A Novel Developmental Role for Dopaminergic Signaling to Specify Hypothalamic Neurotransmitter Identity

Yu-Chia Chen, Svetlana Semenova, Stanislav Rozov, Maria Sundvik, Joshua L. Bonkowsky, and Pertti Panula

From the Neuroscience Center and Department of Anatomy, University of Helsinki, 00290 Helsinki, Finland and the Department of Pediatrics, University of Utah School of Medicine, Salt Lake City, Utah 84113

Hypothalamic neurons expressing histamine and orexin/hypocretin (hcrt) are necessary for normal regulation of wakefulness. In Parkinson’s disease, the loss of dopaminergic neurons is associated with elevated histamine levels and disrupted sleep/wake cycles, but the mechanism is not understood. To characterize the role of dopamine in the development of histamine neurons, we inhibited the translation of the two non-allelic forms of tyrosine hydroxylase (th1 and th2) in zebrafish larvae. We found that dopamine levels were reduced in both th1 and th2 knockdown, but the serotonin level and number of serotonin neurons remained unchanged. Further, we demonstrated that th2 knockdown increased histamine neuron number and histamine levels, whereas increased dopaminergic signaling using the dopamine precursor L-DOPA (L-3,4-dihydroxyphenylalanine) or dopamine receptor agonists reduced the number of histaminergic neurons. Increases in the number of histaminergic neurons were paralleled by matching increases in the numbers of hcrt neurons, supporting observations that histamine regulates hcrt neuron development. Finally, we show that histaminergic neurons surround th2-expressing neurons in the hypothalamus, and we suggest that dopamine regulates the terminal differentiation of histaminergic neurons via paracrine actions or direct synaptic neurotransmission. These results reveal a role for dopaminergic signaling in the regulation of neurotransmitter identity and a potential mechanism contributing to sleep disturbances in Parkinson’s disease.

Dopamine is a critically important neurotransmitter in the vertebrate brain. It is involved in motor functions, conditioned behaviors, and hormone regulation (1). There is also an increasing body of evidence to suggest that dopamine has neurotrophic functions in the central nervous system (2, 3). For example, dopamine from the brain promotes the generation of motoneurons in the zebrafish spinal cord (4). In human disease conditions affecting the dopaminergic system, marked changes in the histaminergic system have been observed (5). Histamine, a modulatory neurotransmitter, is necessary for sleep/wake cycle function, alertness, memory, and hormonal regulation (6), and its functions are disrupted by neurological diseases, including narcolepsy, Gilles de la Tourette syndrome, and schizophrenia (5). In Parkinson’s disease, the nigrostriatal dopaminergic system is severely damaged, and a concomitant increase in brain histamine levels (7) and denser histaminergic fiber networks have been observed (8) in the striatum and substantia nigra. In schizophrenia, the modified dopamine hypothesis proposes decreased dopaminergic activity in some brain regions (9), and an increase in histamine turnover has been reported (10). The potential regulatory role of dopamine for histaminergic neuron development is difficult to study in mammals because tyrosine hydroxylase knockout mice do not survive (11, 12).

In zebrafish (Danio rerio), two non-allelic forms of th are expressed in the brain in a largely complementary manner (13–15). However, the biological roles of the two th forms are not clear. In this report, we studied the roles of th2 in the developing zebrafish brain. We used antisense morpholino oligonucleotides (MOs)2 to knock down th2 gene expression and dopamine receptor ligands to alter dopaminergic signaling and analyzed the effects on the catecholaminergic, histaminergic, serotonergic, and hypocretin systems. Our results show that th2 is essential in dopamine production and reveal a novel regulatory role for th2-dependent dopaminergic signaling during zebrafish development in the specification of hypothalamic histamine and hypocretin neurons.

Results

Hypothalamic Histaminergic and Serotonergic Neurons Are Distinct from th2 Dopaminergic Neurons—Tgf(TH:egfp) transgenic fish express GFP under the control of the fugu th promoter (16). In the 5-dpf zebrafish brain, GFP expression was seen in olfactory bulbs, the preoptic region, the paraventricular organ, and caudal periventricular hypothalamic zones (Fig. 1A). To further characterize the identity of GFP-positive cells in the hypothalamus, we performed in situ hybridization (ISH) with th2 riboprobes, followed by immunohistochemistry (IHC) using TH1, TH2, histamine, or 5-HT antibodies on 5-dpf

2 The abbreviations used are: MO, morpholino oligonucleotide; dpf, day(s) post-fertilization; ir, immunoreactive; his-ir, histamine immunoreactivity; IHC, immunohistochemistry; hpf, hour(s) post-fertilization; WISH, whole-mount in situ hybridization; ISH, in situ hybridization; hcrt, hypocretin; PV/Op, posterior part of the paraventricular organ; qPCR, quantitative PCR; MANF, mesencephalic astrocyte-derived neurotrophic factor; PFA, paraformaldehyde; DIG, digoxigenin; ANOVA, analysis of variance; His, histamine; ctrl, control; L-DOPA, L-3,4-dihydroxyphenylalanine; TH, tyrosine hydroxylase.

