**vglut2** and **gad** Expression Reveal Distinct Patterns of Dual GABAergic Versus Glutamatergic Cotransmitter Phenotypes of Dopaminergic and Noradrenergic Neurons in the Zebrafish Brain

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**ABSTRACT**

Throughout the vertebrate lineage, dopaminergic neurons form important neuromodulatory systems that influence motor behavior, mood, cognition, and physiology. Studies in mammals have established that dopaminergic neurons often use γ-aminobutyric acid (GABA) or glutamatergic cotransmission during development and physiological function. Here, we analyze vglut2, gad1b and gad2 expression in combination with tyrosine hydroxylase immunoreactivity in 4-day-old larval and 30-day-old juvenile zebrafish brains to determine which dopaminergic and noradrenergic groups may use GABA or glutamate as a second transmitter. Our results show that most dopaminergic neurons also express GABAergic markers, including the dopaminergic groups of the olfactory bulb (homologous to mammalian A16) and the subpallium, the hypothalamic groups (A12, A14), the prethalamic zona incerta group (A13), the preoptic groups (A15), and the pretectal group. Thus, the majority of catecholaminergic neurons are gad1b/2-positive and coexpress GABA. A very few gad1/2-negative dopaminergic groups, however, express vglut2 instead and use glutamate as a second transmitter. These glutamatergic dual transmitter phenotypes are the Orthopedia transcription factor–dependent, A11-type dopaminergic neurons of the posterior tuberculum. All together, our results demonstrate that all catecholaminergic groups in zebrafish are either GABAergic or glutamatergic. Thus, cotransmission of dopamine and noradrenaline with either GABA or glutamate appears to be a regular feature of zebrafish catecholaminergic systems. We compare our results with those that have been described for mammalian systems, discuss the phenomenon of transmitter dualism in the context of developmental specification of GABAergic and glutamatergic regions in the brain, and put this phenomenon in an evolutionary perspective. J. Comp. Neurol. 522:2019–2037, 2014.

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**INDEXING TERMS:** hypothalamus; posterior tuberculum; preoptic region; subpallium; noradrenergic; A11 dopaminergic neurons

Understanding the roles of specific neurons in neural circuit function and control of behavior requires identification of the neurotransmitters they express to excite, inhibit, or modulate their target neurons. Recent studies on synaptic transmission support the concept that most—if not all—neurons communicate with more than one neurotransmitter (Burnstock, 2004; El Mestikawy et al., 2011; Hnasko and Edwards, 2012). Surprisingly, neurons sometimes even contain cotransmitters with supposedly opposing effects, such as excitatory glutamate and inhibitory γ-aminobutyric acid (GABA). Granule cells of the mammalian hippocampus, for example,

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release glutamate at synapses connected to CA3 pyramidal neurons. During development and after hyperexcitation or seizure, however, they also discharge GABA (Gutiérrez et al., 2003).

There is evidence that cotransmission is also crucial for neurons that use monoamines as primary transmitter, such as dopamine or noradrenaline (Trudeau, 2004; Chuhma et al., 2009). Dopaminergic (DA) and noradrenergic phenotypes are among the best studied systems in the mammalian brain due to their important modulatory roles in a large variety of neural functions, including the generation and maintenance of emotional states, motivation, motor control, and body homeostasis (Björklund and Dunnett, 2007). In the fore- and midbrain of mammals, 10 major groups of DA neurons have been categorized according to their anatomical location (Dahlstrom and Fuxe, 1964; Hökfelt et al., 1984; Puelles and Verney, 1998). At least during development, a considerable number of these DA groups coexpress a second transmitter, mostly GABA or glutamate. GABA-containing DA neurons, for example, are found in the olfactory bulb A16 group, retinal A17 group, prethalamic zona incerta A13 group, and hypothalamic A12, A14, and A15 group DA neurons (Oertel et al., 1982; Everitt et al., 1984; Kosaka et al., 1987). Only a minority of DA neurons use glutamate as cotransmitter. These neurons are restricted to the mammalian ventral midbrain (A8–10), mainly the ventral tegmental area (VTA; A10) and the substantia nigra pars compacta (SNc; A9) (Descaries et al., 2008; Chuhma et al., 2009), as well as to the diencephalic A11 group (Kawano et al., 2006). The DA neurons of the VTA express vesicular glutamate transporter 2 (VGLUT2) and corelease glutamate during development and adulthood (Kawano et al., 2006; Bérubé-Carrière et al., 2009; Koos et al., 2011). In this case, glutamate serves as a cotransmitter, but in addition facilitates the uptake, packaging, and release of the primary transmitter dopamine, a process called vesicular synergy because of its collaborative effect (El Mestikawy et al., 2011; Hnasko and Edwards, 2012).

Recently, for A16 DA neurons, Borisovska and colleagues (2013) showed that dopamine and GABA are released in distinct modes in these dual transmitter neurons, and may serve temporally distinct roles, with GABA mediating brief and dopamine long-lasting responses. In general, though, the physiological significance of such transmitter dualisms of DA neurons is still fragmentary and their evolution among vertebrates largely unexplored (e.g., Moss et al., 2011).

