fish in a shoal [217,257]. Zebrafish are thus a suitable model for elucidating the complex neurobiology of social behaviour, which is relevant in many human conditions such as autism spectrum disorder [258]. However, not all fish social behaviours can be easily translated into corresponding human behaviours [217]. For instance, there is no obvious equivalent of shoaling in mammals. There are also test paradigms available for other higher brain functions, such as memory and learning, although a detailed presentation of these methods is beyond the scope of this thesis [259].

2.4.3.3 Whole-mount and live brain imaging techniques

When studying the histological structure of the brain in rodents and other models, the brain often needs to be sectioned before analysis. The brain of the larval zebrafish is transparent, which has allowed for the development of histological methods using the whole brain as a sample. For instance, it is possible to do fluorescent immunohistochemistry and *in situ* hybridization on intact brains, although the limited availability of primary antibodies suitable for use in the zebrafish and the limited sensitivity of *in situ* hybridization methods can pose methodological challenges [234,236]. It is thus possible to obtain information about the exact number of neural cell bodies in certain brain areas and visualize their projections, thus providing more detailed anatomical information about the relevant system than a mere study of histological sections [260]. Using transgenic animals expressing fluorescent proteins in certain cells, different *in vivo* imaging setups have also been possible – for instance, activity of individual neurons can be imaged by expressing fluorescent calcium influx reporter genes [261]. Although similar methods are available for other animal models as well, the

zebrafish is currently the most feasible vertebrate option for performing this imaging non-invasively, as the comparable setups in other animals (i.e. mammals) generally either require extensive surgical procedures or are limited to the imaging of superficial brain structures [262-264].

2.4.3.4 Genetic manipulation methods

The easy accessibility of zebrafish embryos has facilitated the development of many different techniques for gene function modification and genetic manipulation. Early studies focused mainly on forward genetic approaches, where male zebrafish are treated with the chemical mutagen N-ethyl-N-nitrosurea (ENU) to induce germline mutations. These fish are then crossed with wild type females and the offspring is subsequently screened for phenotypes [265]. When a phenotype is found, the gene is then identified by cloning. This method was used in a large screen published in a single issue of the journal Development in 1996, in which many genes affecting the development of the zebrafish were identified [266]. However, this method is extremely slow and requires large fish facilities for successful screens.

One of the earliest reverse genetic methods utilized morpholino oligonucleotides that target specific RNA species to transiently inhibit the function of specific genes during development [267]. However, morpholinos also have unspecific off-target effects, and not all morpholino phenotypes have been successfully reproduced when mutants of the same genes have become available [268]. Together with the development of efficient techniques for reverse genetic screening, this has led to a decline in the use of morpholinos. The first widely used method to create constitutive knockout zebrafish of certain genes relied on the TILLING (Targeting Induced Local Lesions in Genomes) approach, in which the offspring of ENU-treated zebrafish are screened for mutations in the gene of interest [213]. However, TILLING also results in numerous off-target mutations, which may affect the vitality and phenotype of the fish strain. Thus, a method for specific mutagenesis was needed. The pioneer method for creating mutations in specific genes of interest used zinc finger nucleases containing a DNA-cleavage domain and a sequence specific DNA-binding domain to induce double-strand breaks in the genome, resulting in mutations in the target site [269]. Recently, however, the zinc finger nucleases have been virtually replaced in the zebrafish field by the CRISPR/Cas9 method, which has been adapted from a prokaryote immune defence mechanism against viruses. This method is based on synthesizing a small guide RNA, which targets a specific sequence in the genome, and Cas9 protein that cleaves both DNA strands at the binding site of the guide RNA [270]. The endogenous repair of this double-strand break is error-prone, often causing deletions and thus resulting in a mutated gene. This method is specific and more efficient than previous methods of site-specific mutagenesis [271]. More recent adaptations of the method have also made it possible to generate insertions using CRISPR/Cas9, opening up new avenues for overexpression studies and transgenics [272].

2.4.3.5 Ethical considerations in the use of zebrafish

When using animal models, it is important to pay attention to ethical use of animals and animal welfare. Having a much less complex central nervous system, the zebrafish is generally considered to be a lower-ordered vertebrate than mammalian models such as the mouse and the rat [273]. This motivates the use of zebrafish models instead of mammals, if a suitable zebrafish model is available. Additionally, many zebrafish studies can be carried out in larvae. According to the 3R guidelines, studies using immature forms of vertebrates are preferable to studies in adult individuals [274]. Experiments done in larvae are also not subjected to legal regulation and do not require an animal experiment permit in the European Union, if the experiments are performed and the larvae are terminated before they start to feed independently (Directive 2010/63/EU). By using larval zebrafish in pilot studies, for instance toxicity screens in drug development, it is also possible to reduce the number of mammalian animals needed in later stage experiments [275]. However, for experiments involving adult fish, such as the generation of genetically modified fish strains, an animal experiment license is required, and the housing conditions, procedures requiring anaesthesia and the termination of fish are strictly regulated by law [276].

3. Aims

Given the increasing pharmacological interest in the histaminergic system, a detailed understanding of the different aspects of the neuropharmacology of histamine is required. A better understanding of this transmitter system in relation to behaviour and other transmitter networks could lead to the discovery of new drug targets in the treatment of psychiatric and neurological conditions. However, in order to make proper hypotheses about possible treatment mechanisms involving the pharmacological modulation of histaminergic signalling, the basic functional properties of the system have to be thoroughly characterized. In this study, we aimed to answer some of the unresolved questions about the histaminergic system, using the zebrafish as a model of the vertebrate brain.

The specific aims of this thesis project were as follows:

1. Describe how the histaminergic system reacts to an acute dose of ethanol in comparison to the dopaminergic system. (I)

2. Investigate factors regulating the expression of *hdc.* (I–III)

3. Investigate the role of VMAT2 in histaminergic neurotransmission in comparison to other neurotransmitter systems. (II)

4. Develop a fluorescent *in situ* hybridization protocol with sufficient sensitivity to co-localize low-abundance transcripts in the zebrafish brain. (II–III)

5. Identify the neurotransmitter phenotype of neurons expressing *hrh3* in the zebrafish brain. (III)

6. Characterize the effect of *hrh3* knockout on behaviour, other transmitter networks and the development of the zebrafish brain. (III)

4. Materials and Methods

4.1 Experimental animals (I–III)

Zebrafish from the wild-type Turku strain were used in all experiments. The mutant strain used in study III was also generated in the Turku strain, and thus shares a similar heritage. This strain was originally established from a mixed population of wild type zebrafish in Turku, Finland, and has since been maintained in our laboratory for almost two decades and used in several publications [20,133,225]. The fish were maintained in a 14/10 h light/dark cycle at a temperature of 27.5–28.5°C. Zebrafish embryos and larvae were raised in 1xE3 medium (5.00 mM NaCl, 0.44 mM $CaCl_2$, 0.33 mM $MgSO_4$ and 0.17 mM KCl) until 8 dpf, when the larvae were transferred into tanks containing fish system water.

Starting at 5 dpf, the fish were fed daily with SDS dry food (SDS Diets, Essex, United Kingdom), with the exception of fish used for experiments and terminated at 7 dpf. After reaching 14 dpf, fish were fed with SDS twice a day and additionally with artemia, which were grown from cysts according to the manufacturer's instructions (Great Salt Lake Artemia Cysts, Sanders, USA). During weekends, fish were fed only once a day with artemia. Fish husbandry was based on the principles described by Westerfield [210].

Larvae were killed in ice-cold water. Genotyping samples were collected by fin clipping under anesthesia with MS-222 (Sigma A5040, Sigma-Aldrich, MO, USA). Deformed and injured fish were not used in the experiments. All animal experiments were authorized by the Animal Experiment Board of the Regional State Administrative Agency of Southern Finland and the experiments were conducted in accordance with the EU Directive 2010/63/EU. All experiments were conducted in the Zebrafish unit of the Neuroscience Center of the University of Helsinki.

4.2 Ethanol treatment (I)

Absolute ethanol (>99.5%) was dissolved into 1xE3 to obtain treatment solutions containing 0.75, 1.50 and 3.00% of ethanol (Aa ethanol, Altia, Finland). The concentrations chosen were based on previous studies on ethanol in the zebrafish, and are similar to the internal concentrations measured in mice after acute administration of ethanol [277-279]. 1xE3 without added ethanol was used for the control group. Treatment solutions were prepared fresh prior to the trial. 1 ml of solution was pipetted into each well of a 48-well plate. One 7 dpf larva was transferred to each well. The treatment durations used were 10 and 30 min. Samples of the larvae used in further experiments were collected immediately following the treatment. No mortality was observed after either treatment.

4.3 Reserpine treatment (II)

The VMAT2 inhibitor reserpine (Sigma 83580, Sigma-Aldrich, MO, USA) was dissolved into 1xE3 at a concentration of 40 mg/L. Since reserpine is poorly soluble in water, the solution was vigorously agitated until no undissolved particles were seen and the solution appeared homogenous. Larvae were treated with reserpine at 4 dpf for either 20 min or 24 h. The 20 min treatment and the chosen reserpine concentration were based on a previous study, where adult zebrafish were treated with reserpine [280]. The longer, 24 h treatment was included to evaluate the possible differences between a short and long reserpine treatment. During treatment, larvae were kept at a density of 30 larvae / 3 ml treatment solution, and this density was also applied for larvae in the control group. After the treatment, the larvae were washed three times with 1xE3 and transferred to a clean plate. No difference in mortality was seen between controls and treated larvae.

4.4 Behavioural tests (I–III)

4.4.1 Locomotion effect of acute ethanol (I)

7 dpf larvae were transferred to a 48-well plate, with each well containing 1 ml of ethanol solution (0% for controls, 0.75%, 1.50% or 3.00%). Group sizes were equal (12 larvae per group). After transferring the larvae to the plate, the locomotion behaviour of the larvae was tracked for 10 min using the EthoVision 3.1 software (Noldus, Wageningen, The Netherlands). The experiment was conducted under light conditions, with an illumination of approximately 330 lux in accordance with previous studies [133]. In order to avoid light reflection artefacts in the tracks, the walls of the wells were coated manually with parafilm. The tracks were analysed by the software. Only tracks with no tracking artefacts and a detection rate over 90% were included in the analysis. Total distance moved, mean angular velocity, mean turn angle and mean meander for the trial were computed using a 0.2 mm minimum distance moved filter. The total distance moved was also analysed in 1 min bins. Larvae that did not move at all were considered abnormal and excluded from further analysis. The amount of excluded larvae per group was as follows: control 1/48, 0.75% ethanol 5/61, 1.50% ethanol 7/68 and 3.00% ethanol 2/55).

4.4.2 Locomotion effect of reserpine treatment (II)

The reserpine behavioural tests were conducted at 5 dpf and 7 dpf (24 h and 72 h after administering reserpine, respectively). 16 larvae per group were tracked on a 48-well plate, using the EthoVision XT 11.5 software and DanioVision DVOC-0040 tracking chamber (Noldus, Wageningen, The Netherlands). Before the trial, the larvae were habituated to the plate for 10 min. Basic locomotor activity in normal light conditions

was assessed for 15 min, followed by three periods consisting of 2 min of darkness and 2 min of light. Illumination was set at approximately 330 lux, and light and dark transitions were instantaneous. A 0.2 mm minimum distance moved filter was used for the data analysis. The total distance moved and maximum distance moved per bout was analysed for the initial light period. For the altering light conditions, the distance moved was analysed in 1 min time bins. In order to analyse the dark-flash response, locomotion during the transition from light to dark was analysed in 1 sec bins.

4.4.3 Locomotion of *hrh3* mutant zebrafish (III)

5 dpf wild type, heterozygous and knockout *hrh3* larvae were tracked using the same DanioVision system as described in chapter 4.4.2. Total distance moved, mean turn angle, mean angular velocity and mean meander were analysed during a 15 min tracking period. Dark adaptation was analysed by tracking the larvae for 15 min in 1 min bins after suddenly turning off the lights.