1 To whom correspondence should be addressed: Neuroscience Center and Dept. of Anatomy, POB 63 (Haartmaninkatu 8), 00014 University of Helsinki, Finland. Tel.: 358-9-191-25263; Fax: 358-9-191-25261; E-mail: pertti.panula@helsinki.fi.
Tg(f.TH:egfp) fish brain. We found that GFP-positive cells in the caudal hypothalamus expressed th2 mRNA (Fig. 1, B–D) and showed TH2 immunoreactivity (Fig. 1E). Larger magnification of single-channel and merged images depicted GFP and th2/TH2 co-localization in TH2 group 10b (Fig. 1, D and E), suggesting that GFP-ir cells co-expressing th2/TH2 in group 10b represent th2-containing populations (dopamine cell population 10/10b; the nomenclature used in this study is based on Sallinen et al. (17) and Chen et al. (13); or DC7 group according to Rink and Wullimann (18)). GFP-positive but TH2-negative cells were found in the olfactory bulb, telencephalon, preoptic region, and caudal hypothalamus (Fig. 1, A–D, white arrows). This may be caused by the differences and insufficiency of regulatory motifs driving GFP expression between zebrafish and fugu. Histamine immunoreactivity (his-ir), a histaminergic neuron marker, was confined to neurons in the caudal hypothalamus around the catecholaminergic cell group in the nucleus of the posterior recess (19, 20). The his-ir cells were located peripherally surrounding the GFP-immunoreactive (ir) cells of the population 10/10b in the caudal hypothalamus region (Fig. 1F, larger magnification shown in Fig. 1F'), and no co-existence of GFP and histamine was found in any single optical scanning frame. Serotonergic (5-HT) cells, which also reside in this region (19, 21), did not show any GFP-ir (Fig. 1G; a single section image shown in Fig. 1G'). Furthermore, we performed triple immunostaining on 5-dpf zebrafish brains using anti-TH1, anti-TH2, and anti-5-HT antibodies (Fig. 1H). The staining result showed that none of the TH1-ir or TH2-ir cells were immunoreactive for 5-HT (Fig. 1H'), suggesting that TH2-ir cells are distinct from serotonergic neurons. In summary, GFP-ir-th2/TH2-expressing cells were surrounded by histaminergic neurons and intermingled with serotonergic neurons in the nucleus of the posterior recess in the caudal hypothalamus. These results are also in agreement with TH2-ir distribution in the adult zebrafish brain (22).

Both TH1 and TH2 Contribute to Catecholamine Synthesis—
Based on the amino acid identity and phylogenetic analysis among invertebrate and vertebrate species, zebrafish th1 and th2 are classified as counterparts of the mammalian th (23, 24). However, it is still unclear whether TH2 has tyrosine hydroxylase activity because Ren et al. (25) reported that zebrafish th2 acts as tryptophan hydroxylase during development. To investigate the impact of th1 and th2 on catecholamine and 5-HT metabolism, the concentrations of dopamine, norepinephrine, epinephrine, and 5-HT were measured by HPLC using 5-dpf zebrafish heads following translation inhibition of th1 or th2. The efficacy of MOs used in this study was verified by Western blotting using an anti-TH2 antibody that recognizes both zebrafish TH1 and TH2 populations (22). The robust specific signals around 55 kDa representing the expected sizes of TH1 and TH2 proteins were observed in the 5-dpf head homogenate and th2-ish, TH2-ir, His-ir, and 5-HT-ir in red in the transgenic line (D–G). 5-HT-ir is shown in green in H. White arrows indicate GFP-positive but th2/TH2-negative populations. Cells labeled with both TH1 and th2 are shown in magenta. Cells labeled with both GFP and TH2 are shown in yellow. 08, olfactory bulb; 3b, preoptic group (Po); 8b, paraventricular organ; 9b, nucleus of lateral recess; 10b, caudal hypothalamus (Hc). TH2 group numbers are based on Ref. 13. Scale bars = 50 μm.