In this study, we mapped dual GABAergic or glutamatergic neurotransmitter phenotypes of DA and noradrenergic neurons in zebrafish. Because of its significance as a vertebrate model in comparative neurogenetics and evolutionary developmental studies, zebrafish catecholaminergic systems have been extensively characterized. Previous work established that most DA groups topologically correspond to those typically found in mammals and other vertebrates (Holzschuh et al., 2001; Kaslin and Panula, 2001; Rink and Wullimann, 2002; Ma, 2003; McLean and Fetcho, 2004a,b; Filippi et al., 2010, 2012; Panula et al., 2010; Yamamoto et al., 2010; Yamamoto and Vernier, 2011). However, a small number of DA groups are either missing or specifically present in zebrafish and other teleost fish. For example, zebrafish lack ventral midbrain DA neurons homologous to the mammalian A8–10 group. Instead, an additional group of DA neurons exists in the zebrafish striatum (dorsal subpallium), which may substitute for some of the mammalian mesostriatal DA input into striatal circuits (Filippi et al., 2010, 2012; Tay et al., 2011). Also, larval and adult zebrafish display a prominent pretectal group that is absent in the mature mammalian brain. Apart from these differences, most DA groups in zebrafish show clear similarities to mammalian systems. As in mammals, dispersed DA neurons are located in the olfactory bulb (OB; mammalian A16) and in the retina (mammalian A17) of zebrafish.

Also, the diencephalic DA clusters, numbered according to their caudorostral position, show conserved molecular characteristics. One of these conserved DA clusters is the prethalamic group (numbered DC1 in

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**Abbreviations**

AC amacrine cells of the retina  
aHyp anterior hypothalamus  
Cerebellum  
Di diencephalon  
DA dopaminergic  
dpf days post fertilization  
Hc caudal zone of periventricular hypothalamus  
Hd dorsal zone of periventricular hypothalamus  
LC locus coeruleus  
LR lateral recess of diencephalic ventricle  
Mesencephalon  
MO medulla oblongata  
OB olfactory bulb  
Phyp posterior hypothalamus  
PM magnocellular preoptic nucleus  
PO preoptic group  
Ppc parvocellular preoptic nucleus, posterior part  
Pr pretectal group  
PR posterior recess of diencephalic ventricle  
PT posterior tuberculum  
PTN posterior tuberal nucleus  
PVD paraventricular organ  
SC suprachiasmatic nucleus  
SP subpallium/subpallial DA group  
R rhombencephalon  
T telenucleus  
Td telencephalic area  
Tv ventral telencephalic area  
Th thalamus  
TL torus longitudinalis  
vDC ventral diencephalic groups  
VL ventrolateral thalamic nucleus
larvae) that depends on expression of the transcription factors arx and islet1 (Filippi et al., 2012). This group is homologous to mammalian neurons of the prethalamic zona incerta (A13), which are defined by the same set of transcription factors during development (Andrews et al., 2003). All DA clusters located in the posterior tuberculum in zebrafish (groups DC 2, 4, 5, and 6) require the activity of the transcription factor Orthopedia (Otp) and express either nkx2.1a or nkx2.1b during development. These groups are homologous to the OTP-dependent A11 DA neurons that form a contiguous band between the pretectum and the thalamus proper in the mammalian brain (Ryu et al., 2007). In contrast, both the DA and pretectum and the thalamus proper in the mammalian larvae (A1–A3 in mammals), as well as medulla oblongata and area postrema groups of the caudal hindbrain (A6 in mammals) have been described.

Transmitter dualism may represent an additional conserved feature of these ancient DA groups. None of these DA groups in zebrafish, however, has been examined with regard to their potential for GABAergic or glutamatergic transmission. We therefore systematically analyzed the distribution of catecholaminergic neurons in zebrafish with respect to markers that indicate GABA or glutamate as second transmitters. In 4-day-old (4 dpf) larvae and in 1-month-old (30 dpf) juvenile zebrafish, we performed fluorescent in situ hybridization (FISH) to detect either the mRNAs of gad1b and gad2, or of vglut2a and vglut2b, both combined with anti-tyrosine hydroxylase (TH) immunofluorescence. Our findings show that coexpression of GABA or glutamate as a second transmitter is a fundamental feature of catecholaminergic neurons in zebrafish. Most importantly, the majority of DA neurons located in the forebrain are GABAergic, whereas only otp-dependent A11 type neurons, located in the posterior tuberculum, express glutamate as a cotransmitter. Our findings further validate previously postulated homologies to DA and noradrenergic groups between zebrafish and mammals (Filippi et al., 2010, 2012; Panula et al., 2010; Tay et al., 2011; Yamamoto and Vernier, 2011).

MATERIALS AND METHODS

Animals

Zebrafish (Danio rerio) were bred and maintained in our animal facility under standard conditions (Westerveld, 2000). Fertilized eggs obtained from AB/TL wild-type matings were raised at 28°C and fixed at larval stages in cold 4% paraformaldehyde in phosphate-buffered saline (PBS). For analyses of gad1b/2 and vglut2 expression in the juvenile brain, fish were raised until 30 days of development (30 dpf, 10-mm stage), deeply anesthetized in 0.02% Tricaine (ethyl 3-aminobenzoate methanesulfonate; Sigma, St. Louis, MO), and sacrificed. The brains were removed from the skull, immediately transferred to cold 4% paraformaldehyde, and fixed for 3 days at 4°C. The samples were then dehydrated to 100% methanol and stored at −20°C until use. In total, 8 juvenile zebrafish brains were analyzed in the present study. All experimental procedures were in accord with the German laws for animal care, and a permit was obtained from the Regierungspräsidium Freiburg to sacrifice fish for organ removal and analysis.