Adult fish of each genotype were tracked in white, cylindrical containers using the EthoVision 3.1 system. In order to evaluate the place preference of the fish, the arena was divided into three virtual zones in the tracking software. The locomotion of the fish was tracked for 20 min in light conditions. The time spent in each of the zones and the same movement parameters as listed above for the larvae were analysed.

4.4.4 Social behaviour of *hrh3* mutant adult zebrafish (III, partially unpublished)

6 adult fish were tracked as a group in the same white cylindrical container used in 4.4.3. The fish were habituated to the arena for 5 min and tracked for 10 min. The individual fish swimming tracks were analysed using idTracker software, and the correlation of leadership scores and probabilities of the fish being in front were computed [257]. The trial was repeated with four different groups of fish for each genotype.

The shoaling behaviour of the *hrh3*-mutant fish was tested using a three-chamber setup developed by Dr. D. Baronio, which is similar to the setup used previously with rodents [281]. A transparent square tank was divided into a rectangular compartment, with two equally sized smaller compartments facing its long side. One of the smaller compartments contained a shoal of fish and the other compartment contained stones. Shoaling preference was measured by counting the amount of time the fish spent in proximity to the tank containing the shoal of fish during a 10 min trial.

Aggression was tested using the mirror test, where the fish were placed in a transparent rectangular tank with a mirror at one end [282]. The time spent in proximity to the mirror during a 10 min trial was measured. A total of 12 fish of each genotype were analysed.

4.5 RNA extraction and cDNA synthesis (I–III)

5–15 larvae were pooled into a microcentrifuge tube in order to obtain one RNA sample. Excess water was removed, and the sample was flash-frozen in liquid nitrogen or immediately processed through the RNA isolation protocol. RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany). In studies II and III, a DNase I treatment was included in order to verify the removal of all genomic DNA, as some of the PCR primers showed a strong tendency to amplify genomic DNA in pilot experiments of these studies. cDNA was synthesized using the SuperScript III reverse transcriptase kit (Invitrogen) using random hexamer primers in accordance with the kit protocol. In study I, 2 µg of RNA was used for the reverse transcription, in study II, 0.5–1 µg, and in study III 0.5 µg. The reason for using less RNA template is optimization of the qPCR to minimize the number of animals that needed to be pooled for one sample – we discovered during the years this project was carried out that 0.5 µg of RNA was sufficient for obtaining cDNA of a good quality. The cDNA was diluted 10 times in nuclease-free water and this dilution was used as a template for qPCR.

4.6 Quantitative PCR (I–III)

In study I, quantitative PCR analysis was performed using the SmartCyclerII® (Cepheid) cycling platform. SYBR Green premix was obtained from Takara (Takara, Madison, WI, USA). The PCR reaction was set up according to the instructions of the cycler and premix kit. The reaction volume was 25 µl. Ct values were computed using the SmartCyclerII® software.

In studies II and III, quantitative PCR was performed using the LightCycler® 480 platform, with SYBR Green I Master reaction mixture produced by the platform manufacturer (Roche, Mannheim, Germany). All experiments were conducted using white 96-well plates. The reaction was set up according to the instructions of the manufacturer. Cp values (equivalents to the Ct value in other systems) were computed using the LightCycler® software.

Relative expression of genes was calculated using the comparative C(T) method described by Livak and Schmittgen [283]. In study I, beta actin (*bactin*) was chosen as the reference gene, as this gene has been reported to be the most stable reference gene in zebrafish exposed to chemical treatments and has also demonstrated good stability in other comparisons [284]. In studies II and III, ribosomal protein L13 a (*rpl13a*) was used as a reference gene instead, because of a recent report that found it to be more stably expressed across different conditions [285]. For the sequences of the primers used, please consult the original publications.

4.7 Antibodies (I–III)

Please consult the table below for details of the antibodies used for immunohistochemistry and *in situ* hybridization.

Antibody	Dilution used	Supplier	Product number	Reference
Mouse anti-tyrosine hydroxylase	1: 1000	Immunostar	22941	Kaslin and Panula 2001 [225]
Rabbit anti-histamine	1: 10000	Prof. Panula	19C	Panula *et al.* 1990 [15]
Rabbit anti-serotonin	1: 1000	Sigma	S5545	Chen *et al.* 2012 [221]
Alexa 488 goat anti-rabbit	1: 1000	Invitrogen	A 11034	Sundvik *et al.* 2012 [286]
Alexa 488 goat anti-mouse	1: 1000	Invitrogen	A 11029	Sundvik *et al.* 2012 [286]
Alexa 568 goat anti-rabbit	1: 1000	Invitrogen	A 11036	Sundvik *et al.* 2012 [286]
Alexa 568 goat-anti mouse	1: 1000	Invitrogen	A 11031	Sundvik *et al.* 2012 [286]
Sheep anti-digoxigenin-POD	1: 2000	Roche	11 207 733 910	Lauter *et al*. 2011 [287]
Sheep anti-fluorescein-POD	1: 2000	Roche	11 426 346 910	Lauter *et al*. 2011 [287]
Sheep anti-digoxigenin-AP	1: 7500	Roche	11 093 274 910	Thisse and Thisse 2008 [288]

4.8 Immunohistochemistry (I–III)

Larvae were fixed in 4% paraformaldehyde (PFA) diluted in 1xPBS (phosphate buffered saline) overnight at +4°C. For histamine staining, the larvae were fixed with 4% freshly prepared 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 0.1 M phosphate buffer pH 7.00.

The next day, the larvae were washed for 3–4 hours in 1xPBS, after which the brains of the larvae were dissected under a stereomicroscope using #5 and #7 Dumont forceps (Fine Science Tools, Heidelberg, Germany). Brains were collected into meshed baskets kept on a 24-well plate, with each well containing 1 ml of PBS. All subsequent wash steps were carried out in the baskets on a 24-well plate.

The brains were washed 3x30 min with PBS containing 0.3% Triton X-100 detergent (PBSTx) at room temperature (RT). After this, nonspecific binding sites were blocked by incubating the samples in PBSTx containing 4% normal goat serum (NGS, Gibco) and 1% dimethyl sulfoxide, overnight at +4°C. After blocking, the samples were incubated in primary antibody overnight for 16–24 h. The primary antibody was diluted in PBSTx containing 2% NGS. After incubation, the brains were washed in RT for 10 min and 3x30 min in PBSTx in order to remove excess antibody. The brains were then incubated at +4°C with Alexa fluorophore-conjugated secondary antibody diluted in PBSTx with 2% NGS. This was followed by a 10 min wash in PBSTx and

3x30 min washes in PBS, followed by 2x60 min incubations in 50% glycerol in PBS. Finally, the brains were incubated for at least 24 h in 80% glycerol in order for the tissue to be completely infiltrated and optimally cleared by the glycerol. The brains were then mounted between two 24 x 60 mm cover glasses, using 18 x 18 mm cover glasses to make a space between the larger glasses.

4.9 Cloning of zebrafish *vmat1* (II)

A 735 bp fragment of the predicted zebrafish *vmat1* (NCBI accession number XM_009304250.2) was amplified from cDNA obtained from whole zebrafish larvae. The primer sequences are reported in the original publication [289]. The PCR reaction was set up using the DyNAzyme II kit (Thermo Scientific F-551), and the reaction was analysed on a 1% agarose gel. The band was excised from the gel and the DNA isolated with the Min Elute Gel Extraction kit (Qiagen, Hilden, Germany). In order to obtain a plasmid, the PCR fragment was subsequently ligated into the pGEM®-T easy vector (Promega A1360, Fitchburg, WI, USA). The plasmid was then transformed into competent DH5α *E.coli* bacteria. The successful cloning of the sequence was verified by sequencing the insert from both ends using T7 and SP6 primers.

4.10 Riboprobe synthesis (I–III)

All riboprobes used were synthesized from plasmids containing a fragment of the cDNA of the gene of interest as an insert, flanked by a RNA polymerase promoter sequence (T3, T7 or SP6). The plasmids were linearized with an appropriate restriction enzyme. 11-digoxigenin-UTP or 12-fluorescein-UTP labelled riboprobes were synthesized using 10x fluorescein or digoxigenin RNA labelling mix and an appropriate RNA polymerase (Roche, Mannheim, Germany), according to the manufacturer's instructions.

4.11 Chromogenic *in situ* hybridization (I–III)

The hybridization procedure was based on the protocol by Thisse and Thisse [288]. Larvae were fixed in 4% PFA diluted in 1xPBS and dissected as described in chapter 4.7. The dissected brains were collected in methanol and stored at –20°C until used for *in situ* hybridization.

During the *in situ* procedure, the brains were kept in 1.5 ml microcentrifuge tubes containing 1 ml of solution, unless otherwise noted. Brains were progressively dehydrated through a series of 5 min washes in 75%, 50% and 25% methanol diluted with 1xPBS containing 0.1% Tween-20 detergent (PBSTw). This was followed by 4x5 min washes in PBSTw, after which the brains were permeabilized by treating them with proteinase K (1 µg/ml) for 10 min. The proteinase digestion was stopped by post-

fixation with 4% PFA in 1xPBS for 20 min, followed by 4x5 min washes with PBSTw. Samples were then prehybridized in hybridization buffer (HB, 50% deionized formamide, 5x sodium salt citrate (SSC), 0.1% Tween-20, 50 µg/ml heparin, 500 µg/ml tRNA with pH adjusted to 6.00 using citric acid) for 2 hrs at 65–67°C. Afterwards, HB was replaced with 250 µl HB containing 50–100 ng of digoxigenin-labelled riboprobe. Hybridization was done overnight at 65–67°C for a minimum of 16 h.

Following hybridization, the samples were washed at 65–67°C as follows: briefly in HB, then 10 min each in 75%, 50% and 25% HB diluted with 2xSSCT (sodium salt citrate with 0.1% Tween-20), followed by a 10 min wash in 2xSSCT and two 30 min washes in 0.2xSSCT. HB used in these washes did not contain tRNA or heparin; SSCT buffers include 0.1% Tween-20. This was followed by 10 min washes in RT with 75%, 50% and 25% 0.2xSSCT diluted with PBSTw and a final 10 min wash with PBSTw. The samples were blocked with PBSTw containing 2% normal sheep serum (NSS) and 2 mg/ml bovine serum albumin (BSA) for 2 h in RT followed by incubation with F_{ab} fragments of alkaline phosphatase conjugated sheep anti-digoxigenin antibodies, diluted to 1:7500 in blocking solution overnight at +4°C.

On the last day of the protocol, excess antibody was washed away with at least 6 washes in PBSTw, lasting on average 15 min per wash. Afterwards, samples were washed twice for 15 min in alkaline Tris buffer (100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20). The solution was thereafter replaced with staining solution, consisting of 225 mg/l nitro blue tetrazolium chloride (Roche, Mannheim, Germany) and 175 mg/l of 5-bromo-4-chloro 3-indolyl phosphate (Sigma-Aldrich, MO, USA). Staining was monitored under a dissecting microscope. When a sufficient signal was seen, the reaction was terminated by several washes in 1xPBS. The samples were infiltrated with glycerol and mounted as described in chapter 4.7.

4.12 Tyramide conjugate synthesis (II–III)

Tyramides were synthesized following the protocol described by Hopman *et al.* [290]. Succinimidyl esters of the following fluorophores were used in order to make respective tyramide conjugates: DyLight 488, DyLight 550, DyLight 633, DY-490, DY-560, DY-647P1, ATTO 488, ATTO 633, ATTO 647N, 5-(and-6)-carboxyfluorescein (FAM), and 5-(and-6)-carboxytetramethylrhodamine (TAMRA).

The tyramine solution was prepared by dissolving tyramine-HCl in dimethyl formamide at a concentration of 10 g/l (58 mM). The pH of the tyramine solution was adjusted to 7.00–8.00 by adding 10 µl/ml triethylamine into the solution. The succinimidyl esters were dissolved in anhydrous dimethyl formamide at a concentration of 10 g/l. The solutions were immediately mixed so that the molar ratio between ester and tyramine was 1.1. The reaction was allowed to proceed for 2 h at RT. The solution was then diluted to a final concentration of 1 mg/l with absolute ethanol, according to the mass of the unconjugated ester, and stored at –20°C. The

concentrations of tyramide used for the amplification reaction are indicated in the table below.