FIGURE 1. Multiple labeling of catecholaminergic, histaminergic, and serotoninergic neurons and GFP distribution in 5-dpf brains of the Tg(f.TH:egfp) transgenic line and Turku WT. A, ventral view of GFP distribution in a 5-dpf brain. The specimens initially hybridized (ISH) with th2 antisense riboprobes were processed for double immunostaining with chicken GFP and mouse TH1 antibodies (shown in B–D). B, ventral view of triple staining showing GFP-ir in green, TH1-ir in blue, and the th2 mRNA expression pattern in red. C, lateral view of B. The distribution patterns in the diencephalic and hypothalamic regions of GFP and TH1 with TH2, histamine (His), or 5-HT are shown in E–G, respectively. A triple immunostaining image with anti-TH1, anti-TH2, and anti-SHT on a 5-dpf brain is shown in H and H'. Larger magnification and single section images of group 10b (white rectangle) in D–H are shown in D–H', respectively. The GFP-ir signal is shown in green, TH1-ir in blue, and th2-ISH, TH2-ir, His-ir, and 5-HT-ir in red in the transgenic line (D–G). 5-HT-ir is shown in green in H. White arrows indicate GFP-positive but th2/TH2-negative populations. Cells labeled with both TH1 and th2 are shown in magenta. Cells labeled with both GFP and TH2 are shown in yellow. 08, olfactory bulb; 3b, preoptic group (Po); 8b, paraventricular organ; 9b, nucleus of lateral recess; 10b, caudal hypothalamus (Hc). TH2 group numbers are based on Ref. 13. Scale bars = 50 μm.
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(supplemental Fig. S1). On the other hand, several strong bands between 40 and 55 kDa that were detected in the 36-hpf whole-embryo homogenate were clearly less prominent in the 36-hpf deyolked embryo (supplemental Fig. S1), suggesting that these nonspecific signals can be due to the interactions between the TH2 antibody and yolk proteins. In Fig. 2A, besides the specific bands around 55 kDa of TH1/TH2, the uneven signals between 40 and below 55 kDa among groups may thus be due to yolk differences in different morphants. Although the use of deyolked embryo homogenates is possible, the manual deyolking procedure of 5-dpf larvae is time-consuming and may easily cause protein degradation during sample preparation. To avoid preparation bias, we used whole larval protein lysates for the Western blotting analysis in this study. The Western blot showed that the TH1 protein was reduced 70% in the th1 morphants, whereas the amount of TH2 protein was essentially unchanged. On the other hand, only 45% of TH2 protein remained in 5-dpf th2 morphants. When th2 mRNA was co-injected with th2 MO1 + 2 (th2 rescue) in th2 morphants, a 40% increase in TH2 protein was detected in th2 morphants compared with the control-injected group (Fig. 2A). The th2 morphants developed with a normally sized head, eyes, trunk (Fig. 2B, F(5, 169) = 2.139, p = 0.0632), and notochord without any apparent defects or any distinguishable gross phenotype (Fig. 2C). The levels of p53 and delta113p53, recognized as off-targeting markers usually induced by MO knockdown (26), were not elevated in the th2 morphants (Fig. 2D, F(3, 8) = 1.216, p = 0.3648; Fig. 2E, F(2, 8) = 1.394, p = 0.3106), suggesting that th2 MOs specifically knocked down TH2 protein expression without causing obvious off-targeting effects. The HPLC results showed that the dopamine, norepinephrine, and epinephrine levels were significantly decreased in th1 morphants (Figs. 2, F–I). A significant reduction in dopamine level was also detected in th2 morphant groups (Fig. 2F, F(7, 16) = 8.942, p = 0.0002), but the norepinephrine and epinephrine levels were unaffected compared with those of control morphants (Fig. 2G, F(7, 16) = 9.372, p = 0.0001; Fig. 2H, F(7, 16) = 6.754, p = 0.0008). 5-HT levels were unaffected in both th1 and th2 morphants (Fig. 2I, F(7, 16) = 2.575, p = 0.0556). The group treated with p-chlorophenylalanine (an inhibitor of tryptophan hydroxylase) was used as a 5-HT intervention control. The MO efficiency was documented here similarly as described in detail earlier (22). Our data show that TH1 is responsible for catecholamine biosynthesis, including dopamine, norepinephrine, and epinephrine, whereas TH2 is responsible only for dopamine synthesis. This result is concordant with the expression pattern of dopamine beta-hydroxylase (dbh); only th1 is expressed in cells that also express dbh (required for the synthesis of norepinephrine and epinephrine) (19).

Dopaminergic and Serotonergic Markers in th2 Morphants—To study whether loss of TH2 function affects the development of catecholaminergic neurons and neighboring serotonergic neurons expressing tph1a, WISH was performed on 5-dpf fish brains. The dopamine transporter (slc6a3), responsible for dopamine reuptake from the synaptic cleft, is present in many dopaminergic cells and is commonly used as a marker of dopaminergic neurons (27). As shown in Fig. 3, A and B, the expression of catecholamines and serotonin in morphants. A, Western blotting analysis using 5-dpf larvae with anti-TH2 antibody. ProAct membrane stain was used as the loading control. B, quantification of body length. The sample number of each group and mean ± S.E. are indicated in the graph. C, bright-field image of the morphants. D and E, qPCR analysis of p53 and delta113p53 transcript levels (n = 3/group, Student’s t test). Catecholamine and serotonin concentrations were measured using HPLC. Fifteen 5-dpf heads were homogenized for each group, and three individual groups (n = 3) per treatment were analyzed. F, dopamine. G, norepinephrine. H, epinephrine. I, serotonin. th2 rescue is th2 MO1 + 2 co-injected with th2 mRNA. *, p < 0.05; **, p < 0.01; ***, p < 0.001; one-way ANOVA with Tukey’s test. Scale bar = 500 μm.
pattern of slc6a3 mRNA was intact in th2 morphants. Vesicular monoamine transporter 2 (VMAT2) is responsible for reuptake of monoamines, including dopamine, 5-HT, norepinephrine, and histamine, in nerve terminals (28). The VMAT2 mRNA expression pattern was not altered in th2 morphants (Fig. 3, C and D). Tryptophan hydroxylase is involved in the biosynthesis of serotonin. The expression pattern of tph1a (Fig. 3, E and F) and the number of serotonergic neurons in the ventrocaudal hypothalamus (5-HT group 4) (29), shown by 5-HT immunohistochemistry (Fig. 3, G–I; \( p = 0.7953 \), Student’s \( t \) test) were largely unaffected in th2 morphants, in agreement with the unchanged 5-HT concentration in 5-dpf morphant brains (Fig. 2G), indicating that knockdown of th2 did not affect the serotonergic system.

