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Neurotransmitter map of the asymmetric dorsal habenular nuclei of zebrafish

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Abstract

The role of the habenular nuclei in modulating fear and reward pathways has sparked a renewed interest in this conserved forebrain region. The bilaterally paired habenular nuclei, each consisting of a medial/dorsal and lateral/ventral nucleus, can be further divided into discrete subdomains whose neuronal populations, precise connectivity and specific functions are not well understood. An added complexity is that the left and right habenulae show pronounced morphological differences in many non-mammalian species. Notably, the dorsal habenulae of larval zebrafish provide a vertebrate genetic model to probe the development and functional significance of brain asymmetry. Previous reports have described a number of genes that are expressed in the zebrafish habenulae, either in bilaterally symmetric patterns or more extensively on one side of the brain than the other. The goal of our study was to generate a comprehensive map of the zebrafish dorsal habenular nuclei, by delineating the relationship between gene expression domains, comparing the extent of left-right asymmetry at larval and adult stages, and identifying potentially functional subnuclear regions as defined by neurotransmitter phenotype. While many aspects of habenular organization appear conserved with rodents, the zebrafish habenulae also possess unique properties that may underlie lateralization of their functions.

Keywords

Epithalamus; Left-right asymmetry; habenula; interpeduncular nucleus; somatostatin; *ano2*; *mbnl3*; *gng8*

INTRODUCTION

The habenular nucleus influences a wide range of behaviors, from sleep and reward to fear and anxiety (Bianco and Wilson, 2009; Hikosaka, 2010; Hikosaka *et al.*, 2008; Klemm, 2004; Lecourtier and Kelly, 2007; Okamoto *et al.*, 2012; Sutherland, 1982), and has been a

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recent focus in research on addiction and depression (Aizawa *et al.*, 2013; Hikosaka, 2010; Hikosaka *et al.*, 2008; Viswanath et al., 2014). Despite increasing functional studies, our knowledge of its organization and neuronal properties is limited. Moreover, many vertebrates, including zebrafish, display prominent left-right (L-R) differences in this dorsal diencephalic structure (Concha and Wilson, 2001), further complicating our understanding of habenular organization, connectivity and function.

The habenulae of mammals consist of bilaterally paired medial and lateral nuclei (Gurdjian, 1925) that have different afferent input (Herkenham and Nauta, 1977) and efferent connections (Herkenham and Nauta, 1979). The lateral nuclei project to a variety of areas in the midbrain and hindbrain, whereas the medial nuclei chiefly innervate the unpaired interpeduncular nucleus (IPN) in the ventral midbrain (Herkenham and Nauta, 1979). Axons from both nuclei contribute to the fasciculus retroflexus (FR) nerve bundles to form one of the most highly conserved conduction systems in the vertebrate brain (Herrick, 1948). While it was initially suggested that zebrafish only possess a habenular region analogous to the medial nucleus of mammals (Concha and Wilson, 2001), further characterization of molecular and anatomical properties confirmed the presence of both a dorsal and ventral nucleus (Amo *et al.*, 2010), which, respectively, correspond to the medial and lateral habenulae of mammals.

In several vertebrate species, the medial/dorsal and lateral/ventral nuclei have been further subdivided into discrete neuronal populations or subnuclei largely based on morphological criteria (Contestabile *et al.*, 1987; Iwahori, 1977; Marburg, 1944; Quina *et al.*, 2009; Wagner *et al.*, 2014). Four subnuclei were initially recognized in the medial nucleus of the well-studied rat habenula by neuronal shape and packing, neuropil density and neurotransmitter immunoreactivity (Andres *et al.*, 1999; Contestabile *et al.*, 1987; Geisler *et al.*, 2003). Antibody labeling demonstrated that neurons located more dorsally produce substance P (Cuello *et al.*, 1978), while those in more ventral regions are cholinergic (Eckenrode *et al.*, 1987). More recent analyses of gene expression corroborate this major sub-division, as well as demonstrate that genes from the *vesicular glutamate transporter* (*vglut*) family are expressed throughout the habenulae (Aizawa *et al.*, 2012; Quina *et al.*, 2009). The dorsal region can be further divided into glutamatergic-only and substance P-expressing/glutamatergic neuronal populations by the localization of transcripts for *vglut1*and *2* and the substance P precursor, *tachykinin 1* (*tac1*) (Aizawa *et al.*, 2012; Quina *et al.*, 2009). Immunolabeling indicates that the cholinergic neurons largely project to the central core of the IPN, whereas substance P-expressing neurons innervate peripheral subnuclei (Contestabile *et al.*, 1987). Additional combinatorial patterns of gene expression reveal 5 distinct subnuclear regions in the medial habenula of the adult rat (Aizawa *et al.*, 2012), and a recent study suggests a similar organization for the mouse (Wagner *et al.*, 2014).

Discrete subnuclei identified in the dorsal habenulae of amphibians and fish show prominent differences in their size and organization between the left and right sides of the brain (refer to Concha and Wilson, 2001). For example, in *Rana esculenta*, the right dorsal habenula is a single nucleus, while the left contains lateral and medial subnuclei, which innervate the IPN via different routes (Braitenberg and Kemali, 1970; Kemali and Guglielmotti, 1984).

Substance P neurons are present in the left and right dorsal habenulae; however, but only in the lateral subnucleus on the left side (Kemali and Guglielmotti, 1984).

In zebrafish, where the dorsal habenulae have emerged as a powerful genetic model to study the development and function of L-R asymmetry in the brain, subnuclear organization has been defined on the basis of patterns of gene and transgene expression. For example, combinatorial expression of the *potassium channel tetramerization domain containing (kctd)* genes *kctd12.1, ktcd12.2,* and *ktcd8* revealed 6 molecularly distinct domains at the larval stage, with 3 differing in size between the left and right dorsal habenula, 2 unique to the left, and 1 unique to the right (Gamse *et al.*, 2005). The transgenic line *Tg(brn3a-hsp70:GFP),* which was generated using regulatory sequences from the *POU domain, class 4, transcription factor 1* (*pou4f1*) gene, labels neurons in the adult dorsal habenula in a mostly non-overlapping pattern with *kctd12.1* expression (Aizawa *et al.*, 2005). The *brn3a* and *kctd12.1* domains have been proposed to correspond to the medial (dHbM) and lateral (dHbL) subnuclei of the dorsal habenulae, respectively (Aizawa *et al.*, 2005). Integration into the *neuronal activity regulated pentraxin* (*nptx2*) locus generated the *Tg(nptx2:Gal4-VP16)* driver line, which also activates reporter gene expression in a largely complementary pattern to *Tg(brn3a-hsp70:GFP)* in the dHbL of the adult brain (Agetsuma *et al.*, 2010). Habenular axons emanating from the dHbL are thought to innervate the dorsal, intermediate and most dorsal part of the ventral IPN, while those originating from dHbM neurons project to the intermediate and ventral IPN (Agetsuma *et al.*, 2010; Aizawa *et al.*, 2005; Gamse *et al.*, 2005).

Transgenic lines have also been used to assess habenular L-R asymmetry. The *Tg(brn3a-hsp70:GFP*) labeled neuronal population is larger in the right habenula while, conversely, in *nptx2:Gal4; UAS:DsRed2* double heterozygous fish more DsRed2-positive neurons are located in the left habenula. The size asymmetry between the dHbM and dHbL subnuclei has been attributed to a higher number of early born dHbL neurons in the left dorsal nucleus and later born dHbM neurons enriched on the right (Aizawa *et al.*, 2007). Prolonging the early period of neurogenesis may also increase the dHbL subnuclei and decrease the dHbM, resulting in bilaterally symmetric dorsal habenulae (Doll *et al.*, 2011).

The goal of the present study was to determine how distinct neuronal populations defined by neurotransmitter phenotype correspond to the previously designated subnuclear regions of the dorsal habenulae. Through double labeling in the larval and adult brain, we provide a consolidated molecular map of the dorsal habenular subnuclei and demonstrate that L-R asymmetric cholinergic and peptidergic subregions only partially overlap with the previously proposed dHbL and dHbM subnuclear organization. By comparing the results in zebrafish with published studies on the medial habenulae of the adult rodent brain, we find that specific neurotransmitter-expressing domains and their spatial relationships are mostly conserved, even though L-R differences are not. Precise definition of subnuclei is important when monitoring neuronal activity and interpreting behavioral responses in efforts to understand how the habenular region of the brain regulates its diverse functions. Moreover, performing such analyses using the zebrafish model could shed light on the significance of directional asymmetry in an essential neural pathway.

MATERIALS AND METHODS

Zebrafish

Zebrafish were raised and housed at 27°C on a 14/10 h light dark cycle. We used the wild-type AB strain (Walker, 1999) and the transgenic lines *TgBAC(gng8:Eco.NfsB- 2A-CAAX-GFP)*^{c375} (deCarvalho *et al*., 2013), *Tg(vglut2a: loxP-DsRed-loxP-GFP)*^{nns9} (Miyasaka *et al.*, 2009), *Tg(brn3a-hsp70:GFP)*^{rw0110b} (also known as *Tg(pou4f1-hsp70l:GFP);* Aizawa *et al*., 2005), *Tg(nptx2:Gal4-VP16)*^{rw0143a} (*also known as Tg(narp:Gal4-VP16);* Agetsuma *et al*., 2010), *Tg(UAS:DsRed2)*^{rw0135} (Agetsuma *et al*., 2010), *Tg(4xnrUAS:GFP)*^{c354} (Akitake *et al.*, 2008) and *Tg(14xUAS:BGi-NLS-emGFP)*^{y262} (H. Burgess, personal communication). For simplicity, we refer to these transgenic lines as *Tg(gng8:GFP*), *Tg(vglut:DsRed), Tg(brn3a:GFP), Tg(nptx2:Gal4), Tg(UAS:DsRed2), Tg(4xUAS:GFP)* and *Tg(14xUAS:GFP)*. Maintenance of zebrafish and experimental procedures on larvae and adults were carried out in accordance with the protocol approved by Carnegie Institutional Animal Care and Use Committee.