Whole-mount fluorescent in situ hybridization

Tyramide signal amplification (TSA)-based fluorescent in situ hybridization was performed as previously described for embryos (Filippi et al., 2007). The same protocol was applied to larvae and whole brains. On the first day, the methanol-stored specimens were rehydrated to PBS containing 0.1% Tween 20 (PBST) and incubated in 1% H2O2 in PBST for 30 minutes, to quench endogenous peroxidase activity. They were then permeabilized by proteinase K treatment (10 μg/ml). The prehybridization was carried out at 65°C in Hybridization Mix (HM: 50% formamide, 5X standard saline citrate [SSC], 50 μg/ml heparin, 5 mg/ml torula RNA, 0.1%...
Tween 20), and the hybridization was carried out in HM with the specific digoxigenin-labeled antisense probes. On the second day, the probes were removed by formamide/SSC washing series at 65°C (Hauptmann and Gerster, 1994), and the last wash was performed with TNT buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl,
TABLE 2. Expression of gad Genes and vglut2 Genes in Catecholaminergic Groups of the Zebrafish Brain

| Larva (4 dpf) | Juvenile brain (30 dpf) | mammalian CA groups |
|---------------|-------------------------|---------------------|
| gad           | vglut2                  |                     |
| Offactory bulb| OB                     | + (1,2)             |
| Subpallium    | SP                     | + (1,2)             |
| Preoptic area | PO                     | + (2)               |
| Pretectum     | Pr                     | + (1,2)             |
| Prethalmal    | DC-1                   | + (1,2)             |
| Posterior tuberculum/hypothalamus | DC-2 | -       |
| Hypothalamus  | DC-3                   | + (2)               |
| Locus coeruleus | LC                  | + (1);             |
| Medulla oblongata | MO                  | -                  |
|                | OB                     | + (1,2)             |
|                | SP                     | + (1,2)             |
|                | PPa                    | + (2)               |
|                | PM                     | + (1,2)             |
|                | Pp                     | + (1,2)             |
|                | Pr                     | + (1,2)             |
|                | PG                     | + (1,2)             |
|                | VL (Gr. 0)             | + (1,2)             |
|                | PT (Gr. 1)             | + (1,2)             |
|                | PT (Gr. 2)             | -                   |
|                | PT (Gr. 4)             | -                   |
|                | PT (Gr. 5)             | -                   |
|                | PVO (Gr. 3)            | + (2)               |
|                | PR (Gr. 7)             | + (2)               |
|                | LC                     | + (1)               |
|                | MO                     | -                  |

1 Larval brain at 4 dpf: abbreviations (see list) indicate catecholaminergic cell groups using the nomenclature and numbering by Rink and Wullimann (2002). Juvenile brain at 30 dpf: abbreviations indicate catecholaminergic cell groups using the nomenclature and numbering by Rink and Wullimann (2002), who suggested the group numbering, and Filippi et al. (2010). Note that the prethalamic larval DC-1 group corresponds to the juvenile and adult prethalamic group 0. Ma (2003) named PT Gr. 1 as TPpd, PT Gr. 2 and 4 as PVOa, PT Gr. 5 & 6 as PTN, and the hypothalamic PVO as TPpv. The gad column indicates whether gad1b (1) or gad2 (2) or both (1,2) are coexpressed with TH. Detection of expression is indicated as follows: +, coexpression; -, no coexpression; and +; , gad1b/2; vglut2 coexpression only in a subset of TH-immunoreactive neurons of this group. The rightmost column shows the mammalian catecholaminergic group numbers (Hüfelfelt et al., 1984) correlating with the zebrafish groups. n.a., not analyzed.

Figure 1. Expression of GABAergic markers gad1b/2 in relation to TH immunoreactivity at 96 hpf. A: Schematic lateral (top) and dorsal (bottom) views of the zebrafish larval brain showing the distribution of catecholaminergic groups detectable by immunohistochemistry at 4 dpf (modified from Tay et al., 2011). B1: Lateral overview of a larval brain immunostained for TH (red), showing the distribution of DA neurons in the olfactory bulb (OB), subpallium (SP), preoptic area (PO), pretectum (Pr), prethalmalum (group 1), posterior tuberculum/medial hypothalamus (altogether labeled here vDC and comprising DA groups 2–6), and posterior hypothalamus (group 7), as well as the noradrenergic group in the locus coeruleus (LC). B2 Same image, indicating the dorsoventral level of the confocal planes shown in C–J. C–J: Fluorescent in situ hybridization to gad1b (C,D,F,H,J, green) or gad2 (E,G, green) combined with anti-TH immunofluorescence (red). Left panels (C–J) show overview of dorsal views for planes encompassing different TH-ir clusters. Right panels (C1–C3 through J2) show enlargements of the areas framed by white boxes in the overview views. Double labeling revealed that the majority of DA groups expressed both gad isoforms, which had mostly overlapping expression patterns. gad expression and TH immunoreactivity were decodetected for the pretectal group (Pr; C–C3), the prethalmal group (group 1; E,E2), the telencephalic groups (SP and OB; F,F2 and H,H2, respectively), and the preptic group (PO; J center, J1,J2). The noradrenergic neurons of the locus coeruleus expressed only gad1b (D–D3, arrowheads), although not all of them did so (arrows in D1–D3). The noradrenergic neurons in the medial hypothalamus (group 3; G–G3) and a subset in the posterior hypothalamus (group 7; I–I3) appear to weakly express only gad2. The arrowheads point at a TH-immunoperoxidase (B1,B2), C–E, E2 (applies to E1,E2,F, G, G3 (applies to G1–G3), I, J, J3 (applies to J1–J3).
Figure 2.
GenBank accession no. NM_194419; gad2/gad65 (Martin et al., 1998) covered nucleotides 921–1610 of GenBank accession no. NM_001017708.

To detect glutamatergic neurons, a mixture of antisense digoxigenin-labeled riboprobes against vglut2a/slc17a6b and vglut2b/slc17a6a were used (Higashijima et al., 2004). The riboprobes were as follows: vglut2a/slc17a6b comprised nucleotides 454–1387 of GenBank accession no. NM_001128821; vglut2b/slc17a6a comprised nucleotides 572–1026 of GenBank accession no. NM_001009982.