**TH2 Plays a Role in the Regulation of Histaminergic Neuron Development**—To study whether th2 deficiency affects the development of histaminergic neurons, hdc ISH and double-labeling immunohistochemistry were performed on 5-dpf brains. The number of histaminergic neurons (Fig. 4, A, B, and G (\( F_{(5, 48)} = 12.58, p < 0.0001 \)); Fig. 4, D, E, and H (\( F_{(5, 39)} = 21.58, p < 0.0001 \)) was significantly higher in th2 morphants than in control morphants. th2 mRNA co-injection efficiently normalized the number of histaminergic neurons in th2 morphants (Fig. 4, C and F–H), showing that the phenotype was specific for th2 MO1 + 2. Moreover, the histamine level measured with HPLC was significantly increased in th2 morphants compared with that in control morphants or in the th2 morphant mRNA rescue group (Fig. 4I, \( F_{(5, 12)} = 8.649, p = 0.0011 \)).
These data indicate that the development of histaminergic neurons is driven in part by the catecholaminergic TH2 neurons in the zebrafish brain.

Effects of th2 Knockdown on Orexin/Hypocretin (hcrt) Neuron Development—It has been reported that the histaminergic neurons regulate the development of hcrt neurons through histamine receptor H1 in zebrafish (20). Here, lack of th2 expression was associated with an increased number of histaminergic neurons. To learn whether hcrt neuron development was affected, possibly as a consequence of the histaminergic neuron alteration as described earlier (20), in the th2-deficient morphants, hcrt ISH was performed on 5-dpf fish brains. As shown in Figs. 5, A and B, a significant increase in hcrt-positive cells was observed in th2 MO1 + 2-injected fish (Fig. 5C, p = 0.0313, Student’s t test) as the histaminergic hdc-containing cell number was increased (Fig. 4).

Dopamine Regulates Histaminergic and Hypocretin Neurons—The dynamic changes of histaminergic neurons in th2 morphants suggest that dopamine might be important for the development of target neuron populations. Dopaminergic signaling is mediated by G protein-coupled dopamine receptors, which are grouped into two main subtypes.
The D1-like receptors comprise D1 and D5 receptors coupled to stimulatory G proteins to activate adenylyl cyclase. The D2-like receptors, including D2, D3, and D4 receptors, are coupled to inhibitory G proteins and inhibit CAMP synthesis (1). To study whether dopamine or its precursor L-DOPA and specific subtypes of dopamine receptors are involved in the regulation of the histaminergic neuron development, 24-hpf fish embryos were treated with 10 mM L-DOPA (dopamine precursor), 10 μM SKF38393 (D1-like receptor agonist), 10 μM SCH23390 (D1-like receptor antagonist), 7.5 μM quinpirole (D2-like receptor agonist), and 7.5 μM haloperidol (D2-like receptor antagonist) until 5 dpf. Relevant drug concentrations were determined in preliminary experiments (data not shown) based on previous publications (4, 30). The drug effect on the histaminergic system was studied by counting the histamine-ir cells. We found that wild-type larvae treated with L-DOPA, quinpirole, and SKF38393 showed a significant reduction in number of histaminergic hdc-expressing cells compared with the untreated control fish (Fig. 6, A, D, G, J, and O; F(7, 63) = 11.06, p < 0.0001), whereas a significant increase in TH1-ir cells was observed in L-DOPA- and quinpirole-treated groups (Fig. 6, B, E, H, K, and M; F(7, 56) = 15.60, p < 0.0001); Fig. 6N (F(7, 56) = 12.51, p < 0.0001) in the posterior part of the paraventricular organ (PVO) and diencephalic complex, including TH1 populations 10 and 13, where histaminergic neurons are neighboring (population number based on earlier descriptions). Interestingly, dopamine receptor antagonist treatment normalized the alterations of cell numbers caused by dopamine receptor agonist administrations, although antagonists alone did not affect the dopaminergic and histaminergic neurons (Fig. 6, M–O). Furthermore, L-DOPA and quinpirole treatment restored (normalized) the increased histaminergic neuron numbers in th2 morphants (Fig. 6P, F(3, 31) = 12.5, p < 0.0001), indicating that histaminergic neurons were affected by dopamine signaling activity during zebrafish development.

