An atlas and database of neuropeptide gene expression in the adult zebrafish forebrain

Running title: Zebrafish atlas and database of neuropeptide

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Author contribution
T. H-K. and Y. Y. designed the study. T. H-K., R. A., Y. Y., N. M., and N. W. conducted experiments. T. H-K. acquired the data, conducted the analysis, and prepared the draft manuscript and figures. H. I. and S. O. designed and constructed the database. Y. Y., N. M., S. O., H. I., R. A., and N. W. edited the manuscript and figures. All the authors have read and approved the final version of the manuscript.

Conflict of interest
The authors report no conflict of interest.

Data availability statement
High-resolution figures and raw data will be uploaded to the CBS Data Sharing Platform. All scan data of glass slides can be viewed and downloaded at the Adult Zebrafish Brain Gene Expression

Database (The address will be updated soon).

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Ethics approval statement

All experimental procedures were approved by the Animal Care and Use Committee of RIKEN.

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Abstract

Zebrafish is a useful model organism in neuroscience; however, its gene expression atlas in the adult brain is not well developed. In the present study, we examined the expression of 38 neuropeptides, and glutamatergic neuron marker gene mix (*slc17a6a, slc17a6b, slc17a7a,* and *slc17a7b*) in the adult zebrafish brain using *in situ* hybridization. The results are summarized as

an expression atlas in 19 coronal planes of the forebrain. Furthermore, the scan data of all sections were made publicly available as a database. Based on these data, we performed detailed neuroanatomical analyses of the hypothalamus. By scrutinizing and comparing the expression patterns of neuropeptides, we found that several regions described as one nucleus in the reference zebrafish brain atlas contain two or more subregions with significantly different neuropeptide/neurotransmitter expression profiles, and we proposed them as novel subnuclei. Subsequently, the expression data obtained in this study were compared with those in mice, and a cluster analysis was performed to examine the similarities. As a result, several nuclei in zebrafish and mice were clustered in close vicinity: zebrafish ventral part of the anterior part of the parvocellular preoptic nucleus (PPav)/magnocellular preoptic nucleus (PM) and mouse paraventricular hypothalamic nucleus (Pa), zebrafish posterior part of the parvocellular preoptic nucleus (PPp) and mouse medial preoptic area (MPA), zebrafish dorsal part of the ventral zone of periventricular hypothalamus (Hvd)/anterior tuberal nucleus (ATN) and mouse ventromedial hypothalamic nucleus (VMN). The present expression atlas, database, and anatomical findings will contribute to future neuroscientific research using zebrafish.

Key points

- The expression of 38 neuropeptides and GABAergic/glutamatergic neuronal marker genes in adult zebrafish forebrain was examined and compiled as an atlas.
- All scanned brain section data were published as a database.
- Based on the expression data obtained, multiple subnuclei in the zebrafish hypothalamus were proposed, and comparisons with the mouse hypothalamus were conducted.

Keywords

adult zebrafish brain, gene expression atlas, neuropeptide, gabaergic and glutamatergic neuron marker, subnuclei, comparison with mouse

1. Introduction

Zebrafish (*Danio rerio*) is widely used as a vertebrate animal model in various biology research fields. It has several advantageous properties, such as external fertilization, large clutch size, transparency of eggs and larvae, rapid development, and feasibility for both forward and reverse genetics (Kalueff *et al*., 2014; Stewart *et al*, 2014; Gerlai, 2020). Recently, whole-brain calcium imaging and high-throughput behavioral assays have been successively developed for larval zebrafish, leading to breakthroughs in neuroscience research (Orger and Polavieja, 2017; Vanwalleghem *et al*., 2018; Loring *et al*., 2020; Lee *et al*., 2022). In addition, multiple brain databases have been developed for larval zebrafish, such as the gene expression atlas of 2-, 3-, and 4-days post-fertilization (dpf) larval brains (ViBE-Z, Ronneberger *et al*., 2012), the brain atlas of single neuron tracings and images of transgenes and *in situ* hybridizations in 6-dpf larvae (MapZebrain, Kunst *et al*., 2019), and transgene expression- and immunohistochemistry-based atlas in 6-dpf larvae (Z-Brain Atlas, Randlett *et al*., 2015; Zebrafish Brain Browser 2 (ZBB2), Tabor *et al*., 2019). However, the number of available brain database resources on adult zebrafish is limited so far, which include the reference brain atlas (Wullimann *et al*., 1996), a database of transgene expression patterns in enhancer-trap lines (ZeBrain), magnetic resonance histology-based 3D atlas (Ullmann *et al*., 2010), and 3D atlas of immunohistochemical images of ten marker genes (AZBA, Kenney *et al*., 2021). Thus, no brain atlas maps gene expression patterns on a large scale. As a promising animal model in behavioral neuroscience, adult zebrafish display more diverse and complex behaviors than larval zebrafish, including associative learning, perceptual decision-making, social behavior, and reproductive behavior (Norton and Bally-Cuif, 2010; Oliveira RF, 2013; Yabuki *et al*., 2016; Chou *et a*l., 2016; Stednitz *et al*., 2018, Okamoto *et al*., 2021). Furthermore, as animal models for various neurological and psychiatric diseases, zebrafish have been actively used in the field of translational research in recent years (Kalueff *et al*., 2014; Stewart *et al*., 2014; Fonseka *et al*., 2016; Fontana *et al*., 2021; Kumar *et al*., 2021). A gene expression atlas of adult zebrafish is needed to expand on these trends.

Here, we report an atlas summarizing the expression patterns of 38 neuropeptide genes and GABAergic/glutamatergic neuronal markers in the adult zebrafish forebrain. Neuropeptides are relatively small peptides that are highly conserved from invertebrates to vertebrates (Holmgren and Jensen, 2001; Elphick *et al*., 2018). They function as neuromodulators and play important roles in various physiological phenomena (Hökfelt *et al*., 2000; van den Pol, 2012; Sohn, 2015; van den Burg and Stoop, 2019). As neuropeptides tend to be expressed in specific types or small localized populations of neurons in the brain, they can also be used as markers for groups of neurons of interest and as genetic tools for visualizing and manipulating these neurons. Indeed,

many transgenic zebrafish have been produced using the enhancer/promoter regions of various neuropeptide genes (Liu *et al*., 2003; Levitas-Djerbi *et al*., 2015; Förster *et al*., 2017; Koide *et al*., 2018; Shainer *et al*., 2019; Song *et al*., 2021). By closely examining the expression patterns of neuropeptides and neurotransmitters, we found that some of the hypothalamic nuclei described in the zebrafish reference brain atlas (Wullimann *et al*., 1996) contained multiple subregions with different expression patterns of neuropeptides, which we defined as subnuclei. Furthermore, by comparing the expression patterns of the neuropeptides in the zebrafish hypothalamus with those in the mouse hypothalamus, we identified several nuclei that exhibited similar expression patterns across species.

2 Materials and Methods

2.1 Animals

Zebrafish (*Danio rerio*) were maintained at 28°C under 14-hour light and 10-hour dark cycle and fed with brine shrimp two to three times a day. Adult wild-type zebrafish (RIKEN-Wako) were used in all the experiments. All experimental procedures were approved by the Animal Care and Use Committee of RIKEN.

2.2 cRNA probe synthesis

Coding regions (500–1000 bp) of neuropeptide and neurotransmitter marker genes were PCR-amplified from zebrafish brain cDNA or genomic DNA and cloned into the pGEM-T Easy vector (Promega). Three glutamate decarboxylase genes (*gad1a*, *gad1b*, and *gad2*) were used as marker genes for GABAergic neurons. For the glutamatergic neurons, four vesicular glutamate transporter (*vglut*) genes (*slc17a6a*, *slc17a6b, slc17a7a*, and *slc17a7b*) were used. The genes analyzed and primers used for PCR cloning are listed in Table 1.

The names of the individual genes followed the ZFIN nomenclature, with a few exceptions, for which the correspondences to previous designations are listed in Table 1. One of the *cart* genes was missing from the ZFIN database, and the ZFIN nomenclature did not match the results of the phylogenetic analysis. Therefore, we followed the nomenclature of Cai *et al*. (2015), in which a detailed phylogenetic analysis of *cart* genes was conducted. Zebrafish harbor two galanin genes (*gal* and *gall*); however, the molecular evolutionary positioning of the second galanin-like gene (*gall*, also called *si:rp71-1c10.8*) remains unclear. Hence, we carried out phylogenetic and syntenic analyses of *gall* and revealed that the second galanin-like gene in zebrafish and other teleost species was not a teleost-specific paralogous gene for *gal*, but an orthologous gene for *Galp* in tetrapods (Supplemental Fig. 1). Therefore, it was designated *galp*.

Digoxigenin (DIG)-labeled antisense cRNA probes were synthesized using T7, SP6, or T3 RNA polymerases (Promega) and purified through Micro Bio-Spin Columns with Bio-Gel (BIO-RAD). Plasmids containing *slc17a6a*, *slc17a6b*, and *slc17a7a* genes were kindly provided by Dr. Higashijima (Higashijima *et al*., 2004).

2.3 *In situ* hybridization

Brains of adult male zebrafish (3–12 months old) were sampled during the light period and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C overnight. The brains were then incubated in 50%, 70%, 100% ethanol, and Lemosol (Wako) for dehydration,

232 embedded in paraffin, and sectioned into 10 μm-thick serial coronal sections. The sections were
233 deparaffinized in Lemosol and 100%, 90%, 70%, and 50% ethanol. After washing in PBS, the
234 sections were treated using 1 μg/mL proteinase K (Invitrogen) in PBS at 37°C for 15 min and re-
235 fixed in 4% PFA in PBS for 10 min at room temperature (RT). The sections were acetylated,
236 followed by incubation in pre-hybridization buffer [50% formamide, 5× saline sodium citrate
237 (SSC), 50 μg/mL heparin, 0.1% Tween] at 60°C for 1 h. The sections were then hybridized using
238 DIG-labeled neuropeptide gene probes (0.01 ng/μL for the *oxt* gene, 0.5 ng/μL for the rest of the
239 genes) in hybridization buffer [100 μg/μL RNA from yeast (Roche) added to pre-hybridization
240 buffer] at 60°C overnight. The *gad* mix (a mixture of *gad1a*, *gad1b*, and *gad2* probes; 0.5 ng/μL
241 each) was used as a marker for GABAergic neurons. Moreover, *vglut* mix (a mixture of *slc17a6a*,
242 *slc17a6b, slc17a7a*, and *slc17a7b* probes; 0.5 ng/μL each) was used as a marker for glutamatergic
243 neurons. The sections were then washed in 5× SSC buffer and 0.2× SSC at 60°C. After washing
244 in 0.2× SSC and maleic acid buffer (MAB; 0.1 M maleic acid, 0.15 M NaCl, 0.19 M NaOH), the
245 sections were blocked with 2% blocking reagent (Roche) and 5% normal goat serum in MAB for
246 30 min at RT and incubated with alkaline phosphatase-conjugated anti-DIG antibody (1:10000
247 for *oxt*, 1:1000 for other genes, Sigma-Aldrich) at 4°C overnight. For color development, the
248 sections were washed in MAB and color development buffer (0.1 M Tris-HCl pH 9.5, 0.05 M
249 $MgCl_2$, 0.01 M NaCl) and then incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue
250 tetrazolium (BCIP/NBT) solution at 30°C for 0.5–7 h. The color development was stopped by
251 washing with PBS and water. Some sections (at least one individual fish for each gene) were
252 counterstained using 1% methyl green stain solution (Muto Pure Chemicals) for 3–5 min and
253 washed in water. The sections were then mounted using Aqua-Poly/Mount (Polysciences) or
254 Immu-mount (Thermo Fisher Scientific).
255
256 **2.4 Image acquisition**
257 Whole images of brain sections on glass slides were captured using a NanoZoomer HT slide
258 scanner (Hamamatsu Photonics). The pictures were cut out for each brain plane using NDP.view2
259 (Hamamatsu Photonics) and trimmed using Adobe Photoshop CS6 (Adobe Inc.).
260
261 **2.5 Database construction**
262 The Adult Zebrafish Brain Gene Expression Database (https://ssbd.riken.jp/azebex/) was
263 constructed based on the SSBD (Systems Science of Biological Dynamics) platform (Tohsato *et*
264 *al*., 2016). SSBD:repository was used as the database infrastructure. OMERO (Open Microscopy
265 Environment) system (Allan *et al*., 2012) was used to preview the images.
266
267 **2.6 Phylogenetic and syntenic analyses**

Phylogenetic and syntenic analyses were performed to examine homologous relationships between the two galanin-like genes in various animal species. The GenBank accession numbers of the genes used for phylogenetic analysis are summarized in Supplemental Table 1. The amino acid sequences of the galanin-like genes in each species were aligned using ClustalW, and a phylogenetic tree was constructed using the maximum likelihood method. The analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA11) software (version 11) (Tamura *et al*., 2021). For syntenic analysis, genomic information for each species was obtained from the Ensembl or NCBI databases.

2.7 Comparison of the hypothalamic nuclei between zebrafish and mouse

To examine the similarity in the hypothalamic nuclei between zebrafish and mice, we compiled a table of gene expression in each nucleus (expressed = 1, not expressed = 0). Comparisons were made for genes that were expressed in both the zebrafish and mouse hypothalamus. Expression profiles of neuropeptide genes in the mouse hypothalamus were obtained from the Allen Brain Atlas (Lein *et al*., 2007) and from previous studies (*Agrp*, Deem *et al*., 2021; *Avp*, Smith *et al*., 1996; Otero-García *et al*., 2015; *Crh*, Alon *et al*., 2009; Keegan *et al*., 1994; *Galp*, Juréus *et al*., 2001; *Oxt*, Otero-García *et al*., 2015; *Pmch*, Croizier *et al*., 2010; Diniz *et al*., 2019; *Npb*, Tanaka *et al*., 2003; *Hcrt*, Peyron *et al*., 1998; *Pomc*, Williams *et al*., 2010; Cowley *et al*., 2001; *Tac1*, Navarro *et al*., 2014; *Tac2*, Duarte *et al*., 2005). The expression of the zebrafish *gad* mix was compared to that of *Gad1* and *Gad2* in mice. Zebrafish *vglut* mix expression was compared with *Vglut2* expression in mice because *Vglut1* was not expressed in the mouse hypothalamus. Because *gad*/*Gad* and *vglut*/*Vglut* are more or less expressed in almost all regions, the expression patterns of *gad* and *vglut* were compared, and those with a higher number of expressing neurons were counted as positive. Both were considered positive when they were equally expressed. When neither was expressed, both were considered negative. The nomenclature of the mouse hypothalamic nuclei follows that of Paxinos and Franklin (2019). Zebrafish often have multiple homologous genes corresponding to a single mouse gene, mainly because of teleost-specific genome duplication (Jaillon *et al*., 2004, Kassahn *et al*., 2009). Teleost-specific paralogous genes expressions, that have been shown to have arisen from teleost-specific genome duplication, were added together (*ccka*/*cckb*, Dupré and Tostivint, 2014; Kurokawa *et al*., 2003; *crha*/*crhb*, Grone and Maruska, 2015; *penka*/*penkb*, Bojnik *et al*., 2011; *pomca*/*pomcb*, Forlano and Cone, 2007; *tac3a*/*tac3b*, Zhou *et al*., 2012; *vipa*/*vipb*, Nam *et al*., 2009). The *cart* genes were expressed in both zebrafish and mice but were not included in the comparison analysis because none of the *cart* genes found in the present zebrafish genome data were considered homologous to the mouse *Cart* gene (Cai *et al*., 2015). Furthermore, zebrafish and mouse hypothalamic nuclei and neuropeptide/neurotransmitter genes were clustered using Ward's method based on Euclidean

distance using the hclust function in R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing).

3. Results

3.1 Neuropeptide expressions in the adult zebrafish forebrain

Expression patterns of 38 neuropeptide genes, *agrp*, *asip2b*, *avp*, *calca*, *cart2a*, *cart2b*, *cart3a*, *cart3b*, *cart4*, *cart5*, *ccka*, *cckb*, *crha*, *crhb*, *gal*, *galp*, *gnrh2*, *gnrh3*, *grp*, *kiss1*, *kiss2*, *npb*, *npvf*, *npy*, *hcrt*, *oxt*, *pdyn*, *penka*, *penkb*, *pmch*, *pmchl*, *pomca*, *pomcb*, *tac1*, *tac3a*, *tac3b*, *vipa*, *vipb*, glutamatergic neuron marker gene mix (*slc17a6a*, *slc17a6b*, *slc17a7a*, and *slc17a7b*), and GABAergic neuron marker gene mix (*gad1a*, *gad1b*, and *gad2*) were examined by *in situ* hybridization throughout the adult zebrafish forebrain (telencephalon and diencephalon). Fig. 1 shows a schematic of the zebrafish brain and the location of each plane. Figs. 2–20 show schematics of the coronal planes and microscopic images of the genes that are expressed in each plane. Schematics were based on the zebrafish brain atlas (Wullimann *et al*., 1996) with several modifications. The division of nuclei in the dorsal telencephalic area was modified according to Ganz *et al.* (2014) and Yáñez *et al*. (2022) (Figs. 3–10). The intermediate nucleus of the ventral telencephalon (Vi) was added, as described by Biechl *et al*. (2017) (Figs. 9, 10). Subregions in the dorsal habenula (Had), medial Had (Hadm), and lateral Had (Hadl) were added according to Agetsuma *et al*. (2010) (Figs. 11–13). The nucleus lateralis tuberis (NLT) in the hypothalamus was added as in recent papers in zebrafish (Akash *et al*., 2014) or other teleost species (Karoubi *et al*., 2016) (Figs. 14, 15). The intercalated nucleus (IC) was added, as described by Mueller and Guo (2009). The intermediate nucleus (IN) was added, and the positions of the paraventricular organ (PVO) and posterior tuberal nucleus (PTN) were altered according to Rink and Wullimann (2001) (Figs. 15, 16). The neuropeptide expression profiles in each nucleus are summarized in Tables 2–6. All scanned data of the whole glass slides are available in the Adult Zebrafish Brain Gene Expression Database (https://ssbd.riken.jp/azebex). The database allows users to view whole-brain scan data online. Raw data in .ndpi format can also be downloaded from the database and viewed using NDP.view2, which is free software. The database also provides images of sections in the hindbrain and expression data in female zebrafish.

3.1.1 Telencephalon

3.1.1.1 Olfactory bulb

In the zebrafish olfactory bulb, the glomerular layer (GL) and the external cellular layer (ECL) contain two types of projection neurons, mitral cells and ruffed cells, and two types of interneurons, periglomerular cells and short-axon cells. The internal cellular layer (ICL) mainly contains GABAergic interneurons and granule cells (Olivares and Schmachtenberg, 2019). The distribution of GABAergic and glutamatergic neurons was consistent with that of these distinct cell types (Figs. 2 and 3, Table 2). *crhb* was strongly expressed in a small number of large-

diameter cells in ECL/GL, which presumably are a subset of mitral cells, as was reported in mammals (Bassett *et al*., 1992) (Figs. 2, 3, Table 2). *penkb* was expressed in small-diameter cells surrounding glomeruli in GL/ECL and ICL, which are likely periglomerular cells and granule cells, respectively, as is the case in mammals (Matsutani *et al*., 1989) (Figs. 2, 3, Table 2). *npy*-positive cells in GL/ECL might be short-axon cells, as described in mammals (Scott *et al*., 1987). *cart3b*, *cckb*, and *tac1* were expressed intensely and exclusively in ICL cells, which are likely granule cells. *gnrh3* was expressed in terminal nerve neurons, with a very small number of cells located in the ventral-most region of the olfactory bulb (Figs. 2 and 3, Table 2).

3.1.1.2 Dorsal telencephalon

The dorsal telencephalic area (D) contains nine nuclei, including several newly defined nuclei (Ganz *et al*., 2014; Yáñez *et al*., 2022) (Figs. 3–10, Table 2). All nuclei showed distinct neuropeptide expression patterns, some of which validated the new definition of nuclei (Figs. 3–10, Table 2). For example, *cart3b* and *tac3b* were selectively expressed in the dorsal part of the lateral zone of D (Dld), but not in the ventral part of the lateral zone of D (Dlv) (Figs. 3 and 4, Table 2). *tac1* was strongly expressed in the posterior part of the medial zone of D (Dmp) but not in the medial zone of D (Dm) (Figs. 4–10, Table 2). Dmp was also characterized by the absence of *cckb* expression (Fig. 8–10, Table 2). The dorsal telencephalon was characterized by high *vglut* expression and low *gad* expression (Figs. 3–10). The dorsal telencephalon expressed fewer neuropeptides than the ventral telencephalon; however, *cckb* and *cart3b* were commonly expressed in most of the dorsal telencephalic areas, except that *cckb* was absent in Dmp, whereas *cart3b* was absent in Dlv and the posterior zone of D (Dp) (Figs. 3–10, Table 2).