**Antibody characterization**

We used the anti-zebrafish TH primary antibody generated against a protein containing the amino acids 92–365 encoded by the tyrosine hydroxylase (th) gene (GenBank accession no. NM_131149), expressed in a bacterial system and purified (Ryu et al., 2007). The detailed characterization of the antibody has been previously published (Filippi et al., 2010). The specificity and identity of the antibody was verified by combined fluorescent whole-mount in situ hybridization using a th antisense probe (GenBank accession no. NM_131149) combined with anti-TH immunohistochemistry. Anti-TH immunoreactivity was observed in somata as well as in axons projecting from th mRNA-expressing cells, and the anti-TH antibody did not stain any cell somata in the brain that did not express th (see in Filippi et al., 2010). This antibody has been shown to be specific for the product of the th gene and does not detect the paralogous TH2 protein (Filippi et al., 2010) (Table 1).

**Immunohistochemistry**

After FISH, the specimens were equilibrated with PBS containing 0.1% Tween 20 and 1% dimethyl sulfoxide (DMSO; PBTD), and preincubated for at least 2 hours with blocking solution (10% goat serum and 1% protease-free bovine serum albumin [BSA] in PBTD). They were then incubated overnight with a 1:500 rabbit polyclonal anti-TH primary antibody (Ryu et al., 2007), subsequently detected by a 1:1,000 Alexa 555-conjugated secondary goat anti-rabbit antibody (Life Technologies, Bethesda, MA; cat. no. A-21428).

**Vibratome sections**

Stained brains were embedded in 3% agarose (molecular biology grade, BIORON, Ludwigshafen Germany; cat. no. 604005) dissolved in PBS and sectioned by using a Leica VT1000S Vibratome. Then 80-μm slices were cut and mounted in Aqua-Poly/Mount (Polysciences, Warrington, PA) for confocal imaging.

**Microscopy and imaging**

For microscopic observation, larvae were mounted in 80% glycerol/PBS containing 1% agarose. Confocal z-stacks of whole-mount larvae and sectioned brains were recorded by using a Zeiss LSM 5 DUO laser scanning confocal microscope. Then z-projections from subsets of focal planes were generated with the Zeiss LSM software and exported as image format files. The images were assembled into figures and processed with the Adobe (San Jose, CA) Photoshop CS2 9.0 software. Adjustments to contrast and brightness were made by using the Photoshop Levels functions. All adjustments were made to whole images including the enlarged sections, except for Figures 111–I3, 3C1–C3, 3F1–F3, and 4F1–F3, for which in the whole enlarged section red and green levels were adjusted to better visualize red and green channel colocalization.

**RESULTS**

To analyze the distribution of GABAergic and glutamatergic cells with relation to DA neurons, we performed FISH to detect GABAergic or glutamatergic markers, coupled with immunofluorescence for TH to detect DA neurons. As markers for GABAergic neurons we used glutamate decarboxylase 1 (gad1b/gad67) and glutamate decarboxylase 2 (gad2/gad65). These genes...
encode two isoforms of the enzyme that catalyzes the conversion of L-glutamic acid into the neurotransmitter GABA (Martin et al., 1998). To visualize glutamatergic neurons, we used probes for the zebrafish paralogous genes vesicular glutamate transporter 2a (vglut2a) and vesicular glutamate transporter 2b (vglut2b), which code for transporters for uptake of glutamate into synaptic vesicles prior to their release into the synapse (Higashijima et al., 2004). We performed our analysis on whole-mount 4-dpf larvae, when all major larval catecholaminergic (CA) groups are clearly detectable by immunohistochemistry (Holzschuh et al., 2001; Kaslin and Panula, 2001; Rink and Wullimann, 2002). Moreover, to uncover potential changes in neurotransmitter distribution in relation to CA neurons during development, we decided to perform the analysis also in 30-dpf juvenile brains. At this stage of development, the brain is morphologically already very similar to the adult brain while still being small enough to be amenable to whole mount staining.

We used a mixture of vglut2a and vglut2b as riboprobe in all our experiments, and therefore will refer to

![Figure 3. Localization of gad1b/2- or vglut2-expressing cells and TH-ir neurons in the telencephalon of the juvenile brain. A: Lateral overview of a juvenile brain stained for th by in situ hybridization (image taken from Filippi et al., 2010), which serves as anatomical reference to indicate the levels of the sections shown in B–G. B–G: Fluorescent in situ hybridization to detect gad1b/2 (C,F; green) or vglut2 (D,G; green) expression combined with anti-TH immunofluorescence (red). In each row, the image at the left shows an overview of the considered section and displays only TH immunostaining. Higher magnifications of the approximate area framed by the white square are shown to the right, together with either gad1b/2 (C,F) or vglut2 (D,G) staining. All images are 6–10-μm confocal z-projections of areas comprising TH-ir cells in the olfactory bulb (OB; B–D) and in the subpallium (SP; E–G). All telencephalic THir neurons express gad1b/2 (C,F) but not vglut2 (D,G), indicating that they are GABAergic. To better display the colocalization of the markers, C1–C3 and F1–F3 show the individual channels of the areas framed in C and F, respectively. For abbreviations, see list. A magenta–green copy of this figure is available as Supporting Information Figure S3. Scale bar = 50 μm in B–G.](image-url)
them together as vglut2. gad1b and gad2 were analyzed together in juvenile brains, as well as individually in larval and juvenile brains, to highlight potential differences in the localization of the two isoforms.