Wnt Signaling Affects Dopaminergic and Histaminergic Neuron Development—The Wnt signaling cascade is essential for hypothalamic progenitor differentiation (31), and overexpression of the Wnt antagonist dickkopf1 (dkk1) mRNA elevates the number of dopaminergic th1-expressing neurons in 2-dpf zebrafish brains (32). To study whether Wnt signaling is involved in the regulation of histaminergic neuron development, his-ir and TH1-ir cells were counted following double staining of histamine and TH1 in 5-dpf fish brains after DKK1 mRNA injections in the yolk at the one-cell stage. The inhibition effect on Wnt signaling was verified by testing the expression of the Wnt downstream targets zic2a and zic5 (33). A decreased signal of zic2a (Fig. 7I) and zic5 (data not shown) in the telencephalon, midbrain, and hindbrain was detected in the DKK1 mRNA-injected group. We next found that TH1-ir cells were robustly increased in the PVO and diencephalic complex in TH1 cell populations 10 and 13 (Fig. 7, B, E, J, and K; p < 0.0001, Student’s t test), which supports the result of Russek-Blum et al. (32). Moreover, a significant increase in th2-containing cell numbers was detected in TH2 cell populations 10b and 11b–9b (Fig. 7, G and H; p < 0.0001 and p = 0.0096, Student’s t test). These cell populations are relevant for histaminergic neurons because these dopaminergic neurons are surrounded by histaminergic neurons (Fig. 1F). In contrast to the effect on TH1 populations, histaminergic neurons were significantly decreased in number in PVO (Fig. 7, A, D, and L; p = 0.0033, Student’s t test), the only site where histaminergic neurons are found in the zebrafish brain. An overlay image of histamine-ir and TH1-ir is shown in Fig. 7, C and F.

We further used qPCR to examine the expression levels of representative genes of the histaminergic, hypocretin, and dopaminergic systems, including receptors and neurotrophic factors important for the dopaminergic system. The th1 and th2 transcripts in the larval heads were not altered, although the TH1 and th2-containing cell numbers in cell groups 10/10b and 13/8–9b were increased by about 36% and 15%, respectively, following DKK1 overexpression. The reason for the evident discrepancy between the cell counting result and the qPCR may be that only two populations adjacent to histaminergic neurons were counted in the brain, but the qPCR was done on the whole head, so that the total change could simply be too subtle to be detected in the overall th expression. A significant reduction of drd3 mRNA was also detected (Fig. 7P, p = 0.0258), and the expression level of hdc and hctr transcripts declined significantly (Fig. 7P, p = 0.0368 and p = 0.026, respectively), confirming the reduction of the His-ir and hctr neurons. Mesencephalic astrocyte-derived neurotrophic factor (MANF) is recognized as a dopaminergic neurotrophic factor (34, 35). Remarkably, a significant increase in manf transcripts was found in the DKK1 overexpression group (Fig. 7P, p < 0.001). To study whether MANF affects histaminergic neuron development, we knocked down manf expression by MOs and compared the number of hdc-expressing cells between control.
MO-injected, and mRNA-rescued morphant groups. We found that the number of hdc-containing cells was significantly higher in the MANF-deficient morphants than in the control (ctrl) MO group and manf mRNA rescue morphants (Fig. 7O, $F_{(2,29)} = 6.104, p = 0.0061$). MANF deficiency thus not only causes the reduction in th1 and th2-containing cells (35) but also leads to a significant increase in hdc-expressing cell numbers found in this study. Moreover, DKK1 overexpression associated with a robust increase in manf transcripts and TH1-containing cells and a reduction in histaminergic cell number suggests that MANF has an impact on histaminergic system development.

**Discussion**

Here we report that dopaminergic signaling controls the numbers of histaminergic and hypocretin neurons in the vertebrate CNS. Using differential knockdown of the zebrafish th genes th1 and th2, we showed that TH2 acts as the mammalian tyrosine hydroxylase in dopamine synthesis and is involved in the regulation of histaminergic and hcrt neuron numbers. We confirmed our findings using experimental manipulations of dopaminergic signaling, including the use of dopamine agonists and Wnt antagonism.

The anatomy of the catecholaminergic systems in both larval and adult zebrafish brain has been well studied by tract tracing (18), in situ hybridization (27), and immunohistochemistry using tyrosine hydroxylase (TH) and dopamine antibodies (15, 17, 19, 21, 27, 36–39). In the embryonic zebrafish brain, th1 and th2 show a complementary expression pattern, and th1 expression is more widespread than th2 (13, 14). th1 shares higher amino acid sequence similarity with mammalian th genes, and comparative analysis of TH1-ir populations between zebrafish and mammals has revealed many conserved features and some differences (17–19).