RNA hybridization and immunofluorescence

Larvae and dissected adult brains were fixed in 4% paraformaldehyde (in PBS) at 4°C overnight. Single and double label colorimetric RNA *in situ* hybridization experiments were performed as described previously for whole larvae (Gamse *et al.*, 2003) and modified for adult brain tissue (Gorelick *et al.*, 2008). Single and double fluorescent RNA *in situ* hybridization (FISH) or FISH coupled with immunolabeling for green fluorescent protein (anti-GFP rabbit antibody; Torrey Pines TP401) was carried out as in a prior study (deCarvalho *et al.*, 2013).

To synthesize antisense RNA probe for *amine oxidase, copper containing 1* (*aoc1*), a 1019 base-pair fragment was PCR-amplified from the cDNA clone MGC154101 (Thermo Scientific) using primers GTCACTGAATACATCGTTGGCCC and TTGTAGACTGTAGATGTAGTTCTGATC and sub-cloned into the pCRII-TOPO vector using the TOPO TA Cloning kit (Invitrogen). pCRII-TOPO-*aoc1* was linearized with *Apa*I and RNA transcribed with SP6 RNA polymerase. For *anoctamin 2* (*ano2*), an *Eco*RI *to Not*I fragment from the cDNA clone MGC171498 (Open Biosystems) was subcloned into the pBluescript SK(−) vector, linearized with *Eco*RI and transcribed with T3 RNA polymerase. A zebrafish homologue to *Drosophila muscleblind-like 3* (*mbnl3*) was obtained from a zebrafish kidney cDNA library and cloned into the *Eco*RI and *Xho*I sites of the pBK-CMV vector. pBK-CMV-*mbnl3* was linearized with *Sal*I and RNA transcribed with T7 RNA polymerase.

Methods for cloning and probe synthesis have been described previously for *guanine nucleotide binding protein (G protein), gamma 8* (*gng8;* Thisse and Thisse, 2004), *kctd12.1* (previously known as *leftover*; Gamse *et al*., 2003), *kctd8* (previously known as *dexter*; Gamse *et al*., 2005), *kctd12.2* (previously known as *right on*; Gamse *et al*., 2005), *solute carrier family 18 (vesicular acetylcholine), member 3b* (*slc18a3b;* commonly known as *vachtb*; Hong *et al*., 2013), *somatostatin1.1* (*sst1.1*; Thisse and Thisse, 2004) and *tac1* (Hong *et al.*, 2013).

Sectioning and microscopy

Larval and adult brains were embedded in 4% low melt agarose (Lonza) in PBS (100g/ml) and sectioned with a VT1000S vibratome (Leica Microsystems, Inc.) from 50 to 250 μm as indicated. For labeling cell nuclei, sections were stained in 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies) in PBS with 0.1% tween for 20 minutes and rinsed in PBS. Sections were mounted in either 50% glycerol (1:1 vol/vol with H_2O), Aqua-Poly/Mount (Polysciences, Inc.) or Prolong Gold (Invitrogen) anti-fade mounting media under cover slips.

Bright-field images were captured by an Axiocam HRc digital camera mounted on an Axioskop (Carl Zeiss). Fluorescent images were collected using a Leica SP5 confocal microscope and processed either with ImageJ (National Institutes of Health) or Imaris (Bitplane).

RESULTS

As summarized in Table 1, an increasing number of genes have been found to be expressed in the bilaterally paired dorsal and ventral habenulae of larval and adult zebrafish, and many of their homologues are transcribed in the corresponding medial and lateral habenular nuclei of rodents. For example, similar to expression of *anoctamin 1* (*ano1*) in the medial habenula of the mouse (Quina *et al.*, 2009), *ano2* is transcribed in a bilaterally symmetric pattern throughout the dorsal nucleus of larval zebrafish (Fig. 1a), and is a useful marker for demarcating its boundaries. In zebrafish, the ventral nucleus was defined by its expression of the *amine oxidase, copper containing 1* (*aoc1*) gene (Amo *et al*., 2010 and refer to Fig. 1b). We find that a zebrafish homologue of the *muscleblind-like 3* (*mbnl3*) gene also exhibits bilaterally symmetric expression throughout the presumptive ventral nucleus (Fig. 1c, d), providing further evidence that the ventral habenulae have a distinct molecular identity. However, not all genes are transcribed exclusively in the dorsal or ventral nucleus. Transcripts for the *guanine nucleotide binding protein (G protein), gamma 8* (*gng8*) gene, for instance, are distributed in a bilaterally symmetric pattern similar to *ano2* (Fig. 1e, f) but are also found in a subset of neurons in both ventral nuclei (arrowheads in Fig. 1e). A transgenic line produced by integration of membrane-tagged green fluorescent protein gene into the *gng8* locus (deCarvalho *et al.*, 2013) labels the same pattern of dorsal and ventral habenular neurons (refer to Fig. 2a).

Additionally, a number of genes show L-R asymmetric expression in the dorsal habenular nuclei (e.g., Agetsuma *et al.*, 2010; Aizawa *et al.*, 2005; Amo *et al.*, 2010; Gamse *et al.*, 2005; Kuan *et al.*, 2007; Taylor *et al.*, 2011 and refer to Table 1). Members of the *potassium channel tetramerization domain containing* gene family were first discovered to exhibit L-R differences, with transcripts for *kctd12.1* and *kctd8* more extensive on the left and right sides, respectively (Gamse *et al*., 2005 and Fig. 1g, i). Double labeling confirms that expression of both genes is confined within the limits of the *ano2*-expressing dorsal habenular nuclei (Fig. 1h, j). *kctd12.2* is not only expressed to a greater extent in the right dorsal nucleus (Fig. 1k), but also bilaterally in a subset of cells in the ventral habenular nuclei (Fig. 1l, arrowheads). Although gene expression patterns such as these appear to

demarcate discrete subregions within the dorsal habenulae, how such asymmetric domains correlate with functional subnuclei is unclear.

Discrete asymmetric cholinergic and peptidergic subnuclei of the larval dorsal habenulae

In mammals, as described above, habenular subnuclei have been identified on the basis of neurotransmitter phenotype and neuronal connectivity. Recent work by Hong *et al*. (2013) demonstrated that the zebrafish larval habenula contains glutamatergic and cholinergic neurons as early as 4 days post-fertilization (dpf). We determined the position of these neuronal groups more systematically using the *gng8:GFP* transgenic background or with respect to the asymmetric expression of *kctd12.1*. In *Tg(gng8:GFP)* larvae, neurons labeled with membrane-tagged GFP are found throughout the dorsal habenulae (Fig. 2a, b) and more sparsely in the ventral nucleus (arrowheads). Co-labeling with *vglut2a:DsRed*,, indicates that these neurons are all glutamatergic (Fig. 2c, d). In the ventral nucleus, *vglut2a:DsRed* expression is more widespread than *gng8:GFP*.

Expression of the *vesicular acetylcholine transporter b* gene (*vachtb*) demarcates the L-R asymmetric cholinergic regions of the dorsal habenulae as well as the bilaterally symmetric ventral nuclei (Hong *et al.*, 2013). Double fluorescence RNA *in situ* hybridization demonstrates that *vachtb* and *kctd12.1* have complementary patterns of expression (Fig. 2e, f); thus, the *kctd12.1*-positive region corresponds to the non-cholinergic territory of the dorsal habenulae. In mammals, this non-cholinergic region contains substance P-expressing neurons. Transcripts for the zebrafish substance P precursor *tachykinin* (*tac1*) are not detected in the habenular region as early as 4 dpf, although they are present elsewhere in the brain (data not shown). However, we find that the neuropeptide encoding gene *somatostatin1.1* (*sst1.1)* is expressed at this stage, in a lateral subdomain of the dorsal habenula (Fig. 2g, h). The *sst1.1* neuronal population is located within the non-cholinergic *kctd12.1*-expressing territory and is larger on the right side of the brain than the left (Fig. 2i, j). Analyses of the combinatorial gene expression patterns in 4 dpf larvae, therefore, indicate that neurons of the dorsal habenulae are all glutamatergic, with a portion of them possessing either a cholinergic or *sst1.1* identity. In the larval brain, the non-overlapping cholinergic and somatostatin neuronal populations are significantly larger in the right dorsal habenula compared to the left.