\textit{gad1/2} colocalizes with TH in most larval DA clusters

gad1b and gad2 expression has been shown to largely overlap in the developing brain of several model organisms (Ahman et al., 1996; Dupuy and Houser, 1996; Martin et al., 1998). Indeed, a gross comparison of their expression patterns by FISH showed that they are both expressed in a similar manner in major areas of the zebrafish brain at 4 dpf (data not shown). We focused our analysis on the potential colocalization of gad1b/2 with TH immunoreactivity in larval DA clusters, and detected some differences in the distribution of gad1b/2 transcripts in specific nuclei of the brain. Our results are shown in Figure 1, organized from dorsal to ventral (see Fig. 1A, B1, B2 for orientation on the distribution of TH-immunoreactive clusters), and summarized in Table 2.

In the diencephalon, pretectal DA neurons strongly expressed both gad genes (shown for gad1b in Fig. 1C–C3). The same was true for prethalamic group 1 DA neurons, which reside in a region characterized by strong gad expression (shown for gad2 in Fig. 1E–E2). The Otp-dependent DA neurons (groups 2, 4–6) develop in the posterior tuberculum, and none of them expressed either gad1b or gad2 (Fig. 1E–E2). The large pear-shaped neurons belonging to group 2 were surrounded by GABAergic cells, but did not appear to express gad (Fig. 1G–G3). In the hypothalamus, the liquor-contacting group 3 DA neurons weakly expressed gad2, but did not express gad1b (Fig. 1G–G3). Similarly, group 7 in the posterior hypothalamus appeared to express gad2 only in some (Fig. 1I–I3, arrowhead) but not all neurons (Fig. 1I–I3, arrow). The larval preoptic group, which contributes to the adult suprachiasmatic nucleus, was only gad2-positive (Fig. 1J–J2). The telencephalic subpallial and olfactory bulb DA groups express both GAD isoforms (Fig. 1F–F2, H–H2), and develop in anatomical areas with prominent populations of gabaergic neurons.

In the hindbrain, most noradrenergic neurons of the locus coeruleus expressed gad1b (Fig. 1D–D3, arrowheads), but, interestingly, some cells were negative for both gad transcripts (Fig. 1D–D3, arrows). This suggests that the locus coeruleus group may be a potentially heterogeneous population of noradrenergic neurons.

Finally, the DA amacrine cells of the retina detectable at this stage appeared to be adjacent to cells expressing gad, but no colocalization was observed (Fig. 1J). This confirms previous findings based on double immunohistochemistry for TH and GABA or GAD in zebrafish and trout (Arenzana et al., 2006; Candal et al., 2008).

\textit{vglut2} is expressed in larval Otp-dependent DA neurons

When considering colocalization with TH immunoreactivity, the distribution of vglut2-expressing cells in the larval brain was generally complementary to that of gad. We did not observe colocalization of vglut2 and TH in the pretectal group (Fig. 2C). In the hindbrain, many cells surrounding the noradrenergic neurons of the locus coeruleus did express vglut2 (Fig. 2D). Our careful analysis of confocal images, however, revealed that TH-immunoreactive (TH-ir) noradrenergic neurons of the LC did not coexpress vglut2 (Fig. 2D1–D3). The prethalamic area around group 1 DA neurons appeared essentially vglut2-free (Fig. 2E–E2). We also did not detect any vglut2 expression in the liquor-contacting hypothalamic groups 3 and 7 (Fig. 2G–G2, I–I2), the preoptic group (Fig. 2J–J2), the telencephalic subpallial (Fig. 2F–F2), or the olfactory bulb DA neurons (Fig. 2H–H2), although they resided in areas adjacent to vglut2-positive cells. We found vglut2 expression in the ganglionic cell layer of the retina, but not in the amacrine cell layer, where DA neurons develop (Fig. 2J).

In contrast, the posterior tubercular region was characterized by intense vglut2 expression (Fig. 2E). All Otp-dependent DA neurons were double labeled for vglut2 (groups 2, and 4–6; Fig. E–E2, G–G2, arrowheads). Thus, among all DA neurons of the larval brain, coexpression of vglut2 and TH was only observed in the Otp-dependent, A11-type neurons residing in the posterior tuberculum and hypothalamus.

Expression of \textit{gad} and \textit{vglut2} in juvenile brains

Consistent with earlier reports, we detected DA neurons in the olfactory bulb mainly in the glomerular and mitral cell/plexiform layers (Byrd and Brunjes, 1995) (Fig. 3B). In mammals, GABAergic neurons have been shown to develop in the glomerular, mitral, and granule cells layers (Parrish-Aungst et al., 2007), whereas glutamatergic neurons were detected in the external plexiform, glomerular, and mitral cells layers, but not granule cell layer (Brill et al., 2009). All of the TH-ir cells detected in the juvenile olfactory bulb were gad-positive (Fig. 3C–C3) and vglut2-negative (Fig. 3D). Similarly, all TH-ir neurons in the subpallium (Fig. 3E)
Figure 4. 

[A detailed image with labeled areas and markers, including TH, gad/TH, and vglut2/TH across different regions such as PM, VL/SC, Pr, and PT.]
expressed gad (Fig. 3F–F3), but not vglut2 (Fig. 3G), consistent with earlier reports on gad expression (Mueller and Guo, 2009).

The preoptic area is characterized by the presence of three main DA clusters, one populating the anterior parvocellular preoptic nucleus (PPa), one that reaches from the magnocellular preoptic nucleus (PM; Fig. 4B) to the posterior parvocellular preoptic nucleus (PPp; Fig. 4E), and one in the suprachiasmatic nucleus (SC; Fig. 4E) (Kaslin and Panula, 2001; Rink and Wullimann, 2001; Ma, 2003; Filippi et al., 2010). All these areas were strongly labeled by gad expression, and almost all TH-ir neurons were also gad-positive (Fig. 4C,F and data not shown). Only in the SC did we find a few TH-ir cells that did not appear to express gad (Fig. 4F–F3, arrows). No coexpression between TH and vglut2 was observed in these preoptic clusters (Fig. 4D,G, and data not shown).