Ren et al. (25) used the Tg(Etvmat2-GFP) transgenic line having GFP expression in vmat2 neurons as a reference marker to indirectly show that th2 and 5-HT were co-localized in the ventral diencephalon and the caudal hypothalamus. The conclusion is contradictory to our current findings and several other reports, which confirm the fact that zebrafish th2 has tyrosine hydroxylase activity in dopamine synthesis (39, 40). In addition, VMAT2 is found in all aminergic neurons, including dopaminergic, histaminergic, noradrenergic, and serotonergic neurons (28, 41). Based on the conserved structure and functional domain comparison of tyrosine hydroxylase among species (22), the co-existence of dopaminergic cell markers with th2 (14, 15, 27), the intensive dopamine immunoreactivity in neurons which express th2 (22, 27, 40), and the functional assays provided in this study strongly indicate that TH2 has tyrosine hydroxylase activity and contributes to the synthesis of dopamine. Moreover, no specific co-localization exists among TH2-ir, histaminergic, and serotonergic markers in the caudal hypothalamus in the 5-dpf and adult zebrafish brain (22). In this study, we provide high-resolution images with multiple labels using Tg(f.TH:egfp) transgenic fish having GFP expressed in th2-expressing cells to prove that th2- and serotonin-containing cells are distinct in the caudal hypothalamus. We also measured endogenous brain catecholamine and 5-HT levels by HPLC. The 5-HT level and tph1a mRNA expression were not affected in th2MO1 + 2 morphants, whereas dopamine levels declined, and the effects were rescued by th2 mRNA co-injection. On the other hand, in the study by Ren et al. (25), neither endogenous tph1a activity and expression level nor dopamine concentration in th2 morphants were analyzed. Additionally, we also analyzed and reported the primary structure of zebrafish TH1 and TH2 (22). In brief, TH2 possesses the arginine residues needed for feedback inhibition by dopamine, and the Leu, Trp, and Asp residues required for substrate (tyrosine) binding are conserved. Importantly, the Leu residue Leu-294 is present in zebrafish TH1 and TH2, whereas, in tryptophan hydroxylase, this is replaced by a Tyr residue. Our findings strongly support that TH2 can function as an active tyrosine hydroxylase, a conclusion that could be further verified by studying a stable th2 mutant fish when one becomes available.

The hypothalamus is responsible for regulating metabolic processes, sleep, and circadian cycles, in which dopamine is also involved (42). Here we first reported that loss of th2 expression caused a significant increase in histaminergic and hypocretin cells as well as histamine level in larval brains, suggesting that th2-expressing neurons affect the development of the adjacent neurons, possibly by direct innervation through synaptic neurotransmission or paracrine actions. This provides evidence that dopamine actively limits the number of histaminergic neurons and fibers, which is in agreement with findings reported in post-mortem brains with Parkinson’s disease. The density of histaminergic fibers in the substantia nigra pars compacta is increased (8), and an increase in histamine level is restricted to brain areas that are affected by lack of dopamine (7).

There is good evidence of dopaminergic regulation of histamine neurons from rodent studies (43). This study showed that rodent histamine neurons are excited by l-DOPA and both dopamine D1 and D2 receptor agonists and that they express both D1- and D2-type dopamine receptors. These results are in full agreement with our results on zebrafish. There is also a growing body of evidence indicating that histamine receptor 3 directly interacts with dopaminergic neurotransmission and forms heterodimers with dopamine receptors in the rodent dorsal striatal target neurons. In zebrafish, it is still unclear whether similar interactions occur in the hypothalamus, where histamine receptor 3 and dopamine receptors are found.

To our knowledge, the developmental regulation of hdc-expressing neurons is largely unknown, except that γ-secretase

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**FIGURE 6. Dopamine receptor agonists affect the histaminergic and dopaminergic neuron numbers in 5-dpf fish brains.** The cell numbers were quantified following histamine and TH1 co-immunostaining. A–C, no treatment. D–F, 10 μM L-DOPA treatment. G–I, 7.5 μM quinpirole treatment. J–L, 10 μM SKF38393. Quantification of TH1-ir cell numbers after dopamine receptor agonist and antagonist administration is shown in PO (TH1 group 10, M) and in the diencephalic complex (TH1 group 13, N). Quantification of his-ir cell numbers is shown in Q. Quantification of his-ir cell numbers in th2MO1 + 2 morphant brains after drug treatments is shown in P. TH1 group numbers 10 and 13 are based on Ref. 61, corresponding to DC7 and DC6 in Ref. 36. His-ir positive cells are shown in green. TH1-labeled cells are shown in red. The number of brains analyzed and the mean value of the cell number are shown in the columns. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; one-way ANOVA with Tukey’s test. Scale bar = 100 μm.
FIGURE 7. Dynamic effects of Wnt signaling on dopaminergic and histaminergic neurons. The images of His-ir and TH1-ir cells are depicted in the wild-type group (A–C) and the DKK1 mRNA overexpression group (D–F). Images of th2-FISH distributions are shown in G and H. I, overexpression of Dkk1 mRNA down-regulates zic2a expression in the telencephalon (tel), midbrain (m), and hindbrain (H) regions compared with the WT. Comparisons of TH1-ir cell numbers between WT and DKK1 mRNA overexpression brains in PVOp (TH1 group 10) and in the diencephalic complex (TH1 group 13) are shown in J and K, respectively. Quantification of His-ir cell numbers is shown in L. Quantification of th2 expression cell numbers in PVOp (TH2 group 10b) and in the diencephalic complex (TH2 groups 8b and 9b) are shown in M and N, respectively. Quantification of hdc-positive cell numbers in the ctrl MO, manf MO, and manf rescue morphant brain is demonstrated in O. P, levels of mRNA expression by qPCR (n = 3). Fold changes were calculated relative to the average expression of wild-type groups. TH1 and TH2 group numbers are based on Ref. 61. TH1-labeled cells are shown in red. His-ir positive cells are shown in green. Arrows indicate regions where zic2a expression is reduced. The number of brains analyzed (n) and the mean value of the cell number are shown in the columns. *, p < 0.05; **, p < 0.01; ***, p < 0.001; Student’s t test or one-way ANOVA with Tukey’s test. Scale bars = 100 μm.
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Experimental Procedures