Asymmetric neurotransmitter-specific subpopulations are retained in the adult dorsal habenulae

We next examined whether L-R asymmetric neuronal populations are maintained during the morphological changes that accompany formation of the adult zebrafish brain (refer to Amo *et al*., 2010). As in the larva, neurons throughout the dorsal and ventral habenular nuclei of adult zebrafish are glutamatergic as revealed by DsRed labeling from the *vglut2a* transgenic reporter (Fig. 3a). In the adult, the cholinergic territory is more extensive in the left habenula (Fig. 3b, c) compared to the larval stage (Fig. 2e, f). At the border, cholinergic and non-cholinergic cells are clearly distinct populations in the left dorsal nucleus, whereas some intermixing of *vachtb* and *kctd12.1*-expressing cells is observed in the right habenula (arrowhead, Fig. 3c). The *sst1.1* and *vachtb* expression domains are also non-overlapping in the left habenula (Fig. 3d, e), although a subset of cells appears to express both genes in the

right dorsal habenula (Fig. 3e, arrowhead). The neuropeptide encoding genes *sst1.1* and *tac1* are expressed by distinct neuronal populations within the *kctd12.1*-positive, non-cholinergic territory (Fig. 3f, g and 3h, i, respectively), (Fig. 3j, k). As in the larva, the *sst1.1*-expressing domain is larger in the right habenula (Fig. 3f, j). Overall, the cholinergic and peptidergic territories are preserved between the larval and adult stages; however, some neurons in the right habenula exhibit an overlap in *sst1.1* and *vachtb* expression that was not detected at the larval stage.

Partial correspondence of neurotransmitter-expressing subdomains with habenular transgenic reporters

Zebrafish transgenic lines that label habenular neurons with fluorescent proteins have been used to define dorsal habenular subregions, to trace axonal connections to the IPN, and in behavioral studies following neuronal ablation or inactivation (Agetsuma *et al.*, 2010; Aizawa *et al.*, 2005; Lee *et al.*, 2010). Notably, *Tg(brn3a:GFP)* was designated as labeling the dHbM subnucleus and *Tg(nptx2:Gal4*) as driving expression in the dHbL (Agetsuma *et al.*, 2010; Aizawa *et al.*, 2005; Okamoto *et al.*, 2012). After establishing the gene expression patterns and neurotransmitter identity of habenular subregions, we sought to determine how this regionalization corresponds with habenular labeling by these transgenes.

We mated *Tg(brn3a:GFP)* and *Tg(vglut2:DsRed)* carriers and observed that all GFP-positive cells in the habenulae of their larval progeny co-express DsRed (Fig. 4a, b), indicating that *brn3a*:GFP-positive cells are glutamatergic. In the dorsal habenulae of adults, the *brn3a:GFP* labeled region was found to overlap only partially with the cholinergic territory as defined by *vachtb* expression (Hong *et al.*, 2013). We observed a similar pattern at the larval stage, with distinct neuronal populations that were either co-labeled, *vachtb* positive/*brn3a:GFP* negative (Fig. 4c, arrowhead) or *vachtb* negative/*brn3a:GFP* positive (Fig. 4c, arrow). Thus, the dorsal habenular subregion labeled by the *brn3a:GFP* transgene does not fully encompass a cholinergic subnucleus. Although the *brn3a*-GFP labeled neurons were designated as a distinct subnucleus in the adult habenula from that labeled by *Tg(nptx2:Gal4); Tg(UAS:DsRed2*) (Agetsuma *et al.*, 2010), some neurons co-label with both fluorescent proteins in the larval dorsal habenula (Fig. 4d, arrowheads).

Consistent with their location in the *kctd21.1* positive non-cholinergic territory, *sst1.1*-expressing neurons were not co-labeled by the *brn3a*:GFP transgene (Fig. 4e). These cells were found in a similar region of the dorsal habenula as neurons labeled by the *nptx2:Gal4* and *UAS:DsRed2* transgenes. Upon determining whether the *sst1.1* neurons co-express *nptx2:Gal4*, we unexpectedly found that the extent of labeling varied significantly depending on the UAS-regulated transgenic reporter that was activated by this Gal4 driver (compare Fig. 4f and g, h). However, despite the variability in labeling between lines, only a subset of *sst1.1*-expressing neurons were co-labeled by the *nptx2:Gal4* driver for the three UAS-regulated reporters tested (Fig. 4i). Thus, expression driven by *Tg(nptx2:Gal4)* does not represent a discrete somatostatin neuronal population.

Habenular connectivity with the IPN

Although transgenic labeling in the dorsal habenulae does not appear to correspond to specific neuronal populations defined by neurotransmitter/neuropeptide expression, it is still useful for examining connectivity with the IPN. In zebrafish larvae and adults, the *gng8:GFP* transgene labels all dorsal habenular neurons (Fig. 2a) and their GFP-positive axons that terminate throughout the IPN (Fig. 5a, d, g, j). Because GFP is membrane-tagged in this line, discrete axonal densities can be recognized in a stereotypic pattern in sagittal sections of the adult IPN (Fig. 5a), with a minimum of 2 dorsal (D-i and D-ii) and 3 ventral clusters (V-i, V-ii and V-iii). An intermediate (I), horseshoe-shaped zone of innervating fibers separates the dorsal and ventral axonal bundles. The stereotypic morphology of axonal bundles at the IPN is already apparent at 4 dpf (Fig. 5j).

Using *Tg(brn3a:GFP)* zebrafish, it had been previously shown that GFP labeled habenular axons predominantly terminate in the intermediate and ventral regions of the adult IPN (Aizawa et al., 2005) and axons labeled by *Tg(nptx2:Gal4);Tg(UAS:DsRed2*) innervate the dorsal, intermediate and ventral IPN (Agetsuma *et al.*, 2010). We corroborated these results and found that, more specifically, in the ventral IPN of adult zebrafish, the *brn3a:GFP* terminals primarily contribute to the V-i and V-iii axon bundles (Figs. 5b, e) and those labeled by the *nptx2:Gal4* driver and *UAS:DsRed2* reporter innervate the V-ii bundle (Figs. 5c, f).

By mating *Tg(brn3a:GFP)* and *Tg(nptx2:Gal4);Tg(UAS:DsRed2)* carriers, the relative extent of GFP and DsRed2 axon terminals along the dorsoventral IPN was examined in transgenic larvae. At 4 dpf, a largely similar pattern of innervation was found in IPN subregions as in the adult (Fig. 5h, k). Because neurons in the left habenula were more extensively labeled when the *nptx2:Gal4* transgenic driver was used to drive *4xUAS:GFP* (Fig. 4g, h), we compared the profiles of innervating axons at the IPN using the two fluorescent reporter lines. Habenular efferents were weakly labeled by the *UAS:DsRed2* transgene in subregions of the dorsal and ventral IPN (Fig. 5h, k). Significantly more GFP-labeled axon terminals were observed in the D-i, D-ii and, I regions with the *4xUAS:GFP* line (Fig. 5i, l), consistent with this reporter being activated to a greater extent in the left habenula (Fig. 4g, h).

Because *brn3a:GFP* labels many cholinergic neurons but not *sst1.1*-expressing neurons, and the axons of *brn3a:GFP* positive neurons predominantly innervate the D-ii, I, V-i and V-iii regions of the IPN, we expect that these IPN regions receive cholinergic input. With only partial overlap between *sst1.1*- and *nptx2:Gal4*-expressing populations, it is difficult to infer the precise pattern of connectivity of this neuronal subgroup with certainty; however, efferents from the somatostatin neurons are expected to contribute to some of the same subregions of the IPN as the neuronal populations labeled by the *nptx2* driver (Fig. 5k, l).

DISCUSSION

In the nervous system, it is generally assumed that discrete clusters of neurons exhibiting the same neurotransmitter phenotype fasciculate to innervate specific target regions and function together. In addition to their shared expression of particular neurotransmitters or

neuropeptides, brain nuclei or their component subnuclei are defined by neuroanatomical features such as cell cluster size, density of cell packing, neuropil structure or stereotypic position. The significance of nuclear designations is especially relevant when modern genetic methods are employed to eliminate or modify brain regions for insights into their roles in physiology and behavior. These approaches have been applied to the study of the habenular nuclei in mammalian and non-mammalian systems (Agetsuma *et al.*, 2010; Lee *et al.*, 2010; Quina *et al.*, 2009; Yamaguchi *et al.*, 2013); however, detailed information about the precise subregions that are affected would allow more rigorous functional correlations.

The goal of this study was to generate a consolidated map of the habenular region in larval and adult zebrafish, by reexamining the expression of widely used genetic markers, describing the spatial expression patterns of newly identified genes, and determining how this information corresponds with neurotransmitter profiles and cell populations labeled by transgenic reporters. In addition, we further characterized the extent of L-R asymmetry in the dorsal habenular nuclei.

Designation of habenular subnuclei

The dorsal habenulae in the adult zebrafish brain have been subdivided into medial and lateral subnuclei on the basis of *Tg*(*brn3a:GFP*) labeling and by expression of *kctd12.1* or directed by the *Tg(nptx2:Gal4)* driver (Agetsuma *et al.*, 2010; Aizawa *et al.*, 2005). The designation of the medial subdivision is further supported by experiments showing that *brn3a:GFP* positive and negative neurons have different birthdates, and that L-R differences in the timing of neurogenesis later contributes to the formation of asymmetric *brn3a*-labeled populations. Several lines of evidence, however, suggest that the regions defined by these transgenic tools may not represent discrete, functional subnuclei. Although initially defined as corresponding to distinct subnuclei, the *kctd12.1* and *brn3a* domains do not appear to be completely segregated in the adult dorsal habenulae (Aizawa et al., 2005). In addition, the reported patterns of *brn3a:GFP* and *nptx2:Gal4; UAS:DsRed2* labeled neurons fail to recapitulate endogenous gene expression, which appears more widespread in the habenular region for both genes (Agetsuma *et al.*, 2010; Aizawa *et al.*, 2005; Aizawa *et al.*, 2007). Partial overlap in labeling from the *brn3a:GFP* and *nptx2:Gal4* transgenes in the larval habenula further suggests that expression of these markers does not correspond to discrete subnuclei. While the *brn3a:GFP* population significantly overlaps with *vachtb*-expressing neurons in the larval dorsal habenula, a subset of cholinergic neurons are not labeled by this transgene. Similarly, the region of the dorsal habenulae labeled by the *nptx2:Gal4* transgenic driver only partially overlaps with *somatostatin1.1*-expressing neurons. Together, these findings suggest that caution should be taken in interpreting *brn3a:GFP* and *nptx2:Gal4; UAS:DsRed2* transgenic labeling as representative of functional habenular subnuclei at least for larval stages.