Fluorophore	Supplier of succinimidyl ester (product number)	Dilution used for TSA
DyLight 488	Thermo Fisher Scientific, Waltham, USA (46402)	1:250 (4 mg/l)
DyLight 550	Thermo Fisher Scientific, Waltham, USA (62262)	1:250 (4 mg/l)
DyLight 633	Thermo Fisher Scientific, Waltham, USA (46414)	1:167 (6 mg/l)
DY-490	DyOmics, Jena, Germany (490-01)	1:250 (4 mg/l)
DY-560	DyOmics, Jena, Germany (560-01)	1:250 (4 mg/l)
DY-647P1	DyOmics, Jena, Germany (647P1-01)	1:250 (4 mg/l)
Atto 488	ATTO-TEC, Siegen, Germany (AD 188-31)	1:250 (4 mg/l)
Atto 633	ATTO-TEC, Siegen, Germany (AD 633-31)	1:250 (4 mg/l)
Atto 647N	ATTO-TEC, Siegen, Germany (AD 647N-31)	1:250 (4 mg/l)
FAM	Thermo Fisher Scientific, Waltham, USA (C1311)	1:250 (4 mg/l)
TAMRA	Thermo Fisher Scientific, Waltham, USA (C1171)	1:250 (4 mg/l)

4.13 Fluorescent *in situ* hybridization (II–III, partially unpublished)

The basis of the protocol for fluorescent *in situ* hybridization was adapted from Lauter *et al.* [291]. The basic outline of the protocol is highly similar to the one described for chromogenic *in situ* hybridization in chapter 4.8. The differences between the protocols are briefly outlined here.

Before rehydration, endogenous peroxidase was inactivated by incubating the samples for 20 min in 2% H_2O_2 in methanol. Hybridization was done at 60°C. The hybridization buffer contained 5% dextran sulphate but was otherwise identical to that used for chromogenic *in situ*. The post hybridization washes were performed at 60°C as follows: quick wash in HBW (50% deionized formamide, 2xSSC, 0.1% Tween-20), 2x30 min in HBW, 1x15 min in 2xSSCT and 2x30 min in 0.2xSSCT. Thereafter the samples were washed 4x10 min in PBSTw, followed by blocking with PBSTw containing 8% NSS for 1–2 h in RT. Detection of the probe was performed using F_{ab} fragments of peroxidase conjugated sheep anti-digoxigenin antibodies, diluted 1:500. After overnight incubation at +4°C, excess antibody was washed 6x15 min with PBSTw, followed by 2x15 min washes in TSA (tyramide signal amplification) buffer (0.1 M borate pH 8.5, 0.1% Tween-20). The probe was then detected by incubating the sample with tyramide reagent diluted in TSA working solution (0.45 g/l 4-iodophenol, 2% dextran sulphate, 0.003% H_2O_2, 0.1% Tween-20) for 15 min. The reaction was stopped by four quick washes with PBSTw, where the tubes were vigorously shaken manually several times during each wash.

When two transcripts were detected in the samples (two-channel *in situ*), the peroxidase in the first antibody was now inactivated by incubating samples for 10–15 min in 0.1 M glycine-HCl buffer, pH 2.00. To remove all glycine, samples were washed 4x5 min in PBSTw. The samples were then blocked and incubated with the second antibody as described above. The second riboprobe was labelled with fluorescein and detected using peroxidase-conjugated sheep anti-fluorescein antibodies, diluted at 1:500. After the final detection, the samples were washed 4x5 min 1xPBSTw and then mounted in 75% glycerol containing 40 mM $NaHCO_3$, in order to ensure a pH above 8 and optimal fluorophore performance.

4.13.1 Optimizations tested for the fluorescent *in situ* protocol

The protocol listed above resulted in sub-optimal detection results for low-abundance transcripts, such as *hrh3*. In order to optimize detection results and the signal-to-noise ratio, several optimizations to the protocol were tested. These have been previously described by King and Newmark for the planarian *S. mediterrania* [292]. The following alternative protocols were tested:

1. Replacing the proteinase K treatment with heat-induced antigen retrieval, performed by boiling the samples in 10 mM sodium citrate buffer pH 6.00, followed by 20 min incubation in PBSTw (no PFA postfixation).

2. Using 0.3% Triton X-100 as a detergent instead of 0.1% Tween-20.

3. Using a higher antibody dilution (1:2000 instead of 1:500)

4. Investigating the difference between using higher (100 ng) and lower (50 ng) amounts of riboprobe.

5. Using 100 mM NaN_3 instead of glycine buffer for inactivation of the first peroxidase antibody. In order to completely remove NaN_3, samples were washed 8x5 min with PBSTw after this step.

6. Testing several different tyramide reagents.

4.14 High-performance liquid chromatography (HPLC) (I–III)

10–20 larvae were pooled per sample and flash-frozen in liquid nitrogen. The larvae were homogenized in 2% perchloric acid by sonication while keeping the samples on ice. The samples were then centrifuged for 30 min at 15 000 g. The supernatant was collected and 10 µl loaded as a sample for HPLC. Catecholamines, serotonin and their metabolites were measured using an electrochemical detector. The detailed procedure has been described earlier [234,239]. Histamine was measured using a Shimadzu HPLC system equipped with a fluorescent detector setup according to the method described by Yamatodani [293]. The pellet was resuspended in 5% SDS and the protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Thermo

Fisher Scientific 23227, Waltham, USA). The measured neurotransmitter amounts were normalized to the protein levels.

4.15 Generation of a *hrh3* knockout zebrafish (III)

The *hrh3* mutant line was generated using the CRISPR/Cas9 system [271]. A 20 nucleotide targeting sequence 5'-GGTGAGTGGAGACTGGGCAG was synthesized by annealing the oligonucleotides 5'-TAGGTGAGTGGAGACTGGGCAG and 5'-AAACCTGCCCAGTCTCCACTCA. This sequence targets the second exon of the zebrafish *hrh3* gene (NCBI Gene ID 561773). The targeting sequence was then cloned into the single-guide RNA expression vector pDR274 (Addgene plasmid #42250). The resulting construct was linearized by digestion with DraI and used as a template for single-guide RNA synthesis with the MAXIscript T7 kit. Cas9 mRNA was synthesized using the mMESSAGE T7 ULTRA kit, using a Cas9 expression plasmid (pMLM3613, Addgene plasmid #42251) linearized with PmeI as template. In order to induce the desired mutation, 26 pg single-guide RNA and 600 pg Cas9 mRNA were injected simultaneously into zebrafish embryos in the one-cell stage. The development of the injected embryos was observed, and normally developed embryos were analysed for mutations at the target site using T7 Endonuclease I assay and high-resolution melt curve analysis PCR (HRM-PCR, primers 5'-CGTACGTCCTGACGGGTG and 5'-ACGCGGTGCACAACATATAATC). The target locus of potential founders was cloned into the pGEM®-T easy vector, as described in chapter 4.9.

Three mutations were successfully identified. Out of these, a line with a 5 nucleotide deletion was chosen. This mutation results in a frameshift mutation and nonsense protein primary sequence after amino acid 133 and a premature stop codon at amino acid 312. The nonsense mutation causes a loss of 5 out of 7 transmembrane domains, the domain responsible for constitutive activity of *hrh3* and the intracellular C-terminal domain. For a more detailed comparison of the sequences, please refer to the original publication (III).

The fish were genotyped using the same HRM-PCR assay as described above. All comparisons between genotypes were performed using sibling fish.

4.16 Microscopy and imaging (I–III)

Light microscopy samples were analysed and imaged using a Leica DM IRB inverse bright-field microscope with an attached digital camera (Leica, Wetzlar, Germany). A whole-mount sample was imaged at several focal planes and a multifocus image was constructed using the Leica Application suite. Anatomical regions were identified according to Mueller and Wulliman [294].

Fluorophore labelled samples were imaged using a Leica SP2 AOBS confocal microscope equipped with a 488 nm argon laser, a 561 nm diode laser and a 633 nm

helium/neon laser. Fluorophore emission ranges were set according to the technical details provided by the fluorophore manufacturer. They are described in more detail in the original publications. Cross-talk between channels was excluded by sequential scanning. For visualizing the entire brain, a HC PL APO 20x/0.70 CS objective was used. Higher magnification images for colocalization analysis were taken using HC PL APO 40x/0.75 CS and HCX PL APO CS 63x/1.3 GLYC objectives.

Cell counting was performed manually from image stacks obtained by confocal microscopy using Fiji software, with the counter blinded to the sample type [295]. Colocalization was determined from single optical sections.

4.17 Statistical analyses (I–III)

For all analyses, n was defined as an independent experimental unit. Analyses were in general conducted using 1-way analysis of variance (ANOVA), followed by Tukey's post hoc test. The non-parametric Kruskal-Wallis test and Dunn's post hoc test were used in cases where the variances were significantly different between groups, as determined by Bartlett's test or the Brown-Forsythe test. In study I, the behavioural data was transformed with the function $y=\lg(y)$, in order to make the distribution normal and allow for the use of parametric tests. Values of 0 were substituted with 10^{-4} in order to allow for the logarithm function to be used.

Behavioural data analysed in time bins was analysed using 2-way repeated measures ANOVA followed by Tukey's post hoc test (except in study I, where the Bonferroni post hoc test was used instead, as Tukey's test was not available in the software at that time).

The level of significance for all tests mentioned above was set at $p < 0.05$. All analyses were conducted in Graph Pad Prism version 5 (Study I) or 7 (Studies II–III) (Graph Pad Software, San Diego, CA, USA).

5. Results

5.1 Acute ethanol affects locomotion in a biphasic, dose-dependent manner (I)

Doses of 0.75% and 1.50% ethanol rapidly increased the locomotor activity of 7 dpf zebrafish larvae during the first 10 min following immersion in the treatment solution (Study I, Fig. 1a). This effect was significant for the 1.50% group ($F(3,213) = 8.304$ $p < 0.0001$). Interestingly, larvae treated with 3.00% ethanol seemed to have an equal swimming activity as controls. This was explained by a biphasic effect of 3.00% ethanol – analysis of locomotor activity in 1 min bins revealed that while the 3.00% groups initially showed a similar increase in locomotion as the 1.50% group, locomotor activity declined rapidly during the trial, and the 3.00% group showed significantly decreased locomotor activity in comparison to the control group during the last 5 min of the trial (treatment effect $F(3,213) = 5.865$ $p = 0.0007$; time effect $F(9,1917) = 89.02$ $p < 0.0001$; Study I, Fig. 1e). This result suggests that higher doses of ethanol lead to sedation in zebrafish larvae.

When analysing parameters describing the movement path of the larvae, we observed a significant increase in the mean angular velocity of larvae in the 1.50% group, in comparison to controls ($F(3,213) = 16.18$ $p < 0.0001$; Study I, Fig. 1c). We saw no biologically relevant changes in mean turn angle or meander, which implies that the increased angular velocity in the 1.50% group is due to a higher amount of turning behaviour rather than an increased magnitude of individual turns.

5.2 Transcripts of enzymes synthesizing dopamine and histamine are rapidly upregulated by ethanol (I)

After a 10 min ethanol treatment, mRNA transcripts of both rate-limiting enzymes of dopamine synthesis, *th1* and *th2*, were upregulated in a dose-dependent fashion, with the 3.00% group showing a significant increase of both transcripts (*th1*: $F(3,17) = 3.439$ $p = 0.0405$; *th2*: $F(3,17) = 8.489$ $p = 0.0011$; Study I, Fig. 2). A similar dose-dependent increase was also observed for the histamine synthesizing enzyme *hdc* ($F(3,17) = 5.515$ $p = 0.0079$).