In the dorsal diencephalon, the broad pretectal DA group strongly expressed gad (Fig. 4I–13). Ma (2003) has described the zebrafish pretectal DA group as located exclusively in the ventral part of periventricular periventricular pretectal nucleus (PPv). According to Mueller and Guo (2009), who reported gad67 expression in PPv only (PPv being gad67-free), the overlap between gad and TH indicates that DA neurons in the pretectum belong to the dorsal subdivision of periventricular pretectal nucleus (PPd and not PPv). Some cells laterally adjacent to pretectal TH-ir neurons expressed vglut2 (Fig. 4I). In addition, we found strong vglut2 expression in the thalamus, a region of the dorsal diencephalon known to be predominantly glutamatergic (Bae et al., 2009; Scholpp et al., 2009).

The larval prethalamic group DA neurons populate the ventrolateral thalamic nucleus (VL) in the juvenile and adult brain (Rink and Wullimann, 2001; Filippi et al., 2010). As in larvae, the whole juvenile prethalamus was predominantly GABAergic; thus, not surprisingly, we found that all VL DA neurons expressed gad (Fig. 4E,F). Caudal to them, another group of cells characterized by similar morphology could be discerned, just anterior and dorsal to the large pear-shaped group 2 DA neurons (Fig. 4K). Like the prethalamic neurons, these cells were embedded in a GABAergic territory, and they expressed gad (Fig. 4L–L3) but not vglut2 (Fig. 4M).

The most rostral Otp-dependent DA neurons (larval group 2; Fig. 5B) expressed vglut2 (Fig. 5D–D3). These neurons, furthermore, did not express gad despite their proximity to gad-expressing cells (Fig. 5C, see also Fig. 6C). The more caudally located Otp-dependent DA groups in the posterior part of the PT and posterior tuberal nucleus (PTN) also expressed vglut2b during larval stages (larval groups 4–6; Fig. 5E,H) and in the mature brain (Fig. 5G–G3,J–J3). None of these groups expressed gad, whose expression domains were restricted to more ventral regions, namely, the dorsal and caudal zones of the periventricular hypothalamus (Fig. 5F,I).

In the periventricular hypothalamus, two main groups of liquor-contacting DA neurons were detected in the paraventricular organ (PVO; Fig. 6B) and in the caudal hypothalamus, lining the posterior recess of the third ventricle (PR; Fig. 6E). Both these regions were almost completely devoid of vglut2 transcripts (Fig. 6D,G), whereas they were mostly gad-positive (Fig. 6C–C3,F–F3). Expression analysis of individual gad isoforms revealed that only gad2 was expressed in these two groups (data not shown; Table 2).

In the hindbrain, the noradrenergic neurons of the locus coeruleus (Fig. 6H) appeared to be sandwiched between areas of medial gad expression and lateral vglut2 expression (Fig. 6, compare I with J). Locus coeruleus noradrenergic neurons expressed gad, and were surrounded by both gad- and vglut2-positive cells (Fig. 6I–I3). Also in this case, expression analysis of individual gad isoforms revealed that only gad1b was expressed in the locus coeruleus (Table 2).

In the medulla oblongata (Fig. 7B), whereas no overlap was observed between TH and gad (Fig. 7C), we...
Figure 5. Localization of gad1b/2 or vglut2 expression and TH immunoreactivity in Otp-dependent DA neurons of the juvenile brain. A: Overview of section planes shown in B–J (for details, see Fig. 3A legend). B–J: Fluorescent in situ hybridization to detect gad1b/2 (C,F,I; green) or vglut2 (D,G,J; green) expression combined with anti-TH immunofluorescence (red). The posterior tubercular and hypothalamic Otp-dependent DA neurons are shown. The overviews in B, E, and H are 2–10-μm confocal z-projections encompassing the large pear-shaped DA neurons in the posterior tuberculum (PT; B,E) and the group in the posterior tuberal nucleus (PTN; H). Higher magnifications of the approximate areas framed by the white squares in the overviews are shown at their right, together with either gad1b/2 (C,F,I) or vglut2 (D,G,J) staining (3–16-μm z-projections). All Otp-dependent DA neurons express vglut2 (D,G,J) but not gad1b/2 (C,F,I), indicating that they are also glutamatergic. Individual channels demonstrating coexpression of TH with vglut2 are shown for each group in D1–D3, G1–G3, and J1–J3. For abbreviations, see list. A magenta–green copy of this figure is available as Supporting Information Figure S5. Scale bar = 50 μm in B–J.
clearly detected coexpression of TH with vglut2 in most noradrenergic neurons (Fig. 7D–D3). A minor number of cells, however, were free of vglut2-expression. However, due to the poor quality of the sections obtained for the posterior hindbrain, the data collected for the noradrenergic group of the medulla oblongata were not...
complete, and we were not able to determine whether those few vglut2-free cells were GABAergic instead.

**DISCUSSION**

Expression studies over the past 30 years have revealed that in mammals the coexistence of two or more different neurotransmitters within a single neuron is a common phenomenon (Burnstock, 2004), including in catecholamine systems (Kosaka et al., 1987). Secondary transmitters are often expressed in higher concentrations during development and after seizure, when cotransmitters are suspected of participating in neuronal plasticity and synapse formation (Bérubé-Carrière et al., 2009; Fortin et al., 2012). Secondary transmitters in mammals also engage in neurotransmission proper and can be found in dual transmitter phenotypes in the mature brain (Seal and Edwards, 2006; Descarries et al., 2008; Stuber et al., 2010). A group of DA neurons of the mammalian ventral tegmental area (VTA), for example, contains glutamate, which facilitates vesicular uptake and storage of dopamine, but also acts as a true transmitter (Alsio et al., 2011; El Mestikawy et al., 2011). The role of GABA in DA neurons, however, has not been well studied. Investigations on mammalian retinal explants and olfactory bulb brain slices suggest participation in neural transmission (Maher and Westbrook, 2008; Hirasawa et al., 2012).

