Localization of Glutamatergic, GABAergic, and Cholinergic Neurons in the Brain of the African Cichlid Fish, *Astatotilapia burtoni*

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Neural communication depends on release and reception of different neurotransmitters within complex circuits that ultimately mediate basic biological functions. We mapped the distribution of glutamatergic, GABAergic, and cholinergic neurons in the brain of the African cichlid fish *Astatotilapia burtoni* using in situ hybridization to label vesicular glutamate transporters (*vglut1, vglut2.1, vglut3*), glutamate decarboxylases (*gad1, gad2*), and choline acetyltransferase (*chat*). Cells expressing the glutamatergic markers *vgluts 1–3* show primarily nonoverlapping distribution patterns, with the most widespread expression observed for *vglut2.1*, and more restricted expression of *vglut1* and *vglut3*. *vglut1* is prominent in granular layers of the cerebellum, habenula, preglomerular nuclei, and several other diencephalic, mesencephalic, and rhombencephalic regions. *vglut2.1* is widely expressed in many nuclei from the olfactory bulbs to the hindbrain, while *vglut3* is restricted to the hypothalamus and hindbrain. GABAergic cells show largely overlapping *gad1* and *gad2* expression in most brain regions. GABAergic expression dominates nuclei of the subpallial ventral telencephalon, while glutamatergic expression dominates nuclei of the pallial dorsal telencephalon. *chat*-expressing cells are prominent in motor cranial nerve nuclei, and some scattered cells lie in the preoptic area and ventral part of the ventral telencephalon. A localization summary of these markers within regions of the conserved social decision-making network reveals a predominance of either GABAergic or glutamatergic cells within individual nuclei. The neurotransmitter distributions described here in the brain of a single fish species provide an important resource for identification of brain nuclei in other fishes, as well as future comparative studies on circuit organization and function. J. Comp. Neurol. 525:610–638, 2017.

INDEXING TERMS: acetylcholine; chat; gad; glutamate; in situ hybridization; neurotransmitter; social decision making network; teleost; vglut; RRID:AB_514497; RRID:SCR_014329; RRID:SCR_014199

The central nervous system (CNS) in vertebrates uses a variety of neurotransmitters to mediate diverse functions from sensory perception to autonomic regulation to control of complex behaviors. To understand the role of specific neurons within integrated neural circuits requires identification of the transmitters they use to modulate other target neurons in the circuit. Some of the most common and widespread neurotransmitters in the brain of vertebrates are glutamate, γ-Aminobutyric acid (GABA), and acetylcholine (ACh), each of which can be identified by labeling with specific markers used in the synthesis or packaging of each transmitter type. For example, glutamatergic neurons express vesicular glutamate transporters (vGluts) that package glutamate into synaptic vesicles for release at the axon terminals, and exist in three main classes: vGlut1, vGlut2, and vGlut3 (Fremeau et al., 2001; Gras et al., 2002; Takamori, 2006). These three vGlut forms often show nonoverlapping distribution patterns and distinct subcellular localization that helps identify subpopulations of glutamatergic neurons (Kaneko and Fujiyama, 2002; Gras et al., 2005; Takamori, 2006; Liguz-Lecznar and Skangiel-Kramska, 2007). Glutamic acid decarboxylase

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(Gad) is the rate-limiting enzyme that converts glutamate into GABA, and is commonly used as an indicator of GABAergic neurons. In most vertebrates, including teleost fishes, Gad exists in two highly conserved isoforms encoded by the *gad1* (GAD67) and *gad2* (GAD65) genes (Bosma et al., 1999; Lariviere et al., 2002). While both Gads synthesize GABA, GAD1 synthesizes cytoplasmic GABA for extrasynaptic and metabolic functions, and GAD2 regulates the vesicular pool for release (Kaufman et al., 1991; Soghomonian and Martin, 1998; Tian et al., 1999). A third novel Gad paralog (GAD3) originally described in the deep-sea armed grenadier fish also exists in most other vertebrates, but little is known about its distribution and function (Bosma et al., 1999; Lariviere et al., 2002, 2005; Grone and Maruska, 2016). In general, glutamate is an excitatory transmitter, while GABA is typically an inhibitory transmitter. However, there are also cases in which GABA is excitatory (Hales et al., 1994; Watanabe et al., 2014) and glutamate inhibitory (Lee and Sherman, 2009), situations where individual neurotransmitter roles change during development, with circadian cycles, or with neurological disorders (Wagner et al., 1997; Han et al., 2002; Maqueda et al., 2003; Ben-Ari et al., 2012), and examples of neurons that can release both glutamate and GABA, depending on context (Gutierrez et al., 2003; Zimmermann et al., 2015).