5.3 Ethanol transiently decreases dopamine levels with no effect on histamine levels (I)

A 10 min treatment with 3.00% ethanol significantly reduced dopamine levels by approximately 30% in zebrafish larvae ($F(3,24) = 6.174$ $p = 0.0029$; Study I, Fig. 7). The 1.50% group showed a similar decrease trend, while dopamine levels were unaltered in the 0.75% group. No significant differences were observed in the

concentrations of noradrenaline or the dopamine metabolites DOPAC and HVA across groups, although HVA levels appeared slightly lower in the ethanol-treated groups. As transcripts of *th1* and *th2* were upregulated at this same point in time, we hypothesized that this upregulation might reflect a compensatory response to the loss of dopamine. Thus, we analysed dopamine levels after a longer, 30 min treatment. At this point in time, no differences in dopamine levels were observed across groups.

As for histamine levels, we found no significant change in any of the groups after a 10 min treatment, although the histamine levels were slightly lowered in the 1.50% and 3.00% groups.

5.4 Unaltered morphology of histaminergic and dopaminergic networks after ethanol treatment (I)

In order to investigate if the transcriptional changes reflected global or local changes in the dopaminergic and histaminergic systems, we analysed the distribution of *th1*, *th2*, and *hdc* mRNA in the brains of zebrafish larvae after a 10 min ethanol treatment. The expression patterns were unaltered between groups, suggesting that the transcriptional changes occurred in the same cells that express the mRNA species analysed under normal conditions. Th1- and histamine immunohistochemistry also showed unaltered cell population sizes, fibre projections and cellular morphology, supporting these conclusions (Study I, Fig. 3–4). As histological changes might become apparent on a longer time scale than molecular ones, we performed the same analyses using larvae treated with 1.50% ethanol for 30 min (Study I, Fig. 4 and 6). However, the longer treatment also showed no changes in the histaminergic and dopaminergic networks.

5.5 Reserpine alters behavioural responses to sudden darkness (II)

During normal light conditions, larvae treated with reserpine (both the 20 min and 24 h group) were hypoactive in comparison to controls (Study II, Fig. 2). The hypoactivity was more pronounced in the group treated for 24 h. This effect was long-lasting and observed at both 24 h and 72 h after administration of reserpine ($H(2) = 23.9$ $p < 0.0001$ and $F(2,141) = 22.07$ $p < 0.0001$, respectively). The maximum distance moved per bout of motor activity was unaltered or slightly increased in the reserpine-treated groups, suggesting that the neuromuscular pathways responsible for movement were intact.

Zebrafish larvae increase their locomotion in response to sudden darkness in two phases [251]. First, the larvae show a dark-induced flash response, a spike of locomotor activity during the first 500 ms of darkness. A 24 h reserpine treatment abolished the dark-flash response almost completely both at 24 h and 72 h after administration of reserpine, in comparison to controls (Tukey's $p < 0.0001$ for all points in time, except in 3a where $p = 0.0108$; Study II, Fig. 3). The 20 min treated larvae showed

significantly decreased dark-flash responsiveness only at 72 h after administration of reserpine (Study II, Fig. 3d–f; Tukey's $p < 0.0001$ except in 3d where $p = 0.0339$). The dark-flash response is of particular interest with regard to the study of the histaminergic system, as previous studies have demonstrated that decreased histamine levels inhibit this response [133,296].

After the dark-flash response, zebrafish larvae show increased locomotor activity during the first several minutes following sudden onset of darkness, through a poorly understood neural mechanism [251]. When analysing locomotor activity during 2 min periods of alternating light and darkness, we found that this response was significantly aggravated in both reserpine-treated groups 24 h after administration of reserpine (interaction effect $F(22,1551) = 24.91$ $p < 0.0001$; Study II, Fig. 2). When returning to light conditions, the reserpine treated larvae rapidly returned to baseline activity, being hypoactive in comparison to controls. At 72 h after administration of reserpine, both reserpine-treated and control larvae showed similar levels of locomotor activity.

5.6 Neural histamine is depleted by reserpine (II)

Immunohistochemical analysis revealed that histamine immunoreactivity in the brains of larvae was strongly decreased in both reserpine-treated groups at 72 h after administration of reserpine (Study II, Fig. 4). Importantly, immunoreactivity was almost completely absent in the fibre projections of the histaminergic neurons, suggesting that histamine was absent especially from the nerve terminals. The reserpine-treated larvae also showed a significantly decreased number of histamine immunoreactive neurons both at 24 h and 72 h after administration of reserpine, with a similar 40% decrease observed in both the 20 min and 24 h treated groups ($F(2,35) = 13.9$ $p < 0.0001$ and $F(2,43) = 26.34$ $p < 0.0001$, respectively; Study II, Fig. 4). Although some neurons retained immunoreactivity in the soma, the projections of these cells did not show any detectable immunoreactivity.

In order to verify that the decrease in histamine immunoreactive neurons was not due to reserpine toxicity, we analyzed the number of *hdc* mRNA positive neurons in the treated brains and found this to be the same as in controls. Thus, we concluded that the decrease in immunoreactivity reflects a decrease in histamine levels in the otherwise intact histamine neurons.

As some of the histaminergic neurons retained immunoreactivity, we investigated whether the neurons were expressing different vesicular transporters. However, we saw that almost all *hdc*-positive neurons were also positive for *vmat2* (Study II, Fig. 5). Additionally, we saw no expression of *vmat1* mRNA in any region of the brain. This strongly suggests that *vmat2* acts as the main vesicular transporter in all *hdc*-positive neurons, and the uneven depletion of histamine after reserpine treatment is due to a mechanism other than the presence of another transporter.

5.7 Depletion upregulates synthesis of monoamines but not histamine (II)

As expected from studies in other vertebrates, reserpine strongly diminished levels of dopamine, noradrenaline and serotonin in the zebrafish (Study II, Fig. 7). The magnitude of the decrease was similar at both 24 h and 72 h after administration of reserpine. Dopamine (24h treatment: $H(2) = 8.28$ $p = 0.0062$; 72h treatment: $H(2) = 9.42$ $p = 0.0024$) and noradrenaline levels (24h treatment: $H(2) = 10.22$ $p = 0.0006$) were most sensitive to reserpine treatment, with both 20 min and 24 h treatments resulting in a 80–90% decrease of both transmitters. Serotonin levels were significantly decreased only by the 24 h treatment, while the 20 min treatment resulted in a 40–70% decrease that was not significant ($H(2) = 10.21$ $p = 0.0007$ and $H(2) = 6.86$ $p = 0.0236$ for each point in time, rescpetively). Levels of histamine showed a similar sensitivity to reserpine as serotonin, with a significant 70–80% decrease observed only for the 24 h group at both points in time ($H(2) = 8.271$ $p = 0.0065$ and $H(2) = 12.5$ $p < 0.0001$, respectively).

In study I, decreased levels of dopamine were associated with increased *th1* and *th2* transcripts, suggesting that depletion of dopamine upregulates this synthesis. Thus, we investigated whether reserpine-induced depletion of histamine and monoamines upregulated their synthesis by measuring relevant mRNA species by qPCR at 72 h after administration of reserpine (Study II, Fig. 6). We found that *th1* ($F(2,11) =$ $9.F(2,11) = 9.515$ $p = 0.0040$) and *tph1a* ($F(2,11) = 4.552$ $p = 0.0363$) were upregulated in the reserpine-treated groups, while *th2* and *tph2* were not, suggesting that these transcripts are differentially regulated by the depletion of their end product. Interestingly, *dbh* was extremely strongly upregulated, showing a 300% increase in the 24 h treated group ($F(2,11) = 29.98$ $p < 0.0001$) and an increase trend in the 20 min treated group. In contrast to the other amines, histamine synthesis was not upregulated, showing an unchanged expression of *hdc* in the reserpine-treated groups. We also investigated if the monoamine reuptake mechanisms were upregulated. Only the noradrenaline transporter *net* was upregulated in the reserpine-treated groups ($F(2,11) = 8.178$ $p = 0.0067$), while the serotonin and dopamine transporters showed a similar level of expression as controls. We also saw no change in the expression of *vmat2*. As reserpine inhibits *vmat2* irreversibly, this might in part explain why the effects of reserpine are extremely long lasting.

5.8 Optimized fluorescent *in situ* hybridization protocol for detection of low-abundance mRNA species (II–III, development of protocol unpublished)

Although many protocols for fluorescent *in situ* hybridization are available for the zebrafish, many of these have mainly been tested with genes that are expressed at a relatively high level, such as transcription factors that show abundant expression during development. However, transcripts with a lower level of expression, such as genes responsible for neurotransmitter synthesis and neurotransmitter receptors, are

more difficult to visualize, particularly when detecting two or more transcripts. Using the protocol described by Lauter *et al.* as a starting point, we performed several optimizations to develop a protocol that could be used to reliably detect transcripts with lower levels of expression. Firstly, we discovered that the tyramide signal amplification reaction produced less background when using a higher dilution of the peroxidase-conjugated antibody than recommended by the manufacturer (1:2000 instead of 1:500).

Secondly, we tested a large repertoire of different tyramide reagents (listed in chapter 4.12). We found no better alternative for the red wavelength channel than the TAMRA conjugate described by Lauter *et al*. In the far-red channel, however, we found that the DY-647P1 tyramide worked much better than the previously described DyLight 633 tyramide, showing a much clearer signal (Figure 2). In the green channel, the Atto488 tyramide slightly outperformed the previously described FAM tyramide, showing better bleach resistance. Although both the Atto488 and DY-647P1 fluorophores have been available for some time, they had not been previously used successfully for whole-mount *in situ* hybridization.

Whole-mount *in situ* hybridization in zebrafish has traditionally included a proteinase K treatment in order to permeabilize the tissue to the riboprobe. We tested an alternative permeabilization method described by King and Newmark for the planarian *S. mediterrania*, where the samples are permeabilized by boiling them in citrate buffer [292]. Surprisingly, we found that this heat-induced antigen retrieval method resulted in a greatly improved signal compared to the proteinase K treatment, allowing improved visualization of less abundant transcripts (Figure 3). We also found that, in accordance with King and Newmark, inactivating the first peroxidase-conjugated antibody using 100 mM NaN_3 instead of acidic glycine resulted in more consistent detection of the second riboprobe, although this improvement was less dramatic. We attempted replacement of the Tween-20 detergent with Triton X-100, as King and Newmark had discovered that this resulted in better detection. However, we noted that a change of detergent mostly increased background staining.

Using these optimizations, we were able to reliably colocalize specific neural markers, as can be seen from our results in studies II and III.