Distinct neuronal populations in the zebrafish dorsal habenula defined by neurotransmitter identity

The significance of subdomains of gene expression in the zebrafish habenulae is largely unknown. As a first step, we sought to determine how they might correlate with specific neuronal subgroups by examining the expression of zebrafish homologues for genes

indicative of neurotransmitter identity. This approach avoids potential problems associated with using antibodies that are typically generated against mammalian antigens. We examined larval and adult stages, as some neurons are known to undergo changes in neurotransmitter phenotype over time (e.g., Landis and Keefe, 1983; Spitzer, 2012) and new neuronal subgroups may also arise.

The analysis of larval zebrafish revealed that cholinergic neurons are a distinct population from cells expressing *kctd12.1*, which encodes a protein shown to associate with gamma-aminobutyric B ($GABA_B$) receptors to influence their pharmacology and signaling properties (Ivankova *et al.*, 2013; Schwenk *et al.*, 2010) and to promote formation of dense neuropil in the zebrafish dorsal habenula (Taylor *et al.*, 2011). Instead, we found that the *kctd12.1* domain contains *somatostatin1.1*-expressing peptidergic neurons.

All neurons of the zebrafish dorsal and ventral habenulae are glutamatergic. In the asymmetric dorsal habenulae of larval zebrafish, the left habenular nucleus is mainly composed of a glutamatergic-only subregion with additional small cholinergic and *sst1.1* clusters. In contrast, the right nucleus largely consists of expanded cholinergic and *sst1.1* subregions.

During maturation of the zebrafish brain to its adult form, the presumptive ventral nuclei become repositioned ventromedially (Amo *et al.*, 2010), but the relative positions of subnuclear territories within the dorsal nuclei largely appear the same. Left-right differences in the size of neurotransmitter expressing populations, however, are less pronounced. Notably, at 4 dpf there is only a small cluster of cholinergic neurons in the left dorsal habenula, but by adulthood the proportion of neurons that express cholinergic genes is more similar between the left and right nucleus. In adults, both habenula also acquire a *tac1*-expressing subdomain (Hong *et al.*, 2013), in addition to the cholinergic, *sst1.1* and glutamatergic-only subregions.

Curiously, while the boundaries between these four neuronal populations are discrete in the left dorsal habenula, they are not as well demarcated on the right, due to intermixing of neurons expressing different neurotransmitters. It is the left habenula of zebrafish that receives preferential innervation from the parapineal organ, an accessory to the pineal organ, which in most individuals is asymmetrically positioned to the left of the pineal. The parapineal influences the gene expression and neuroanatomical properties of the left dorsal habenula that distinguish it from the right (Concha *et al.* 2003; Gamse et al., 2003). It is tempting to speculate that parapineal neurons may also serve to refine subnuclear organization, ensuring the formation of precise boundaries between peptidergic and cholinergic clusters in the left habenula. In certain frogs, the photoreceptive frontal organ sends asymmetric projections across the left habenular nucleus (Eldred *et al.*, 1980; Guglielmotti and Cristino, 2006; Kemali and De Santis, 1983) and the left habenula contains distinct cholinergic and substance P subnuclei (Kemali and Guglielmotti, 1984; Marin *et al.*, 1997). The right habenula also has cholinergic and substance P neurons (Kemali and Guglielmotti, 1984; Marin *et al.*, 1997); however, as in zebrafish, the boundaries between these neuronal populations are not as clearly delineated as on the left. In mammals, where there is no structure analogous to the parapineal, deep pineal neurons project to both medial

habenular nuclei (Korf *et al.*, 1990), and both exhibit distinct cholinergic and substance P subnuclei (Cuello *et al.*, 1978). Whether innervating parapineal or pineal neurons play a role in the refinement of the habenular map into discrete subnuclear compartments remains to be demonstrated experimentally.

From the analysis of neurotransmitter and neuropeptide producing neuronal populations, we present a schematic map of the dorsal habenulae of larval and adult zebrafish (Fig. 6a). It is important to stress that this is only a preliminary map, as it is highly likely that additional neuropeptides or transmitters are co-expressed with glutamatergic neurons and located in other subregions of the dorsal habenulae. For example, cells immunoreactive for thyrosine hydroxylase important in L-DOPA synthesis have been identified in the medial habenulae of alpaca (Marcos *et al.*, 2013) and mRNA encoding orphanin FQ as well as this neuropeptide were localized to the rat medial habenulae (Neal *et al.*, 1999). Indeed, as indicated in Table 1, genes encoding *tachykinin 3a* (Biran *et al.*, 2012; Ogawa *et al.*, 2012) and receptors for glutamate (Haug *et al.*, 2013), serotonin (Norton *et al.*, 2008) and hypocretin (Appelbaum *et al.*, 2009) show enriched expression in subregions of the zebrafish dorsal habenulae, suggestive of other neuronal specializations.

Designating subnuclei in the zebrafish habenulae on the basis of neurotransmitter identity enables a more direct comparison with mammalian brains, in efforts to uncover the habenular subregions and circuitry modulating a wide variety of behaviors. In rodents, the medial habenulae are bilaterally symmetric and contain glutamatergic neurons (Barroso-Chinea *et al.*, 2007; Qin and Luo, 2009), which have been subdivided into dorsal and ventral subnuclei depending on whether they utilize substance P or acetylcholine (Aizawa *et al.*, 2012; Contestabile *et al.*, 1987; Cuello *et al.*, 1978; Lecourtier and Kelly, 2007; Quina *et al.*, 2009). Recent work reveals an additional glutamatergic-only subnucleus, which is found medially to the substance P subnucleus (Aizawa *et al.*, 2012). The location and organization of subnuclei designated by neurotransmitter phenotype in the dorsal habenulae of adult zebrafish is strikingly similar to that of the rodent medial habenulae. An exception is the presence of somatostatin neurons, which has not yet been described for the rodent medial habenula. Somatostatin-immunopositive fibers are found only in the lateral habenulae of the postnatal rat (Shiosaka *et al.*, 1981). However, images from the Allen Brain Atlas indicate that the *sst* gene is transcribed in neurons in the medial habenula of the mouse, in the same region where cholinergic neurons are located. Further studies are needed to ascertain the location of these neurons relative to the cholinergic population, in order to determine whether there is a distinct somatostatin-producing subnucleus as in the zebrafish dorsal habenula.

Connectivity of dorsal habenular subnuclei with the IPN

Neurons of the medial habenula of mammals project their axons through the fasciculus retroflex to terminate at the interpeduncular nucleus (refer to Kappers *et al.*, 1936; Sutherland, 1982)). From the accumulations of terminating fibers, the structure of the IPN has been well defined in rodents as consisting of 3 unpaired (rostral, apical, central) and 4 paired (intermediate, lateral, rostral lateral, dorsal lateral) subdivisions in a consensus nomenclature put forward by Lenn and Hamill (1984) Lesioning studies, histochemistry and

immunoreactivity have revealed the presence of a wide variety of neurotransmitters and neuropeptides in axons terminating within particular subdivisions of the IPN (reviewed by Morley, 1986).

Visualization of a transgenic line with expression of membrane-tagged GFP under the control of *gng8* regulatory sequences (deCarvalho *et al.*, 2013) provides a complete picture of innervation of the zebrafish IPN by dorsal habenular neurons. As with previous dye labeling and immunolabeling experiments (Aizawa *et al.*, 2005; Gamse *et al.*, 2005; Tomizawa *et al.*, 2001), the axon terminals of habenular neurons can be readily distinguished as contributing to bundles in dorsal, intermediate and ventral regions of the larval and adult IPN. However, because our understanding of the zebrafish is far from complete, it is premature to assign a nomenclature analogous to that proposed for mammals (Lenn and Hamill, 1984). We, therefore, designated IPN afferent input as consisting of two dorsal (D-i and D-ii) and three ventral (V-i, V-ii, and V-iii) axonal densities and an intermediate fiber bundle (I) that separates them on the basis of the stereotypic morphology of GFP labeled afferents (and refer to Fig. 11 in Agetsuma et al., 2010). Moreover, we find that the pattern of innervating fibers is already in place in the IPN of 4 dpf larvae. This simplified naming strategy must be modified as more information is obtained about the precise homology with mammalian IPN subdivisons and the distribution of synaptic terminals expressing different neurotransmitters.