Another common neurotransmitter in the vertebrate central and peripheral nervous system is acetylcholine, which is also the primary excitatory transmitter released by motor neurons at the skeletal neuromuscular junction. Acetylcholine is synthesized from choline and acetyl CoA by the transferase enzyme choline acetyltransferase (ChAT), a specific marker commonly used as a reliable indicator of cholinergic neurons (Kimura et al., 1981; Eckenstein and Thoenen, 1983). In the brain, cholinergic neurons are implicated in diverse functions such as learning and memory (Gold, 2003; Braida et al., 2014) and control of hormone release (Richardson et al., 1980; Egozi et al., 1986), and ACh is also thought to function as a neuromodulator that can alter the excitability state of entire neuronal networks (Picciotto et al., 2012).

Teleost fishes are the most speciose and behaviorally diverse group of vertebrates, which makes them ideal models for testing hypotheses on the relationships between neural function and behavior, as well as comparative studies to better establish brain homologies across taxa. For example, the social decision-making network (SDMN) is a collection of interconnected nuclei composed of the social behavior network and mesolimbic reward system that is proposed to mediate complex social behaviors in all vertebrates (O'Connell and Hofmann, 2011, 2012). However, focused tests of this model in nonmammalian vertebrate groups are limited, partly due to our current lack of knowledge on homologous brain regions across taxa, particularly in the forebrain, which develops by eversion in ray-finned (actinopterygian) fishes and inversion (or evagination) in most other vertebrates (Wullimann and Mueller, 2004). To remedy this, we need more studies on the distribution of both widespread (i.e., glutamate, GABA, ACh) and more specific (i.e., transcription factors, neuropeptides, biogenic amines) neurochemical markers that can facilitate our identification of homologous brain nuclei between teleosts and other vertebrates. While the distribution of glutamatergic (Higashijima et al., 2004; Filippi et al., 2014), GABAergic (Anglade et al., 1999; Martyniuk et al., 2007; Trabucchi et al., 2008; Mueller and Guo, 2009) and cholinergic (Ekström, 1987; Brantley and Bass, 1988; Perez et al., 2000; Mueller et al., 2004; Lopez et al., 2013) neurons were examined separately in several different fish species using various markers, there are often species-specific distribution patterns that may vary among the >30,000 different species of fishes, likely with important functional implications. Further, no previous study has examined these multiple neurotransmitter types in the brain of a single fish species.

The African cichlid fish *Astatotilapia burtoni* has become a valuable model in social neuroscience (reviewed in Fernald and Maruska, 2012; Maruska and Fernald, 2013, 2014; Maruska, 2015). The wealth of previous information on neural, behavioral, and physiological plasticity coupled with the recent genome sequencing of this and several other African cichlid species (Brawand et al., 2014) propels this model to the forefront of behavioral and evolutionary neuroscience. For *A. burtoni* to also become a powerful model for neural circuit function, however, we must identify which neurotransmitters are expressed in different brain nuclei. This is especially important in light of the many recent studies that use expression of immediate early gene (IEG) markers (e.g., *cfos, egr-1*) as proxies for neural activation in response to varying social conditions (Maruska et al., 2013a,b; O'Connell et al., 2013). Functional interpretations of IEG activation data are often hindered by a lack of information on the neuronal phenotype that is activated. The accumulating resources in *A. burtoni* that show localization of different signaling neurochemicals and their receptors in the brain (Munchrath and Hofmann, 2010; O'Connell et al., 2011; Huffman et al., 2012; Loveland et al., 2015), however, increases the utility of this model system for future functional studies. By mapping the distribution patterns of excitatory, inhibitory, and modulatory neurochemicals in the brain we can better infer the type of neural circuitry activated during complex behaviors,

and how it compares to similar studies performed in other vertebrates.