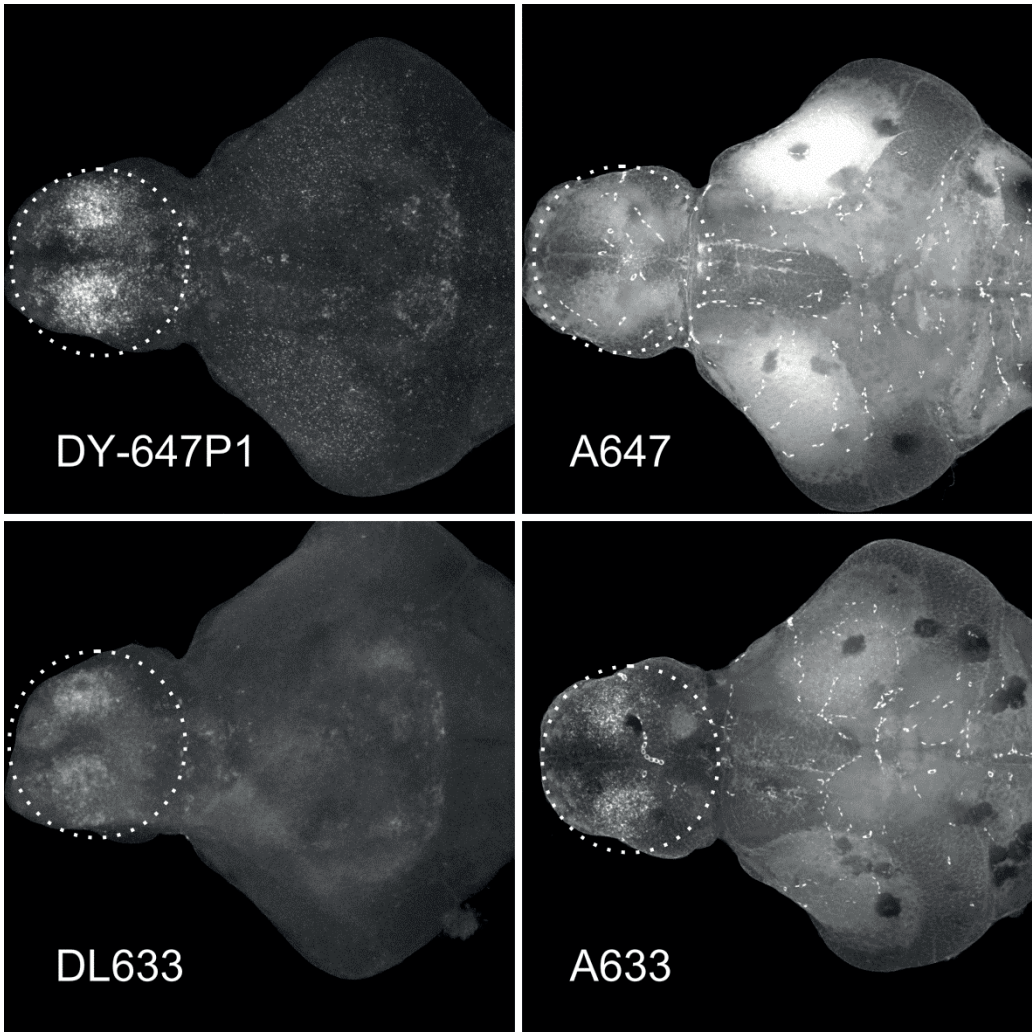

Figure 2. Comparisons between different far-red tyramides. Note how the *hrh3* signal in the dorsal telencephalon (outlined area) is far clearer when using the DY-647P1 tyramide in comparison to the DyLight 633 (DL633) tyramide. We also tested long-wavelength Atto tyramides, but these showed a high background staining.

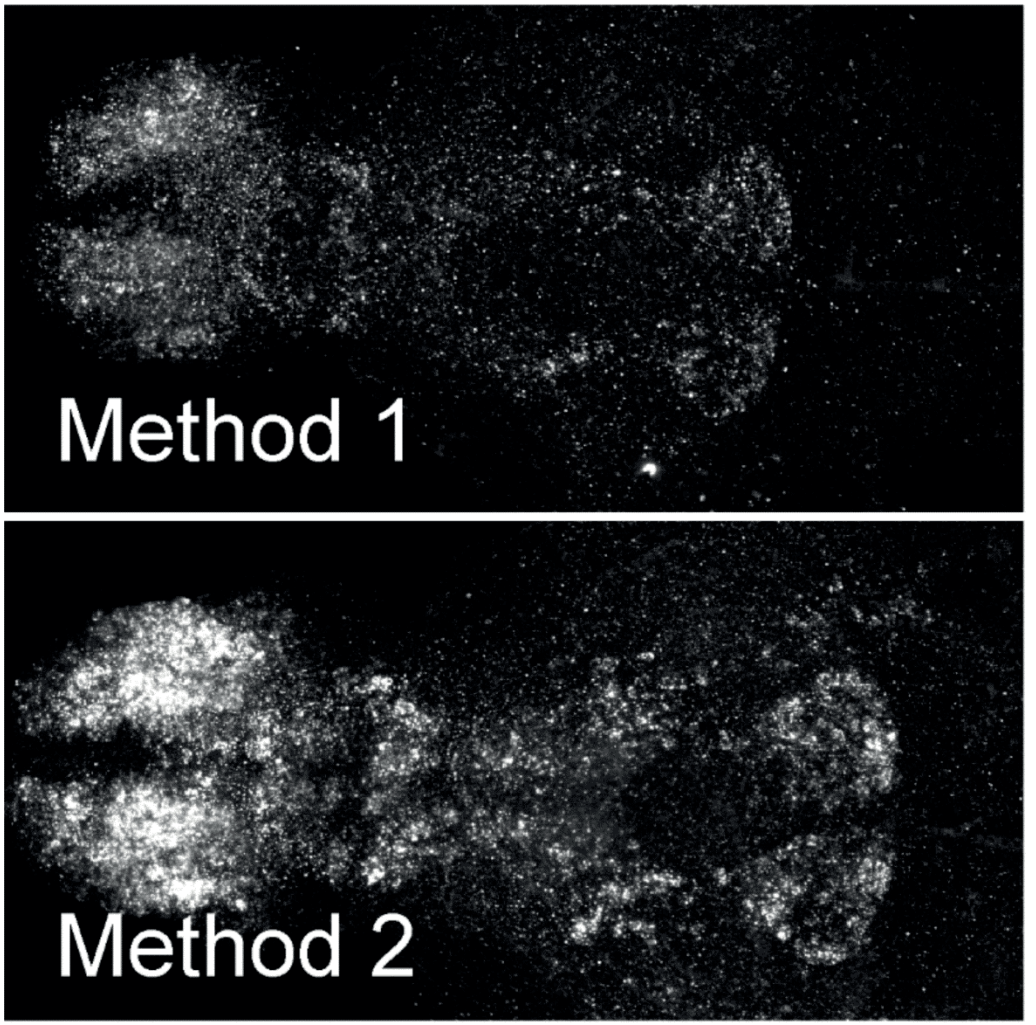

Figure 3. Comparison between heat-induced antigen recovery in citrate buffer (method 2) and proteinase K treatment (method 1) for permeabilization. Note the stronger signal and more clearly identifiable cellular structures obtained by method 2.

5.9 Expression of *hrh3* in the zebrafish brain (III)

hrh3 expression in the zebrafish has previously been poorly characterized, with previous reports showing moderate expression in the dorsal telencephalon [133]. We discovered that the expression of *hrh3* was much more widespread in the brain (Study III, Fig. 1). The strongest expression was consistently observed in the dorsal telencephalon (Study III, Fig. 1). However, we also saw prominent expression in the optic tectum, thalamic region and hypothalamus (Study III, Fig. 1). The hypothalamic expression was localized to the posterior hypothalamus, the region containing the

histaminergic neurons, and two bilaterally located populations in the anterior hypothalamus.

During development, prominent *hrh3* expression was firstly detected at 3 dpf (Study III, Fig. 1A–C). At this stage, prominent expression was seen in the pallium of the dorsal telencephalon, with weaker expression also seen in the posterior hypothalamus and the thalamic region. At 5 dpf, the anterior hypothalamic populations were detectable (Study III, Fig. 1F), and expression in the optic tectum became prominent at 14 dpf (Study III, Fig. 1G–H). 30 dpf brains showed expression in the same regions as 14 dpf brains (Study III, Fig. 1J–M).

Although *hrh3* expression has been characterized in several species, no direct histological evidence of the transmitter phenotype of neurons expressing *hrh3* is available. Using our optimized fluorescent *in situ* hybridization protocol, we were able to identify the *hrh3*-positive cells of the pallium to be mostly glutamatergic, expressing the vesicular glutamate transporter *vglut2.2* (Study III, Fig. 2A–C). The dorsal pallium also contains individual *gad2* mRNA positive GABAergic neurons [297]. Interestingly, these neurons showed no *hrh3* expression (Study III, Fig. 2D–F). Coexpression between *hrh3* and *gad2* was, however, seen in individual neurons in the ventral areas of the pallium (Study III, Fig. 2G–I). Our results thus indicate that the majority of the target neurons of *hrh3* in the dorsal telencephalon are glutamatergic. We were further able to demonstrate that the zebrafish histaminergic neurons express *hrh3*, consistent with observations in other species (Study III, Fig. 2J–L). Due to the lower sensitivity of the fluorescent *in situ* protocol, we were unable to reliably identify the neurotransmitter phenotype of *hrh3*-expressing cells in other brain regions.

5.10 *hrh3* knockout larvae adapt faster to darkness (III)

Using the CRISPR/Cas9 method, we successfully generated a *hrh3* knockout strain of zebrafish (see chapter 4.15 and Study III, Fig. 3 for details). Larvae lacking *hrh3* displayed baseline-like locomotor activity at 5 dpf. Additionally, we saw no differences in parameters describing the swimming path of the larvae, such as meander and angular velocity. This indicates that the circuitry controlling basal movement coordination was intact in *hrh3* knockout larvae (Study III, Fig. 4C–D, F–G). The *hrh3* knockout larvae also show a normal dark-flash response and showed a similar increase in locomotor activity upon sudden darkness as wild type and heterozygous siblings (Study III, Fig. 4A–B). However, we discovered that *hrh3* larvae remained hyperactive for a shorter time after onset of sudden darkness, returning significantly faster toward baseline locomotor activity than wild type siblings (genotype effect $F(2,91) = 3{,}259$ $p = 0.0430$; Study III, Fig 4A). These results suggest that *hrh3* can regulate adaptation of motor responses to sudden changes in the environment.

We also analysed basic locomotion behaviour of adult zebrafish. We found that adult *hrh3* knockout fish were significantly hypoactive in comparison to wild type siblings ($F(2,27) = 4.23$ $p = 0.0252$; Study III, Fig. 5A). We saw no differences in swimming-

path parameters such as meander (Study III, Fig. 5B). A previous study found that depletion of histamine in adult zebrafish decreased the time spent in proximity to the edge of the tank, a characteristic behaviour known as thigmotaxis [247]. We found that the time spent close to the edge of the tank was unaltered between *hrh3* knockout fish and wild type siblings, suggesting that histamine does not regulate thigmotaxis through *hrh3* (Study III, Fig 5C).

As recent studies in rodents have implicated *hrh3* in the regulation of social behaviour, we also analysed several metrics of social behaviour. We found that *hrh3* knockout and wild type fish show a similar correlation between the leadership score of an individual and the probability of said individual to be swimming in front (Study III, Fig. 5D–F). Additionally, we have seen no differences in shoaling preference ($t(18.99) = 1.584$ $p = 0.1298$) and aggressive behaviour ($F(2,41) = 0.5261$ $p = 0.5948$) between *hrh3* knockout and wild type fish (Puttonen *et al*, unpublished results, data not shown). This suggests that knockout of *hrh3* does not significantly affect social behaviour of the zebrafish.

5.11 Lower levels of serotonin and dopamine in *hrh3* knockout larvae (III)

A multitude of previous studies have shown that *hrh3* regulates aminergic neurotransmission, as reviewed in chapter 2.1.5.4. However, these systems have not been previously described in *hrh3* knockout animal models. We addressed this question using several methodological approaches. First, we analysed the expression of key genes in monoaminergic neurotransmission in all three genotypes using quantitative PCR. This included enzymes of monoamine synthesis (*th1*, *th2*, *dbh*, *tph1a*, *tph2*, *ddc*) and high-affinity monoamine transporters (*dat*, *serta*, *sertb*, *net*, *vmat2*). Additionally, we analysed the expression of *hdc* and the dopamine receptors (*drd1*, *drd2a*, *drd2b*, *drd3*, *drd4b*). We noted, however, that the expression of the genes mentioned above was completely unaffected by the *hrh3* genotype.

Next, we analysed the levels of monoamine transmitters by HPLC. We found that both dopamine and serotonin levels were significantly decreased in *hrh3* knockout larvae ($H(2) = 8.06$ $p = 0.0092$ and $F(2,13) = 3.948$ $p = 0.0457$, respectively; Study III, Fig. 6A, D). Levels of noradrenaline, adrenaline and the catecholamine and serotonin metabolites were not significantly altered across groups (Study III, Fig. 6B–C, E–G).

In order to investigate whether the decreased transmitter levels resulted from structurally altered dopaminergic and serotonergic networks, we visualized these systems using Th1 and serotonin immunohistochemistry. We found that all Th1 and serotonin populations were identifiable in the brains of both *hrh3* knockout and wild type larvae, and appeared to be of similar size (Study III, Fig. 7). The dopaminergic and serotonergic fibre projections also appeared intact. These results suggest that the decreased levels of serotonin and dopamine reflect functional changes in otherwise intact networks, and that *hrh3* knockout does not significantly alter the development of these transmitter networks.