Previous work had demonstrated that that *brn3a:GFP* and *nptx2:Gal4* transgenic lines primarily label different neuronal populations in the dorsal habenulae of adult zebrafish, which innervate largely distinct dorsoventral regions of the IPN (Agetsuma *et al.*, 2010). Overall, our findings support this connectivity map for the adult, with the potential for overlapping input in the D-ii and I IPN subregions (shown in Fig. 6b). In 4 dpf larvae, these transgenes label innervating fibers throughout the IPN, although they are more concentrated in certain subregions at this early stage. For example, *brn3a:GFP* labeled axons predominate in the V-i density and to a lesser extent in V-iii. Because the pattern of neuronal labeling in the larval habenula depends on the UAS reporter under *nptx2:Gal4* control, the location of labeled axons also varied between lines. With the *UAS:DsRed2* reporter that labeled neurons sparsely in both habenulae, axonal endings were enriched in the D-i and V-ii bundles, relative to the *brn3a:GFP* labeled terminals. However, when a significantly larger group of neurons in the left habenula was labeled by *4xUAS:GFP*, innervating axons were primarily detected in the D-i, D-ii and I regions of the IPN.

Differences in the pattern of IPN innervation between larval and adult stages, such as the strongly labeled V-ii terminal bundle present in *nptx2:Gal4* transgenic adults or the increase in *brn3a:GFP* labeled terminals in the V-iii region, are likely the outcome of differential neurogenesis in subregions of the developing dorsal habenular nuclei and continued axonal outgrowth to target regions. Understanding how and when new neuronal populations arise, such as the expanded cholinergic cluster on the left or the bilateral *tac1*-expressing groups, will help clarify how the habenulo-interpeduncular connectivity map evolves over time.

We can predict the most likely sites of cholinergic and peptidergic afferent input to the IPN from the partial overlap between neurotransmitter expression and transgene labeling in

dorsal habenular neurons. As depicted schematically in Fig. 6c, cholinergic neurons are expected to terminate in the D-ii, I and V IPN regions, owing to the majority of them being labeled by *brn3a:GFP*. Based on partial overlap with the *nptx2:Gal4* driver and lack of co-expression with *brn3a:GFP*, the contribution of *sst1.1*-expressing neurons would be limited to the dorsal IPN, intermediate IPN, and V-ii region of the ventral IPN. A similar profile is expected for substance P neurons in the adult brain based on the location of *tac1*-expressing neurons within the *brn3a:GFP* negative region (Hong *et al.*, 2013). While the proposed connectivity map provides a useful guide, it is an oversimplification that rests on indirect inferences and will need to be validated by the production and analysis of transgenic lines that selectively label each neurotransmitter-expressing population of the dorsal habenulae.

L-R asymmetry in IPN connections

The value of having a framework to classify afferent input is that it helps resolve a discrepancy concerning how left and right habenular neurons connect with the IPN in the zebrafish brain. One model suggests that the left habenula primarily projects to the dorsal IPN and the right habenula innervates the ventral IPN in a laterotopic manner (Aizawa *et al.*, 2005; Bianco *et al.*, 2008). Results from dye labeling, immunolabeling and transgenic labeling of habenular axons, however, indicate a more complicated scenario with differing proportions of neurons from the left and right sides of the brain contributing to both the dorsal and ventral IPN (Agetsuma *et al.*, 2010; Beretta *et al.*, 2012; Gamse *et al.*, 2005; Kuan *et al.*, 2007; Okamoto *et al.*, 2012). For the right habenula of the larval brain, we hypothesize that the significantly larger cholinergic subnucleus innervates the ventral IPN. In the left habenula, the smaller cholinergic neuronal cluster would also project to the ventral IPN while the larger population of non-cholinergic neurons would innervate the dorsal IPN.

This revised model can explain two previous puzzling observations. First, when the parapineal is ablated and the left dorsal habenula adopts the molecular profile and neuroanatomical properties of the right dorsal habenula, the vast majority of habenular efferents project to the ventral IPN (Gamse *et al.*, 2005; Kuan *et al.*, 2007). These projections are presumed to emanate from the large cholinergic subnucleus that, following loss of the parapineal, is present in the left as well as the right dorsal habenula (K. Santhakumar, T. deCarvalho and M.E. Halpern, unpublished observations). However, a small axonal bundle remains at the dorsal IPN (Gamse *et al.*, 2005) that may represent the efferents of peptidergic neurons present in both habenulae. Second, a study analyzing the projections of individual neurons described distinct axonal morphologies for cells labeled in the left dorsal habenula versus the right (Bianco *et al.*, 2008). Axons with the left morphology (L-typical) exhibit a greater degree of arborization and innervate an extensive region of the IPN and those with the right morphology (R-typical) show less axonal branching and innervate a more dorsoventrally restricted region. Even though neurons with a L-typical arbor predominate in the left habenula and those with a R-typical arbor are the majority in the right habenula, neurons having the opposite axonal morphology were also observed (Bianco *et al.*, 2008). We propose that the subset of neurons in the right habenula with L-typical arbors contain the *sst-1* subpopulation that innervates the dorsal IPN; whereas neurons in the left habenula with R-typical arbors correspond, in part, to the small

cholinergic group that projects ventrally. In this model, neurons from both sides of the brain that utilize the same neurotransmitter innervate the same regions of the IPN.

The neurotransmitter profile of the zebrafish dorsal habenular nuclei serves as a useful guide to understand habenular function, but also points to many outstanding questions. Knowledge of how subsets of habenular axons form connections at the appropriate subregion of the IPN is lacking. Selective expression of Neuropilin1a in only the left dorsal habenula allows neurons to respond to Semaphorin3b signaling and directs them to innervate the dorsal IPN (Kuan *et al.*, 2007). However, more cues must be involved in steering habenular axons at the IPN and determining their precise connectivity along its dorsoventral axis. Once at the target, the interplay between habenular efferents and IPN neuronal populations is largely unknown. In larval zebrafish, IPN neurons can differ greatly in their morphology (Bianco *et al.*, 2008) and cells with elaborate processes may receive diverse input from innervating habenular fibers. Although recent studies have focused on the modulatory role of dorsal habenular neurons in fear responses (Agetsuma *et al.*, 2010; Lee *et al.*, 2010), the exact neuronal subtypes involved remain unclear. Specific functions and microcircuits involving the distinct cholinergic and somatostatin-expressing subsets of neurons must also be determined. A greater understanding of the neurotransmitter identity, subnuclear organization, and connectivity of the zebrafish dorsal habenulae will ultimately reveal why the left and right nuclei differ and how this specialization might influence behavior.

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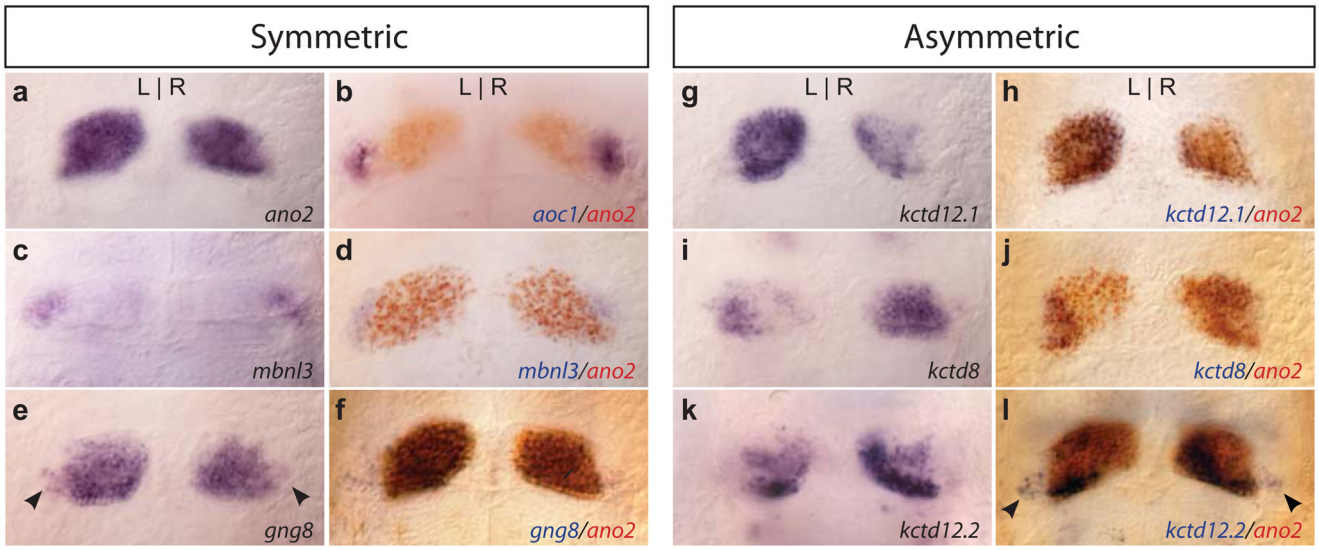

FIG. 1. Bilaterally symmetric and left-right asymmetric gene expression in the larval habenular nuclei

(**a**) Symmetric expression of the *ano2* gene delineates the boundaries of the dorsal habenular nuclei, whereas (**b**) *aoc1* (Amo *et al*., 2010) and (**c**, **d**) *mbnl3* transcripts localize to the ventral habenular nuclei. (**e**, **f**) Symmetric expression of *gng8* encompasses the entire dorsal habenulae, and is found in a subset of cells in the ventral nucleus (arrowheads). (**g**) The *kctd12.1* and (**i**,) *kctd8* genes show predominant expression in the left or right side, respectively (Gamse *et al*., 2005), which is confined within the boundaries of the dorsal nuclei (**h, j**). (**k**) *kctd12.2* transcripts are more abundant in the right dorsal nucleus (Gamse *et al*., 2005), and (**l**) also found bilaterally in the ventral nuclei (arrowheads). All larvae are 4 dpf.