3.1.1.3 Ventral telencephalon

The ventral telencephalic area (V) contains nine nuclei (Figs. 3–10, Table 3). In contrast to the abundant presence of glutamatergic neurons in the dorsal telencephalon, most neurons in the ventral telencephalic area were GABAergic inhibitory neurons (Figs. 3–10, Table 3). The dorsal nucleus of V (Vd) and supracommissural nucleus of V (Vs) are anteroposteriorly adjacent structures in the dorsomedial part of the ventral telencephalon and show exactly the same pattern of neuropeptide expression, implying similar functional features of these nuclei (Figs. 3–7. Table 3). The postcommissural nucleus of V (Vp) also expressed a similar repertoire of neuropeptides as Vd and Vs but additionally expressed *cart5* and *npb* (Figs. 8, 9, Table 3). The intermediate nucleus of V (Vi) is located in the most posterodorsomedial part of the ventral telencephalon, and its presence has recently been proposed in zebrafish (Biechl *et al*., 2017). Vi shared the expression of many neuropeptides with Vd, Vs, and Vp but was characterized by the lack of *crhb* and *npy* (Figs. 9 and 10, Table 3). The ventral part of the entopeduncular nucleus (ENv) and dorsal part of

379 the entopeduncular nucleus (ENd) differed greatly in the neurotransmitter profiles: ENd was
380 mostly occupied by GABAergic neurons (Figs. 5-8), while ENv was composed predominantly of
381 glutamatergic neurons (Figs. 8–10). In addition, *crhb* and *npy* were selectively and strongly
382 expressed in ENd, but not in ENv (Figs. 5–10, Table 3).
383
384 **3.1.2 Diencephalon**
385 3.1.2.1 Preoptic area
386 Four nuclei were observed in the preoptic area, all of which expressed numerous neuropeptides
387 (Figs. 7–12, Table 4). All nuclei commonly expressed *ccka*, *crhb*, *npy*, *pdyn*, *penka*, *penkb*, *tac1*,
388 and *tac3a* (Figs. 7–12, Table 4). The anterior part of the parvocellular preoptic nucleus (PPa)
389 expressed 21 neuropeptides among the 38 tested, with the largest number in the preoptic area
390 (Figs. 7–9. Table 4). The dorsal and ventral parts of the PPa showed different neuropeptide
391 expression patterns (see Section 3.2.1). The posterior part of the parvocellular preoptic nucleus
392 (PPp) was characterized by densely packed GABAergic neurons, and its neuropeptide-expressing
393 cells were sparser than those in other preoptic nuclei (Figs. 10–12). The magnocellular preoptic
394 nucleus (PM) contained *avp*-, *oxt*-, *crhb*-, or *penka*-expressing magnocellular cells, as well as
395 parvocellular cells positive for *asip2b*, *cart5*, *ccka*, *gal*, *galp*, *npy*, *pdyn*, *penkb*, *tac1*, and *tac3a*
396 (Figs. 10 and 11). In addition, cells with very weak expressions of *crha* and *npb* were also
397 observed (Figs. 10 and 11).
398
399 3.1.2.2 Hypothalamus
400 The hypothalamus contains nine nuclei (Figs. 12–20, Table 4). NLT, which is a thin cell layer at
401 the outer rim of the hypothalamus, expressed *gal*, *npy*, *pmch*, *pmchl*, and *pomca* (Figs. 12, 13,
402 Table 4). The ventral zone of the periventricular hypothalamus (Hv), lateral hypothalamic nucleus
403 (LH), dorsal zone of the periventricular hypothalamus (Hd), and caudal zone of the periventricular
404 hypothalamus (Hc) commonly expressed *cart3b*, *cart5*, *ccka*, *cckb*, *crhb*, *kiss2*, *npb*, *npy*, *pdyn*,
405 *penka*, *penkb*, *pomcb*, *tac3a*, and *vipa* (Figs. 12–20, Table 4). Furthermore, Hv expressed 23
406 neuropeptides, many of which were localized ventrally (see 3.2.2) (Figs. 12–16). Neuropeptide
407 expression in Hd and Hc was also not uniform, with distinct subregions within the nucleus that
408 displayed different neuropeptide expression profiles (see 3.2.3 and 3.2.4) (Figs. 14–18). The IN
409 is adjacent to the PTN and is distinguishable from it by the predominance of GABAergic neurons,
410 while PTN neurons are glutamatergic (Figs. 15, 16). The anterior tuberal nucleus (ATN) differed
411 significantly from other hypothalamic nuclei in that it expressed only two neuropeptides, *tac1* and
412 *tac3a*, and contained only glutamatergic neurons (Figs. 13–16), which is a feature similar to the
413 mammalian ventromedial hypothalamic nucleus (VMN) (see 3.3). Furthermore, the central (CIL)
414 and diffuse (DIL) nucleus of the inferior lobe differed from other hypothalamic nuclei in the

415 predominance of glutamatergic neurons and low neuropeptide expression (Figs. 14–20). A large
416 part of the inferior lobe, including the CIL and DIL, has recently been shown to be of
417 mesencephalic origin, and it has been debated whether these nuclei should be included or
418 excluded from the hypothalamus (Bloch *et al*., 2019). Our results also suggest that these nuclei
419 differ significantly from other hypothalamic nuclei in terms of neuropeptide expression profiles.
420
421 3.1.2.3 Prethalamus
422 Prethalamus contains four nuclei (Figs. 10–13, Table 5). The rostrolateral nucleus (R) did not
423 express any of the neuropeptides examined (Fig. 11). The intermediate thalamic nucleus (I),
424 ventromedial thalamic nucleus (VM), and ventrolateral thalamic nucleus (VL) are located adjacent
425 to each other; however, their neuropeptide expression patterns differed. The three nuclei
426 commonly expressed *cart3b* and *penka* (Figs. 10–13). VM expressed seven neuropeptides, among
427 which *npb*, *pdyn*, *vipa*, and *vipb* were present only in the VM among the prethalamic nuclei (Figs.
428 1–13). The VL had a higher percentage of glutamatergic neurons than the VM, and *cckb*
429 expression was specific to the VL in the prethalamus (Figs. 11–13). *npy* and *penkb* were expressed
430 only in I among the prethalamic nuclei (Fig. 11).
431
432 3.1.2.4 Posterior tuberculum
433 The periventricular nucleus of the posterior tuberculum (TPp), PVO, and PTN in the posterior
434 tuberculum, which are known to contain many dopaminergic neurons (Rink and Wullimann,
435 2001), also showed expression of multiple neuropeptides (Figs. 13–18, Table 5). Of note is the
436 specific expression of *calca* only in the TPp of the forebrain (Figs. 14 and 15). Furthermore, *hcrt*
437 and *npvf* were selectively localized to the PVO in the forebrain (Figs. 14 and 15).
438
439 3.1.2.5 Thalamus
440 The zebrafish thalamus has seven nuclei (Figs. 10–14, Table 5). The habenula is a conserved
441 structure among all vertebrates. In teleost, it is divided into the dorsal (Had) and ventral (Hav)
442 habenular nuclei, which differ in neuronal connectivity and function (Okamoto *et al*., 2012).
443 Neuropeptide expression also differed between Had and Hav, with *kiss1* being the only
444 neuropeptide expressed in Hav (Figs. 11–13). Had can be further divided into Hadm and Hadl
445 (Agetsuma *et al*., 2010). *pdyn* and *tac1* were expressed in Hadl, whereas *tac3a* was expressed in
446 Hadm (Figs. 11 and 12). The dorsal posterior thalamic nucleus (DP) was the only nucleus other
447 than the hypothalamus expressing *asip2b* (Fig. 16). IC was recently defined based on its high
448 expression in *gad* (Mueller and Guo, 2009). Strong expression of *npy* and *penkb* also
449 distinguished this nucleus from other adjacent nuclei (Figs. 14,15).
450

3.1.2.6 Preglomerular complex

Neuropeptide expression was low in the nuclei of the preglomerular complex. The subglomerular nucleus (SG), tertiary gustatory nucleus (TGN), anterior preglomerular nucleus (PGa), caudal preglomerular nucleus (PGc), and corpus mamillare (CM) did not express any of the neuropeptides examined (Figs. 13–20, Table 6). In contrast, a few neuropeptides were expressed in the lateral (PGl) and medial (PGm) preglomerular nuclei. In addition, the torus lateralis (TLa) and posterior thalamic nucleus (P) only expressed *tac1* (Figs. 15–17).

3.1.2.7 Pretectum

Neuropeptide expression was low in the nuclei of the pretectum (Figs. 10–14, Table 6). The dorsal accessory optic nucleus (DAO), magnocellular superficial pretectal nucleus (PSm), posterior pretectal nucleus (PO), and ventral accessory optic nucleus (VAO) did not express the examined neuropeptides. The remaining nuclei expressed neuropeptides with distinct expression patterns.

3.1.3 Individual differences

For each gene, we examined the brains of 3–5 male zebrafish and found that, in most cases, the expression patterns were consistent across individuals (although not compared quantitatively). Therefore, only representative pictures from one fish for each gene are shown in Figs. 2–20. In contrast, several genes showed marked individual differences in their expression patterns, as summarized in Supplemental Fig. 2. *asip2b* in PPa was observed in only two of the four fish examined. *asip2b* in DP was also observed in only two of the four fish examined. As *asip2b* (also referred to as *agrp2*) has been reported to play a role in background adaptation (Zhang *et al*., 2010) and stress axis regulation (Shainer *et al*., 2019), these individual differences might be related to these factors. *agrp* was expressed in Hv in all four fish examined; however, the signal densities were highly variable. As for *pomca*, in four fish examined, one showed expression in both NLT and Hv, while the other three showed expression only in Hv. As *agrp* and *pomca* expression is known to be regulated by feeding conditions (*agrp*, Jeong *et al*., 2018; *pomca*, Löhr *et al*., 2018), these individual differences may reflect different feeding statuses.

3.2 Novel subnuclei revealed by neuropeptide expressions in the hypothalamus

Neuropeptides are prominently expressed in the hypothalamus and are, therefore, considered to play crucial roles in various hypothalamic functions. Our scrutinized observation of the expression patterns of neuropeptides in the hypothalamus revealed that within some of the hypothalamic nuclei defined by the zebrafish brain atlas (Wullimann *et al*., 1996), there were distinct subnuclei that were not annotated, but displayed distinct neuropeptide expression patterns.

487 **3.2.1 Subnclei in the PPa**

488 The anterior part of the parvocellular preoptic nucleus (PPa) is a relatively large region covering
489 all of the anterior preoptic area (Fig. 21 A). Within the PPa, there is a dense mass of neurons in
490 the dorsal part of the PPa (PPad), segregated from the ventral part of the PPa (PPav) by a thin
491 blank area with few neurons (Fig. 21, see the counterstain). The expressions of four neuropeptides
492 (*cart5*, *ccka*, *gnrh3*, and *vipa*) were restricted to the PPav (Figs. 7-9), as represented by *ccka* and
493 *vipa* in Fig. 21. Although other neuropeptides present in PPa were expressed in both PPad and
494 PPav, their expression patterns were not uniform: some genes such as *gal* and *pdyn* were expressed
495 predominantly in PPav, while others such as *cart3b* and *tac3a* were expressed more prominently
496 in PPad (Fig. 21). In addition, glutamatergic neurons were mostly restricted to PPav, whereas
497 GABAergic neurons were present in both PPad and PPav (Fig. 21). Thus, based on the expression
498 patterns of neuropeptides/neurotransmitters, we proposed two subnuclei in PPa: PPad and PPav.
499

500 **3.2.2 Subnuclei in Hv**

501 The ventral zone of the periventricular hypothalamus (Hv) is a layer of neurons surrounding the
502 diencephalic ventricle (DiV) (Fig. 22). Most of the neuropeptides present in the Hv (*agrp*, *asip2b*,
503 *cart3b*, *cart3a*, *ccka*, *crha*, *crhb*, *gnrh3*, *npb*, *penka*, *penkb*, *pomcb*, and *vipa*) were expressed
504 exclusively in the ventral part of Hv (Hvv) (Figs. 12-16), as represented by *agrp* and *ccka* in Fig.
505 22. In contrast, four genes (*pdyn*, *tac1*, *tac3a*, and *tac3b*) were expressed in the dorsal part of Hv
506 (Hvd) (Figs. 12-16) as represented by *pdyn* and *tac3a* in Fig. 22. The boundary between Hvd and
507 Hvv was clearly defined by *pdyn* expression (Fig. 22 B). The genes expressed in Hvd and Hvv
508 were mutually exclusive, with only one exception: *cckb*, which was highly expressed in Hvv and
509 slightly expressed in the anterior part of Hvd (Fig. 22). Hvd and Hvv also differed in
510 neurotransmitter expression patterns: Hvd contained mostly glutamatergic neurons, whereas Hvv
511 harbored mostly GABAergic neurons (Fig. 22). Thus, we propose that Hv can be divided into two
512 subnuclei, Hvd and Hvv.
513

514 **3.2.3 Subnuclei in Hd**

515 The dorsal zone of the periventricular hypothalamus (Hd) is the nucleus that surrounds the lateral
516 recess of the DiV (LR). At the anterior end (Fig. 14; plane 13 in Fig. 23), Hd was located medially,
517 adjacent to Hv. More posteriorly (Fig. 15; plane 14 in Fig. 23), Hd extends laterally and continues
518 caudally through the lateral part of the hypothalamus. The expression of neuropeptide genes
519 differed along the mediolateral and dorsoventral axes of Hd (Fig. 23). Genes such as *cart5*, *gal*,
520 *kiss2*, *npy*, *pdyn,* and *tac3a* were expressed only in the medial part of Hd (Hdm) (Figs. 14 and 15),
521 as represented by *npy* in Fig. 23. Within the lateral part of the Hd, the neuropeptide expression
522 patterns were different between the dorsal and ventral parts. *cart3b* and *npb* were only expressed

in the dorsolateral part of Hd (Hddl) (Figs. 15–20), as represented by *cart3b* in Fig. 23. In contrast, *ccka*, *cckb,* and *pomcb* were only expressed in the ventrolateral part of Hd (Hdvl) (Figs. 15–20), as represented by *ccka* in Fig. 23. The boundary between Hddl and Hdvl is clearly delineated by *ccka* expression. Five genes (*galp*, *penka*, *penkb*, *tac1*, and *vipa*) were expressed in all the Hd subnuclei (Figs. 14–20), as shown by *penka* and *vipa* in Fig. 23. Both Hddl and Hdvl were *gad*-positive but not *vglut*-positive (Fig. 23), whereas Hdm harbored both *gad*-positive and *vglut*-positive neurons. Altogether, we propose that Hd be divided into three subnuclei: Hdm, Hddl, and Hdvl.

3.2.4 Subnuclei in Hc

The caudal zone of the periventricular hypothalamus (Hc) refers to the hypothalamic area caudal to the Hv and LH (Fig. 24). The anterior part of Hc is not recessed, while the posterior part surrounds the posterior recess (PR) (Fig. 24). The expression of neuropeptides in the anterior and posterior parts of Hc was distinct. In the anterior part of Hc, seven genes (*cart5*, *kiss2*, *npy*, *penkb*, *pmchl*, *pomcb*, and *tac3a*) were expressed only in the dorsal part (the anterodorsal part of Hc, Hcad) (Figs. 17, 18), as shown by *pomcb* and *tac3a* in Fig. 24. Underneath Hcad, there was a triangular dense cell population (the anterointermediate part of Hc, Hcai). *npb* and *cckb* were expressed only in Hcai (Figs. 17 and 18), as represented by *npb* in Fig. 24. *agrp* and *tac1* were expressed in the ventral cell population (the anteroventral part of Hc, Hcav).

The posterior part of the Hc was divided into three regions. The dense neuronal population surrounding the PR was divided into two parts: the subregion dorsal to the PR (posterointermediate part of the Hc, Hcpi) and the subregion ventral to the PR (posteroventral part of the Hc, Hcpv). Dorsal to the Hcpi is another subregion with a lower cell density (the posterodorsal part of Hc, Hcpd). These three subregions showed different neuropeptide expression patterns. Nine genes were expressed only in Hcpd (*cart5*, *gal*, *kiss2*, *npy*, *pdyn*, *pomcb*, *tac1*, *tac3a*, and *vipa*) (Fig. 19), as represented by *pomcb*, *tac3a,* and *pdyn* in Fig. 24. Three genes were expressed in Hcpd and Hcpv (*cart3b*, *cckb*, and *penkb*) (Fig. 19), as represented by *cckb* and *penkb* in Fig. 24. Four genes (*ccka*, *crhb*, *npb*, and *penka*) were expressed in all three regions (Fig. 19), as shown by *penka* and *npb* in Fig. 24. Genes expressed in both Hcpi and Hcpv were not uniformly expressed; all neuropeptides, except *penka*, were more abundantly expressed in Hcpv. *vglut* was expressed in Hcpd and in some cells in Hcpv, but not in Hcpi. *gad* was expressed in all three subregions. Thus, based on the differential expression patterns of neuropeptide genes, we propose the delineation of six subnuclei in the Hc: Hcad, Hcai, Hcav, Hcpd, Hcpi, and Hcpv.

3.3 Comparison of neuropeptide expressions in the hypothalamus between zebrafish and mouse

Using the data obtained in this study, we next compared the neuropeptide expression profiles in the hypothalamic nuclei of zebrafish and mice (Fig. 25). Hypothalamic nuclei were hierarchically clustered based on the expression matrix. Several hypothalamic nuclei in zebrafish were highly clustered with those in mice and showed high similarity in neuropeptide expression. These include the zebrafish Hvd/ATN with mouse ventromedial hypothalamic nucleus (VMN), zebrafish PPav/PM with mouse paraventricular hypothalamic nucleus (Pa), and zebrafish PPp with mouse medial preoptic area (MPA) (Fig. 25). Both zebrafish Hvd and ATN had only one gene that differed from the mouse VMN in either the presence (*Cck*) or absence (*Pdyn*) of expression, respectively. The expression profiles of zebrafish PM and mouse Pa were mostly identical, except that *Galp* and *Vip* were expressed only in zebrafish PM. The zebrafish PPav also showed similar expression patterns to the mouse Pa, but *Vip* and *Pomc* were only expressed in the PPav. In addition, zebrafish PPav contained high numbers of both GABAergic and glutamatergic neurons, whereas mouse Pa predominantly contained glutamatergic neurons. The zebrafish PPp and mouse MPA were also remarkably similar, except that *Vip* and *Gal* were expressed only in mouse MPA.

4. Discussion

4.1 Mapping of neuropeptide and neurotransmitter marker gene expressions

The expression of 38 neuropeptide genes and GABAergic/glutamatergic neuron marker genes was mapped throughout the adult zebrafish forebrain. This study presents the first large-scale atlas and database of gene expression in the adult zebrafish brain. Although expression of some of the neuropeptide genes was previously analyzed by *in situ* hybridization in the adult zebrafish brain in separate papers (*agrp*, Jeong *et al*., 2018; *avp*, Carreño Gutiérrez *et al*., 2019; *cart2b*, Akash *et al*., 2014; *cart3a*, Akash *et al*., 2014; *cart3b*, Akash *et al*., 2014; *cart5* Akash *et al*., 2014; *crha*, Grone, and Maruska, 2015; *crhb*, Alderman and Bernier, 2007; *gnrh2*, Steven *et al*., 2003; *gnrh3*, Steven *et al*., 2003; *kiss1*, Servili *et al*., 2011; *kiss2*, Servili *et al*., 2011; *hcrt*, Appelbaum *et al*., 2009; *npy*, Jeong *et al*., 2018; *npvf*, Marvel *et al*., 2018; *oxt*, Carreño Gutiérrez *et al*., 2019; *pmch*, Berman *et al*., 2009; *pmchl*, Berman *et al*., 2009; *tac1*, Ogawa *et al*., 2012; *tac3a*, Biran *et al*., 2012; Ogawa *et al*., 2012; *tac3b*, Ogawa *et al*., 2012; *gad1b*, Mueller *et al*., 2009), it has been difficult to perform detailed comparisons due to different sampling and staining conditions. The gene expression atlas is a fundamental resource in neuroscience research and will be useful for a variety of studies, including searching for marker genes for groups of neurons of interest, detailed anatomical analysis of each brain area, and investigation of homologous relationships with other species. The latter two points were further analyzed in the present study.

4.2 Subnuclei in the hypothalamus

The hypothalamus plays an important role as a central hub that controls the autonomic nervous system, endocrine system, and behavior. However, in teleost species, the anatomical relevance of the hypothalamic nuclei to these functions remains unclear. In addition, the definition of nuclei in the zebrafish hypothalamus is relatively simple compared with that in mammals. The zebrafish hypothalamus is divided into ten nuclei in the commonly used brain atlas (Wullimann *et al*., 1996), whereas the mouse hypothalamus is divided into over 25 nuclei in the brain atlas (Paxinos and Franklin, 2019). In this study, we attempted to define "subnuclei" in the zebrafish hypothalamus based on differential neuropeptide and neurotransmitter expression profiles to further increase the resolution of the current atlas. The information on these subnuclei defined in the present study will provide useful insights for future research on the neuroanatomy and functions of the teleost hypothalamus.