6. Discussion

6.1 Dopaminergic and histaminergic mechanisms in acute effects of ethanol (I)

In this thesis, we discovered that a short, 10 min treatment with acute ethanol upregulated expression of rate-limiting genes in dopamine and histamine synthesis, *th1*, *th2* and *hdc*. Dopaminergic mechanisms underlying acute effects of ethanol have been readily studied previously, and recently the histaminergic system has also attracted more attention in this field. However, previous studies have mainly focused on the neurochemical dynamics of these systems, with the molecular mechanisms having received considerably less attention.

A few rodent studies have reported increased *Th* mRNA levels in the VTA following chronic ethanol treatment setups [298,299]. Our results suggest that upregulation of *th* occurs rapidly after ethanol exposure, even in animals that have not previously encountered the substance. This is supported by the only mammalian study on the subject, where increased *Th* expression was observed in rats 1 h after administration of a single dose of ethanol [300]. We already saw a significant increase after a 10 min treatment, suggesting an even more rapid transcriptional response than reported previously. Additionally, we found the magnitude of this upregulation to be dose-dependent. Our *in situ* hybridization and immunohistochemistry results suggest that the transcriptional changes were not confined to any specific cell population, suggesting that the dopaminergic network was diffusely affected. This is also supported by the fact that both *th* isoforms were upregulated, as these isoforms are partly expressed in different brain regions – for instance, *th2* is absent in the proposed zebrafish functional homolog of the mammalian mesencephalic dopaminergic groups [232,234]. Although we analysed only transcript levels in this study, a later report proved that Th protein was increased by acute ethanol, supporting our results [301].

We reported for the first time that acute ethanol upregulated *hdc*, following a similar dose-dependency as the upregulation of the *th* isoforms. In rats, increased histamine levels and histidine decarboxylase activity in the hypothalamus were reported in an early study [173]. However, since the hypothalamus of rats also contains some mast cells, it was unclear whether these changes reflected alterations in mast cells or in neurons. In the zebrafish, however, *hdc* has been found solely in neurons [20]. Thus, we present the first evidence of the upregulation of neuronal histamine synthesis after acute ethanol. As this upregulation is extremely rapid, it suggests that the histaminergic network itself is acutely modulated by ethanol and that the histaminergic system is not merely modulated by the other neurotransmitter systems. This is supported by the observation that histamine seems to be necessary for ethanol-induced hyperactivity in mice [169]. In mice, acute ethanol does not alter histamine release in the nucleus accumbens, while dopamine release is increased in this region [171]. However, ethanol-related histaminergic dynamics in other brain regions has yet to be investigated.

Elevated brain histamine levels have been associated with alcohol preference in rats, and a single study has found increased levels of histamine in certain types of human alcoholics [302]. Thus, the role of neural *hdc* in the pathophysiology of alcoholism should be further studied.

The mechanism underlying the upregulation of histamine and dopamine synthesis is still unclear. We noted that dopamine levels in zebrafish larvae were decreased after a 10 min ethanol treatment with the 1.50% and 3.00% doses. Ethanol is known to increase dopamine in other animals, and this depletion could be caused by a rapid increase in dopamine release. As the *th* isoforms were upregulated at this time, this would suggest a compensatory upregulation of synthesis in response to rapid dopamine depletion. This is supported by our observation that dopamine levels were equal across groups at 30 min after ethanol treatment. Additionally, this mechanism would explain the discrepancy between our results and previous studies in zebrafish that have consistently reported increased levels of dopamine following an acute dose of ethanol, as these studies have measured dopamine levels after a longer, 1 h treatment [303,304]. The mechanism mediating the increase in transcription remains to be elucidated. A recent study showed that ethanol increases *Th* transcription in cultured midbrain dopaminergic neurons through the cAMP/PKA and MAPK/Erk kinase pathways [305]. As the cAMP/PKA pathway is known to be an important downstream target of ethanol, it is also possible that *th* transcription is activated directly by ethanol, independently of dopamine depletion [306,307]. It should, however, be noted that this effect was demonstrated by exposing the cells to a very high, 100 mM dose of ethanol, which cannot be considered physiological in mammals. Histamine levels were not significantly altered, and thus it seems unlikely that increased *hdc* transcription would be triggered by a compensatory mechanism. As the cAMP/PKA pathway stimulates histamine synthesis, the transcriptional effects of ethanol on *hdc* may also be related to this mechanism, although this needs to be studied further [25].

As reported in previous studies, acute ethanol treatment increased locomotor activity of the larval zebrafish [277,278]. We additionally demonstrated that higher ethanol doses had a biphasic effect on locomotion, showing an initial hyperactivity, which was rapidly followed by hypoactivity, similar to results in rodents [308]. As these locomotor changes were associated with robust upregulation of both the dopaminergic and histaminergic systems, it suggests that these networks are involved in the locomotor response. Earlier studies have shown that both of these networks are relevant in the regulation of zebrafish locomotor activity. Both inhibition of *hdc* translation and pharmacological antagonism of histamine receptor signalling decreases locomotion of larval zebrafish [133,226]. As in mammals, amphetamine-induced dopamine release causes hyperactivity, and inhibition of dopamine signalling causes hypoactivity [280,309]. Recently, it was shown that inhibition of Drd2 and tyrosine hydroxylase phosphorylation reduces alcohol-induced hyperactivity in the zebrafish [310,311]. DRD2 inhibition also has a similar effect in mice, suggesting that the pathways mediating ethanol-induced hyperactivity are at least partially conserved [312]. Thus, the dopaminergic mechanisms

may be more relevant to the observed locomotor response. The findings discussed here are summarized in Figure 4.

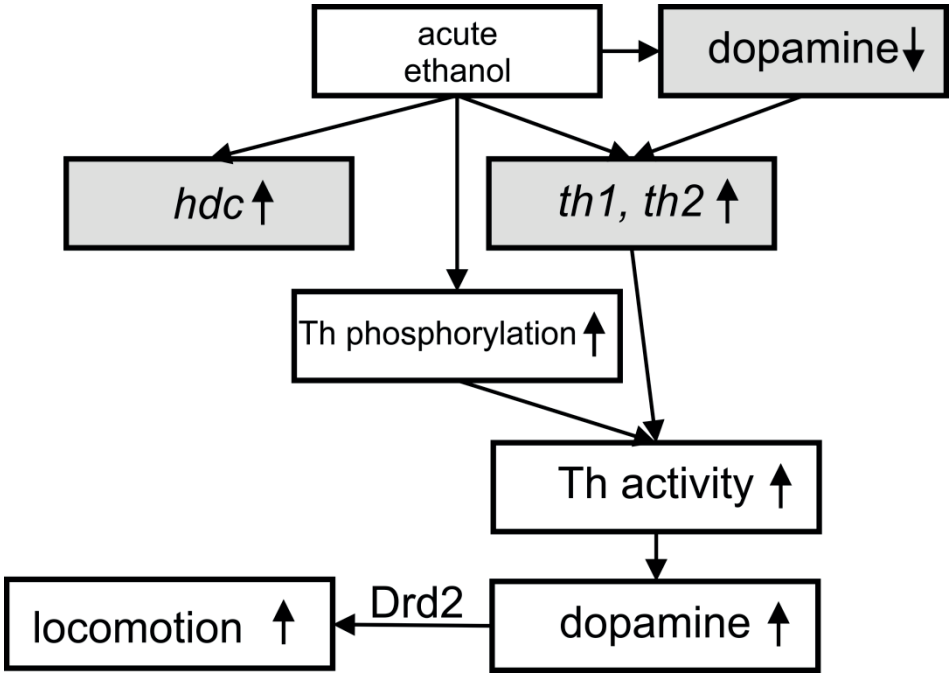

Figure 4. Summary of currently known effects of acute ethanol on the histaminergic and dopaminergic systems in the zebrafish. Discoveries from Study I are indicated by a light-grey background.

6.2 New aspects of the regulation of amine neurotransmitter synthesis (I–III)

One of the aims of this study was to identify factors regulating the expression of neural *hdc*. In addition to ethanol treatment, we examined the effect of depletion of neural histamine by reserpine and knockout of the *hrh3* autoreceptor from the histaminergic neurons. This question is of relevance, as the alterations in brain histamine levels have been described in many different brain disorders, and transcriptional regulation of *hdc* is still poorly understood [58].

Although reserpine-treated larvae showed decreased histamine levels, the expression of *hdc* was unaltered. Previous studies have shown that reserpine treatment in mice increases the activity of HDC, but the mechanism behind this has not been studied [35]. Our results suggest that the underlying mechanism is likely to be related to post-translational modification of HDC, rather than increased transcription. This is the case in the periodic regulation of HDC activity in the mouse brain, where *Hdc* mRNA levels remain stable but enzyme activities show diurnal fluctuation [313].

In contrast to the histaminergic system, reserpine treatment upregulated genes involved in the synthesis of other amine neurotransmitters. Interestingly, we only saw an increase in the expression of *th1*, while *th2* remained unchanged. Previous studies in reserpinized rodents have also suggested that different dopaminergic populations react differently to dopamine depletion [314,315]. For instance, expression in locus coeruleus is increased, while expression in the substantia nigra is not. The zebrafish *th1* is expressed in the noradrenergic locus coeruleus and in the majority of the dopaminergic populations of the brain [232]. Additionally, we found that the expression of *dbh* was greatly upregulated, suggesting that noradrenaline depletion results in a strong compensatory transcriptional response. Our results also suggest that it is unlikely that the increase in *th1* and *th2* mRNA expression following an acute dose of ethanol can be merely explained as a compensatory response to dopamine depletion, as the dopamine depletion observed after acute ethanol was much smaller in magnitude but still caused a much larger transcriptional response. Also, reserpine-induced dopamine depletion did not affect *th2*, while ethanol also upregulated the *th2* transcript.

We also observed a similar effect in serotonin synthesis – *tph1a* was upregulated, whereas *tph2* was not. This is the first study to show differential upregulation of *tph* mRNA upon serotonin depletion in any species. This suggests that different serotonin populations show different transcriptional responses to serotonin depletion, as in the case of dopamine. This may have importance in understanding functional differences among serotonergic neurons, as recent studies have identified distinct subtypes of serotonergic neurons [316].

In order to further understand transcriptional mechanisms in relation to monoamine depletion, we also analysed expression of the high-affinity monoamine transporters. We found, however, that only the noradrenaline transporter *net* was upregulated, whereas the serotonin and dopamine transporters were not. Together with the fact that *dbh* showed stronger upregulation than other rate-limiting enzymes, this suggests that noradrenaline depletion induces stronger compensatory transcriptional mechanisms than depletion of the other monoamines. This might reflect the importance of maintaining sufficient levels of noradrenaline, as knockout of noradrenaline synthesis is fatal in mice [317].

We additionally investigated whether knockout of *hrh3*, the autoreceptor of histaminergic neurons, affected *hdc* transcription. However, *hrh3* knockout zebrafish larvae showed normal expression of all enzymes of monoamine synthesis and all monoamine transporters, suggesting that removal of *hrh3* does not affect monoamine synthesis at a transcriptional level.

Taken together, we discovered that depletion of histamine does not induce compensatory changes in mRNA expression, while depletion of other aminergic neurotransmitters resulted in the upregulation of at least one synthesizing enzyme. These findings are summarized in Figure 5. Additionally, we described novel differences in the regulation of *th* and *tph* isoforms in the zebrafish, which is relevant for further studies into the characteristics of these enzymes.