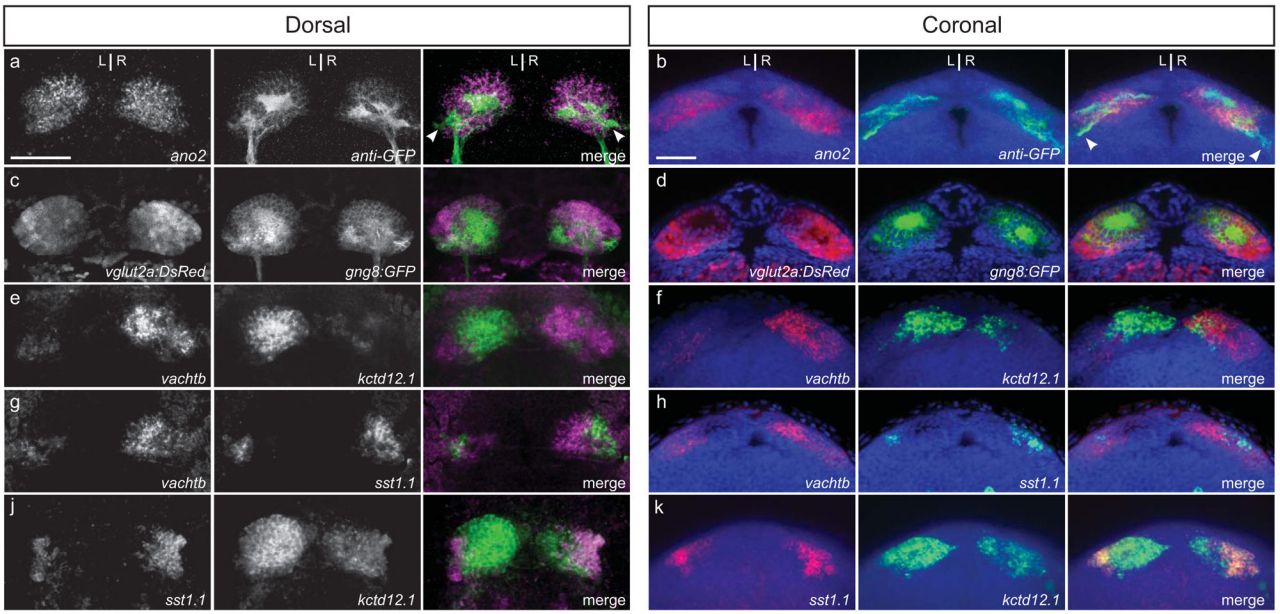

FIG. 2. L-R asymmetric cholinergic and peptidergic subdomains of the larval dorsal habenulae

(**a**, **b**) *gng8:GFP* labeling detected by anti-GFP immunofluorescence is coextensive with *ano2* in the dorsal habenular nuclei. The membrane-tagged GFP also labels a subpopulation of neurons in the ventral nucleus (arrowheads). (**c**, **d**) *vglut2a:DsRed* labels neurons throughout the dorsal and ventral habenulae. (**e, f**) *vachtb* and *kctd21.1* are expressed in different neuronal populations, which are more intermingled in the right habenula than the left. (**g, h**) *vachtb* is transcribed in a non-overlapping pattern with *sst1.1*-expressing neurons located in a lateral subregion of the dorsal habenula. (**i**, **j**) The *sst1.1*-expressing cells are confined within the *kctd12.1* domain and are more abundant in the right dorsal habenula than the left. All larvae are 4 dpf. Scale bars are 30 μm.

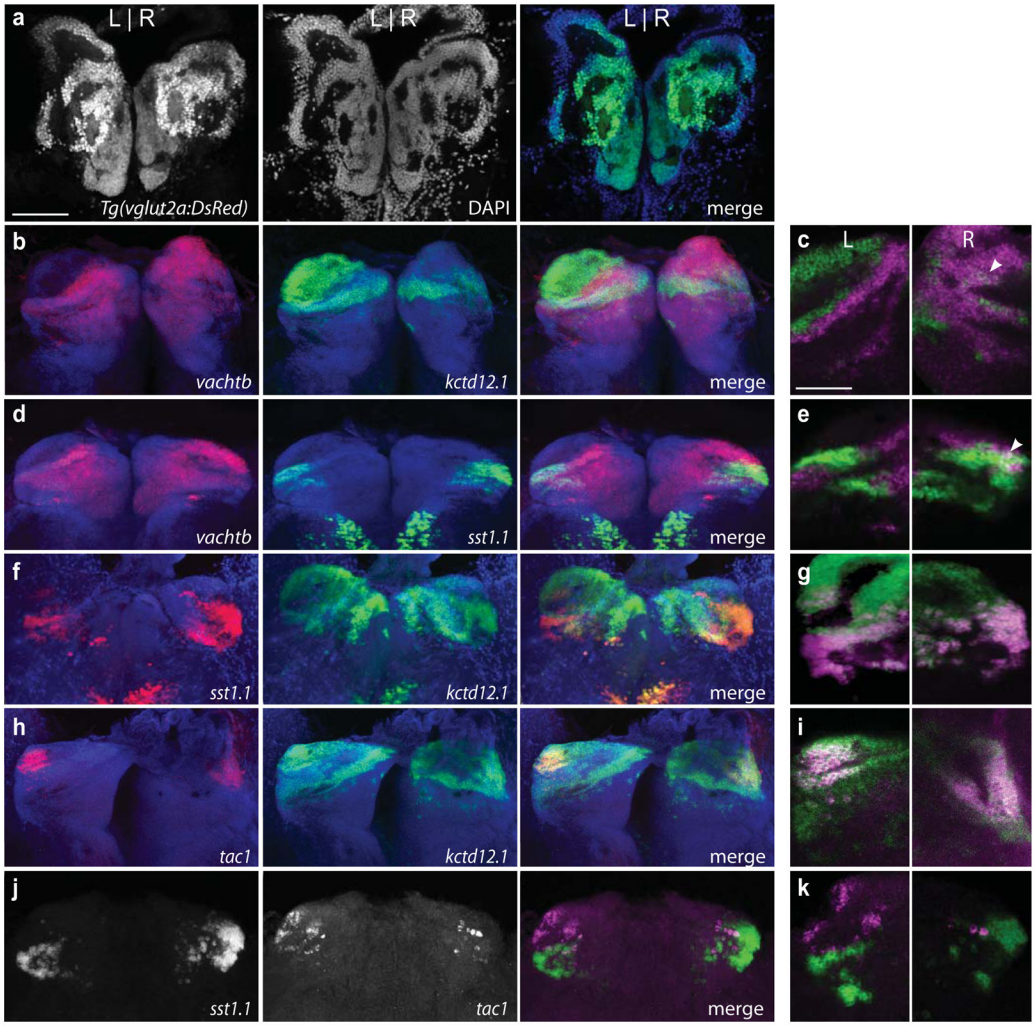

FIG. 3. Neurotransmitter subpopulations of the adult dorsal habenulae

(**a**) *Tg(vglut2a:DsRed)* is expressed throughout the dorsal and ventral habenulae. **b, d**, **f**, **h**, **j** are z-stack maximum projections of the dorsal habenula. **c**, **e**, **g**, **i**, **k** are magnified views of a single focal plane. (**b**) Complementary expression pattern of *vachtb* and *kctd12.1* (z volume = 56 μm) with (**c**) no overlap on the left but some intermingling of cells in the right nucleus (arrowhead). (**d**) *vachtb* and *sst1.1* are mainly expressed in different neuronal populations (z volume = 53 μm) except for (**e**) a small subset of co-expressing cells in the right habenula (arrowhead). (**f, g**) *sst1.1* is mainly co-expressed within the *kctd12.1* domain (z volume= 69 μm). (**h, i**) *tac1* is also co-expressed with *kctd12.1*, (z volume= 64 μm) (**j**) *sst1.1* and *tac1* are transcribed in distinct neuronal populations (z volume= 14 μm). All images are of coronal sections and, except for Figs. **j, k**, were stained with DAPI, represented in the blue channel. Scale bar is 100 μm for **a**, **b, d**, **f**, **h**, **j** and 50 μm for **c**, **e**, **g**, **i**, **k.**

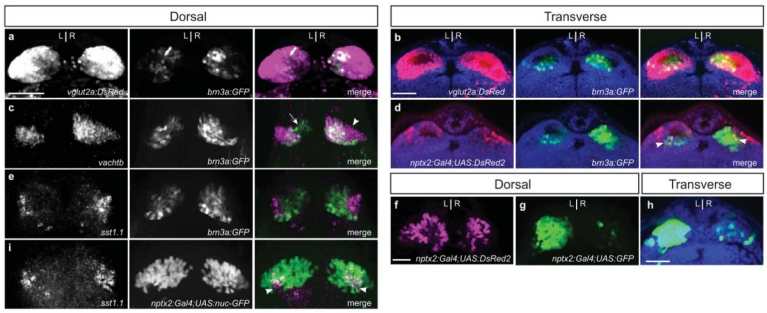

FIG. 4. Partial correspondence of neurotransmitter subdomains and habenular transgenic reporters

(**a**, **b**) Coextensive labeling of habenulae by *brn3a:GFP* and *vglut2:DsRed* indicates that *brn3a:GFP*-positive cells are glutamatergic. (**c**) Detection of *vachtb* RNA by *in situ* hybridization and *brn3a:GFP* by anti-GFP immunolabeling reveals *vachtb*-positive/*brn3a:GFP*-negative (arrowhead), *vachtb*-negative/*brn3a:GFP* positive (arrow) as well as region of co-expression. (**d**) *nptx2:Gal4;UAS:DsRed2*-labeled neurons have areas of overlap at the boundaries of the *brn3a:GFP*-labeled population (arrowheads) (**e**) *sst1.1*-expressing neurons are distinct from the *brn3a:GFP*-labeled population, which were detected by anti-GFP immunolabeling. (**f**) The *nptx2:Gal4;UAS:DsRed2* transgene labels neurons sparsely in the left and right habenulae. (**g, h**) *4xUAS:GFP* activated by *nptx2:Gal4* labels relatively more neurons in the left and less in the right dorsal habenular nucleus compared with *UAS:DsRed2*. (**i**) *sst1.1* expression is confined to the lateral subregions of the *nptx2:Gal4;14xUAS:GFP* dorsal habenular domain (arrowheads) detected by anti-GFP immunolabeling. All larvae are 4 dpf. Scale bars are 30 μm.