4.2.1 The anterior part of the parvocellular preoptic nucleus (PPa)

The neuropeptide expression patterns revealed that PPa could be clearly divided into two subnuclei: PPad and PPav. Previous studies have shown that the expression of genes other than

609 neuropeptides is also different between PPad and PPav, although differences in preparation make
610 it difficult to compare the exact location of expression. Estrogen receptor 1 (*esr1*) and androgen
611 receptor (*ar*) are mostly expressed in PPav (*esr1*, Diotel *et al*., 2011; *ar*, Gorelick *et al*., 2008).
612 Cholinergic neurons are predominantly localized to PPad (Clemente *et al.*, 2004), whereas
613 dopaminergic neurons are confined to PPav (Yamamoto *et al*., 2011). Orthopedia a (*otpa*), a
614 transcription factor important for the development of neurosecretory neurons, is also found only
615 in PPav (Eugenin von Bernhardi *et al*., 2022). Recently, PPa has been shown to be critical in
616 zebrafish social behavior (Nunes *et al*., 2021; Tallafus *et al*., 2022). PPad and PPav may play
617 different roles in social information processing.
618
619 **4.2.2 The ventral zone of the periventricular hypothalamus (Hv)**
620 Based on the expression patterns of neurotransmitters and neuropeptides, we proposed two
621 subnuclei in Hv: Hvd and Hvv. The Hv in the teleost (also referred to as the NLTv, ventral part of
622 the lateral tuberal nucleus) is considered homologous to the mammalian arcuate nucleus (Arc),
623 based on the shared expression of feeding-related neuropeptide genes such as *agrp*, *pomc*, *npy*,
624 and *cart* (Forlano and Cone, 2007; Soengas *et al*., 2018). All of these feeding-related
625 neuropeptides are expressed exclusively in the Hvv, but not in the Hvd, suggesting a role for Hvv
626 in the control of feeding. However, as Hvv also expresses many other neuropeptide genes, such
627 as *ccka*/*b*, *crha*/*b*, *npb*, *penka*/*b*, and *vipa*, its function may not be confined to feeding. The
628 function of Hvd is not yet clear, but its neuropeptide and neurotransmitter expression patterns
629 resemble those of the mammalian ventromedial hypothalamus (VMN), as discussed below.
630
631 **4.2.3 The dorsal zone of the periventricular hypothalamus (Hd)**
632 Hd can be divided into three subnuclei, Hdm, Hddl, and Hdvl. Recently, it has been reported that
633 Hd is activated by feeding behavior (Wee *et al*., 2018) or food-related cues (Muto *et al*., 2017;
634 Wakisaka *et al*., 2017). However, the subnuclei in the Hd that are activated and the types of
635 neurons that are involved in feeding remain unknown. The neuropeptide expression atlas of the
636 present study will help to clarify these important questions in the near future.
637
638 **4.2.4 The caudal zone of the periventricular hypothalamus (Hc)**
639 Our analysis revealed that Hc could be divided into six subnuclei: Hcad, Hcai, Hcav, Hcpd, Hcpi,
640 and Hcpv. The posterior region of the Hc has been reported to harbor cerebrospinal fluid (CSF)-
641 contacting cells that produce dopamine and/or serotonin, which are absent in mammals (Xavier
642 *et al*., 2017). Within the posterior part of the Hc, the expression patterns of neuropeptides varied
643 greatly among the three subnuclei, suggesting that CSF-contacting cells in the posterior Hc are
644 not uniform. In addition, the colocalization of two dopamine-synthesizing enzymes, tyrosine

645 hydroxylase (TH) 1 and TH 2, is confined to Hcpv (Yamamoto *et al*., 2011). Functional
646 differences in the Hc subnuclei have also been suggested in a previous study with larval zebrafish,
647 in which the Hcpi and Hcpv responded differently to the visual cues of conspecifics (Tunbak *et*
648 *al*., 2020). These regions might play distinct roles in the social reward system.
649
650 **4.3 Comparison of hypothalamic neuropeptide expression patterns between zebrafish and**
651 **mouse**
652 To date, few studies have compared the structure of the teleost hypothalamus with that of other
653 vertebrate lineages, and homologous relationships are not yet clear. In the present study, a
654 comprehensive and detailed examination of the expression profiles of a large number of
655 neuropeptides and neurotransmitters in the same preparation enabled us to conduct the most
656 extensive comparison of the hypothalamic nuclei between zebrafish and mice to date and to reveal
657 several similarities across species.
658
659 **4.3.1 Zebrafish magnocellular preoptic nucleus (PM), ventral part of the anterior part of**
660 **the parvocellular preoptic nucleus (PPav), and mouse paraventricular hypothalamic**
661 **nucleus (Pa)**
662 It has been considered that the teleost PM and mammalian Pa are homologous because they share
663 many features, such as the existence of avp- and oxt-expressing magnocellular neurons (Goodson
664 and Bass, 2001; Olivereau and Olivereau, 1988), innervation by *agrp* and *pomc* neurons (Forlano
665 and Cone, 2007), and the expression of other marker genes such as *nurr1* (Kapsimali *et al*., 2001),
666 *ccka*, *nts*, *penka*, *penkb*, *vipa*, *sst1.1*, *crhb*, and *otpa* (Herget *et al*., 2014). Consistently, the
667 zebrafish PM was clustered closest to the mouse Pa in our analysis. Fifteen of the 17 neuropeptide
668 genes examined showed identical expression status between PM and Pa, suggesting highly similar
669 functionality. Zebrafish PPav also clustered close to the PM. In the brain atlases of some fish
670 species, a portion of the PM is considered to be parvocellular, referred to as the parvocellular part
671 of the PM (PMp), and it is located anteriorly adjacent to the magnocellular part of the PM (PMm)
672 (medaka, Anken and Bourrat, 1998; goldfish, Braford and Northcutt, 1983). In the zebrafish
673 reference atlas (Wullimann *et al*., 1996) it was considered that the PMp was not evident; however,
674 the PPav may correspond to the PMp in other fish species and is closely related to the PM, and
675 therefore, highly similar to Pa in mice.
676
677 **4.3.2 Zebrafish posterior part of the parvocellular preoptic nucleus (PPp) and mouse medial**
678 **preoptic area (MPA)**
679 Zebrafish PPp clustered close to the mouse MPA. The preoptic area as a whole is thought to be
680 conserved between teleosts and mammals (Goodson, 2005; O'Connell and Hofmann, 2011);

however, the homology of specific nuclei or regions within the preoptic area has not been discussed. The fact that the neuropeptide expression profile of zebrafish PPp was particularly similar to that of mouse MPA may indicate functional similarities between these nuclei. In mammals, MPA harbors estrogen receptor (*Esr1*)-expressing neurons, which play important roles in sexual and parental behaviors (Tsuneoka and Funato, 2021). Although there is no available data on the presence or absence of *esr1* in zebrafish PPp, *esr1* expression in the PPp has been reported in several fish species, such as Atlantic croaker, sea bass, and medaka (Hawkins *et al*., 2005; Muriach *et al*., 2008; Zempo *et al*., 2013). In contrast, *esr1* is also expressed in the PPa and PM in many teleost species. Furthermore, galanin, which is excessively expressed in mammalian MPA (Wu *et al*., 2014; Moffitt *et al*., 2018), was not observed in zebrafish PPp but was abundantly expressed in PPa. Collectively, it may be more accurate to consider that many of the cell populations corresponding to mammalian MPA are located in the PPp in zebrafish, but some are also distributed in other regions of the preoptic area.

4.3.3 Zebrafish dorsal subnucleus of the ventral zone of periventricular hypothalamus (Hvd), anterior tuberal nucleus (ATN), and mouse ventromedial hypothalamic nucleus (VMN)

Zebrafish Hvd and ATN clustered close to the mouse VMN. The similarity between the ATN and VMN has been proposed previously, based on the location, expression of *esr1*, and connections with the preoptic area (Forlano *et al*., 2005; Goodson, 2005; Forlano and Bass, 2011; O'Connell and Hofmann, 2011). Hvd is located medially adjacent to the ATN, but the homologous relationship between Hvd and VMN has not been reported so far. Our data showed that *pdyn*, which is present predominantly in the dorsomedial part of the mouse VMN (Allen Brain Atlas), is strongly expressed in zebrafish Hvd but not in ATN, indicating that Hvd and ATN might represent homologs of different subnuclei in the VMN. Other genes that are highly expressed in the mouse VMN include *Adcyap* and *Bdnf* (Khodai *et al*., 2021). *adcyap1b* is expressed in both the Hvd and ATN in teleosts (Nakamachi *et al*., 2018), and *bndf* is predominantly expressed in the Hvd(Cacialli *et al*., 2016).

Mammalian VMN controls a variety of behavioral and physiological traits such as sexual behavior, aggression, fear response, and energy homeostasis (Silva *et al*., 2013; Hashikawa *et al*., 2017; Khodai *et al*., 2021). In teleost fishes, it has been reported that the Hv and ATN in males are activated by the courtship of competing males (midshipman fish, Petersen *et al*., 2013), and during the change from subordinate to dominant (cichlid fish, Maruska *et al*., 2013), suggesting a role for Hv and ATN in social information processing. Further studies are required to elucidate the degree to which mammalian VMN and fish Hvd/ATN functions are conserved.

5. Conclusion

We constructed an expression atlas and database of 38 neuropeptide genes and GABAergic/glutamatergic neuron marker genes in the forebrain of adult zebrafish. By utilizing these data, we proposed subnuclei in the hypothalamic nuclei and compared them with mouse hypothalamic nuclei. This atlas and database will become a useful resource that will contribute to the future development of neuroscience research using zebrafish.

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Tables

Table 1. Genes examined in this study and primers used for *in situ* hybridization (ISH) probe synthesis.

Table 2. The summary of gene expressions in the olfactory bulb and dorsal telencephalon.

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Table 4. The summary of gene expressions in the preoptic area and hypothalamus.

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Supplemental Table 1. The database of *gal* and *galp* genes in various species was used for phylogenetic tree analysis.

Figure Legends

Figure 1. Schematic of the zebrafish brain viewed from the lateral side. The anterior is to the left, and the dorsal is to the top. Lines 1–19 indicate the locations of each plane in Figs. 2–20.

Figure 2. Schematic of plane 1 and photographs of ISH results of genes expressed in plane 1. Scale bar = 100 μm.

Figure 3. Schematic of plane 2 and photographs of ISH results of genes expressed in plane 2. Scale bar = 200 μm.

Figure 4. Schematic of plane 3 and photographs of ISH results of genes expressed in plane 3. Scale bar = 200 μm.

Figure 5. Schematic of plane 4 and photographs of ISH results of genes expressed in plane 4. Scale bar = 200 μm.

Figure 6. Schematic of plane 5 and photographs of ISH results of genes expressed in plane 5. Scale bar = 200 μm.

Figure 7. Schematic of plane 6 and photographs of ISH results of genes expressed in plane 6. Scale bar = 200 μm.

Figure 8. Schematic of plane 7 and photographs of ISH results of genes expressed in plane 7. Scale bar = 200 μm.

Figure 9. Schematic of plane 8 and photographs of ISH results of genes expressed in plane 8. Scale bar = 200 μm.

Figure 10. Schematic of plane 9 and photographs of ISH results of genes expressed in plane 9. Scale bar = 200 μm

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Figure 12. Schematic of plane 11 and photographs of ISH results of genes expressed in plane 11. Scale bar = 200 μm.

Figure 13. Schematic of plane 12 and photographs of ISH results of genes expressed in plane 12. Scale bar = 200 μm.

Figure 14. Schematic of plane 13 and photographs of ISH results of genes expressed in plane 13. Scale bar = 200 μm.

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Figure 16. Schematic of plane 15 and photographs of ISH results of genes expressed in plane 15. Scale bar = 200 μm.

Figure 17. Schematic of plane 16 and photographs of ISH results of genes expressed in plane 16. Scale bar = 200 μm.

Figure 18. Schematic of plane 17 and photographs of ISH results of genes expressed in plane 17. Scale bar = 200 μm.

Figure 19. Schematic of plane 18 and photographs of ISH results of genes expressed in plane 18. Scale bar = 200 μm.

Figure 20. Schematic of plane 19 and photographs of ISH results of genes expressed in plane 19. Scale bar = 200 μm.

Figure 21. Subnuclei in the anterior part of the parvocellular preoptic nucleus (PPa). (A) The PPa (grey) in the original zebrafish brain atlas. (B) The PPa can be divided into two subnuclei: the ventral part of PPa (PPav, pink) and dorsal part of PPa (PPad, purple). Neuropeptides such as *ccka* and *vipa* and *vglut* mix are exclusively expressed in the PPav. *gal* and *pdyn* are expressed in both the PPad and PPav, but predominantly in the PPav. *cart3b* and *tac3a* are also expressed in the PPav and PPad, but predominantly in the PPad. *gad* mix is expressed in both the PPad and PPav. Scale bar = 100 μm.

Figure 22. Subnuclei in the ventral zone of the periventricular hypothalamus (Hv). (A) The Hv (grey) in the original zebrafish brain atlas. (B) The Hv can be divided into two subnuclei: the ventral part of Hv (Hvv, purple) and the dorsal part of Hv (Hvd, pink). Neuropeptides such as *pdyn* and *tac3a* are expressed only in the Hvd, whereas *agrp* and *ccka* are expressed only in the Hvv. *cckb* is expressed in both the Hvd and Hvv. *gad* mix is predominantly expressed in the Hvv and the *vglut* mix is more densely expressed in the Hvd. Scale bar = 100 μm.

Figure 23. Subnuclei in the dorsal zone of the periventricular hypothalamus (Hd). (A) The Hd (grey) in the original zebrafish brain atlas. (B) The Hd can be divided into three subnuclei: the medial (Hdm, pink), dorsolateral (Hddl, purple), and ventrolateral (Hdvl, yellow) part of Hd. Some neuropeptides are expressed solely in the Hdm, Hddl, or Hdvl (represented by *npy*, *cart*, and *ccka*, respectively). Neuropeptides such as *penka* and *vipa* are expressed in all three subnuclei. *gad* mix is also expressed in all subnuclei. *vglut* mix is absent in all subnuclei. Scale bar = 100 μm.

Figure 24. Subnuclei in the caudal zone of the periventricular hypothalamus (Hc). (A) The anterior part of Hc (grey) in the original zebrafish brain atlas. (B) The anterior part of Hc can be divided into three subnuclei: the anterodorsal (Hcad, yellow), anterointermediate (Hcai, blue), anteroventral (Hcav, pink) part of Hc. Neuropeptides such as *pomcb* and *tac3a* are solely expressed in the Hcad. Neuropeptides such as *npb* and *cckb* are expressed exclusively in the Hcai. *agrp* and *tac1* are only expressed in the Hcav. Neuropeptides such as *cckb*, *pdyn*, and *penka* are expressed across multiple subnuclei. *gad* mix and *vglut* mix are expressed in all three subnuclei. (C) Posterior part of Hc (grey) in original zebrafish brain atlas. (D) The posterior part of Hc can be divided into three subnuclei: the posterodorsal (Hcpd, green), posterointermediate (Hcpi, light green), posteroventral (Hcpv, purple) part of Hc. Neuropeptides such as *pomcb*, *tac3a*, and *pdyn* are solely expressed in the Hcpd. *cckb*, *penkb*, and *vglut* mix are expressed in the Hcpd and Hcpv. *penka*, *npb*, and *gad* mix are expressed in all three subnuclei. Scale bar = 100 μm.

Figure 25. Comparison between zebrafish and mouse hypothalamic nuclei based on their neuropeptide expression patterns. Expression of neuropeptides (green) and neurotransmitter markers (purple) in the hypothalamic nuclei of zebrafish (light yellow) and mice (light blue) were extracted from the present ISH results (zebrafish), the Allen Mouse Brain Atlas, and previous papers (mouse) and summarized in a table (expressed = 1, not expressed = 0). Ward's method hierarchically clustered zebrafish and mouse nuclei and expressed genes.

1264 **Supplemental Figure 1.** Phylogenetic tree and synteny analyses of *gal* and *galp* gene families in
1265 vertebrates. A: Phylogenetic tree of GAL and GALP amino acid sequences. The accession
1266 numbers of the sequences used in this analysis are listed in Supplementary Table 1. Sequences
1267 are aligned using ClustalW, and a phylogenetic tree is constructed by the maximum likelihood
1268 method using the Molecular Evolutionary Genetics Analysis (MEGA11) software. Numbers at the
1269 branch nodes represent the bootstrap confidence level (500 replicates). The scale bar indicates the
1270 number of substitutions per site. B: Schematic showing the genes in the genomic area around *gal*
1271 and *galp* genes. The arrowhead represents the orientation of the gene. Orthologous genes among
1272 vertebrate species are indicated in the same color.
1273
1274 **Supplemental Figure 2.** Summary of individual differences in neuropeptide expression.
1275 Photographs of four individual fish are shown for each gene for which individual differences in
1276 expression are observed. Some sections are subjected to methyl green nuclear staining. Scale bar
1277 = 100 μm.

Table 1. Genes examined in this study and primers used for in situ hybridization (ISH) probe synthesis.

Gene	Peptide	Synonim	Forward primer	Reverse primer	NCBI gene ID	mRNA GenBank accession no.
agrp	agouti-related neuropeptide		AGGATCTGGGCAAAGCTGTCCACCTGCAGAGAAGAG	GCAATAATCAGAGTTTATTGAATGCATATATACAAA	100149042	NM_001328012.1
asip2b	agouti-signaling protein 2b	agrp2	CTTCATAAAAACTCCCCTTTATCAGGTCGG	AAGCTTGCAGCTATGTGCTGTCATTATGGC	796595	NM_001271291.1
avp	arginine vasopressin	arginin vasotocin	AGTGTATGTCGTGTGGCCCCGGGGGTGTGGGCCGC	CTGAATCGCAGCAGATGCCCTCAGCGGCGCAGCG	352922	NM_178293.2
calca	calcitonin related polypeptide alpha	cgrp	GGTCACCAGTCCACACGTCCACAGTTCCTCTTTG	CTTGCTCAAATTCACAATTGTTACAATTTGTAATTC	436744	NM_001002471.1
cart2a	cocaine- and amphetamine-regulated transcript 2a		ATGTTTAGCAGACGGATTGTCTTTTTCGCTGCTGCTTCTGCCC	TTAAAGGCATTTTAAAATAAAAAAATGGCATGATGTTCCTTGTGG		OQ363808
cart2b	cocaine- and amphetamine-regulated transcript 2b	cart1	CCACATGAATTTTGCTAGCTGGGATATGACGC	ATTTGATGATGGTAAACAACACACTGGAGC	562883	XM_021473557.1
cart3a	cocaine- and amphetamine-regulated transcript 3a	cart3	TTGCGCTGTGCTGCTCTCCTGCATACAGGC	ATCTTCAAACGCATCTGATAGTATCATAGC	557301	NM_001327818.1
cart3b	cocaine- and amphetamine-regulated transcript 3b	cart2	TCCAGTCTGAGGATGCGCATGGCAGTG	GCGGACAGTCGCACATCTTGCCGATTC	550232	NM_001017570.2
cart4	cocaine- and amphetamine-regulated transcript 4	cartl	ATGACGAGCTCTGAGATGCTGATGACAGGACTCCTCGTAGCGGG	TCAGATGCATTTTAAGAGAAATGAGTTACAGTTTGATCCTCTGCC	100329270	NM_001386407.1
cart5	cocaine- and amphetamine-regulated transcript 5	cart4	GCACACAAGAGCTGGGAAACGCTTAG	ACAACAGAGTCAGTAGTAGGTCACC	799423	NM_001082932.1
ccka	cholecystokinin a		GTGTTTCTCCAGCTCTCTCTGCGTCTCTGC	GTGACTCTGTACAGATGATCGGCTAATCGG	100007763	NM_001386383.1
cckb	cholecystokinin b		AGGCTCCGTTCGCCGGAACTCCTCGATGAACAGCC	TAGGGTTTAAGGCCAAAGTTGAATGCTGACCTG	100000095	XM_002665615.6
crha	corticotropin-releasing hormone a		AGATTACCGTGAATTCCCCTATGAATCTGCACGTGG	TATTTCACTCAGTTTGGGTTTAAATAACCC	796284	XM_009298729.3
crhb	corticotropin-releasing hormone b		CAGATTCTCCTCGCCACTTTTTGACATGAAGCTC	GAAAAATATAAATATAAGACATTCTCTTGC	492507	NM_001007379.1
gal	galanin		GAATCCACAGGAGCATCAGAGGACCAGCAGCAG	GAATGCCATTTATGAGTTTTATTTTTGAATCC	100006361	XM_005162992.4
galp	galanin-like peptide	gall, si:rp71-1c10.8	GCAAACCTGTTATTTGAAGGTTACAGGCGG	CCATGATTCAGTAAAGGGGAGAGATGGAGG	100538216	NM_001327976.1
gnrh2	gonadotoropin-releasing hormone 2		GCTGGATGCCCTCATTCGTGATTTCCAAAAGAG	CAAAATACCAAAATAAAGCCTTTATTGTAGGAAC	353222	NM_181439.4
gnrh3	gonadotoropin-releasing hormone 3		ATCTTGAACAAACACAGCAGTTTTAGCATGGAGTG	GTTCCTTCAGTGAAGTGTATTTATTAAATCCATAA	360141	NM_182887.2
grp	gastrin-releasing peptide		GGCTGAGGACTTGTTACGGGCTTTAAGAATGCAG	TCAAATGATATGCAAAACAGTCATTTTTTATAAACC	559978	NM_001161350.1
hcrt	hypocretin	orexin	AGAAGCTCCAGGTGCTCGTCTTCATGGCGCTGC	TAGCGACAAGTGTCATCGTTTTTCATAACTGTCC	613239	NM_001077392.2
kiss1	kisspeptide 1		TATGAAGCGAACACAAGCTCCATACCTGC	AACATGAAGGCAAATACCTCAGAGAGGACC	100134982	NM_001113489.1
kiss2	kisspeptide 2		ATATAAGCTCTACAATTCAGCAATATGAATACC	TAGGCTACAATAACTCAATAAAACAAAGTGCGC	100216472	NM_001142585.1
npb	neuropeptide b		ACAACAGAGAACACTATCACCCGAGCGCGC	TGTCAACAGCACGTCTGGCTGCCTCGGCGG	563766	XM_009296109.3
npvf	neuropeptide VF	gnih	TCAGAGAATGCTCAAGAGATTCCCCGCAGTCTGGAG	CCTTTCTTTTGGAAATGATGGTGATTCAGGTGTGCG	100002108	NM_001082949.1
npy	neuropeptide y		CCCACAGAGCAAGAATTCCAATCAAGATCTC	TCAGGGGCAAAAGACAGCAAACAAAGACGAC	30281	NM_131074.2
oxt	oxytocin	isotocin	ACGCGTCCGCAACTCTTCTTCTTCTTCTTCTCC	TCTGCACTAATGTACAGTCAAGCTAATCATCAG	352920	NM_178291.2
pdyn	pro-dynorphin		GACTTGTACTTTAGAGTGTGAAGGAGCCGTTCCGC	GAGTTCTAGTTGAGTTATCAATGATACAACAGCC	414850	XM_009297105.1
penka	pro-enkephalin a		GGCCTGCACATTAGAATGTGAAGGGTCTGTGGAC	ATTCTGTACAGCTAATAGCTCAAAATGTTGCCTCG	368228	NM_200083.2
penkb	pro-enkephalin b		GAATGTGTGTTGGAGTGTGAGGAGCAACCGAACGC	CTGTACAACAAATGACAAGATTCACACTGGTACCC	360136	NM_182883.1
pmch	pro-melanin-concentrating hormone	pmch2	GTGCAAAGCTAGGTTCTGCAACCATCACAGCCG	TCATACATTTCTGCTCGTCATGTTTATATTTGG	100003610	NM_001202542.1
pmchl	pro-melanin-concentrating hormone, like	pmch1	GACAGGAAACAGCAGCCAAACCGCTAAAGC	AGAACAGAACAGAACATGAACAGACCTGTG	100216485	NM_001162488.1
pomca	pro-opiomelanocortin a		GGAATGCATACAATTATGCAGGTCTGAACTTACAG	TAGGAACCTCTCATCCAGAAGTTTGCTGGG	353221	NM_181438.3
pomcb	pro-opiomelanocortin b		GCAGTGTCTCAGAAAGTGCAGATCGGACCAAGAATC	TTAATCTGTGAACTGCTGTCCATTGCCGATGGCG	100034412	XM_005170169.4
tac1	tachykinin 1		GAGTCGTATGAATGGGGAACAGTACAGATCTACGAC	GTTCCACCAATAAAGAAAATATCmAATATCAAGC	555634	NM_001256391.1
tac3a	tachykinin 3a		ATGTACCGTGGACTTGTGTTACTGTTCTTGGTTTTGG	CTATAACCCACGACGAAACCTCAGTTTGCATTTG	100320280	XM_005162221.4
tac3b	tachykinin 3b		CTGAACAGCATCCTGTTAACACCAGCTCATACGAG	TCATACACCACAGCAAAACCTCAGTCTTCCATCC	569642	NM_001256390.1
vipa	vasoactive intestinal peptide a		GCCGCTCTGAGGTCAGTCCGAACGCAAGAAATCAAG	CTTTGAATGGCAAAAATAAAGATTTCCAATGCCCG	795653	NM_001114553.3
vipb	vasoactive intestinal peptide b		GTCAGCGGACAGAGATCGTATACCAACCAC	CTGTGTCATTCACACCAGAGTCGCAATCAC	100000558	XM_021479523.1
gad1a	glutamate decarboxylase 1a		TCTGGCGGCGTTCCGGATCCCAACTC	TGGTAACCGCCGGATTGGAGACCACC	100329827	XM_002663304.5
gad1b	glutamate decarboxylase 1b	gad67	ATGGCGTCTTCTGCACCTTCTTCCTCGGCTGGTG	TTACAGATCCTGACCGAGCCGCTCGATCTCATCG	378441	NM_194419.1
gad2	glutamate decarboxylase 2	gad65	CGGCTGGAAACGGCAGTCAAAGCCCG	GGCTGGTAGCTCACCATGGTCGTGCC	550403	NM_001017708.2
slc17a6a	solute carrier family 17 member 6a	VGLUT2.2, vglut2b	Higashijima et al., 2004	Higashijima et al., 2004		
slc17a6b	solute carrier family 17 member 6b	VGLUT2.1, vglut2a	Higashijima et al., 2004	Higashijima et al., 2004		
slc17a7a	solute carrier family 17 member 7a	VGLUT1, vglut1a	Higashijima et al., 2004	Higashijima et al., 2004		
slc17a7b	solute carrier family 17 member 7b	vglut1b	ATTCCCACTGCGGCACGAATGCACTTCGGC	GCTGTGGAAGTCATTCGTTCATCCCTCTGCCC	100331980	XM_009297642.3

Table 2. The summary of gene expressions in the olfactory bulb and dorsal telencephalon.