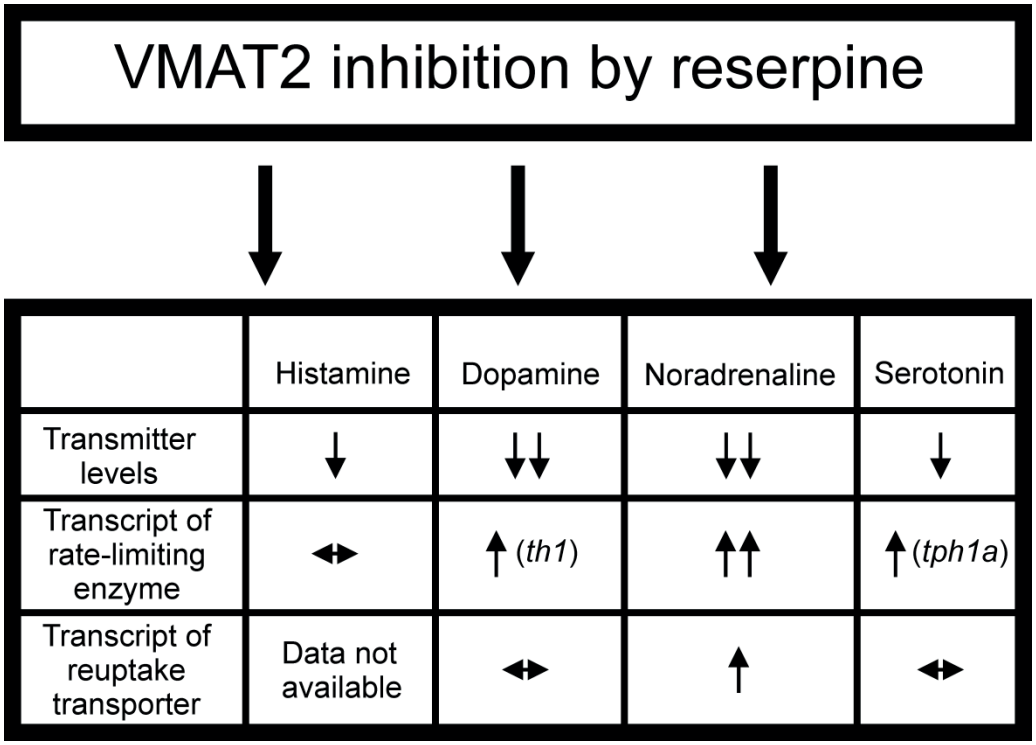

Figure 5. Overview of changes in monoamine levels and related transcripts after reserpine treatment. Double arrows indicate a larger magnitude change. The histaminergic system is the only system not to show any transcriptional changes, while the strongest transcriptional changes occurred in the noradrenergic system.

6.3 The role of VMAT2 in histamine storage (II)

Although histamine transport by *vmat2* has been described previously, its relevance to histamine neurons had not been previously characterized [31]. We discovered that inhibition of *vmat2* by reserpine treatment depleted almost all histamine immunoreactivity in axonal processes of these neurons. This implies that histamine was lacking especially in the axon terminals and in its site of action. Additionally, immunoreactivity in cell bodies was clearly decreased, although a subset of the neurons seemed to retain immunoreactivity. The decrease in histamine was verified independently by HPLC. Thus, we provide compelling evidence of the *vmat2* dependence of histaminergic neurotransmission.

What could then explain the discrepancy in previous studies? Previous studies have relied on the use of HPLC measurements, which are unable to differentiate between neural and extraneural histamine [33-36]. Maldonado and Maeyama tried to account for this by using rats lacking mast cells, and discovered that reserpine decreased histamine levels significantly only in rats with mast cells, which partially led the authors to

suggest that the neural pool of histamine would be relatively resistant to reserpine [35]. However, if mast cell histamine constituted a significant amount of the histamine in the brain, basal histamine levels should be altered between the strains used, although the authors speculated that the lack of mast cell histamine might upregulate neural histamine synthesis by a poorly understood mechanism. Also, histamine synthesis is not restricted only to neurons and mast cells; for instance, neutrophils have recently been shown to produce histamine [318]. Although we observed that histamine levels were less sensitive to reserpine treatment than catecholamine levels, our immunohistochemical results clearly demonstrate that the neural pool of histamine was significantly affected by reserpine. The differences between our studies could theoretically be related to differences in histamine metabolism between the zebrafish and mammals, but at least zebrafish *mao* shows a similar lack of histamine metabolizing activity as its mammalian counterparts [319]. We therefore find it unlikely that the metabolism of histamine would be dramatically different in the zebrafish.

One intriguing discovery was the retained immunoreactivity in a subset of histamine neuron soma. We did not discover the mechanism behind this. One possibility would be that a subset of histaminergic neurons expresses a different monoamine transporter. However, we concluded this to be unlikely, as virtually all *hdc* mRNA positive neurons in the zebrafish brain expressed *vmat2*, and we saw no expression of the other major monoamine transporter, *vmat1* in the brain. This is consistent with observations in other species, showing that *vmat1* is not significantly expressed in the brain [320]. Thus, the reason for this observed heterogeneity remains to be elucidated. However, it is known that histaminergic neurons display heterogeneity in other properties as well. For instance, histamine neurons display differences in cotransmitter profiles and receptor expression [286,321]. Thus, it is possible that differences in histamine synthesis or metabolism between individual histaminergic neurons could explain the uneven loss of histamine immunoreactivity, but this warrants further study.

Recent reports have associated polymorphisms in the VMAT2 gene with the prevalence of several conditions, such as Parkinson's disease, alcoholism, psychotic disorders and cognitive impairment after traumatic brain injury [322-326]. As we have demonstrated conclusively the importance of VMAT2 in neural histamine storage, altered function of the histaminergic system should also be considered as a potential contributor to pathological processes involving VMAT2 polymorphisms. The relevance of altered VMAT2 expression to histaminergic neurotransmission should be further evaluated in different models, such as animals lacking or overexpressing VMAT2 in histamine neurons.

6.4 Histamine and other monoamines as regulators of responses to sudden changes in the environment (II–III)

The histaminergic system is known to be important in regulating the wakefulness of animals. For instance, periods of wakefulness in mice are correlated with histamine release, and HRH1 signalling has been shown to promote wakefulness [327-329]. Importantly, HRH1 antagonists that cross the blood-brain barrier have substantial sedative effects in humans [330]. In the zebrafish, histamine has been shown to regulate responsiveness to environmental cues. Burgess and Granato demonstrated that when illumination is suddenly turned off, zebrafish respond with an instantaneous burst of locomotor activity that lasts for approximately 500 ms [251]. As this response results in a 180° turn referred to as an O-bend, it has been suggested as having a navigational function, orienting the larvae back towards light upon suddenly entering a darker area [251]. The circuitry regulating the dark-flash response is still poorly understood. However, zebrafish with decreased neural histamine have an impaired dark-flash response. This effect has been shown both after inhibition of *hdc* translation by morpholinos and after inhibition of histamine synthesis by alpha-fluoromethylhistidine and also by inhibition of Hrh1 signalling [133]. Additionally, presenilin 1 knockout zebrafish have a deficiency of histaminergic neurons and an inconsistent dark-flash response at 5 dpf, with unaltered morphology of other aminergic networks [296]. Our observation of the lack of a dark-flash response in reserpine-treated animals might thus be explained by impaired histaminergic transmission, which further supports our conclusion that histamine transmission is *vmat2* dependent. We also observed that the dark-flash response was more strongly impaired in larvae receiving a longer reserpine treatment. This also supports the importance of histamine in this response, as only the longer reserpine treatment significantly reduced histamine levels by HPLC. As *hrh1* and *hrh3* are expressed partially in the same area of the dorsal telencephalon, we also investigated the dark-flash response of *hrh3* knockout zebrafish larvae. This was unaltered, suggesting that the dark-flash response is not dependent on *hrh3*.

A recent study described that *hdc* knockout zebrafish that completely lack histamine have an intact dark-flash response, leading the authors to suggest that the previously observed effect of impaired histaminergic transmission might be due to off-target effects of the used methods [248]. It is true that morpholinos are notorious for off-target effects, and many – if not all – pharmacological substances also affect structures other than their intended target. However, the association between impaired histamine signalling and an impaired dark-flash response has been demonstrated with several methods – morpholino knockdown, three structurally different pharmacological compounds and a mutation impairing histamine neuron development [133,296]. It is unlikely that all these methods would have the same off-target effect. Hence, other explanations for the lack of dark-flash impairment in *hdc* knockout zebrafish should be considered. It is known that complete knockout of a gene by a loss of function mutation may cause a different phenotype than partial knockdown of the gene or translation inhibition, for instance by triggering compensatory mechanisms [331]. Further

studies, i.e. with inducible knockout models, should be conducted in order to conclusively settle the question of histamine as a regulator of the dark-flash response.

After the dark-flash response, zebrafish larvae show transiently increased locomotion during adaptation to darkness, which slowly declines to a lower level of activity than during light conditions [251]. This has been speculated as helping the animal move back into lighted environments [251]. The circuitry regulating this behaviour is not known. We discovered that depletion of amine neurotransmitters increased this hyperactivity. However, the exact aminergic network responsible remains to be determined. Histamine is unlikely to be involved, as histamine-depleted animals do not display a similar phenotype. Monoaminergic networks innervated the nucleus of the medial longitudinal fasciculus in the zebrafish midbrain, a region necessary for the control of swimming velocity, and monoamine depletion could thus affect the swimming speed of larvae [332]. The dopaminergic and serotonergic networks in particular also project extensively to many other regions regulating motor activity, such as the subpallium in the telencephalon (homologous to the mammalian basal ganglia) and the motor neurons in the spinal cord [235,333]. Thus, the regulation of locomotion by monoamines occurs on several different levels, and extensive studies will still be necessary to precisely dissect the mechanism by which monoamines regulate hyperactivity after sudden darkness. We were, however, able to contribute one additional piece to this puzzle, as we discovered that the adaptation of this hyperactivity was faster in *hrh3* knockout larvae. This suggests that *hrh3* can regulate adaptation to sudden changes in the environment.

The hyperactivity occurring after darkness has also been suggested as representing anxiety-like behaviour and stress caused by a sudden change in the environment [334,335].Thus, the increased dark-induced hyperactivity observed in the reserpine-treated larvae could suggest increased anxiety-like behaviour. This would be consistent with previous observations in *vmat2* heterozygous fish, which show increased anxiety-like behaviour in a dark avoidance test [336]. The observation that *hrh3* knockout larvae adapt faster to this stimulus could further be interpreted to suggest that *hrh3* could be involved in the regulation of adaption to anxiogenic stimuli in the zebrafish. However, there was no change in thigmotaxis, the preference for the outer rim of a tank, in adult *hrh3* knockout zebrafish (discussed in detail in chapter 6.5). As thigmotaxis is a more established measure of anxiety, it seems unlikely that the faster adaptation to darkness observed in larvae would represent altered anxiety. Also, *hrh3* knockout larvae showed a wild type-like initial hyperactivity, with only the adaptation being altered. Anxiolytic treatments have been shown not to affect the habituation of zebrafish to the environment [337]. Thus, we find it unlikely that the faster adaptation to darkness is related to anxiety.

How, then, does *hrh3* regulate the circuitry relevant to adaptation to darkness? We saw very strong *hrh3* expression in the pallium of the zebrafish. This region contains the areas of the zebrafish brain that are functionally equivalent to the mammalian cortex, amygdala and hippocampus, which are important centers in the regulation of behaviour [219]. We discovered that *hrh3* was expressed predominantly in glutamatergic neurons of

the pallium. In the zebrafish, neurons from the pallium send extensive projections to lower brain regions, and knockout of *hrh3* could alter the function of these neurons [133]. However, we also detected *hrh3* expression in the optic tectum of the zebrafish. This region is involved in the regulation of visually elicited motor responses, and it is thus also possible that *hrh3* alters the adaptation to darkness at this site as well [249].

Regardless of how *hrh3* knockout alters adaptation to darkness, our results imply that *hrh3* regulates the glutamatergic component in the pallial projection neurons, but we do not know with certainty whether Hrh3 protein is localized postsynaptically in the cell bodies or presynaptically in the axon terminals. The HRH3 radioligand binding profile in the zebrafish brain does not show significant binding in the pallium, suggesting that a presynaptic location of HRH3 in the axon terminals of these neurons is more plausible [338]. Interestingly, *hrh1* is also prominently expressed in the pallium, and this area receives dense histaminergic innervation [133]. Thus, it is possible that histamine could modulate the pallial neurons bidirectionally – exciting them at the cell body and dendrites through *hrh1*, and inhibiting them at the axon terminal through *hrh3*. A diagram of this theoretical circuit is outlined in Figure 6. It would be relevant to study this potential mechanism further in mammals, as it could reveal a complex regulatory role of histamine in the function of cortical projection neurons. Although both *HRH1* and *HRH3* are expressed in the mammalian cortex, their precise location in the cortical circuits remains unknown [67].