Our study on cotransmitters in zebrafish catecholaminergic neurons analyzed the dual expression of either GABAergic or glutamatergic markers together with TH in larval and juvenile zebrafish. Our previous report on the distribution of th-expressing cells in 30-dpf juvenile brains showed that all groups described for the adult by immunohistochemistry (Kaslin and Panula, 2001; Rink and Wullimann, 2001; Ma, 2003) were already developed at this stage (Filippi et al., 2010). Recent work suggested that a potential second TH-encoding gene, th2, may mark additional DA groups in the mature brain (Candy and Collet, 2005; Yamamoto et al., 2010), but the protein product is not detected by our TH antibody (Filippi et al., 2010). However, more recent work revealed that the th2 locus does not encode an enzyme with TH activity.

**Figure 7.** Localization of gad1b/2 or vglut2 expression and TH immunoreactivity in the medulla oblongata at 30 dpf. A: Overview of section plane shown in B-D (for details, see Fig. 3A legend). B–D3: Fluorescent in situ hybridization to detect gad1b/2 (C; green) or vglut2 (D; green) expression combined with anti-TH immunofluorescence (red). The overview in B is a 10-μm confocal z-projection showing TH-ir noradrenergic neurons of the medulla oblongata (MO) in the hindbrain. These neurons were intermingled but not colocalizing with gad-expressing cells (C; 3-μm projection). Instead, we detected coexpression of TH with vglut2 (D–D3; 6-μm projection). D1–D3 are single-channel enlarged views of the framed area in D. The arrowheads point at examples of coexpression. A magenta–green copy of this figure is available as Supporting Information Figure S7. Scale bar = 50 μm in B–D.
(Ren et al., 2013). Therefore, our analysis of TH-ir cells likely covers the entire set of dopamine-producing neurons of the juvenile brain.

We show that in 4-day-old larvae and 1-month-old juveniles all DA and noradrenergic neurons have the potential to synthesize GABA or to load glutamate into vesicles, as traced by the expression patterns of \textit{gad1b}/2 and \textit{vglut2a}/b genes versus TH protein. Our results thus demonstrate that in the zebrafish brain most DA neurons can synthesize GABA as a cotransmitter in larvae and mature brain. Prominent groups of \textit{gad1b}/2-expressing DA neurons are located in areas that are predominantly populated by GABAergic neurons like the subpallium, the preoptic region, the prethalamus, and the hypothalamus (groups 3 and 7). Notably, all of these regions are largely free of GABAergic neurons within the locus coeruleus. This cluster has been described as a bipartite structure in the mature brain, one part of which belongs to the prethalamus (group 0) and the other (group 1) to the posterior tuberculum, i.e., the periventricular posterior tubercular nucleus (TPp) (Rink and Wullimann, 2002). Furthermore, Wullimann and Rink (2001) showed that the prethalamic group (0) is intermingled with Pax6-expressing cells during development, whereas group 1, which they interpreted as belonging to the posterior tuberculum, lacks expression of Pax6, and only neurons of this latter region project to the
genic regulation of catecholaminergic phenotypes. For example, all of the DA A11-type neurons of the posterior tuberculum coexpressing \textit{vglut2a}/b require the activity of \textit{orthopedia (otp)}. However, the adjacent hypothalamic DA neurons that express \textit{gad1b}/2 do not express \textit{otp} during development (Ryu et al., 2007). These differences can thus be used as a criterion to separate neurons of the hypothalamus from those of the posterior tuberculum, and distinguishing these regions helps in our understanding of gene expression data. As studies in mammals show, \textit{Neurogenin 1 (Ngn1)} is important for the determination of glutamatergic phenotypes, but is absent in GABAergic phenotypes (Wullimann and Mueller, 2002; Schuurmans et al., 2004; Nakatani et al., 2007). Both the regulation and function of the zebrafish ortholog \textit{ngn1} are largely conserved (Blader et al., 2004). \textit{ngn1} is also a determinant of DA differentiation in the zebrafish basal forebrain (Jeong et al., 2006). During zebrafish development, \textit{ngn1} is expressed in forebrain regions of mostly glutamatergic neurons, including the region of the posterior tuberculum, but not in the hypothalamus (Mueller and Wullimann, 2003). Therefore, we need to assume that only the \textit{vglut2a}/b-expressing DA groups of the posterior tuberculum, but not the \textit{gad1b}/2-expressing ones of the hypothalamus, depend on the activity of \textit{ngn1}.

In contrast, most DA neurons in the zebrafish forebrain reside in regions defined by the expression of \textit{dix2a} and \textit{zash1a (ascl1)}. These regions are the subpallium, the preoptic region, the ventral thalamus, and the hypothalamus (Mueller and Guo, 2009). Orthologs of \textit{dix2a} and \textit{ascl1} are required for the determination of GABAergic phenotypes, as shown in mammalian (Andrews et al., 2003; Roybon et al., 2010; Virolainen et al., 2012) and zebrafish (Macdonald et al., 2013) systems. Significantly, many DA neurons in the zebrafish forebrain express markers specific for precursor cells developing into GABAergic phenotypes, such as \textit{dix2a} and other \textit{dix} genes (Filippi et al., 2012).