The goal of this study was to localize and map the distribution patterns of glutamatergic, GABAergic, and cholinergic neurons in the brain of the model cichlid fish *A. burtoni* using in situ hybridization for the transmitter markers vGlut (glutamatergic), Gad (GABAergic), and ChAT (cholinergic). These distribution patterns will contribute important neuroanatomical data to facilitate future phylogenetic and evolutionary comparisons across all vertebrate taxa.

MATERIALS AND METHODS

Animals

Adult African cichlid fish, *Astatotilapia burtoni*, were maintained in aquaria in mixed-sex social communities under conditions that mimic their natural habitat in Lake Tanganyika, Africa (12/12-hour light:dark; pH 8.0; temp 28–30°C; conductivity 300–500 μS/cm; constant aeration). Aquaria contained gravel-covered bottoms and halved terra cotta pots that served as shelters defended by dominant males. All experiments were performed in accordance with the recommendations and guidelines stated in the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*, 2011. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA.

Tissue collection

Fish were netted from their aquaria, anesthetized in ice-cold cichlid-system water, measured for standard length (SL) and body mass (BM), and killed by rapid cervical transection. Brains were exposed and then fixed in the head overnight at 4°C with 4% paraformaldehyde (PFA) in 1× phosphate-buffered saline (1×PBS), rinsed in 1×PBS, and cryoprotected overnight in 30% sucrose (all solutions were made with RNAse-free 0.25 μm filtered water). Gonads were also removed and weighed (gonad mass, GM) to calculate gonadosomatic index [GSI = (GM/BM)*100]. Brains were then embedded in OCT media (TissueTek, Sakura Fine Tek, Torrance, CA), sectioned in the transverse plane on a cryostat (Leica CM1850 or Cryostar NX50) at 20 μm, and collected onto two alternate sets of charged slides (VWR Superfrost plus, Chicago, IL). Sections were dried flat for 1–2 days at room temperature and then stored at −80°C until processing.

Preparation of DIG-labeled riboprobes for in situ hybridization

Throughout this article, we use standard gene nomenclature: for fishes, gene symbols are lowercase and italicized, while protein names are not italicized with the first letter capitalized and the remainder of the name in lowercase; for other vertebrates, gene symbols are in uppercase and italicized, while protein symbols are identical but not italicized. When referring generally and collectively to a group of transmitter markers without phylogenetic distinction, we use the symbols vGlut, Gad, and ChAT.

To localize *vglut* (*vglut1, vglut2.1, vglut3*)-, *gad* (*gad1, gad2*)-, and *chat*-expressing cells in the brain, we used chromogenic-based in situ hybridization (ISH) with riboprobes on cryosectioned brain tissue similar to our previous work (Grone and Maruska, 2015a,b). Our recent bioinformatic analyses also identified *gad3* in *A. burtoni*, but in situ hybridization and reverse-transcription polymerase chain reaction (RT-PCR) attempts failed to detect transcripts in the brain (Grone and Maruska, 2016). Primers for each mRNA of interest were designed from *A. burtoni* sequences available in GenBank, commercially synthesized (Life Technologies, Bethesda, MD), and are shown in Table 1. Templates for riboprobes were generated by PCR amplification (Platinum SuperMix, Life Technologies) of whole-brain *A. burtoni* cDNA, gene-specific primers, and the following reaction conditions: 95°C for 1 minute, 40 cycles of: (95°C for 15 seconds, 55°C for 15 seconds, 72°C for 1 minute), and 72°C for 1 minute. Purified PCR products (MinElute PCR kit, Qiagen, Chatsworth, CA) were then used as the template in the transcription reaction to incorporate digoxigenin (DIG)-labeled (DIG-labeling mix, Roche, Nutley, NJ) nucleotides into the nucleic acid sequence, followed by probe purification (GE Illustra Probe Quant G-50 microcolumns). Probes were transcribed from the T3 polymerase transcription initiation sequence (aattaaccctcactaaaggg) that was added to the reverse (for antisense probes) or forward (for sense control probes) gene-specific primers. PCR products and final probes were checked on a 1% agarose gel after each step and verified to be bands of the correct size. Probes were diluted with hybridization buffer and stored at −20°C.