Zebrafish Strain and Maintenance—Zebrafish were obtained from our breeding line maintained in the laboratory for more than a decade (17, 19, 29, 54). Fish were raised at 28 °C and staged in hours post-fertilization or days post-fertilization as described previously (55). The Tg(f:TH:egfp) transgenic line was reported previously (Tg(f:TH:egfp)esc60) by Fujimoto et al. (16); the Zebrafish Model Organism Database nomenclature is Tg(True:Th:EGFP)esc6. The permits for the experiments were obtained from the Office of the Regional Government of Southern Finland in agreement with the ethical guidelines of the European convention.

Characterization of Rabbit TH2 Antibody—The TH2 antibodies used in this study were produced against a recombinant protein containing a 150-amino acid N-terminal fragment of TH2 tagged by GST at the N terminus (22). The crude antiserum (TH2 169C) reacted with both forms of zebrafish TH (TH1 and TH2). The TH2 antiserum can be used in combination with a monoclonal antibody targeting TH1 to allow identification of single-stained TH2 cells and double-stained TH1 cells. The full characterization of the antibodies is described in detail elsewhere (22).

Western Blotting—Western blotting analysis was performed as described previously (22). Briefly, 5-dpf zebrafish larvae were collected and manually homogenized on ice in 0.05 M Tris-HCl buffer (pH 7.5) containing Complete Mini protease inhibitors (Roche) and 0.3 mM PMSF. 40 μg of proteins was loaded on each lane, followed by blotting onto PVDF membranes, and the membranes were stained with ProAct membrane stain (M282–1L, Amresco Inc.) as the loading control. Rabbit TH2 antiserum was preadsorbed on PFA-fixed 1-dpf embryos, and primary and secondary antibodies were diluted 1:3000. The intensities of the Western blotting bands were measured by ImageJ 1.49c.

HPLC—The heads of 5-dpf larvae were analyzed following removal of the eyes and trunks on ice. Fifteen dissected heads were grouped and lysed in 150 μL of 2% perchloric acid with sonication. After centrifugation, 10 μL of supernatant was collected and manually homogenized on ice in 0.05 M Tris-HCl buffer (pH 7.5) containing Complete Mini protease inhibitors (Roche) and 0.3 mM PMSF. 40 μg of proteins was loaded on each lane, followed by blotting onto PVDF membranes, and the membranes were stained with ProAct membrane stain (M282–1L, Amresco Inc.) as the loading control. Rabbit TH2 antiserum was preadsorbed on PFA-fixed 1-dpf embryos, and primary and secondary antibodies were diluted 1:3000. The intensities of the Western blotting bands were measured by ImageJ 1.49c.

RNA Isolation and cDNA Synthesis—For quantitative real-time PCR analysis, total RNA was extracted from 15 pooled fish heads collected at 5 dpf (RNeasy mini kit, Qiagen, Valencia, CA). To synthesize cDNA, 2 μg of total RNA was reverse-transcribed using SuperScript™ III reverse transcriptase (Invitrogen) according to instructions provided by the manufacturer. The primers for cloning tph1a, zic2a, and zic5 were as follows: tph1a, 5′-CCATGAACTCGGAATGACTT-3′ and 5′-CTCTGAAACGTGGTGTGATGCA-3′; zic2a, 5′-GGATGTGATCGACGCTTTGC-3′ and 5′-AAATGCCCTGGTTACCCCA-3′; and zic5, 5′-ACATAGCGTTGACGGTGGA-3′ and 5′-ATTTCCTGTCGACGCATCA-3′.

MO Design and Use and mRNA Injections—Antisense MOs (Gene Tools LLC, Philomath, OR) were designed to target the splice donor sites of exon 2 and exon 4 of th1 (th1 MO1, 5′-AT-TATGGTACCTCCTCGAAAACC-3′; th1 MO2, 5′-TATCCAGCACTTACGGGTGATGAC-3′), the splice donor sites of exon 3 and exon 7 of th2 (th2 MO1, 5′-CTGTTGTCATCTACAGGGTGATC-3′; th2 MO2, 5′-TTATGGCATTGACGTTACGTCGAGG-3′), and splice donor sites of exon2 and exon3 of manf (manf MO1, 5′-GACGGGTACTTA-
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CaaTcGGTTTCT-3; manf MO2, 5-TGCAAACACT-CACCGTATTGTGAGT-3). The working concentration was determined by injecting serially diluted MOs. The injection dose of th2 MO1 or th2 MO2 was 8 ng. The combination doses of 3.5 ng each of th1 MO1 and th1 MO2 (th1 MO1 + 2), 4 ng each of th2 MO1 and th2 MO2 (th2 MO1 + 2), and 4 ng each of manf splice-blocking MOs (manf MOs) were found to produce the most effective inhibition (22, 35). A standard control MO (ctrl MO, 5-CCTTACCTACCTGATATTTA-3”) purchased directly from Gene-Tools was injected at 8 ng/embryo. The th2 full-length open reading frame cDNA constructs were prepared by RT-PCR using Phusion High-Fidelity PCR Master Mix (Finnzymes, Espoo, Finland). The primers for each of