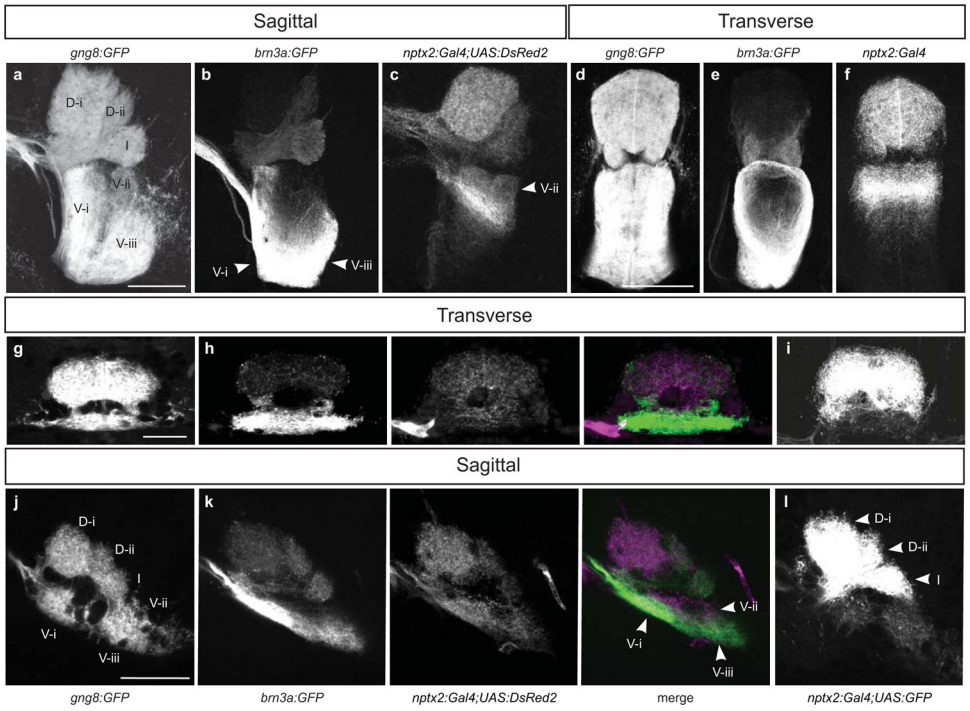

FIG. 5. Habenular connectivity with the IPN revealed by transgenic reporters

gng8:GFP labeled habenular axons terminate in discrete, stereotypic bundles in the dorsal (D), intermediate (I) and ventral (V) IPN of (**a, d**) adult and (**g, j**) larval brains. (**b, e**) In the adult brain, *brn3a:GFP* labeled dorsal habenular neurons predominantly contribute to the D-ii, intermediate and V-i and V-iii bundles and (**c, f**) *nptx2:Gal4;UAS:DsRed2* labeled axons terminate in the D-i, D-ii, intermediate and V-ii IPN bundles. (**h, k)** In the 4 dpf larval brain, IPN innervation patterns are similar to the adult when the *UAS:DsRed2* reporter is used. (**i, l**) However, with *Tg(4xUAS:GFP)* regulated by the *nptx2:Gal4* driver, labeled fibers preferentially innervate the dorsal and intermediate IPN. Scale bar is 100 μm for **a–f** and 30 μm for **g–l**.

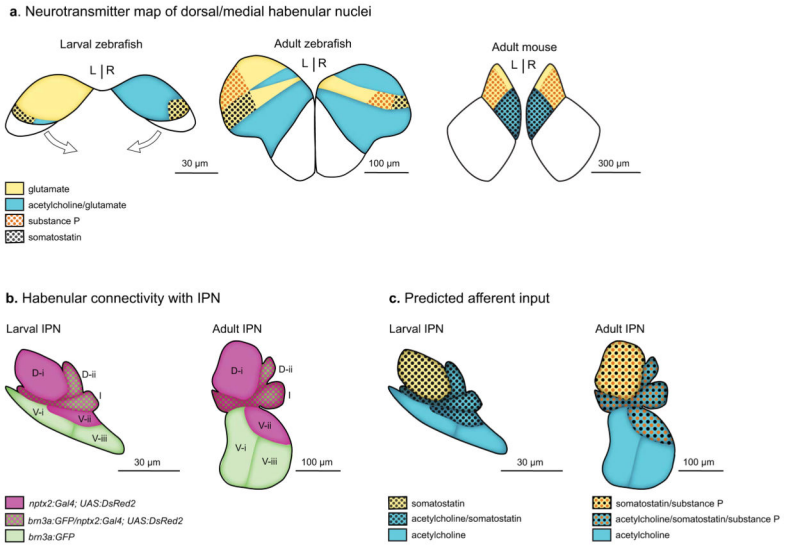

FIG. 6. Model of dorsal habenular neurotransmitter populations and their IPN connectivity

(**a**) Transverse views of the dorsal/medial habenular nuclei. Prominent L- R differences in neurotransmitter distribution in the dorsal habenulae of larval zebrafish become less pronounced in the adult brain. In adult brains, the overall organization of glutamatergic, cholinergic and peptidergic populations is conserved between the dorsal habenula of zebrafish and medial habenula of the mouse (based on neuroanatomical data from Quina *et al*., 2009 and the Allen Mouse Brain Atlas). However, somatostatin and cholinergic neurons are in distinct populations in the zebrafish habenulae, which has not been reported for rodents. (**b, c**) Sagittal views of stereotypic axon terminal bundles at the interpeduncular nucleus of larval and adult zebrafish. In (**b**), habenular axon terminals labeled by either the *brn3a:GFP* or *nptx2:Gal4* transgenes are mostly enriched in different subregions of the IPN, (**c**) Predicted afferent input to the IPN. The projections of cholinergic neurons are limited to the ventral and intermediate IPN; whereas those of peptidergic (*sst1.1* and *tac1*) neurons are expected to be found at the dorsal IPN, intermediate IPN and/or V-ii region of the ventral IPN.

TABLE 1

Gene expression in the zebrafish habenulae and homology with rodents

Listed are genes found to be transcribed in regions of the dorsal (D) and/or ventral (V) habenular nuclei for larval (La) or adult (A) zebrafish, as determined by RNA *in situ* hybridization. Questions marks indicate where published data are unclear. Expression of homologous genes in the medial (M) and/or lateral (L) habenular nuclei of rodents is also noted. References are provided for the zebrafish and rodent studies. AB atlas signifies the Allen Mouse Brain Atlas, available from: http://mouse.brain-map.org, ©2012 Allen Institute for Brain Science.

Zebrafish Gene Name	Gene Symbol	Zebrafish Ha Domain	Stage	Zebrafish References	Rodent Ha Domain	Rodent References
adenylate cyclase activating polypeptide 1a	*adcyap1a*	D (left only)	La	Amo *et al*., 2010	M, L	AB atlas
amine oxidase, copper containing 1	*aoc1*	V	La, A	Amo *et al*., 2010	Neither	AB atlas
anoctamin 2	*ano2*	D	La	This study	M	AB atlas
Ca2+-dependent activator protein for secretion 2	*cadps2*	D	La	Gamse *et al*., 2003; Chen *et al*., 2009	M, L	Sadakata *et al*., 2007
calcium/calmodulin-dependent protein kinase IGb	*camk1gb*	D, V?	La	Tovin et al., 2012	Neither	AB atlas
cAMP responsive element binding protein 1a	*creb1a*	D, V	A	Dworkin *et al*., 2007	Neither	AB atlas
cell adhesion molecule 2a	*cadm2a*	D (L>R), V?	La, A	Pietri *et al*., 2007	M	AB atlas
choline acetyltransferase	*chatb*	D (R>L), V	La, A	Hong *et al*., 2013	M	Lauterborn *et al*., 1993
chemokine (C-X-C motif), receptor 4b	*cxcr4b*	D	La	Carl *et al*., 2007; Roussinge *et al*., 2009	Neither	AB atlas
collapsin response mediator protein	*crmp1*	D	La	Jayasena *et al*., 2011	M	AB atlas
contactin 2	*cntn2*	D (R>L), V?	La	Carl *et al*., 2007	Neither	AB atlas
cytochrome P450, family 19, subfamily A, polypeptide 1	*cyp19a1b*	D, V	A	Goto-Kazeto *et al*., 2004	L	Shinoda et al., 1989
developing brain homeobox 1b	*dbx1b*	D, V?	La	Wu *et al*., 2014	Neither	AB atlas
ets variant gene 1	*etv1*	D (L>R)	La	Roussinge *et al*., 2006	M	Quina *et al*., 2009
extracellular leucine-rich repeat and fibronectin type III domain containing 1b	*elfn1b*	D (L>R)	La	Sollner and Wright, 2009	M, L	Dolan and Mitchell, 2013
F-box protein 7	*fbxo7*	D, V	A	Zhao *et al*., 2012	M	AB atlas
follistatin-like 4	*fstl4*	D, V?	A	Tsuchimoto *et al*., 2005	Neither	AB atlas
G protein-coupled receptor 161	*gpr161*	D, V?	La	Leung *et al*., 2008	Neither	AB atlas
glutamate receptor, ionotropic, AMPA 1a,b	*gria1a,b*	D?, V	La	Hoppmann *et al*., 2008	M, L	AB atlas
glutamate receptor, ionotropic, AMPA 4a,b	*gria4a,b*	D	La	Hoppmann *et al*., 2008	M, L	AB atlas