	Olfactory bulb			Dorsal telencephalon								
	ICL	ECL/GL	Terminal nerve neruon	Dma	Dld	Dlv	Dm	Dd	Dc	Dp	Dl	Dmp
agrp	-	-	-	-	-	-	-	-	-	-	-	-
asip2b	-	-	-	-	-	-	-	-	-	-	-	-
avp	-	-	-	-	-	-	-	-	-	-	-	-
calca	-	-	-	-	-	-	-	-	-	-	-	-
cart2a	-	-	-	-	-	-	-	-	-	-	-	-
cart2b	-	-	-	-	-	-	-	-	-	-	-	-
cart3a	+	+	-	-	-	-	-	-	+	-	-	-
cart3b	+	-	-	+	+	-	+	+	+	-	+	+
cart4	-	-	-	-	-	-	-	-	-	-	-	-
cart5	-	-	-	-	-	-	+	-	-	-	+	-
ccka	-	-	-	-	-	-	-	-	-	-	-	-
cckb	+	-	-	+	+	+	+	+	+	+	+	-
crha	-	-	-	-	-	-	-	-	-	-	-	-
crhb	+	+	-	-	-	-	+	+	+	+	+	-
gal	-	-	-	-	-	-	-	-	-	-	-	-
galp	-	-	-	-	-	-	-	-	-	-	-	-
gnrh2	-	-	-	-	-	-	-	-	-	-	-	-
gnrh3	-	-	+	-	-	-	-	-	-	-	-	-
grp	-	-	-	-	-	-	-	-	-	-	-	-
hcrt	-	-	-	-	-	-	-	-	-	-	-	-
kiss1	-	-	-	-	-	-	-	-	-	-	-	-
kiss2	-	-	-	-	-	-	-	-	-	-	-	-
npb	-	-	-	-	-	-	-	-	-	-	-	-
npvf	-	-	-	-	-	-	-	-	-	-	-	-
npy	-	+	-	-	-	-	-	-	+	-	-	-
oxt	-	-	-	-	-	-	-	-	-	-	-	-
pdyn	-	-	-	-	-	-	-	-	-	-	-	-
penka	+	-	-	-	-	-	+	-	-	-	+	+
penkb	+	+	-	-	-	-	-	-	-	-	-	-
pmch	-	-	-	-	-	-	-	-	-	-	-	-
pmchl	-	-	-	-	-	-	-	-	-	-	-	-
pomca	-	-	-	-	-	-	-	-	-	-	-	-
pomcb	-	-	-	-	-	-	-	-	-	-	-	-
tac1	+	-	-	-	-	-	-	-	-	+	+	+
tac3a	-	-	-	-	-	-	-	-	-	-	-	-
tac3b	-	+	-	-	+	-	-	-	-	-	-	-
vipa	-	-	-	-	-	-	+	-	-	-	+	-
vipb	-	-	-	-	-	-	-	-	-	-	-	-
gad mix	+	+	-	+	+	+	+	+	+	+	+	+
vglut mix	+	+	-	+	+	+	+	+	+	+	+	+

Table 3. The summary of gene expressions in the ventral telencephalon.

	Ventral telencephalon								
	Vl	Vc	Vv	Vd	Vs	Vp	Vi	ENd	ENv
agrp	-	-	-	-	-	-	-	-	-
asip2b	-	-	-	-	-	-	-	-	-
avp	-	-	-	-	-	-	-	-	-
calca	-	-	-	-	-	-	-	-	-
cart2a	-	-	-	-	-	-	-	-	-
cart2b	-	-	-	-	-	-	-	-	-
cart3a	-	-	+	+	+	+	+	+	+
cart3b	-	-	+	+	+	+	+	+	-
cart4	-	-	-	-	-	-	-	-	-
cart5	-	-	+	-	-	+	-	-	+
ccka	-	-	-	-	-	-	-	-	-
cckb	-	+	-	+	+	+	+	+	-
crha	-	-	-	-	-	-	-	-	-
crhb	-	-	+	+	+	+	-	+	-
gal	-	-	-	-	-	-	-	-	-
galp	-	-	-	-	-	-	-	-	-
gnrh2	-	-	-	-	-	-	-	-	-
gnrh3	-	-	-	-	-	-	-	-	-
grp	-	-	-	-	-	-	-	-	-
hcrt	-	-	-	-	-	-	-	-	-
kiss1	-	-	-	-	-	-	-	-	-
kiss2	-	-	-	-	-	-	-	-	-
npb	+	-	+	-	-	+	-	-	-
npvf	-	-	-	-	-	-	-	-	-
npy	-	-	+	+	+	+	-	+	-
oxt	-	-	-	-	-	-	-	-	-
pdyn	-	-	+	+	+	+	+	-	-
penka	+	-	+	+	+	+	+	-	+
penkb	-	+	+	+	+	+	+	-	-
pmch	-	-	-	-	-	-	-	-	-
pmchl	-	-	+	-	-	-	-	-	-
pomca	-	-	-	-	-	-	-	-	-
pomcb	-	-	+	-	-	-	-	-	-
tac1	-	+	+	+	+	+	+	-	-
tac3a	-	-	+	+	+	+	+	-	-
tac3b	-	-	-	-	-	-	-	-	-
vipa	-	-	+	-	-	-	-	-	-
vipb	-	-	-	-	-	-	-	-	-
gad mix	+	+	+	+	+	+	+	+	+
vglut mix	+	-	+	+	+	+	+	-	+

Table 4. The summary of gene expressions in the preoptic area and hypothalamus.

	Preoptic area				Hypothalamus								
	PPa	PPp	SC	PM	NLT	Hv	LH	Hd	Hc	IN	ATN	DIL	CIL
agrp	-	-	-	-	-	+	-	-	+	-	-	-	-
asip2b	+	-	-	+	-	+	-	-	+	-	-	-	-
avp	+	+	-	+	-	+	-	-	-	-	-	-	-
calca	-	-	-	-	-	-	-	-	-	-	-	-	-
cart2a	-	-	-	-	-	-	-	-	-	-	-	-	-
cart2b	-	-	-	-	-	-	-	-	-	-	-	-	-
cart3a	+	+	-	-	-	+	-	-	+	-	-	-	-
cart3b	+	+	+	-	-	+	+	+	+	+	-	-	-
cart4	+	-	-	-	-	-	-	-	-	-	-	-	-
cart5	+	+	+	-	-	+	+	+	+	-	-	-	-
ccka	+	+	+	+	-	+	+	+	+	+	-	+	+
cckb	+	+	-	-	-	+	+	+	+	-	-	-	-
crha	-	-	-	+	-	+	-	-	-	-	-	-	-
crhb	+	+	+	+	-	+	+	+	+	+	-	-	-
gal	+	-	+	+	+	-	+	+	+	-	-	-	-
galp	-	-	-	+	-	-	+	+	-	-	-	-	-
gnrh2	-	-	-	-	-	-	-	-	-	-	-	-	-
gnrh3	+	-	-	-	-	+	-	-	-	-	-	-	-
grp	-	-	-	-	-	-	-	-	-	-	-	-	-
hcrt	-	-	-	-	-	-	-	-	-	-	-	-	-
kiss1	-	-	-	-	-	-	-	-	-	-	-	-	-
kiss2	-	-	-	-	-	+	+	+	+	-	-	-	-
npb	+	-	+	+	-	+	+	+	+	-	-	-	-
npvf	-	-	-	-	-	-	-	-	-	-	-	-	-
npy	+	+	+	+	+	+	+	+	+	-	-	+	-
oxt	+	+	-	+	-	-	-	-	-	-	-	-	-
pdyn	+	+	+	+	-	+	+	+	+	+	-	+	+
penka	+	+	+	+	-	+	+	+	+	+	-	-	-
penkb	+	+	+	+	-	+	+	+	+	+	-	-	-
pmch	-	-	-	-	+	-	+	-	-	-	-	-	-
pmchl	-	-	-	-	+	-	+	-	+	+	-	-	-
pomca	-	-	-	-	+	+	+	-	+	-	-	-	-
pomcb	+	-	-	-	-	+	+	+	+	-	-	+	-
tac1	+	+	+	+	-	+	-	+	+	-	+	-	-
tac3a	+	+	+	+	-	+	+	+	+	+	+	-	-
tac3b	-	-	-	-	-	+	-	-	-	-	-	-	-
vipa	+	-	-	+	-	+	+	+	+	+	-	+	-
vipb	-	-	-	-	-	-	-	-	-	-	-	-	-
gad mix	+	+	+	+	-	+	+	+	+	+	-	+	+
vglut mix	+	+	+	+	-	+	+	+	+	-	+	+	+

Table 5. The summary of gene expressions in the prethalamus, posterior tuberculum, and thalamus.

	Prethalamus				Posterior tuberculum			Thalamus					
	R	VM	VL	I	TPp	PVO	PTN	Had	Hav	A	DP	IC	CP
agrp	-	-	-	-	-	-	-	-	-	-	-	-	-
asip2b	-	-	-	-	-	-	-	-	-	-	+	-	-
avp	-	-	-	-	-	-	-	-	-	-	-	-	-
calca	-	-	-	-	+	-	-	-	-	-	-	-	-
cart2a	-	-	-	-	-	-	-	-	-	-	-	-	-
cart2b	-	-	-	-	-	-	-	-	-	-	-	-	-
cart3a	-	-	-	-	+	-	+	-	-	+	+	-	-
cart3b	-	+	+	+	+	-	+	-	-	-	+	+	-
cart4	-	-	-	-	-	-	-	-	-	-	-	-	-
cart5	-	-	-	-	-	-	-	-	-	-	-	-	-
ccka	-	-	-	-	+	+	+	-	-	-	-	+	+
cckb	-	-	+	-	+	+	+	+	-	+	+	-	+
crha	-	-	-	-	-	-	-	-	-	-	-	-	-
crhb	-	+	+	-	+	+	+	-	-	-	+	+	-
gal	-	-	-	-	-	-	-	-	-	-	-	-	-
galp	-	-	-	-	+	-	-	-	-	-	-	-	-
gnrh2	-	-	-	-	-	-	-	-	-	-	-	-	-
gnrh3	-	-	-	-	-	-	-	-	-	-	-	-	-
grp	-	-	-	-	-	-	-	-	-	-	-	-	-
hcrt	-	-	-	-	-	+	-	-	-	-	-	-	-
kiss1	-	-	-	-	-	-	-	-	+	-	-	-	-
kiss2	-	-	-	-	+	-	-	-	-	-	-	-	-
npb	-	+	-	-	+	-	+	-	-	-	-	+	-
npvf	-	-	-	-	-	+	-	-	-	-	-	-	-
npy	-	-	-	+	+	-	+	-	-	+	-	+	-
oxt	-	-	-	-	-	-	-	-	-	-	-	-	-
pdyn	-	+	-	-	-	+	+	+	-	-	-	+	-
penka	-	+	+	+	+	+	+	-	-	-	-	+	+
penkb	-	-	-	+	+	+	+	-	-	+	-	+	-
pmch	-	-	-	-	-	-	-	-	-	-	-	-	-
pmchl	-	-	-	-	-	+	-	-	-	-	-	-	-
pomca	-	-	-	-	-	+	-	-	-	-	-	-	-
pomcb	-	-	-	-	-	-	+	-	-	-	-	-	-
tac1	-	-	-	-	+	-	-	+	-	-	-	-	+
tac3a	-	-	-	-	+	+	+	+	-	-	-	-	-
tac3b	-	-	-	-	+	-	-	-	-	-	-	-	+
vipa	-	+	-	-	+	-	+	-	-	-	-	-	-
vipb	-	+	-	-	-	-	-	-	-	-	-	-	-
gad mix	+	+	+	+	+	+	+	-	-	+	-	+	-
vglut mix	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 6. The summary of gene expressions in the preglomerular complex and pretectum.

	Preglomerular complex									Pretectum									
	TLa	P	PGl	PGm	SG	TGN	PGa	PGc	CM	CPN	PSp	APN	DAO	PSm	PO	VAO	PPv	PPd	PCN
agrp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
asip2b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
avp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
calca	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cart2a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cart2b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cart3a	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
cart3b	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
cart4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cart5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ccka	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
cckb	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-
crha	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
crhb	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	+	+	+
gal	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
galp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
gnrh2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
gnrh3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
grp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
hcrt	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
kiss1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
kiss2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
npb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
npvf	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
npy	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
oxt	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pdyn	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
penka	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
penkb	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
pmch	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pmchl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pomca	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pomcb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
tac1	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
tac3a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
tac3b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
vipa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
vipb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
gad mix	-	+	+	+	-	+	+	-	-	+	-	+	+	-	+	+	+	+	+
vglut mix	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+

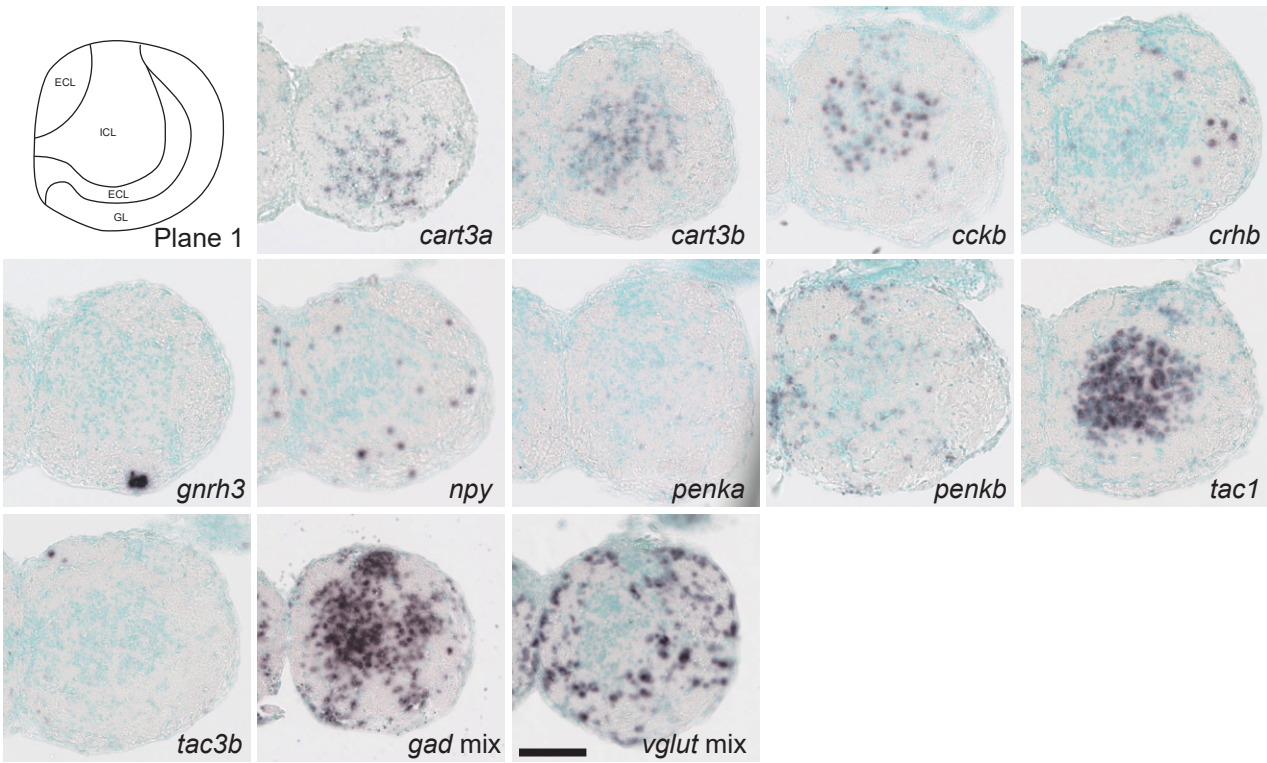


Figure 2

A schematic of plane 1 and photographs of ISH results of genes expressed in plane 1. Scale bar = 100 μm.

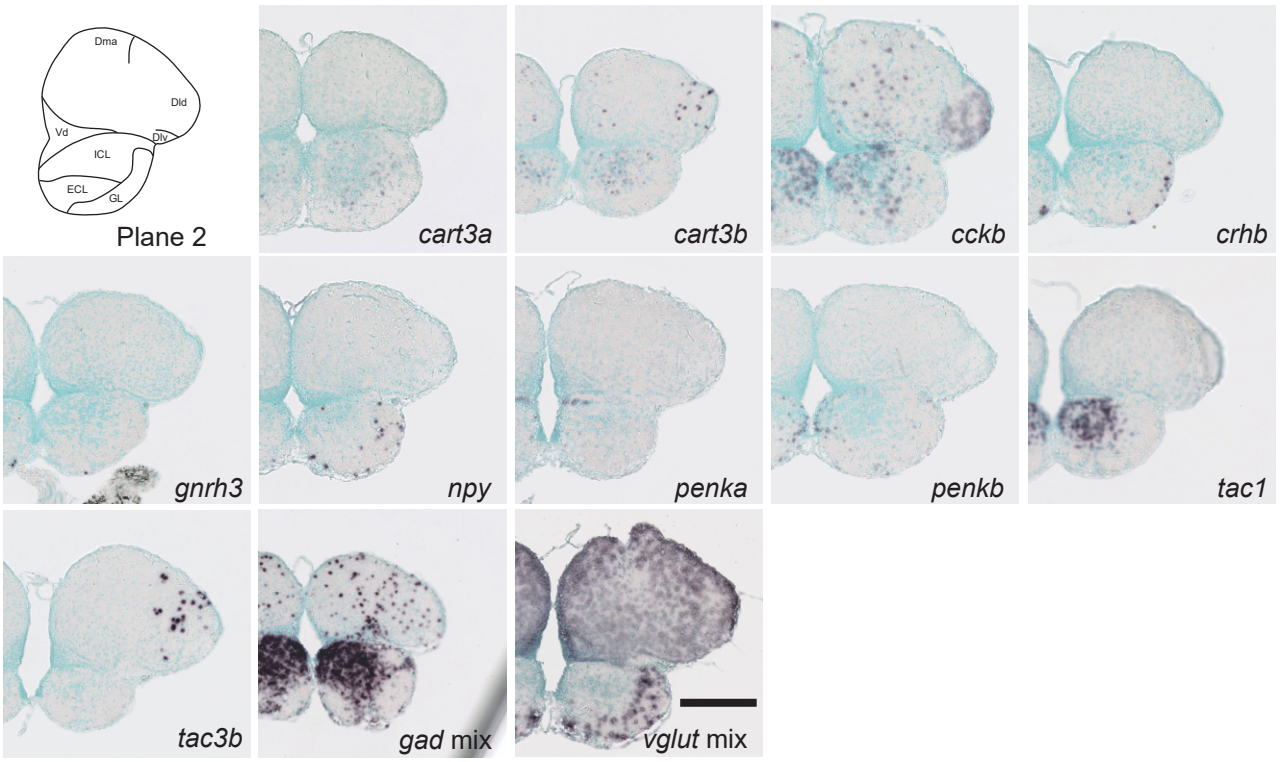


Figure 3

A schematic of plane 2 and microscopic images of genes expressed in the plane 2. Scale bar = 200 μm.