Chromogenic in situ hybridization

To localize and map the distribution of glutamatergic, GABAergic, and cholinergic cells in the *A. burtoni* brain,

Abbreviations

4v	fourth ventricle	ALLn	anterior lateral line nerve
ac	anterior commissure	An	anterior thalamic nucleus
aGn	anterior glomerular nucleus	AON	anterior octaval nucleus

AP	accessory pretectal nucleus
ATn	anterior tuberal nucleus
cc	central canal
CC	cerebellar crest
CCeG	granular layer of corpus cerebelli
CCeM	molecular layer of corpus cerebelli
CCeP	Purkinje cell layer of corpus cerebelli
CG	central gray
CM	corpus mammillare
CON	caudal octavolateralis nucleus
CP	central posterior thalamic nucleus
CZ	central zone of tectum
Dc	central part of the dorsal telencephalon
Dc-1	central part of the dorsal telencephalon, subdivision 1
Dc-2	central part of the dorsal telencephalon, subdivision 2
Dc-3	central part of the dorsal telencephalon, subdivision 3
Dc-4	central part of the dorsal telencephalon, subdivision 4
Dc-5	central part of the dorsal telencephalon, subdivision 5
Dd	dorsal part of the dorsal telencephalon
Dd-d	dorsal part of the dorsal telencephalon, dorsal subdivision
Dd-v	dorsal part of the dorsal telencephalon, ventral subdivision
Dl	lateral part of the dorsal telencephalon
Dl-d	dorsal part of lateral part of the dorsal telencephalon
Dl-g	granular zone of lateral part of the dorsal telencephalon
Dl-v1	ventral part of the lateral part of the dorsal telencephalon, subdivision 1
Dl-v2	ventral part of the lateral part of the dorsal telencephalon, subdivision 2
Dm	medial part of the dorsal telencephalon
Dm-1	medial part of the dorsal telencephalon, subdivision 1
Dm-2r	medial part of the dorsal telencephalon, rostral subdivision 2
Dm-3	medial part of the dorsal telencephalon, subdivision 3
DON	descending octaval nucleus
Dp	posterior part of the dorsal telencephalon
DP	dorsal posterior thalamic nucleus
DT	dorsal tegmental nucleus
DWZ	deep white zone of tectum
E	entopeduncular nucleus
ECL	external cell layer of olfactory bulb
EG	eminentia granularis
GL	glomerular layer of olfactory bulb
Gn	glomerular nucleus
hc	horizontal commissure
IC	intercalated nucleus
ICL	inner cell layer of olfactory bulb
ICo	isthmic commissure
IIIn	oculomotor nucleus
IO	inferior olive
IP	interpeduncular nucleus
IVn	trochlear nerve nucleus
IXm	motor nucleus of glossopharyngeal nerve
IXn	glossopharyngeal nerve (cranial nerve IX)
LL	lateral lemniscus
LT	lateral thalamic nucleus
MgON	magnocellular octaval nucleus
mlf	medial longitudinal fasciculus
MON	medial octavolateralis nucleus
NC	nucleus corticalis
NCIL	central nucleus of the inferior lobe
NDILc	caudal part of the diffuse nucleus of the inferior lobe
NDILl	lateral part of the diffuse nucleus of the inferior lobe
NDILm	medial part of the diffuse nucleus of the inferior lobe
nGMp	magnocellular preoptic nucleus, gigantocellular division
nHd	dorsal habenular nucleus
nHv	ventral habenular nucleus
NI	isthmal nucleus
NLT	lateral tuberal nucleus
NLTd	lateral tuberal nucleus, dorsal part
NLTi	lateral tuberal nucleus, intermediate part
NLTl	lateral tuberal nucleus, lateral part
NLTm	lateral tuberal nucleus, medial part
NLTv	lateral tuberal nucleus, ventral part
NLV	lateral valvular nucleus
NLVa	anterior part of the lateral valvular nucleus
NLVc	central part of the lateral valvular nucleus