In addition, we found in the zebrafish prethalamus another prominent cluster of \textit{gad1b}/2-expressing DA neurons that appears to extend into the posterior tuberculum. This cluster has been described as a bipartite structure in the mature brain, one part of which belongs to the prethalamus (group 0) and the other (group 1) to the posterior tuberculum, i.e., the periventricular posterior tubercular nucleus (TPp) (Rink and Wullimann, 2002). Our findings regarding the molecular phenotype and homology of most of the DA (and noradrenergic) groups are also consistent with gene expression studies of the
subpallium. However, our recent and previous results demonstrate that these two populations of dopaminergic neurons express gad1b/2 and are developmentally defined by similar sets of molecular markers (Filippi et al., 2010, 2012). Our results thus support the hypothesis that these neurons derive from the prethalamus and that they topologically correspond to DA neurons found in the mammalian prethalamus (zona incerta, A13; Filippi et al., 2010, 2012).

Our finding that most DA neurons in the zebrafish brain also express markers for a GABAergic phenotype conforms with studies on various vertebrates including lampreys, amphibians, and mammals (Kosaka et al., 1987; de Rijk et al., 1992; Barreiro-Iglesias et al., 2009). GABAergic phenotypes have been shown specifically for DA groups in the olfactory bulb (A16; Muguinaini et al., 1984; Kosaka et al., 1985; Parrish-Aungst et al., 2007; Maher and Westbrook, 2008), in the hypothalamus (A12; Everitt et al., 1984; Meister and Hökfelt, 1988; Schimchowitsch et al., 1991), the zona incerta of the prethalamus (A13; Oertel et al., 1982), and the amacrine cell layer of the retina (A17; Wässle and Chun, 1988). Whereas spinal DA neurons have not been observed in zebrafish, in lamprey and elasmobranch liquor-contacting spinal DA neurons colocalization of DA and GABA or GAD and TH have been shown (Sueiro et al., 2004; Rodicio et al., 2008). Kosaka and colleagues (1987), however, did not find an overlap of GAD and TH in the far-projecting DA neurons of diencephalic A11 and mesodiencephalic A8–A10 groups, except for some neurons of the A10 group. These results are in line with our observations in zebrafish, in which the only diencephalic groups that do not express gad belogn to the Otp-dependent A11-like groups.

We did not detect any coexpression of gad and TH in the amacrine cell layer of the larval retina of zebrafish, which is consistent with previous findings in adult zebrafish (Arenzana et al., 2006) and other teleosts (Wulle and Wagner, 1990; Candal et al., 2008). However, Huang and Moody (1998) colleges report that GABA and dopamine are coexpressed only transiently in the Xenopus tadpole. In contrast, in amniotes including reptiles, birds, and mammals, coexpression of TH and GABAergic markers in the inner nuclear layer of the retina is common (Kosaka et al., 1987; Wässle and Chun, 1988; Wulle and Wagner, 1990; Andrade da Costa and Hokoç, 2003). Perhaps the dual GABA/DA phenotype regresses during the development of amniotes, as this observation suggests, whereas it remains present in amniotes also in the mature brain. Our study focused on 4-day-old larval zebrafish. This developmental stage may be too progressed to observe a similar coexpression. Additional studies need to investigate whether Gad/GABA and TH in the fish retina are coexpressed transiently as is the case in other vertebrates.

The main mammalian DA systems absent in zebrafish are the mesencephalic A8–A10 systems. There is strong evidence that a subset of these DA groups including VTA neurons also uses glutamatergic transmission (Sulzer et al., 1998; Hnasko et al., 2010) and that dual transmission is significant for physiological function (Chuhma et al., 2009; Stuber et al., 2010; Koos et al., 2011) as well as development (Fortin et al., 2012). Although this midbrain population of DA neurons with ascending striatal projections is absent in zebrafish (for a different view, see Wullimann, 2011), we note that the A11 DA neurons in zebrafish, which provide ascending projections to the subpallium/striatum (Rink and Wullimann, 2001; Tay et al., 2011), also have a glutamatergic dual transmitter phenotype. Thus, at least with regard to transmitter systems, the A11 Otp-dependent DA neurons in zebrafish may be equipped to elicit similar responses in the striatum as in the mammalian nigrostriatal system. Whether posterior tubercular–striatal DA circuits in zebrafish are organized and function similar to mammalian nigrostriatal circuits, as has been suggested for lamprey (Ericsson et al., 2013), remains to be determined. Interestingly, glutamatergic cotransmission is also present in both the mammalian A11 and nigrostriatal systems (Kawano et al., 2006).

To conclude, our study demonstrates that most DA and noradrenergic neurons in the zebrafish brain are dual transmitter types that express either GABA or glutamate. This finding, together with similar works on lamprey, amphibians, and mammals, supports the hypothesis that DA neurons with dual transmitter phenotype evolved in the earliest vertebrates. The pattern of gad1b/2- and vglut2a/b-expressing DA and noradrenergic neurons is consistent between 4-dpf larvae and 30-dpf juveniles. Therefore, this pattern likely remains stable into adulthood, similar to the situation reported for lampreys and mammals. Our study thus provides a solid foundation for physiological studies of GABA and glutamate cotransmitters in DA systems. In particular, the vglut2a/b-expressing A11-type DA neurons of the posterior tuberculum in zebrafish are promising candidates to examine the role of glutamate for the development and function of these important and far-projecting neurons. Similarly, the DA neurons of the hypothalamic and pretectal regions will allow investigation of the physiological significance of the cotransmitter GABA in dual transmission in these conserved parts of the brain.

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CONFLICT OF INTEREST STATEMENT
The authors declare that they have no conflict of interest.

ROLE OF AUTHORS
All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: AF, WD. Acquisition of data: AF. Analysis and interpretation of data: AF, TM, WD. Drafting of the manuscript: AF, TM. Critical revision of the manuscript for important intellectual content: WD. Obtained funding: WD. Study supervision: WD.

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