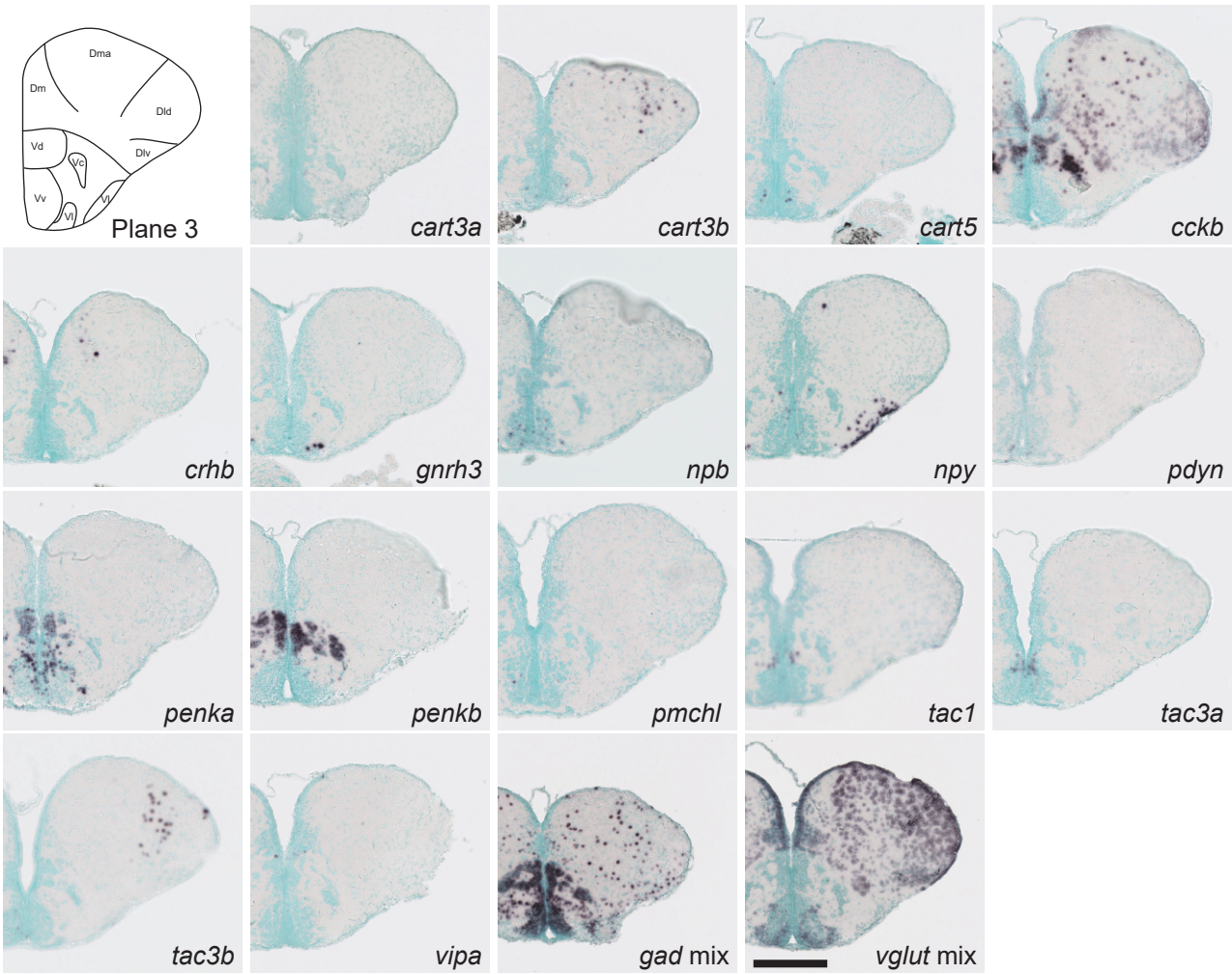


Figure 4

A schematic of plane 3 and microscopic images of genes expressed in the plane 3. Scale bar = 200 μm.

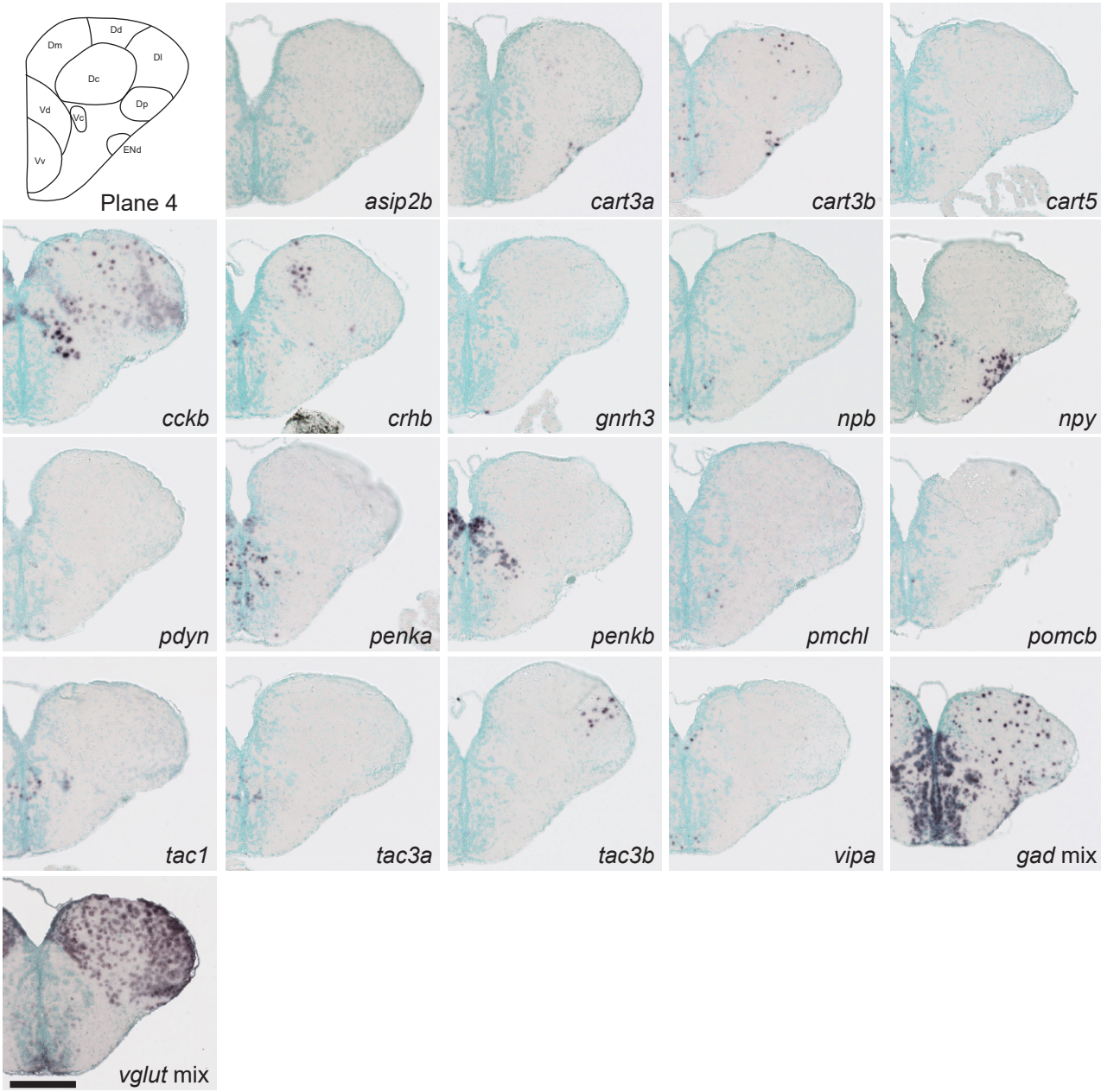


Figure 5

A schematic of plane 4 and microscopic images of genes expressed in the plane 4. Scale bar = 200 μm.

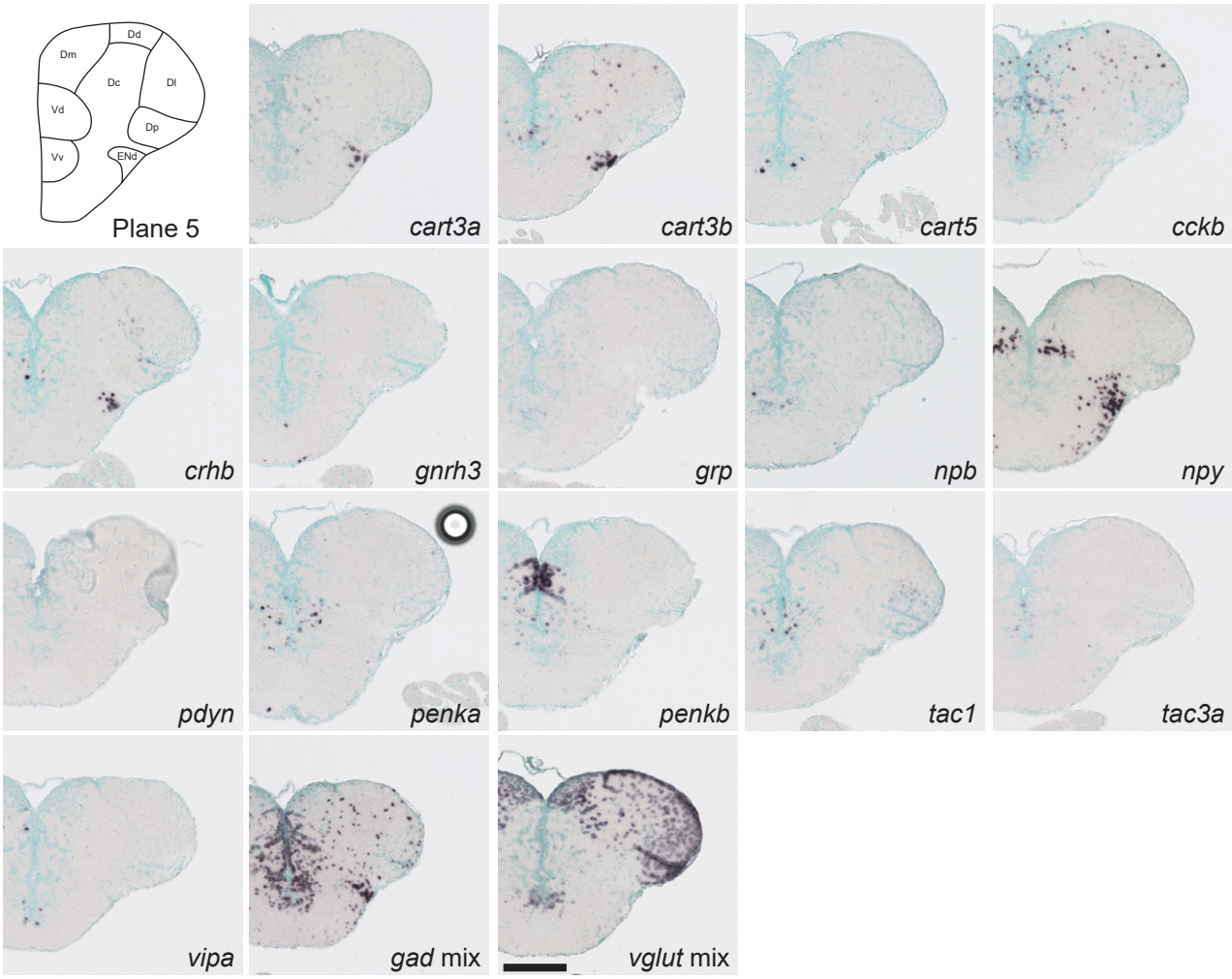


Figure 6

A schematic of plane 5 and microscopic images of genes expressed in the plane 5. Scale bar = 200 μm.

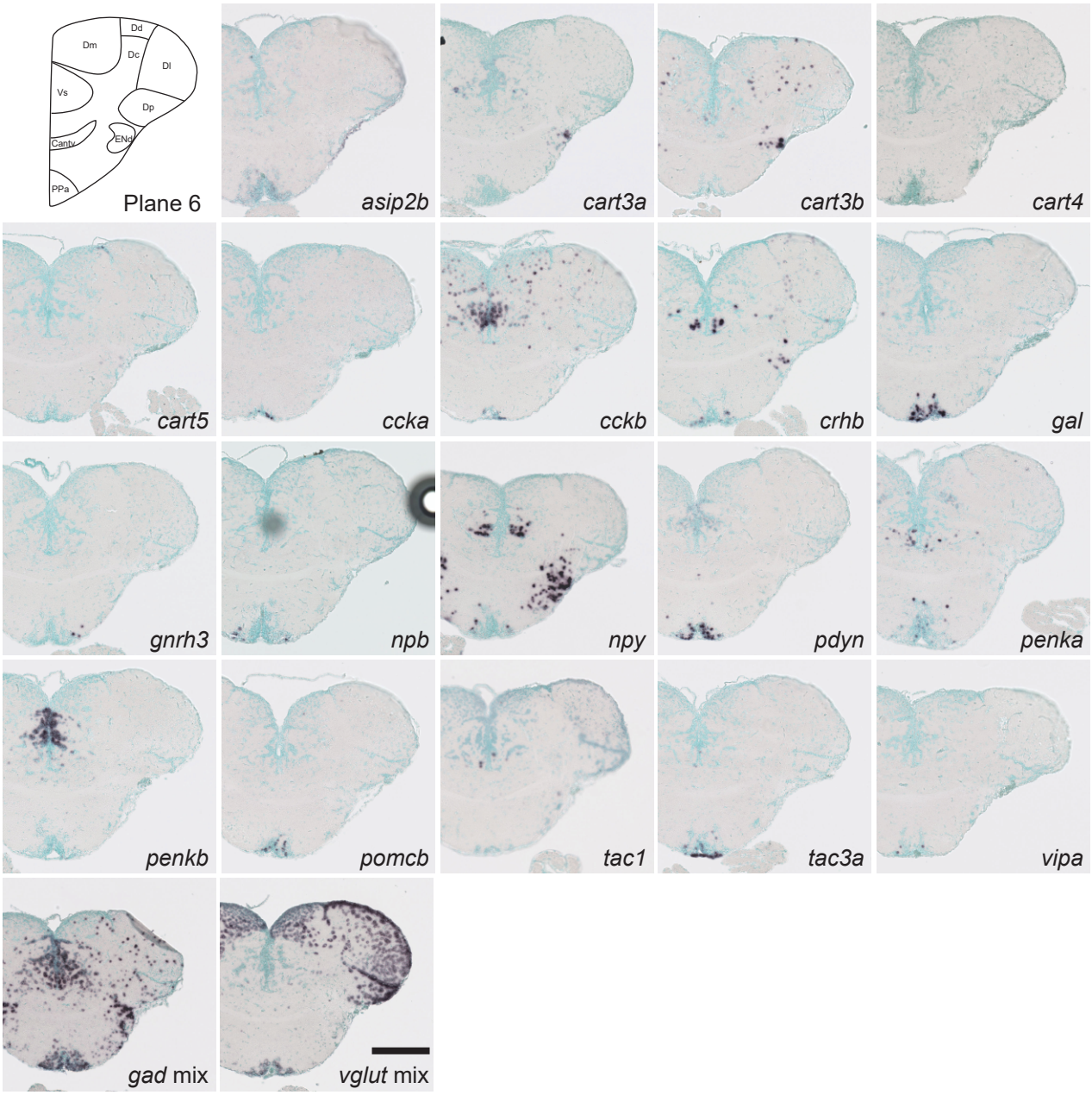


Figure 7

A schematic of plane 6 and microscopic images of genes expressed in the plane 6. Scale bar = 200 μm.

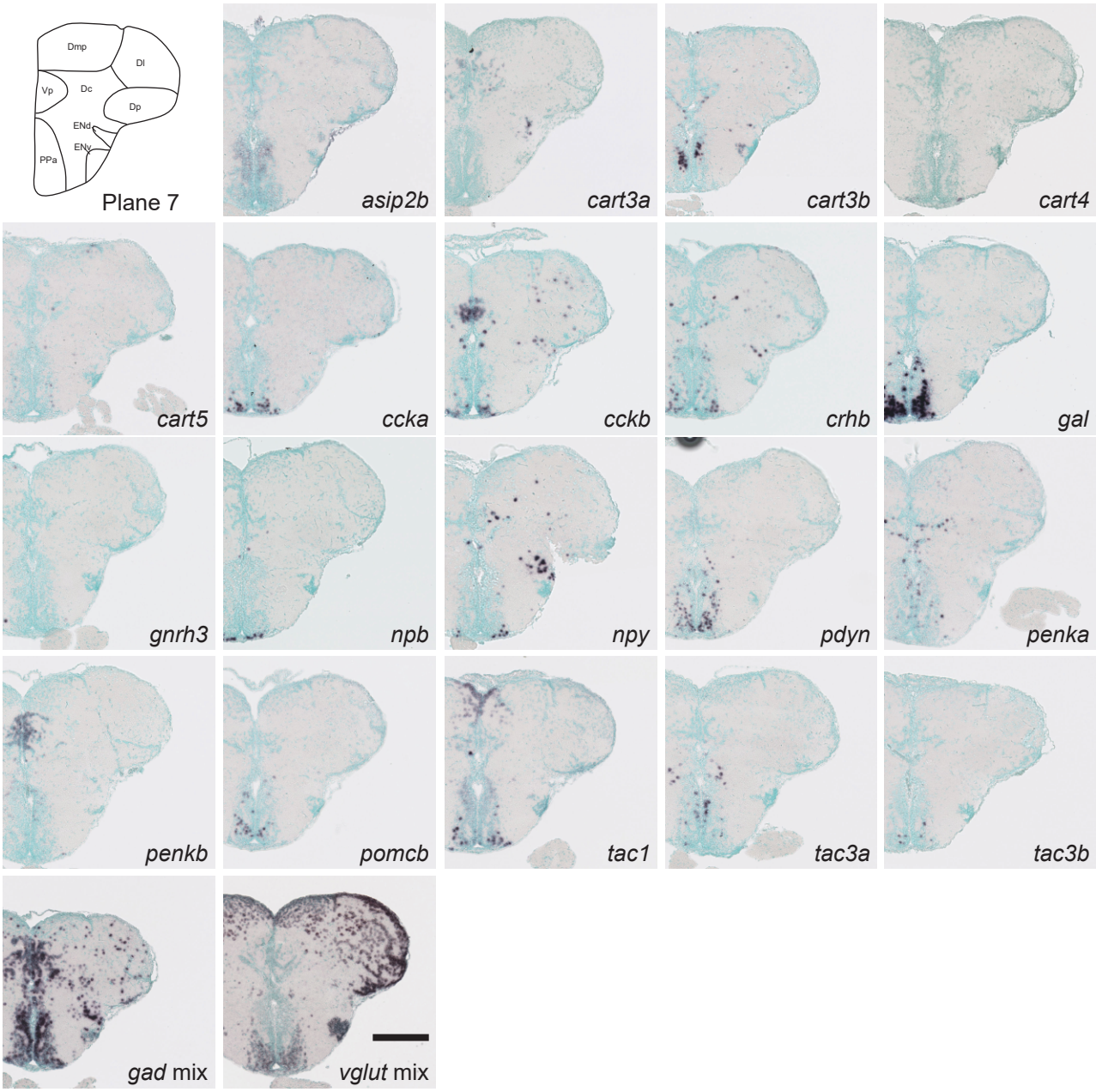


Figure 8

A schematic of plane 7 and microscopic images of genes expressed in the plane 7. Scale bar = 200 μm.

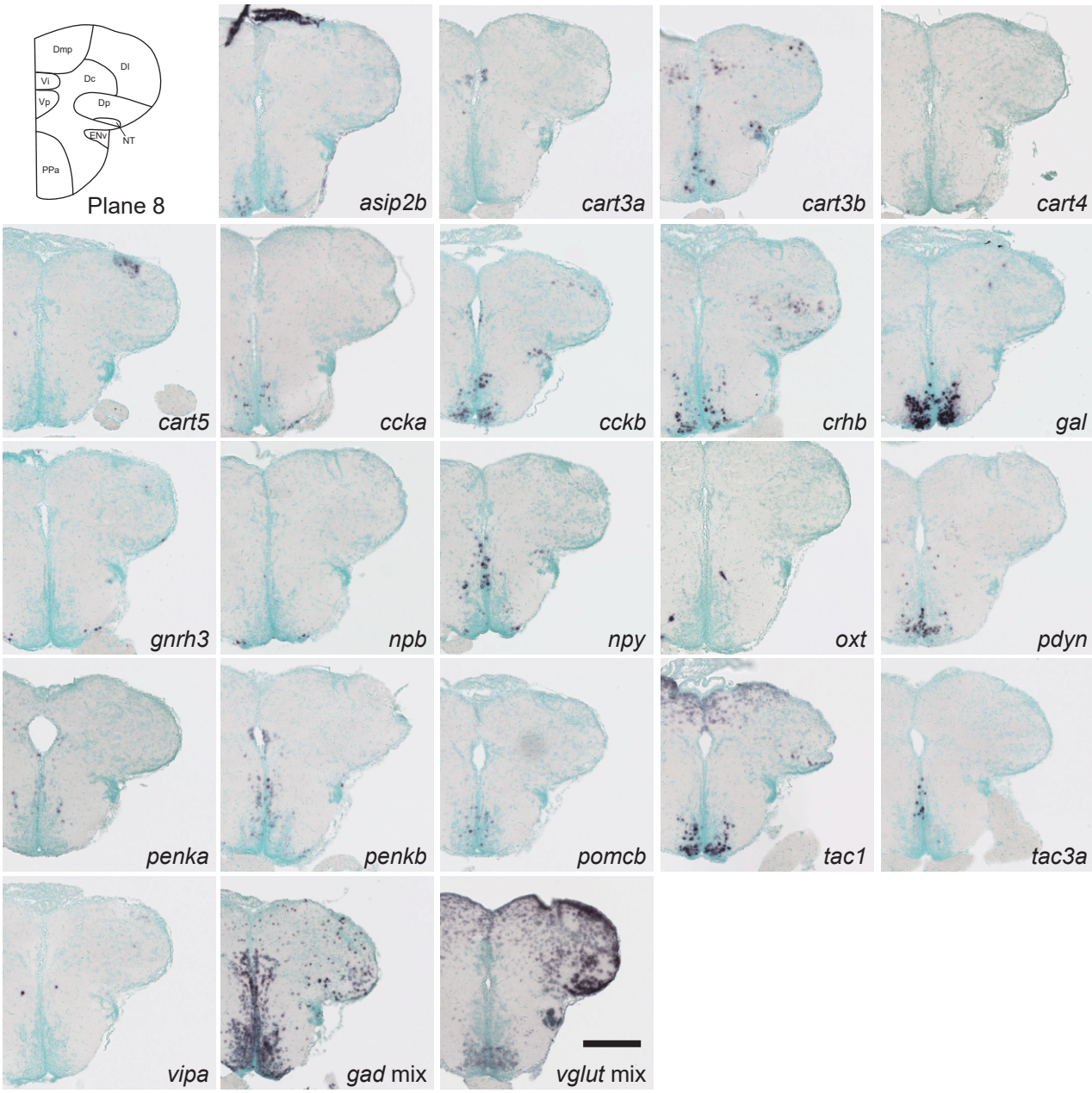


Figure 9

A schematic of plane 8 and microscopic images of genes expressed in the plane 8. Scale bar = 200 μm.