Zebrafish Gene Name	Gene Symbol	Zebrafish Ha Domain	Stage	Zebrafish References	Rodent Ha Domain	Rodent References
guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O, a	*gnao1a*	D, V?	La	Huang *et al.*, 2012	M	AB atlas
guanine nucleotide binding protein, gamma 8	*gng8*	D, V	La	This study	M	Betty *et al.*, 1998
glutamate receptor, metabotropic 3	*grm3*	D (R>L)	La	Haug *et al.*, 2013	M, L	Ohishi *et al.*, 1993
glutamate receptor, metabotropic 4	*grm4*	D, V?	La	Haug *et al.*, 2013	Neither	AB atlas
glutamate receptor, metabotropic 6a	*grm6a*	D	La	Haug *et al.*, 2013	Neither	AB atlas
glutamate receptor, metabotropic 6b	*grm6b*	D (R>L)	La	Haug *et al.*, 2013	Neither	AB atlas
glutamate receptor, metabotropic 7	*grm7*	D	La	Haug *et al.*, 2013	L	Ohishi *et al.*, 1995
glutamate receptor, metabotropic 8b	*grm8b*	V	La	Haug *et al.*, 2013	Neither	AB atlas
5-hydroxytryptamine (serotonin) receptor 1bd	*htr1bd*	D (L>R), V?	La, A	Norton, 2008	Neither	AB atlas
hypocretin receptor	*hcrtr*	D	La, A	Appelbaum *et al.*, 2009	Neither	AB atlas
Kallmann syndrome 1a sequence	*kal1a*	D, V	A	Ayari *et al.*, 2012	NA	
Kallmann syndrome 1b sequence	*kal1b*	D?, V	A	Ayari *et al.*, 2012	NA	
kisspeptin1	*kiss1*	V	A	Kitashi *et al.*, 2009; Ogawa 2012	Neither	AB atlas
kisspeptin1 receptor	*kiss1r*	V	A	Servili *et al.*, 2011	M	AB atlas
Kruppel-like factor 4a	*klf4a*	V	La	Li et al., 2011	Neither	AB atlas
leptin receptor	*lepr*	D, V	A	Liu *et al.*, 2010	Neither	AB atlas
platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit b	*pafah1b1b*	D	La	Drerup *et al.*, 2010	M, L	AB atlas
melanin-concentrating hormone receptor 1a	*mchr1a*	D, V	A	Berman *et al.*, 2009	Neither	AB atlas
monoamine oxidase	*mao*	D, V	A	Anichtchik *et al.*, 2006	M, L	AB atlas
muscleblind-like 3 (Drosophila)	*mbnl3*	V	La	This study	Neither	AB atlas
nudE nuclear distribution gene E homolog like 1 (A. nidulans) A	*ndel1a*	D	La	Drerup *et al.*, 2007	M, L	AB atlas
neuropilin 1a	*nrp1a*	D (left only)	La	Kuan *et al.*, 2007	Neither	AB atlas
neuronal pentraxin 2a	*nptx2a*	D (L>R)	La, A	Agetsuma *et al.*, 2010	M	AB atlas
neuronal pentraxin 2b	*nptx2b*	D?, V	A	Appelbaum *et al.*, 2010		See *nptx2a*
nuclear receptor subfamily, group A, member 2	*nr4a2*	D, V?	La	Blin, 2008	M, L	Quina *et al.*, 2009
opsin 4xa	*opn4xa*	D	La	Matos-Cruz *et al.*, 2011	No homolog	
purkinje cell protein 4a	*pcp4a*	D, V	La, A	Mione et al., 2006	M, L	AB atlas
PTEN induced putative kinase 1	*pink1*	D, V	A	Sallinen et al., 2010	M, L	Taymans *et al.*, 2006

Zebrafish Gene Name	Gene Symbol	Zebrafish Ha Domain	Stage	Zebrafish References	Rodent Ha Domain	Rodent References
potassium channel tetramerization domain 12.1	*kctd12.1*	D (L>R)	La, A	Gamse *et al*., 2003	M	Gamse *et al*., 2005 Metz *et al*., 2011
potassium channel tetramerization domain 12.2	*kctd12.2*	D (R>L), V	La, A	Gamse *et al*., 2005		See *kctd12.1*
potassium channel tetramerization domain 8	*ktcd8*	D (R>L)	La, A	Gamse *et al*., 2005	M	Gamse *et al*., 2005 Metz *et al*., 2011
POU domain, class 4, transcription factor 1	*pou4f1*	D (R>L)	La, A	Amo *et al*., 2010	M, L	Quina *et al*., 2009
prokineticin 2	*prok2*	D, V	A	Ayari et al., 2010	M, L	Cheng *et al*., 2006
protocadherin-10	*pcdh10a*	V	A	Amo *et al*., 2010	L	Aizawa *et al*., 2012
purkinje cell peptide 4	*pcp4a*	D, V	La, A	Mione *et al*., 2006	M, L	AB atlas
reticulon 4 receptor	*rtn4r*	D, V?	La	Brösamle et al., 2009	M	Josephson *et al*., 2002
roundabout homolog 3	*robo3*	D, V?	La	Sollner and Wright, 2009	M	Quina *et al*., 2009
si:ch211-154o6.4		D (R>L), V?	La	Amo *et al*., 2010	No homolog	
solute carrier family 5 (choline transporter) member 7	*slc5a7; hacta*	D (R>L)	La, A	Hong *et al*., 2013	M	AB atlas
solute carrier family 18 (vesicular acetylcholine), member 3b	*slc18a3; vachtb*	D (R>L), V	La, A	Hong *et al*. 2013	M	Quina *et al*., 2009
solute carrier family17 (sodium- dependent inorganic phosphate cotransporter), member 6a	*slc17a6; vglut2b*	D, V	La, A	Appelbaum 2009	M, V	Aizawa *et al*., 2012
solute carrier family17 (sodium- dependent inorganic phosphate cotransporter), member 6b	*slc17a6; vglut2a*	D, V	La, A	Bae *et al*., 2009; Appelbaum 2009	M, V	Aizawa *et al*., 2012
solute carrier family17 (sodium- dependent inorganic phosphate cotransporter), member 7	*slc17a7; vglut1*	D (L>R)	A	Bae *et al*., 2009	M	Aizawa *et al*., 2012
spondin 1b	*spon1b*	D, V	La, A	Gamse *et al*., 2003; Akle *et al*., 2012	M	AB atlas
synuclein, gamma b	*sncgb*	D (L>R)	La, A	Milanese *et al*., 2012 Chen *et al*., 2009	M	Quina *et al*., 2009
tachykinin 1	*tac1*	D (R>L)	A	Hong *et al*., 2013	M	Quina *et al*., 2009; Aizawa *et al*., 2012
tachykinin 3a (formerly tac2a)	*tac3*	D (R>L), V?	La, A	Biran *et al*., 2012; Ogawa *et al*., 2012	No homolog	AB atlas
transient receptor potential cation channel, subfamily C, member 1	*trpc1*	D (L>R)	La	von Niederhäusern *et al*., 2013	M, L	AB atlas
transient receptor potential cation channel, subfamily C, member 5a	*trpc5a*	D, V?	La	von Niederhäusern *et al*., 2013	M, L?	Fowler *et al*., 2007
transient receptor potential cation channel, subfamily M, member 3	*trpm3*	D, V	La	Kastenhuber et al., 2013	M	Oberwinkler *et al*., 2005

Zebrafish Gene Name	Gene Symbol	Zebrafish Ha Domain	Stage	Zebrafish References	Rodent Ha Domain	Rodent References
unc-51-like kinase 2	*ulk2*	D (R>L), V	La	Taylor *et al*., 2011	Neither	AB atlas
uncoupling protein 2	*ucp2*	D, V	A	Tseng *et al*., 2011	Neither	AB atlas
vertebrate ancient long opsin b	*valopb*	D, V?	La	Kojima *et al*., 2008	Neither	AB atlas
wingless-type MMTV integration site family, member 4a	*wnt4a*	D	La	Hendricks *et al*., 2008	Neither	AB atlas