NMIL	medial nucleus of the inferior lobe
nMLF	nucleus of medial longitudinal fasciculus
nMMp	magnocellular preoptic nucleus, magnocellular division
NP	nucleus pretectalis
NPC	central pretectal nucleus
nPMp	magnocellular preoptic nucleus, parvocellular division
nPPa	parvocellular preoptic nucleus, anterior part
nPPp	parvocellular preoptic nucleus, posterior part
NRL	nucleus of the lateral recess
NRP	nucleus of the posterior recess
NT	nucleus taenia
nTE	nucleus of the thalamic eminence
OB	olfactory bulb
OC	optic chiasm
OEN	octavolateralis efferent nucleus
ON	optic nerve
PAG	periaqueductal gray
pc	posterior commissure
PG	periventricular granular cell mass of caudal lobe
PGc	commissural preglomerular nucleus
PGl	lateral preglomerular nucleus
PGm	medial preglomerular nucleus
PGZ	periventricular gray zone of tectum
Pit	pituitary
PLLn	posterior lateral line nerve
PLm	medial part of the perilemniscular nucleus
PN	prethalamic nucleus
POA	preoptic area
PON	posterior octavolateralis nucleus
PPd	dorsal periventricular pretectal nucleus
PPv	ventral periventricular pretectal nucleus
PS	pineal stalk
PSi	superficial pretectal nucleus, intermediate division
PSP	parvocellular superficial pretectal nucleus
PTT	paratoral tegmental nucleus
PVO	paraventricular organ
Ri	inferior reticular nucleus
Rm	medial reticular nucleus
Rs	superior reticular nucleus
RTN	rostral tegmental nucleus
SGn	secondary gustatory nucleus
sgt	secondary gustatory tract
SOd	dorsal part of secondary octaval nucleus
SOv	ventral part of secondary octaval nucleus
spm	spinal motor neurons
SR	superior raphe nucleus
SRd	superior raphe nucleus, dorsal division
SRm	superior raphe nucleus, medial division
STN	sensory trigeminal nucleus
SVn	secondary visceral nucleus
SWGZ	superficial gray and white zone of tectum
T	tectum
TBT	tectobulbar tract
TGN	tertiary gustatory nucleus
TL	torus longitudinalis
TLa	nucleus of the torus lateralis
TON	tangential octaval nucleus
TPp	periventricular nucleus of the posterior tuberculum
TS	torus semicircularis
TSc	central nucleus of torus semicircularis
TSvl	ventrolateral nucleus of torus semicircularis
v	ventricle
Vc	central part of the ventral telencephalon
VCeG	granular layer of valvula cerebelli
VCeM	molecular layer of valvula cerebelli
VCeP	Purkinje cell layer of valvula cerebelli
Vd	dorsal part of the ventral telencephalon
Vd-c	dorsal part of the ventral telencephalon, caudal subdivision
Vd-r	dorsal part of the ventral telencephalon, rostral subdivision
Vde	descending tract of the trigeminal nerve
Vi	intermediate nucleus of the ventral telencephalon
VIIIn	octaval nerve (cranial nerve VIII)
VIIn	facial nerve (cranial nerve VII)
VInc	abducens nerve (cranial nerve VI) nucleus, caudal part
Vl	lateral part of the ventral telencephalon
VL	vagal lobe
Vmd	dorsal motor nucleus of trigeminal nerve
VMn	ventromedial thalamic nucleus
Vmv	ventral motor nucleus of trigeminal nerve
VOT	ventral optic tract
Vp	postcommissural nucleus of the ventral telencephalon
Vs	supracommissural nucleus of the ventral telencephalon
Vs-l	lateral part of the supracommissural nucleus of the ventral telencephalon
Vs-m	medial part of the supracommissural nucleus of the ventral telencephalon
VTn	ventral tuberal nucleus
Vv	ventral part of the ventral telencephalon
Xm	vagal motor nucleus
Xn	vagal nerve (cranial nerve X)

TABLE 1.