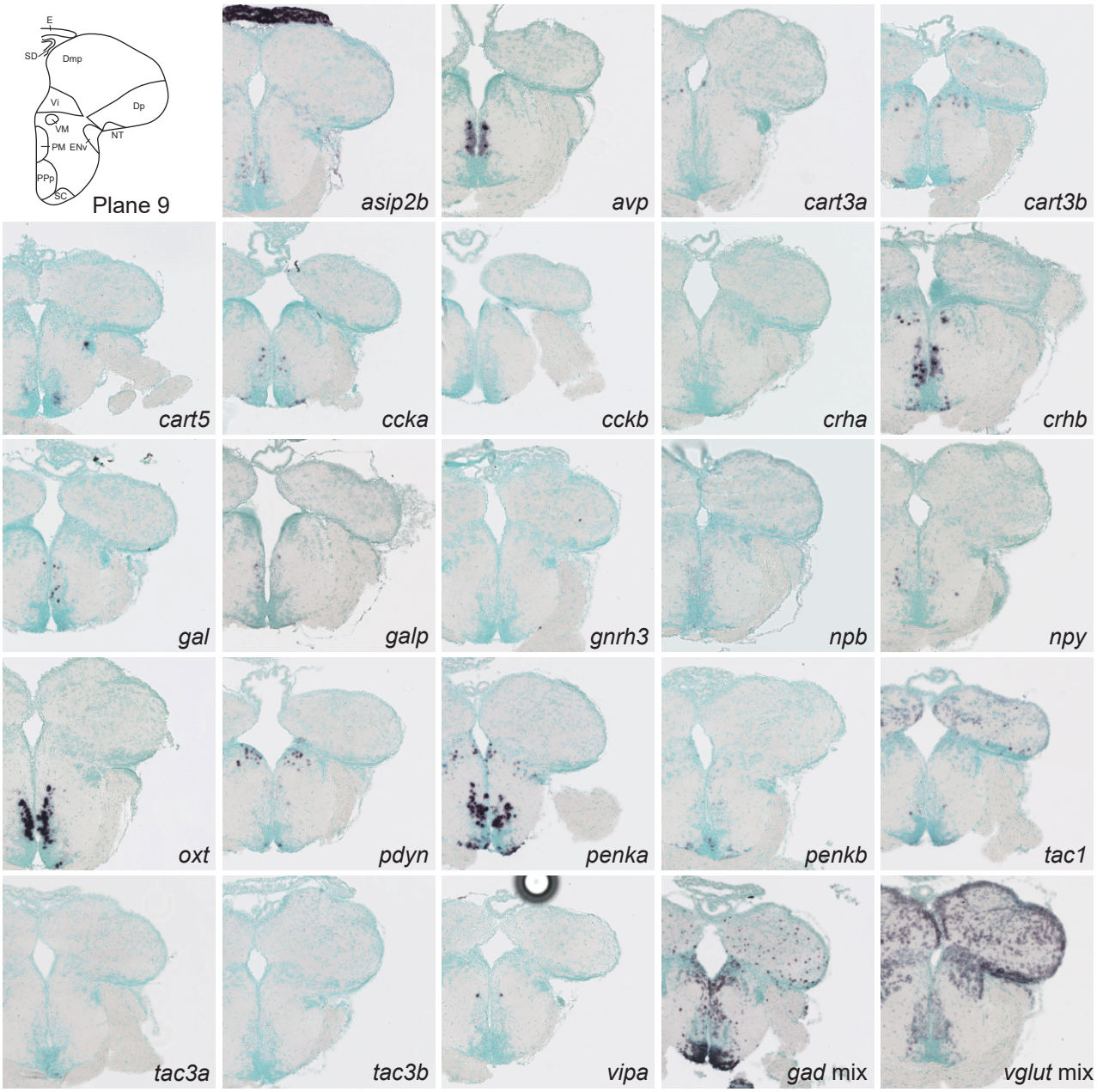


Figure 10

A schematic of plane 9 and microscopic images of genes expressed in the plane 9. Scale bar = 200 μm.

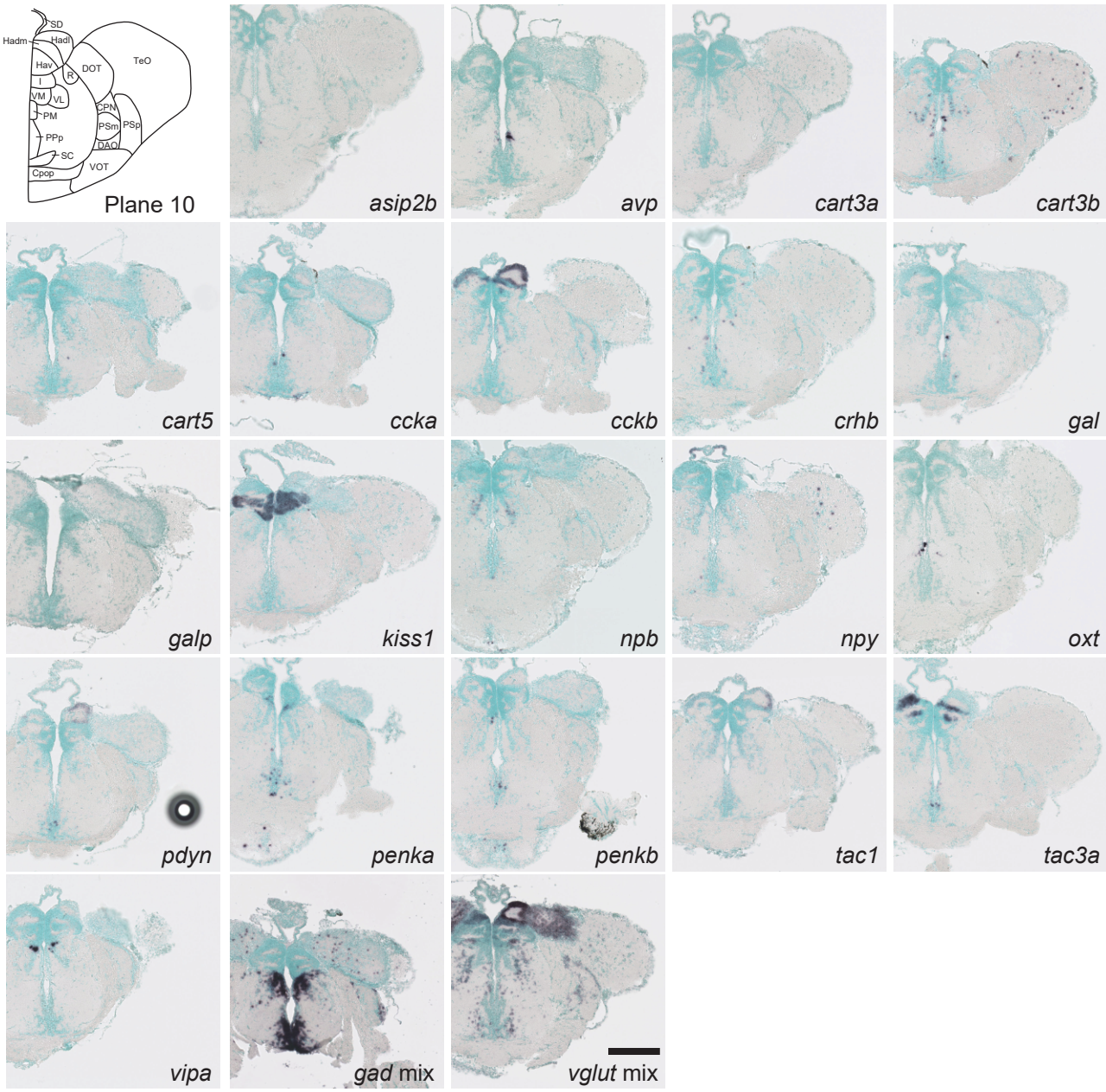


Figure 11

A schematic of plane 10 and microscopic images of genes expressed in the plane 10. Scale bar = 200 μm.

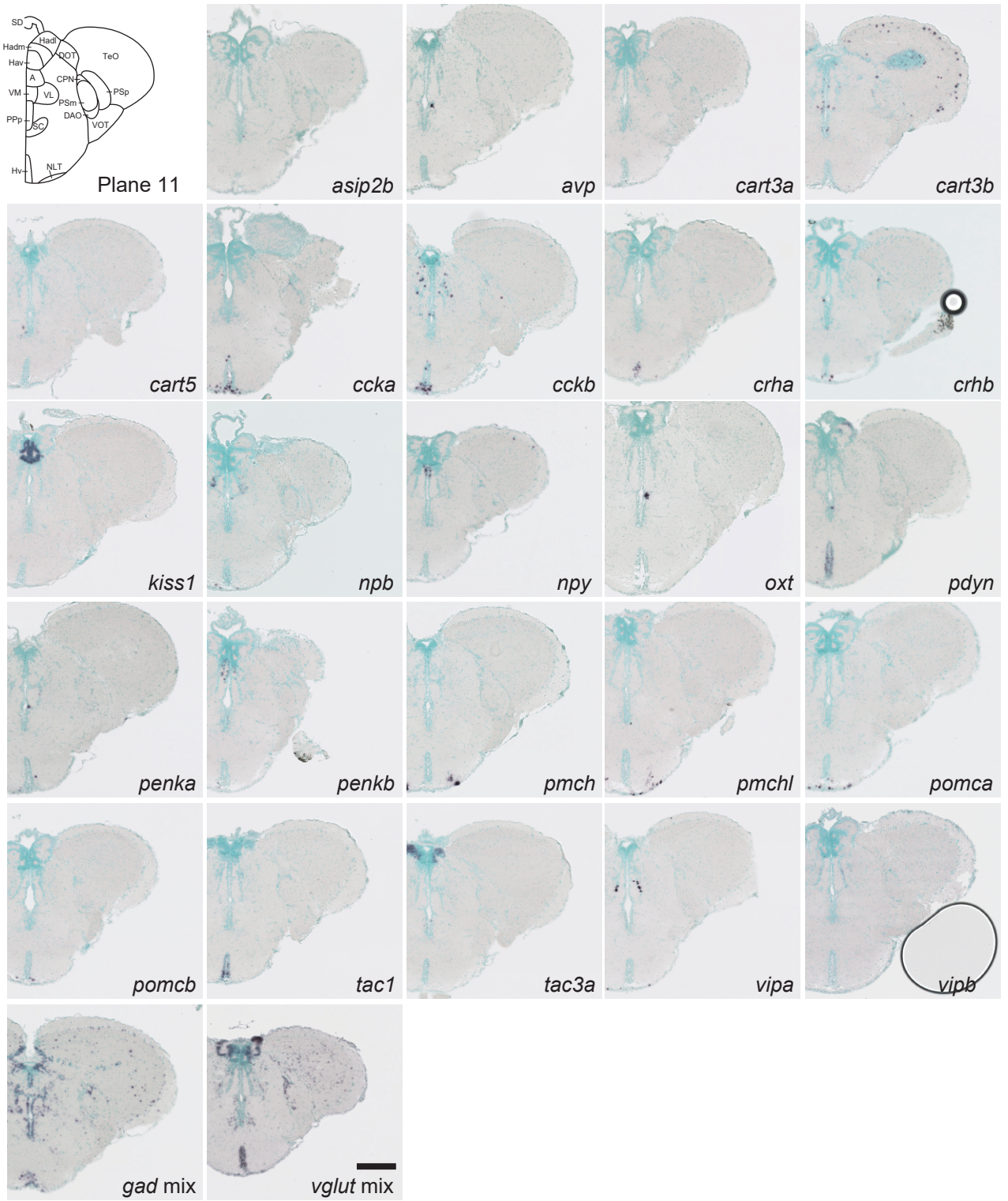


Figure 12

A schematic of plane 11 and microscopic images of genes expressed in the plane 11. Scale bar = 200 μm.

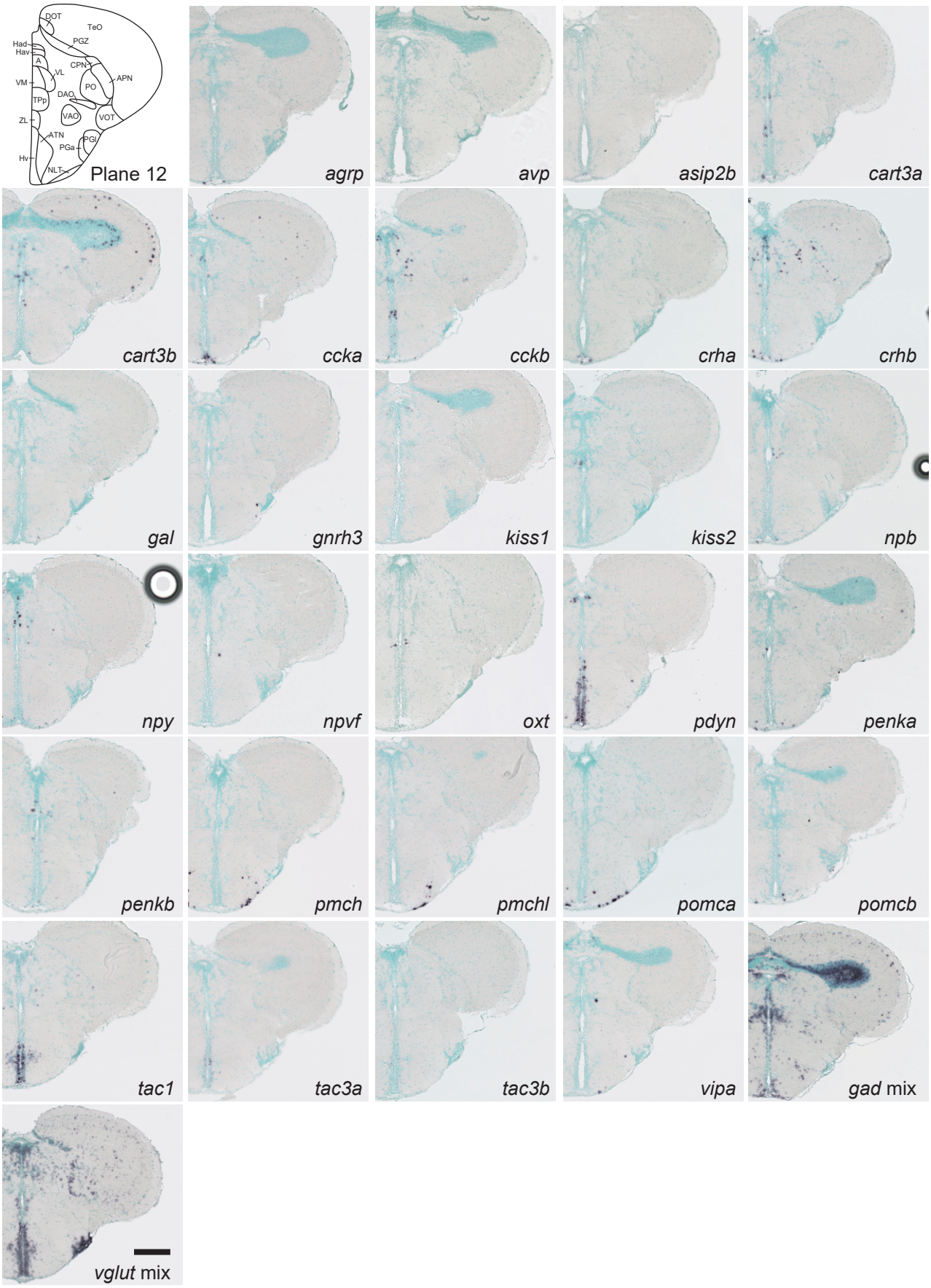


Figure 13

A schematic of plane 12 and microscopic images of genes expressed in the plane 12. Scale bar = 200 µm.

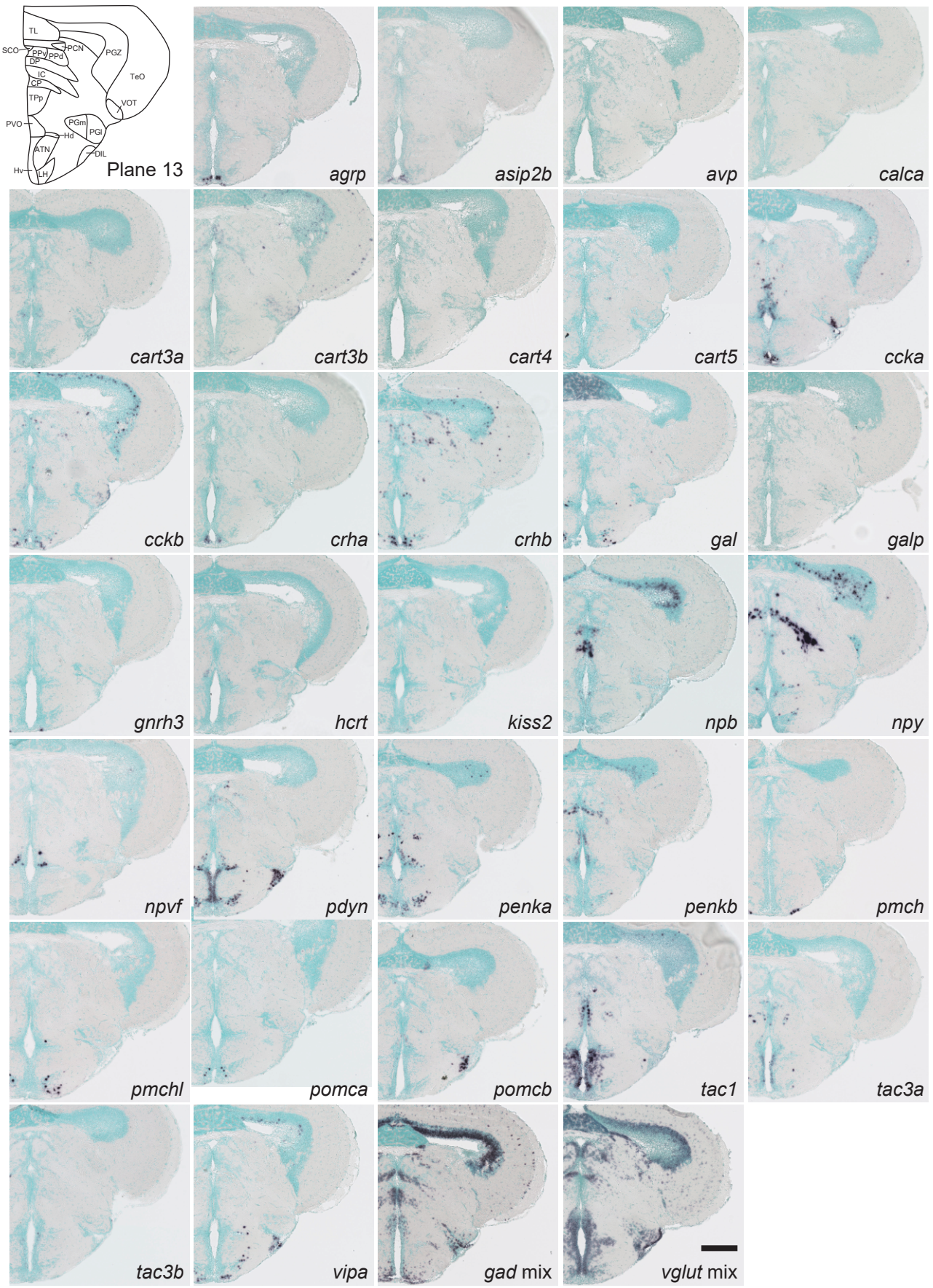


Figure 14

A schematic of plane 13 and microscopic images of genes expressed in the plane 13. Scale bar = 200 μm.

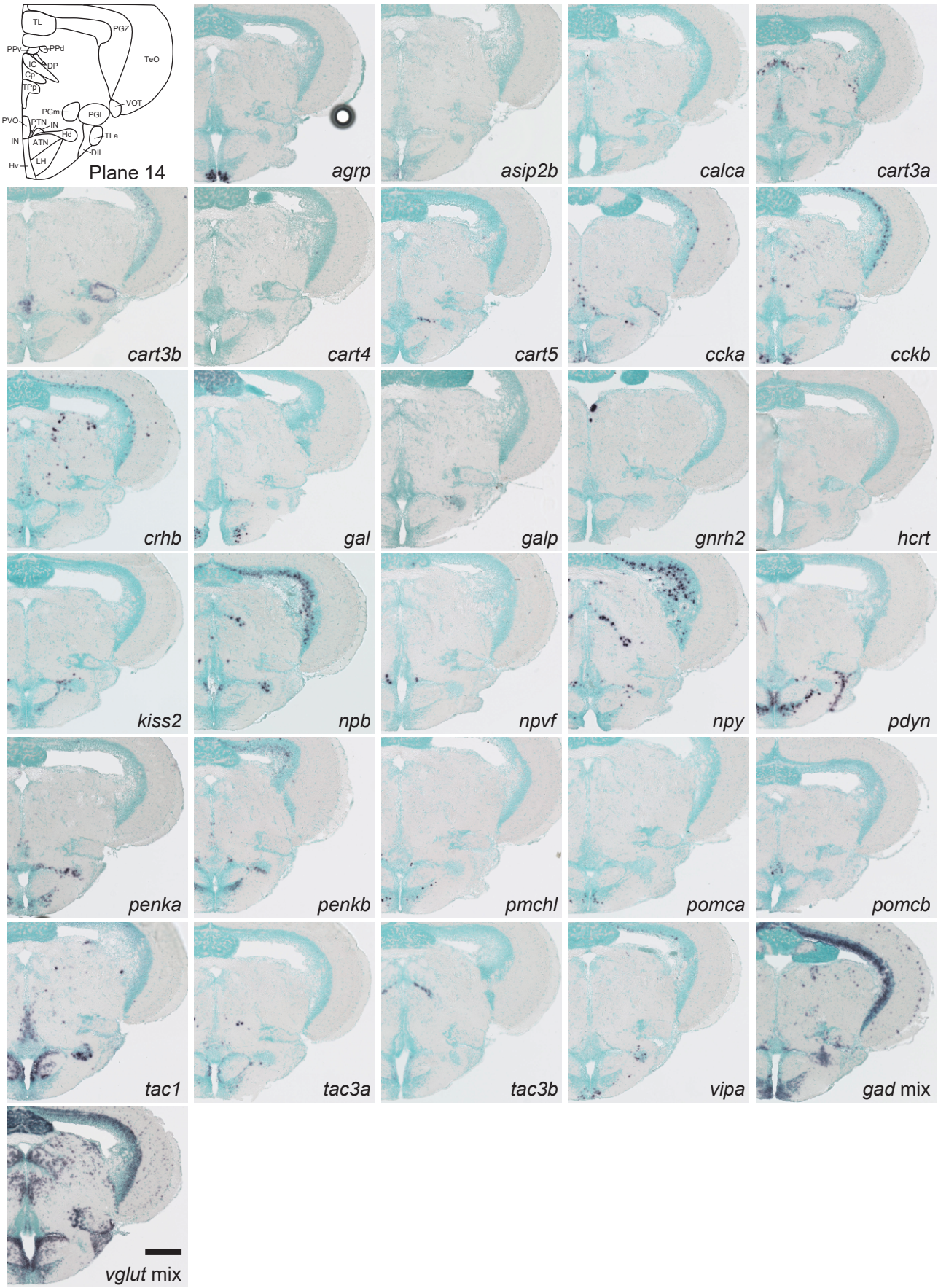


Figure 15

A schematic of plane 14 and microscopic images of genes expressed in the plane 14. Scale bar = 200 μm.