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CATGTTCCGATTTCT-3
CACATC-3
GAGGG-3
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were prepared by RT-PCR using Phusion High-Fidelity PCR Master Mix of the manufacturer. The WISH procedure followed the instructions of the manufacturer. The WISH procedure followed the protocol of Thissex and Hisse (58). Prehybridization and hybridization were conducted at 65 °C for all riboprobes. In situ hybridization signals were detected with sheep anti-digoxigenin-AP Fab fragments (1:10,000, Roche Diagnostics). Color staining was carried out with chromogen substrates (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphatase). For th2 fluorescent in situ hybridization (FISH), samples were incubated in 2% H2O2 in methanol for 20 min to inactivate endogenous peroxidase activity. A th2-DIG-labeled probe was used for hybridization. To visualize the hybridized probe, samples were incubated in peroxidase-conjugated anti-DIG antibody (1:500, Roche, 11207733910) followed by the bench-made carboxyfluorescein tyramide reaction (59).

Immunocytochemistry—Immunostaining was performed on 4% paraformaldehyde (PFA)-fixed 5-dpf dissected brains as described earlier (13). Antisense and sense digoxigenin (DIG)-labeled RNA probes were generated using the DIG RNA labeling kit (Roche Diagnostics) following the instructions of the manufacturer. The WISH procedure followed the protocol of Thissex and Hisse (58). Prehybridization and hybridization were conducted at 65 °C for all riboprobes. In situ hybridization signals were detected with sheep anti-digoxigenin-AP Fab fragments (1:10,000, Roche Diagnostics). Color staining was carried out with chromogen substrates (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphatase). For th2 fluorescent in situ hybridization (FISH), samples were incubated in 2% H2O2 in methanol for 20 min to inactivate endogenous peroxidase activity. A th2-DIG-labeled probe was used for hybridization. To visualize the hybridized probe, samples were incubated in peroxidase-conjugated anti-DIG antibody (1:500, Roche, 11207733910) followed by the bench-made carboxyfluorescein tyramide reaction (59).

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tion Suite software and Corel DRAW X3 software (13). Immunofluorescence samples were examined using a Leica TCS SP2 AOBS confocal microscope. For excitation, an argon laser (488 nm), green diode laser (561 nm), and red HeNe laser (633 nm) were used. Emission was detected at 500–550 nm, 560–620 nm, and 630–680 nm, respectively. Cross-talk between the channels and background noise was eliminated with sequential scanning and frame-averaging as described earlier (17). Stacks of images taken at 0.2- to 1.2-μm intervals were compiled, and the maximum intensity projection algorithm was used to produce final images with Leica confocal software and Imaris imaging software version 6.0 (Bitplane AG, Zurich, Switzerland). Cell numbers were counted in each 1.0-μm optical slice using ImageJ 1.46r software (National Institutes of Health, Bethesda, MD), and all cell counts were performed by an investigator blinded to the sample type.

Pharmacological Treatments—24-hpf wild-type or morphant embryos were manually dechorionated, and 20 embryos/group were raised in 6-well plates containing 3 ml of E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, and 0.33 mM MgSO4 with or without drug additions (L-DOPA, D9628; SKF38398, S101; quinpirole, Q102; haloperidol, H1512; SCH23390, D054; Sigma-Aldrich, St. Louis, MO)). The incubation medium was replaced daily until 5 dpf. For the serotonin pharmacological treatments, 4-dpf fish were exposed to 100 μM serotonin receptor antagonists: buspirone (BSP, R106), WAY-100635 (W102), SCH23390, D054; Sigma-Aldrich, St. Louis, MO). The incubation medium was replaced daily until 5 dpf. For the serotonin measurement control, 4-dpf fish were exposed to 100 μM p-chlorophenylalanine (25920, Sigma-Aldrich) for 24 h.

Statistical Analysis—Data analysis was performed by GraphPad Prism v.4.1 software (San Diego, CA). p Values were generated by one-way analysis of variance (ANOVA) for multiple comparisons using Tukey’s multiple comparison test and Student’s t test (unpaired test) for comparison of two groups. Data are presented as mean ± S.E. p < 0.05 was considered statistically significant.

Author Contributions—Y. C. C. and P. P. conceived and designed the experiments. Y. C. C., S. S., S. R., and M. S. performed the experiments and analyzed the data. J. L. B. contributed the transgenic fish. Y. C. C. and P. P. wrote the manuscript with input from all authors.

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