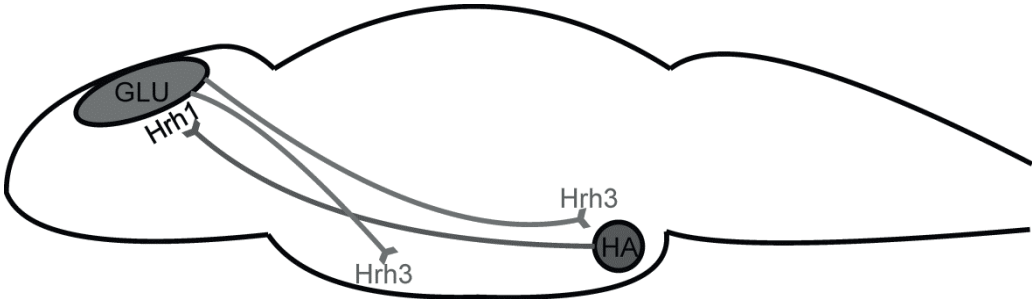

Figure 6. A hypothetical schematic of how histamine receptors could regulate glutamatergic neurons in the dorsal telencephalon. Ascending inputs from the hypothalamic histamine neurons (HA) synapse with the glutamatergic target neurons with Hrh1, which send projections to other brain areas. The axon terminals of the glutamatergic neurons express Hrh3, which regulates transmitter release at these terminals. Projections of neurons are based on earlier studies [133].

6.5 The zebrafish *hrh3* knockout phenotype (III)

Aside from the faster adaptation to darkness described in the previous chapter, *hrh3* knockout had a minimal effect on the behaviour of zebrafish. Adult *hrh3* knockout fish were hypoactive in comparison to controls, but otherwise displayed similar locomotor coordination. This is consistent with studies in mice [154,231]. However, larval zebrafish

displayed completely normal basal locomotion, suggesting a different role of *hrh3* in the locomotor activity of larval and adult zebrafish. This is in contrast to previous results showing that Hrh3 antagonists cause hypoactivity in larval zebrafish [226]. However, this study also reported a similar effect of Hrh3 agonists. Agonists and antagonists of a receptor are unlikely to have the same effect on the animal, and thus it is likely that these pharmacological effects are at least partially explained by nonspecific effects of the compounds. However, the exact binding profile and receptor affinity of these ligands in the zebrafish have not been properly characterized. Differences in HRH3 ligand affinities and effects between species have been previously described between the human and rat isoform of HRH3 [339].

Previous studies have shown histamine and HRH3 to be involved in anxiogenic responses in mice, but this association has remained enigmatic, as described in chapter 2.3.5.2. Histamine has also been suggested as being involved in anxiety in the zebrafish. In adult zebrafish, depletion of histamine increases the time fish spend swimming in the center of the tank (and thus decreases thigmotaxis), which has been interpreted to suggest decreased anxiety-like behaviour [133]. An opposite phenotype is seen in *psen1* knockout fish, which, as adults, have an increased number of histaminergic neurons [296]. We did not find any differences in the place preference of *hrh3* knockout adult fish, suggesting that this response is not mediated by *hrh3*. In order to better understand the involvement of *hrh3* in anxiety, the behavioural response to different anxiogenic conditions and drugs should be evaluated in this model. Additionally, we investigated different metrics of social behaviour in adult *hrh3* knockouts, but found these to be similar to controls. Thus, although *hrh3* inhibitors may have beneficial effects on social behaviour in disease states such as autism [281], it seems that it has a minimal role in regulating social behaviour in the normal brain.

Interestingly, we discovered that *hrh3* knockout larvae had lower levels of dopamine and serotonin than wild type siblings. This effect was not due to an altered morphology of the dopaminergic or serotonergic networks or to altered transcription of enzymes of monoamine synthesis and transport, as all these measures were unchanged between genotypes. This is the first study to characterize monoaminergic systems in *hrh3* knockout animals in detail. Altered transmitter levels in otherwise intact networks suggest that the function of these networks is changed. The mechanism for this remains to be elucidated. However, altered levels of dopamine and serotonin could be of relevance in the observed faster adaptation to darkness. For instance, dopamine is an important regulator of locomotor circuits in the zebrafish, while serotonin regulates light-seeking behaviour in zebrafish larvae [340-342].

In conclusion, the zebrafish *hrh3* mutant displays minimal phenotypes. However, the altered habituation to darkness and decreased dopamine and serotonin levels in *hrh3* knockout zebrafish larvae motivate further studies using this model to better understand how *hrh3* knockout affects the function of the brain. As the zebrafish larva is an exceptionally versatile model for *in vivo* imaging techniques, these could be employed to understand how the brain function is altered at the level of individual neurons [261,263].

6.6 Evaluation of applied methodology (I–III)

The use of zebrafish larvae for these studies minimizes uncontrolled genetic and environmental variation between the animals and experimental groups. Larval zebrafish can easily be raised in standardized conditions on a Petri dish. In our mutant studies, all comparisons were made between siblings, removing the risk of maintaining separate homozygous lines that could have unidentified genetic variation acquired throughout the generations. Thus, the error arising from differences between subjects should be minimal. However, it should be noted that zebrafish strains display a larger genetic variability than inbred rodent strains, which is partially due to a relatively strong inbreeding depression in zebrafish [343]. Although one study has shown preliminary evidence that it might be possible to create inbred zebrafish strains in the future, these strains have yet to be firmly established in the zebrafish research community [344,345]. Therefore, regardless of the experimental setup, zebrafish studies have an inherently larger interindividual genetic variation than mice. It should also be noted that our studies were conducted using a wild type strain that is specific to our animal facility. However, this Turku strain has been used in many publications for over 20 years [20,133,296,297,319,338,346].

Although the need to pool larvae in order to obtain sufficient samples for qPCR and HPLC results in a need for a larger number of animals, this also increases the reliability of the sample, as the measured value will represent a mean of several individuals. However, the small size of the larvae makes it unfeasible to dissect brains from unfixed larvae, and thus, our results may partly be affected by changes in peripheral organs. As *vmat2* and *hrh3* in the zebrafish are both predominantly expressed in the brain, it is unlikely that genetic or pharmacological manipulation of these proteins would result in predominant changes in the periphery [226,347]. Also, histamine and monoamine levels are clearly higher in the brain than in peripheral organs of larvae [237]. Thus, the error introduced into the results by peripheral effects should be minimal.

Our fluorescent *in situ* hybridization protocol allowed for reliable demonstration of coexpression of transcripts, and should also be useful for this purpose in future studies. Of particular interest is the ability to detect transcripts of low abundance, such as GPCR mRNAs. However, as this method still shows less sensitivity than the chromogenic *in situ* hybridization protocol, some regions may be difficult to analyse reliably. In the case of *hrh3*, we were not able to properly evaluate the coexpression of *hrh3* with all relevant markers, such as genes responsible for monoamine synthesis. In our experience, the major obstacle is the significant loss of sensitivity when detecting the second riboprobe. Additionally, *in situ* hybridization is unable to provide information about where in the cell the protein synthesized from the mRNA species is expressed. This would be most properly evaluated by immunohistochemistry, although this method is unfortunately unavailable for many proteins of interest due to the lack of reliable antibodies that work with the zebrafish (although this issue is prominent in most other model organisms as well). The antibodies used in this dissertation have all been thoroughly characterized in previous publications.

As discussed when evaluating the discrepancy in results between comparable knockout, knockdown and pharmacological manipulations, knocking out a gene function completely might lead to compensatory mechanisms in the organism that mask part of the loss of function. Thus, certain normally *hrh3*-dependent mechanisms may still be intact in our knockout model. For instance, lack of this receptor may change the way synapses where this receptor normally is present form during brain development, and thus alter the brain circuitry. The only feasible way to avoid this situation is the creation of inducible knockout lines, where a protein function can be specifically silenced at any stage of development.

7. Conclusions

In this dissertation, I aimed to uncover novel mechanisms in the function of the histaminergic system. Our main conclusions were as follows:

1. Ethanol rapidly upregulates expression of neural *hdc* in a similar manner as *th1* and *th2* are upregulated. Acute ethanol treatment caused a rapid decrease in dopamine levels, which recovered during a longer treatment, suggesting that the upregulation of the *th* mRNA species is a compensatory mechanism. Histamine levels, however, showed no significant changes after ethanol treatment. These results suggest that ethanol rapidly modulates the histaminergic and dopaminergic systems at a transcriptional level. Additionally, ethanol stimulates locomotor behaviour of larval zebrafish, with higher doses being sedative after longer exposure. This is similar to observations in mammals. The rapid changes in the dopaminergic and histaminergic systems occurred in the same time frame as the locomotor activation, suggesting that these networks are likely to be involved in locomotor responses to ethanol in the zebrafish.

2. Inhibition of VMAT2 by reserpine strongly diminished histamine immunoreactivity from both neural soma and axonal projections and decreased total histamine levels. Other monoamine levels were also decreased. However, whereas decreases in other monoamine levels upregulated their synthesis, *hdc* expression was not changed, implying that histamine depletion does not trigger a compensatory upregulation of *hdc*. Additionally, we saw that the histamine-mediated dark-flash response was impaired in reserpinized zebrafish larvae, suggesting that histaminergic transmission was impaired.

3. The optimized protocol for fluorescent *in situ* hybridization with sufficient sensitivity to detect transcripts with low-moderate expression developed in this study was sensitive enough to detect low-level mRNAs. Using this method, we identified most of the *hrh3*-expressing neurons in the dorsal telencephalon to be glutamatergic, while *hrh3* was also detected in individual GABAergic neurons in more ventral regions of the telencephalon. Additionally, we saw expression of *hrh3* in *hdc* mRNA positive neurons.

4. The new zebrafish model with a loss-of-function allele of *hrh3* was phenotyped in this study. The *hrh3* knockout larvae showed a faster habituation to sudden darkness, which suggests a role of *hrh3* in the adaptation and regulation of responses to sudden changes in the environment. Adult *hrh3* knockout fish were hypoactive, while larvae showed normal locomotor activity, suggesting a different role of *hrh3* in regulation of

locomotion in different developmental stages. Social behaviour was unaffected by *hrh3* knockout. *hrh3* knockout did also not affect the development of aminergic networks or the expression of key genes in monoaminergic neurotransmission. However, dopamine and serotonin levels were decreased, which may reflect an altered function of these networks.

8. Questions for Future Research

1. The acute histaminergic responses to ethanol still need to be evaluated further, particularly as regards how ethanol affects the activity of *hdc*. As we discovered that ethanol upregulates *hdc*, it would also be of interest to study possible alcoholism-related polymorphisms in the human *hdc* promoter, to see if this mechanism has clinical significance.

2. The role of VMAT2 in the function of histaminergic neurons needs to be studied further in models lacking and overexpressing VMAT2 in the histamine neurons specifically. This could reveal how VMAT2 affects histamine turnover and metabolism, which could be relevant when considering the pathogenetic mechanisms related to VMAT2 polymorphisms.

3. Differences in histamine metabolism and turnover among histaminergic neurons should be investigated. This could most robustly be done by single-cell sequencing of the transcriptome of these neurons.

4. Inducible knockout models of histamine receptors and *hdc* should be generated, in order to see how removal of specific components of the histaminergic system in a normally developed brain affects behaviour and other parameters. Additionally, the changes in brain activity patterns under different conditions caused by histamine receptor and *hdc* knockout (both inducible and classic ones) should be characterized.

5. In order to better understand the partially contradictory results obtained with our Hrh3 knockout fish and Hrh3 ligands, the properties of zebrafish histamine receptors need to be characterized using *in vitro* expression of the receptors in cells, and the affinities and effects of histamine receptor ligands at the receptors should be determined.

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