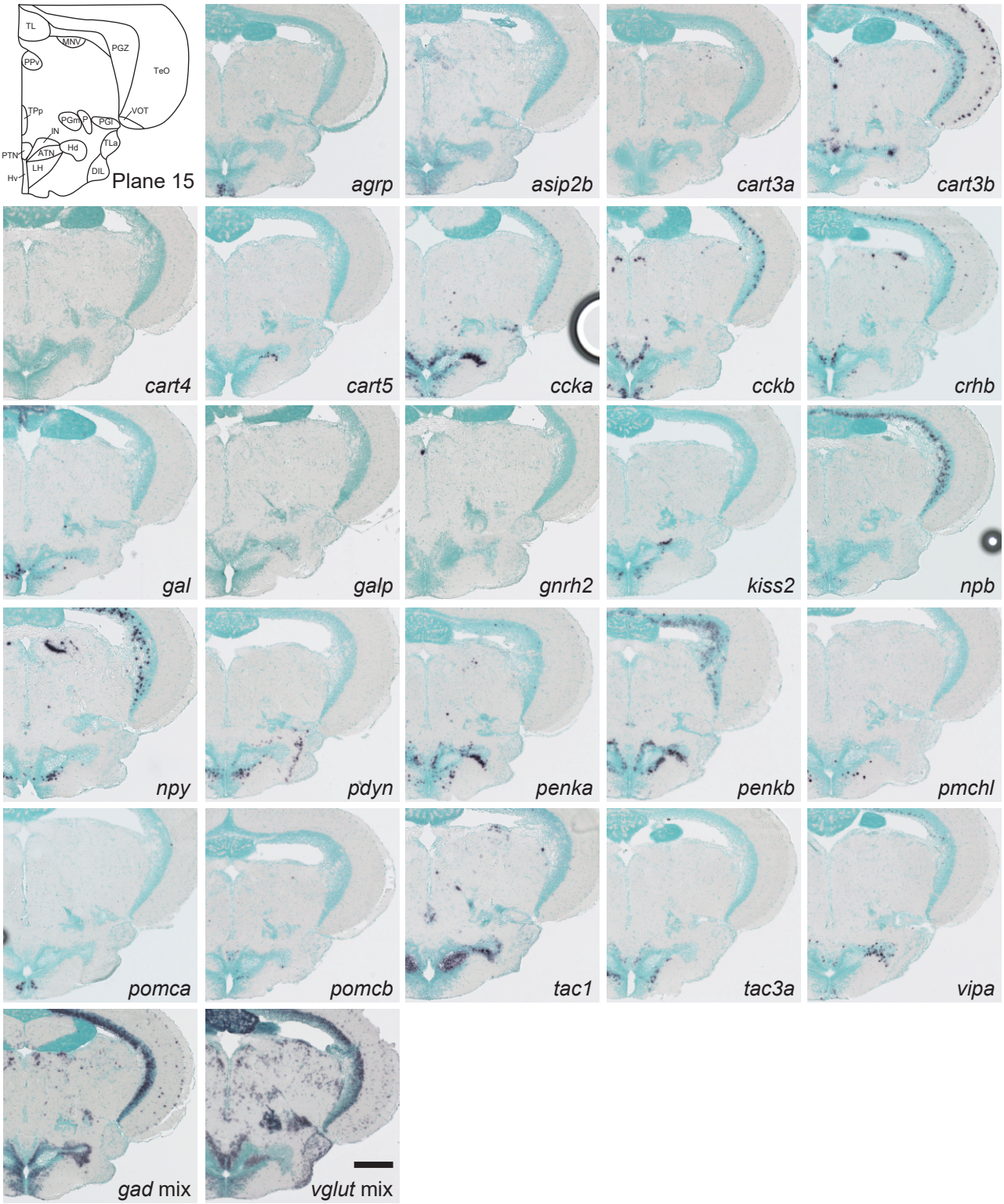


Figure 16

A schematic of plane 15 and microscopic images of genes expressed in the plane 15. Scale bar = 200 μm.

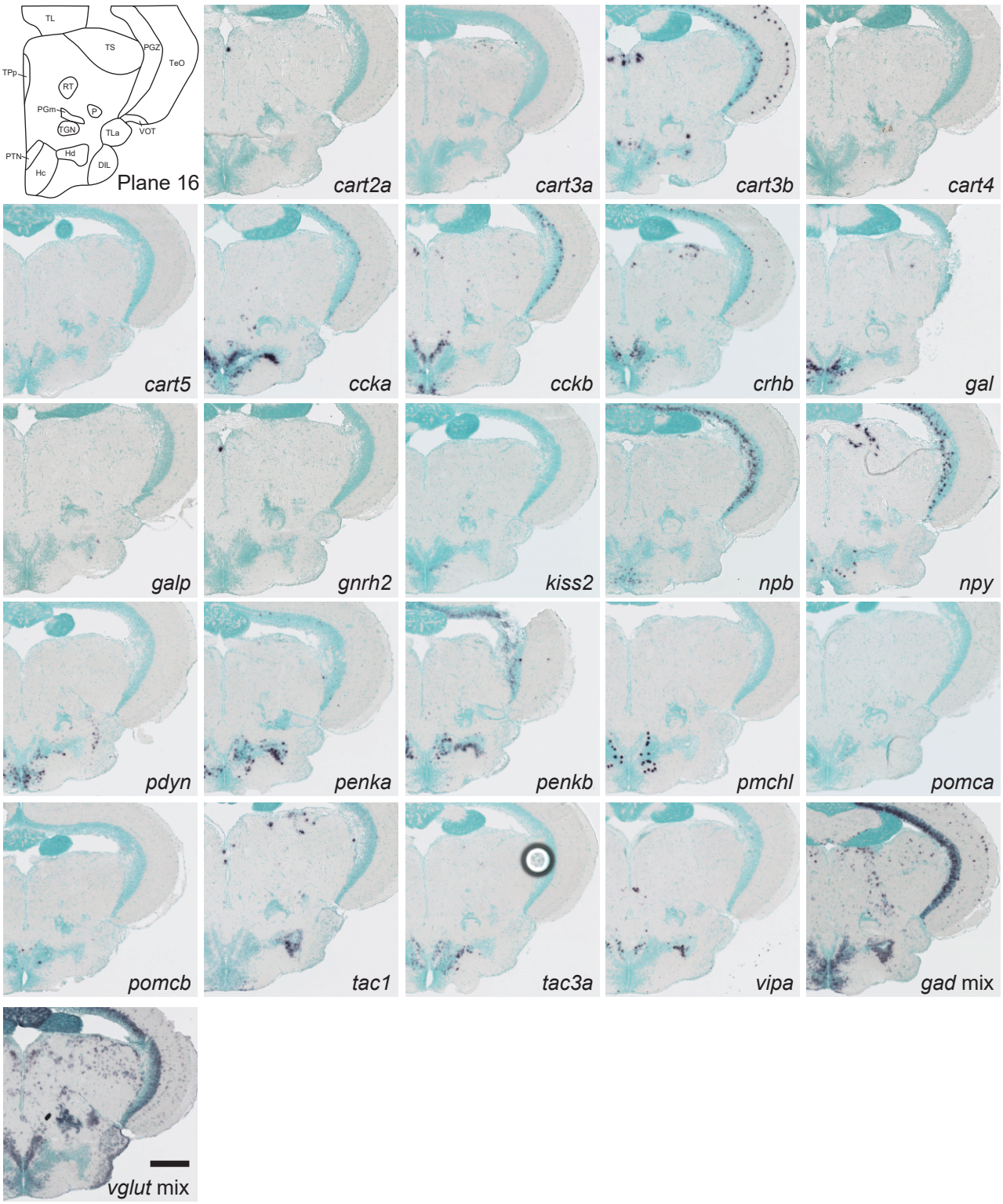


Figure 17

A schematic of plane 16 and microscopic images of genes expressed in the plane 16. Scale bar = 200 μm.

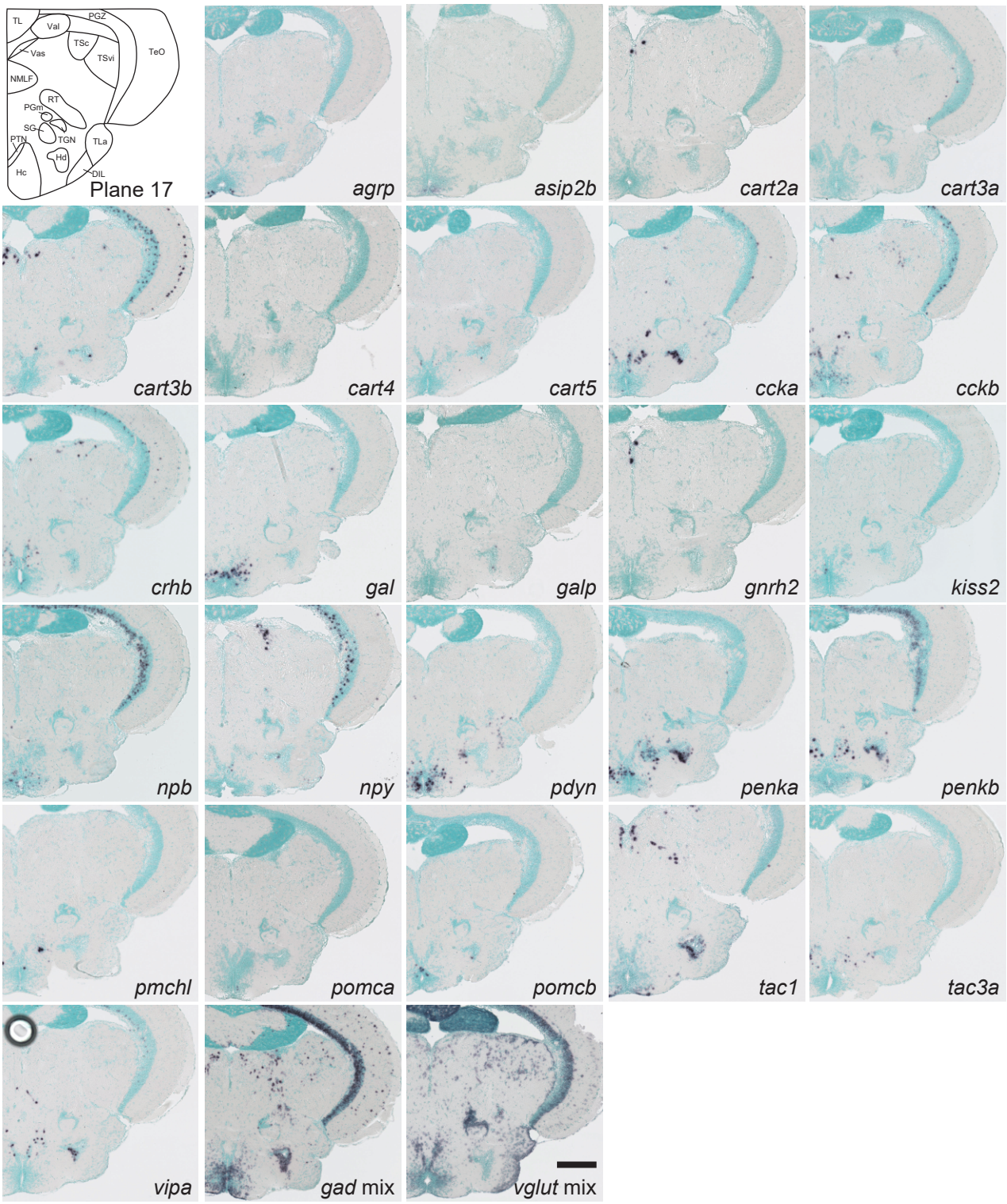


Figure 18

A schematic of plane 17 and microscopic images of genes expressed in the plane 17. Scale bar = 200 μm.

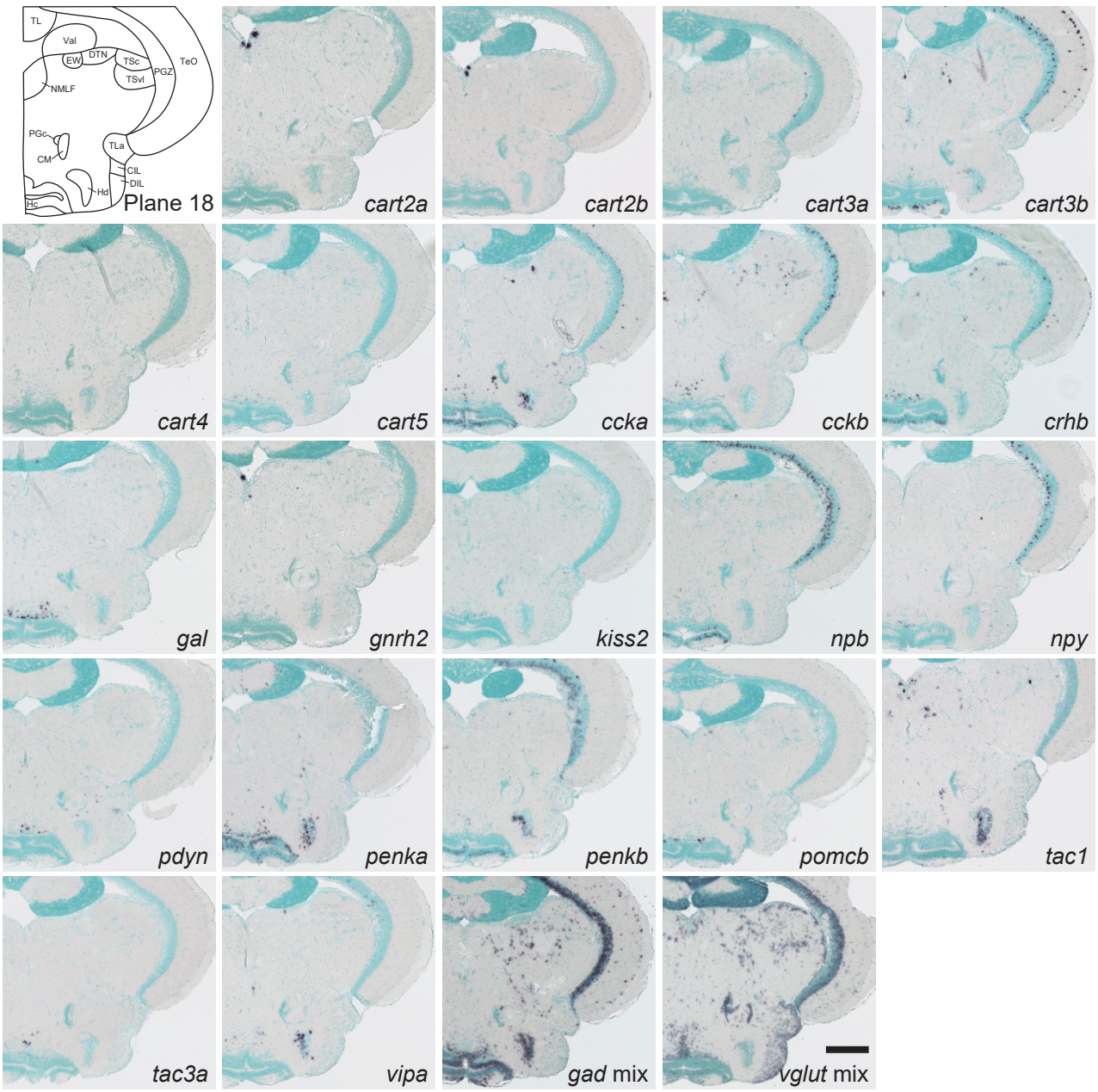


Figure 19

A schematic of plane 18 and microscopic images of genes expressed in the plane 18. Scale bar = 200 μm.

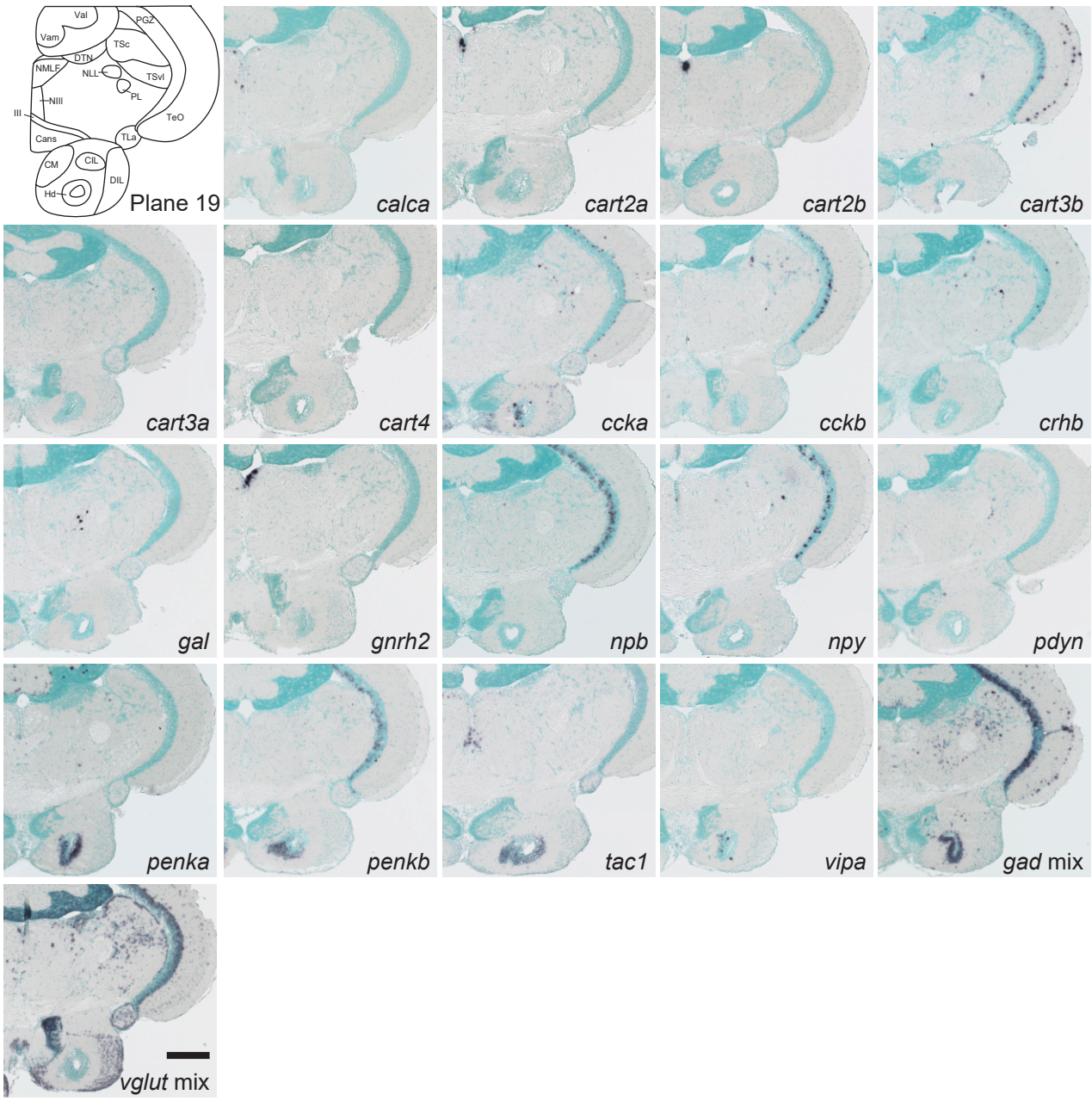


Figure 20

A schematic of plane 19 and microscopic images of genes expressed in the plane 19. Scale bar = 200 μm.

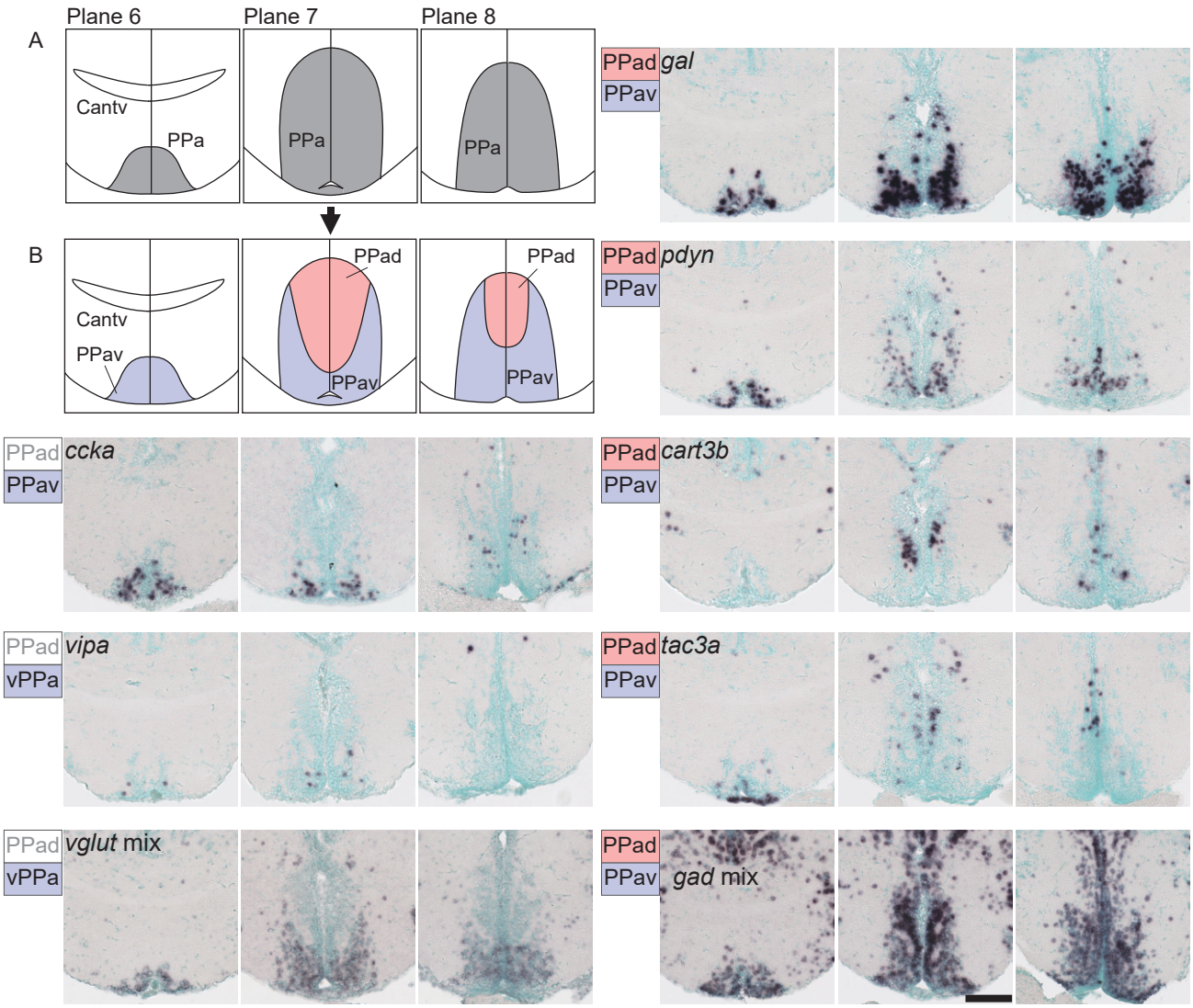


Figure 21

Subnuclei in the anterior part of parvocellular preoptic nucleus (PPa). (A) The PPa (grey) in the original zebrafish brain atlas. (B) The PPa can be divided into 2 subnuclei: the ventral part of PPa (PPav, pink) and the dorsal part of PPa (PPad, purple). Neuropeptides such as *ccka* and *vipa* and *vglut* mix are exclusively expressed in the PPav. *gal* and *pdyn* are expressed in both the PPad and PPav, but predominantly in the PPav. *cart3b* and *tac3a* are also expressed in the PPav and PPad, but predominantly in the PPad. *gad* mix is expressed in both the PPad and PPav. Scale bar = 100 µm.

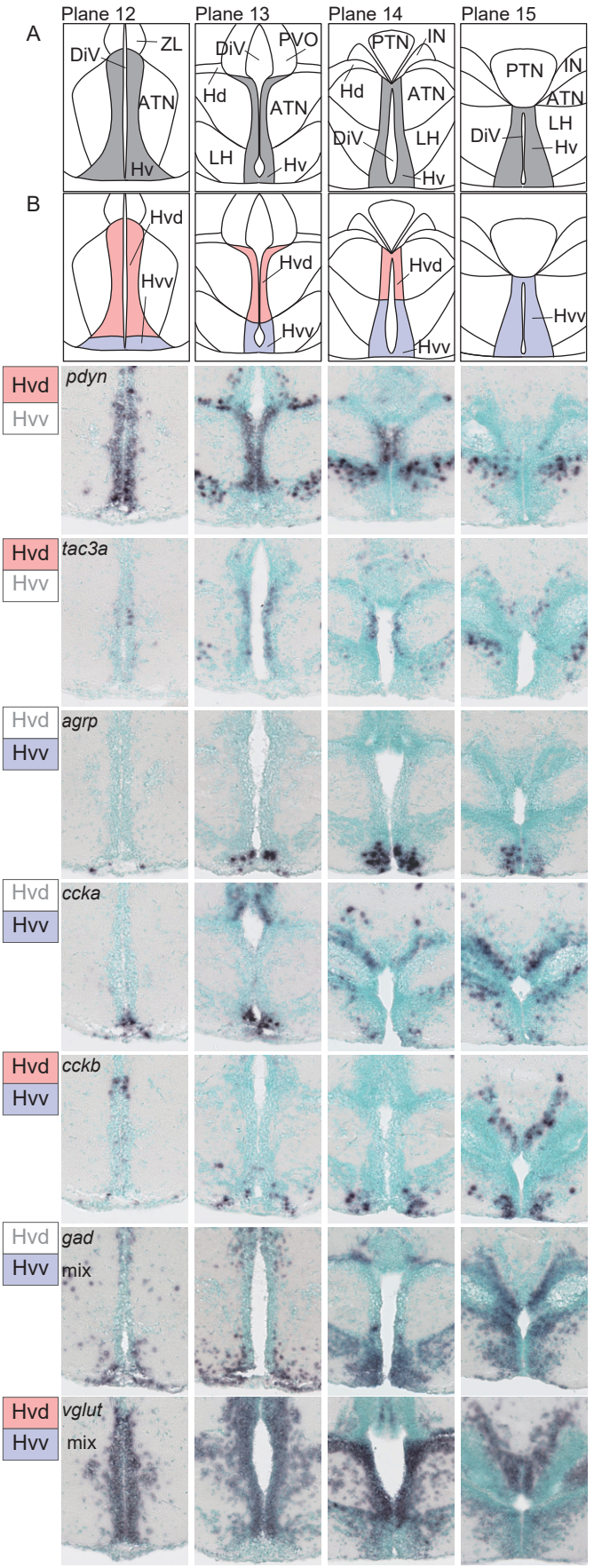


Figure 22

Subnuclei in the ventral zone of periventricular hypothalamus (Hv). (A) The Hv (grey) in the original zebrafish brain atlas. (B) The Hv can be divided into 2 subnuclei: the ventral part of Hv (Hvv, purple) and the dorsal part of Hv (Hvd, pink). Neuropeptides such as *pdyn* and *tac3a* are expressed only in the Hvd, whereas *agrp* and *ccka* are expressed only in the Hvv. *cckb* is expressed in both the Hvd and Hvv. *gad* mix is predominantly expressed in the Hvv and the *vglut* mix is more densely expressed in the Hvd. Scale bar = 100 µm.

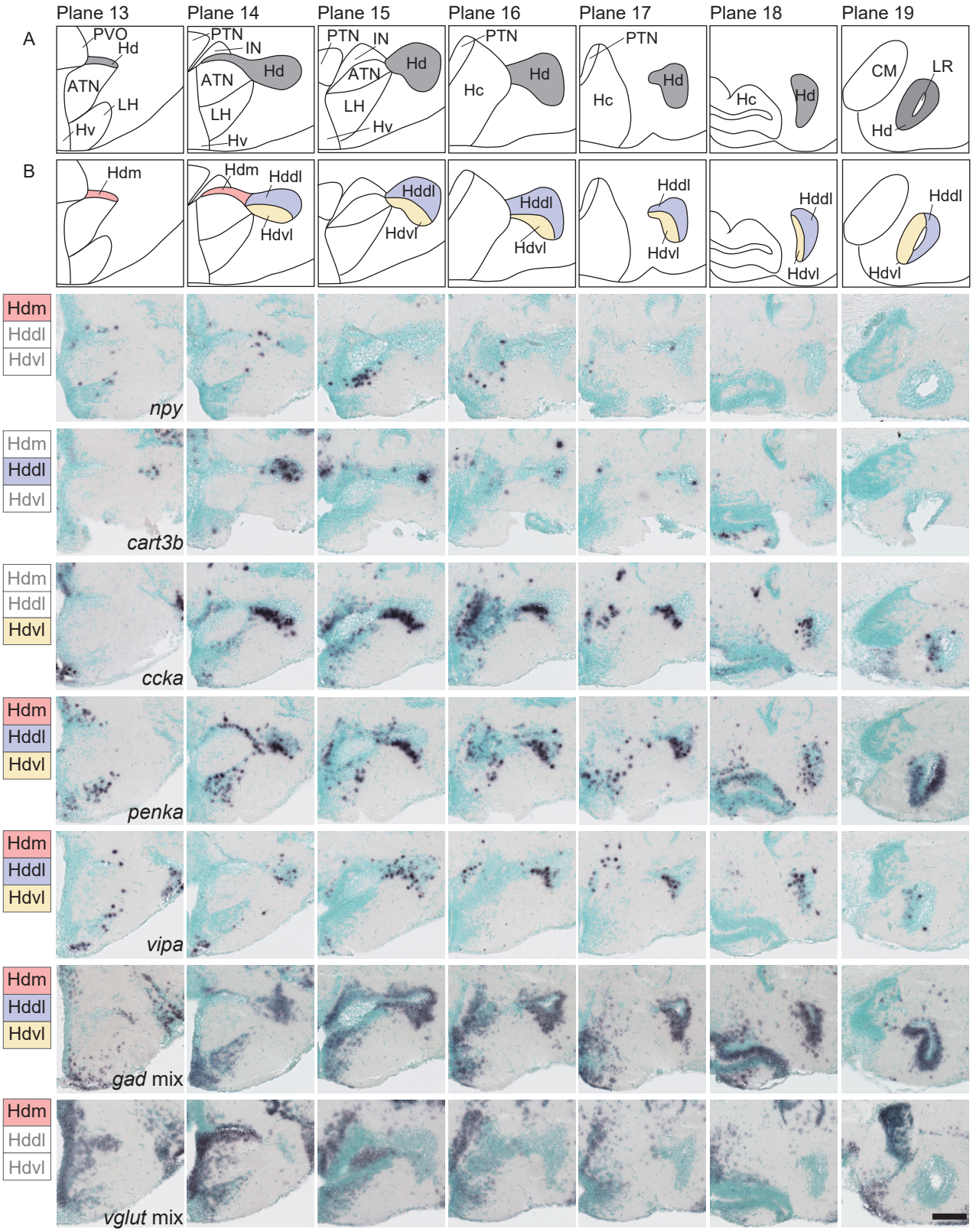


Figure 23

Subnuclei in the dorsal zone of the periventricular hypothalamus (Hd). (A) The Hd (grey) in the original zebrafish brain atlas. (B) The Hd can be divided into 3 subnuclei: the medial part of Hd (Hdm, pink), the dorsolateral part of Hd (Hddl, purple), and the ventrolateral part of Hd (Hdvl, yellow). Some neuropeptides are expressed solely in the Hdm, Hddl, or Hdvl (represented by *npy*, *cart*, and *ccka*, respectively). Neuropeptides such as *penka* and *vipa* are expressed in all three subnuclei. *gad* mix is also expressed in all subnuclei. *vglut* mix is absent in all subnuclei. Scale bar = 100 μm.

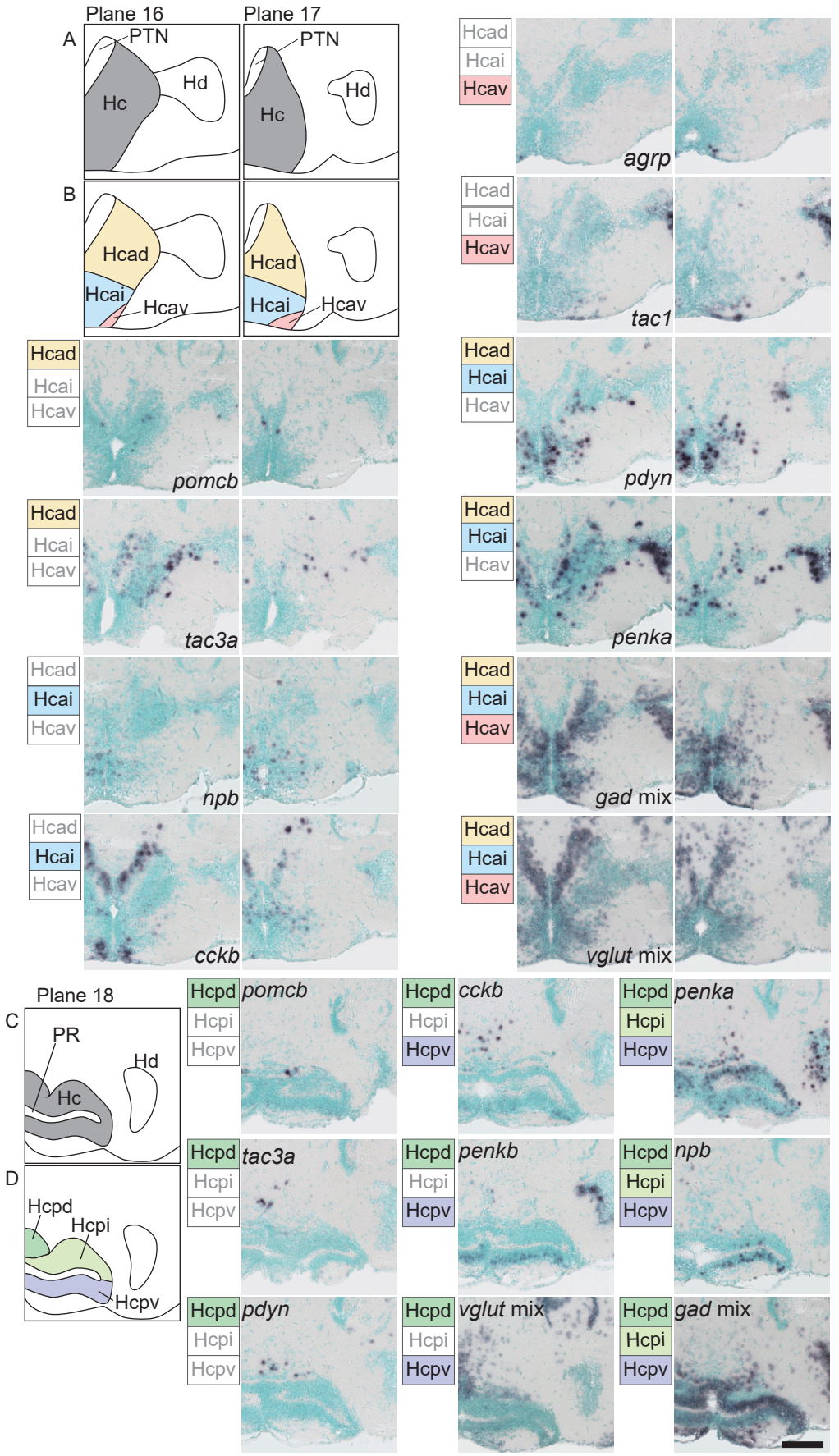
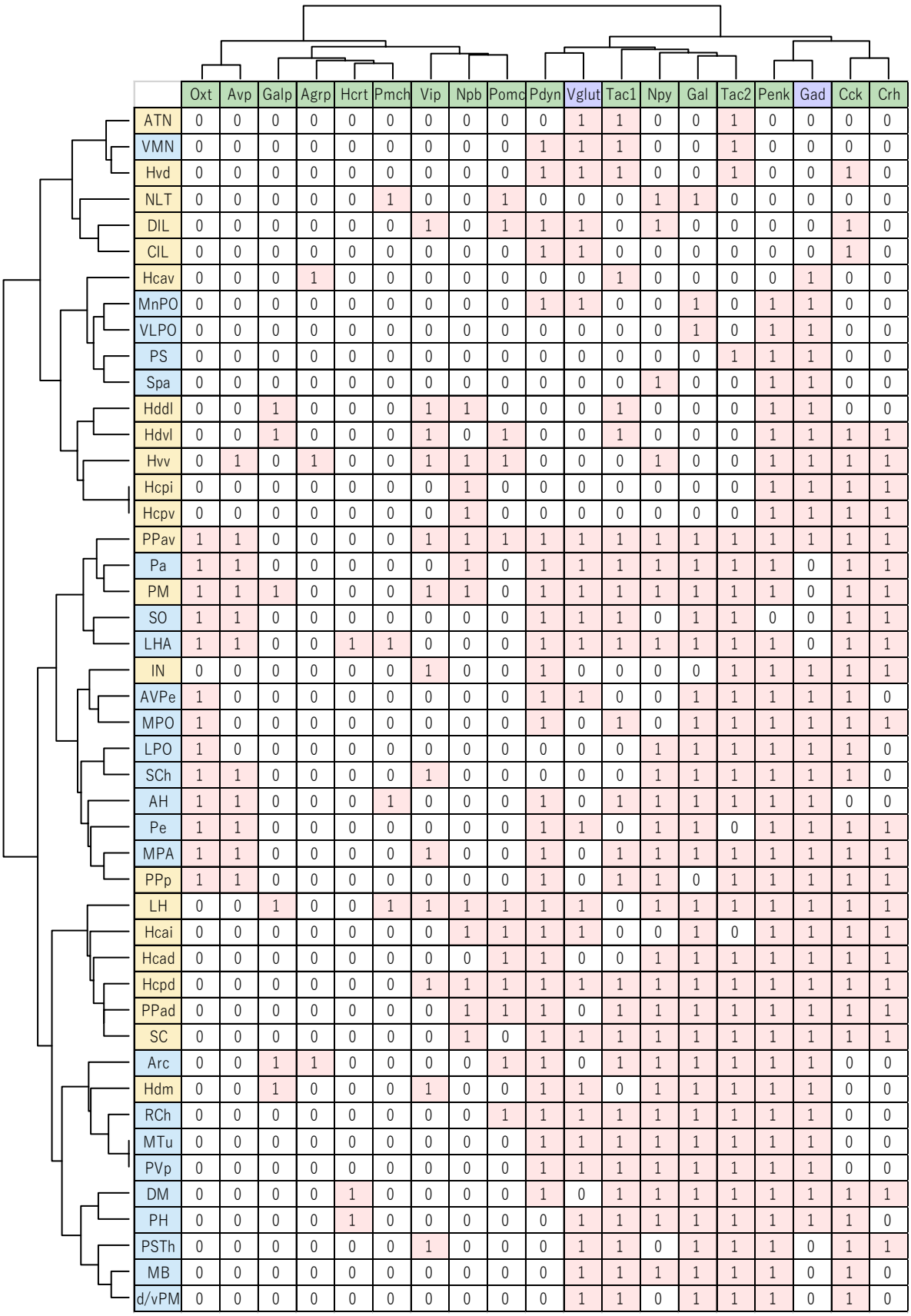

Plane 16
Plane 17
A
PTN
Hc
Hd
B
Hcad
Hcai
Hcav
agrp
tac1
pomcb
pdyn
tac3a
penka
npb
gad mix
cckb
vglut mix
Plane 18
C
PR
D
Hcpd
Hcpi
Hcpv
penkb

Figure 24

Subnuclei in the caudal zone of the periventricular hypothalamus (Hc). (A) The anterior part of Hc (grey) in the original zebrafish brain atlas. (B) The anterior part of Hc can be divided into 3 subnuclei: the anterodorsal part of Hc (Hcad, yellow), the anterointermediate part of Hc (Hcai, blue), and the anteroventral part of Hc (Hcav, pink). Neuropeptides such as *pomcb* and *tac3a* are solely expressed in the Hcad. Neuropeptides such as *npb* and *cckb* are expressed exclusively in the Hcai. *agrp* and *tac1* are only expressed in the Hcav. Neuropeptides such as *cckb*, *pdyn*, and *penka* are expressed across multiple subnuclei. *gad* mix and *vglut* mix are expressed in all three subnuclei. (C) Posterior part of Hc (grey) in original zebrafish brain atlas. (D) The posterior part of Hc can be divided into 3 subnuclei: the posterodorsal part of Hc (Hcpd, green), the posterointermediate part of Hc (Hcpi, light green), and the posteroventral part of Hc (Hcpv, purple). Neuropeptides such as *pomcb*, *tac3a*, and *pdyn* are solely expressed in the Hcpd. *cckb*, *penkb*, and *vglut* mix are expressed in the Hcpd and Hcpv. *penka*, *npb*, and *gad* mix are expressed in all three subnuclei. Scale bar = 100 μm.



	Oxt	Avp	Galp	Agrp	Hcrt	Pmch	Vip	Npb	Pomc	Pdyn	Vglut	Tac1	Npy	Gal	Tac2	Penk	Gad	Cck	Crh
ATN	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0
VMN	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0
Hvd	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
NLT	0	0	0	0	0	1	0	0	1	0	0	0	1	1	0	0	0	0	0
DIL	0	0	0	0	0	0	1	0	1	1	1	0	1	0	0	0	0	1	0
CIL	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0
Hcav	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0
MnPO	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	1	0	0
VLPO	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0
PS	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
Spa	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0
Hddl	0	0	1	0	0	0	1	1	0	0	0	1	0	0	0	1	1	0	0
Hdvl	0	0	1	0	0	0	1	0	1	0	0	1	0	0	0	1	1	1	1
Hvv	0	1	0	1	0	0	1	1	1	0	0	0	1	0	0	1	1	1	1
Hcpi	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	1
Hcpv	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	1
PPav	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
Pa	1	1	0	0	0	0	0	1	0	1	1	1	1	1	1	1	0	1	1
PM	1	1	1	0	0	0	1	1	0	1	1	1	1	1	1	1	0	1	1
SO	1	1	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	1	1
LHA	1	1	0	0	1	1	0	0	0	1	1	1	1	1	1	1	0	1	1
IN	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	1	1	1
AVPe	1	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1	1	1	0
MPO	1	0	0	0	0	0	0	0	0	1	0	1	0	1	1	1	1	1	1
LPO	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0
SCh	1	1	0	0	0	0	1	0	0	0	0	0	1	1	1	1	1	1	0
AH	1	1	0	0	0	1	0	0	0	1	0	1	1	1	1	1	1	0	0
Pe	1	1	0	0	0	0	0	0	0	1	1	0	1	1	0	1	1	1	1
MPA	1	1	0	0	0	0	1	0	0	1	0	1	1	1	1	1	1	1	1
PPp	1	1	0	0	0	0	0	0	0	1	0	1	1	0	1	1	1	1	1
LH	0	0	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1
Hcai	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	1	1
Hcad	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1	1	1	1	1
Hcpd	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
PPad	0	0	0	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1	1
SC	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1
Arc	0	0	1	1	0	0	0	0	1	1	0	1	1	1	1	1	1	0	0
Hdm	0	0	1	0	0	0	1	0	0	1	1	0	1	1	1	1	1	0	0
RCh	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0
MTu	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
PVp	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
DM	0	0	0	0	1	0	0	0	0	1	0	1	1	1	1	1	1	1	1
PH	0	0	0	0	1	0	0	0	0	0	1	1	1	1	1	1	1	1	0
PSTh	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	1	0	1	1
MB	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	0
d/vPM	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	1	0

Figure 25

Comparison of neuropeptide/neurotransmitter gene expression in the hypothalamus between zebrafish and mouse. Expression of neuropeptides (green) and neurotransmitter markers (purple) in the hypothalamic nuclei of zebrafish (light yellow) and mice (light blue) were extracted from ISH results (zebrafish) and the Allen Brain Atlas and previous papers (mouse) and summarized in a table (expressed = 1, not expressed = 0). Zebrafish and mouse nuclei and expressed genes were hierarchically clustered by Ward's method.