Primer Pair Sequences Used to Generate Templates for the Synthesis of Gene-Specific Riboprobes in *Astatotilapia burtoni*

Gene	Forward (5′→3′)	Reverse[1] (5′→3′)	Product size (bp)	Ascension no.
gad1	GTTTCAGGCGAACAGAGT	GACAGATCCAGGCACTTATC	1201	XM_005918145.1
gad2	GACTCGGGTGTTTGGATTT	CAAGCAGAAAGACAGAGGATAG	888	XM_005932121.1
vglut1	CTGGGATACTGGTGCAATAC	GCACATACTCCTCAGACTTATC	897	XM_005945247.1
vglut2.1	GAGTGAAGTTGAACCGTAGAG	CATCCCATTTACACACGAGATA	899	XM_005945601.1
vglut3	GAGAGCACAAGTGAGGATAAA	CAGCAACAGCAAATACATACAA	806	XM_005943686.1
chat	CGTAAGGGATCTGTGCAATAA	CTGGTACAACAGACACAAGAA	807	XM_005913995.1

[1]Reverse primers are shown without the T3 polymerase initiation sequence on the 5′-end (AATTAACCCTCACTAAAGGG). Product sizes also do not include this T3 polymerase sequence.

we performed chromogenic ISH on a total of 30 adult males of mixed social status (SL = 46.90 ± 4.5 mm SD; BM = 3.04 ± 1.41 g SD; GSI = 0.64 ± 0.33 SD): *vglut1* ($n = 5$), *vglut2.1* ($n = 5$), *vglut3* ($n = 4$), *gad1* ($n = 6$), *gad2* ($n = 6$), and *chat* ($n = 4$). There were no obvious differences in overall localization patterns for any gene between subordinate and dominant males, but it was not the focus of this study to quantify status-dependent expression levels. Slides of cryosectioned brains were thawed to room temperature and sections were surrounded by a hydrophobic barrier (Immedge pen, Vector Laboratories, Burlingame, CA) and allowed to dry for 30–45 minutes. Sections were incubated at room temperature in the following RNAse-free solutions: 1×PBS (3 × 5 minutes), 4% PFA (20 minutes), 1×PBS (2 × 5 minutes), proteinase K (10 μg/ml final conc. in proteinase K buffer; 50 mM Tris-HCl pH 7.5, 5 mM EDTA pH 8.0) (10 minutes), 1×PBS (10 minutes), 4% PFA (15 minutes), 1×PBS (2 × 5 minutes), milliQ water (Millipore, Bedford, MA) (1–2 minutes), 0.1M triethanolamine-HCl pH 8.0 with 0.25% acetic anhydride (10 minutes), and 1×PBS (5 minutes). Slides were then incubated in prewarmed hybridization buffer (without probe; 50% formamide, 5× SSC, 0.1% Tween-20, 0.1% CHAPS, 5 mM EDTA, 1 mg/ml torula RNA) inside sealed humidified chambers in a hybridization oven at 60–65°C for 3 hours. Probes were then diluted in hybridization buffer and added to slides, covered with hybrislips (Life Technologies) to evenly distribute probe and prevent drying, and placed in sealed humidified chambers in a hybridization oven at 60–65°C. Following overnight hybridization (12–16 hrs), the following washes were performed at 60–65°C: 2× SSC:formamide with 0.1% Tween-20 (2 × 30 minutes), 2× SSC:Maleate Buffer (MABT; 100 mM maleic acid pH 7.2, 150 mM NaCl, 0.1% Tween-20) (2x15min), and MABT (2 × 10 minutes). Slides were then washed in MABT at room temperature (2 × 10 minutes), incubated in MABT with 2% bovine serum albumin (BSA) to block nonspecific binding (3 hours), and incubated with alkaline-phosphatase-conjugated anti-DIG Fab fragments (1:5,000 dilution; Roche; RRID:AB_514497) overnight at 4°C in a sealed humidified chamber. Slides were then rinsed with MABT (3 × 30 minutes) and incubated in alkaline phosphatase buffer (2 × 5 minutes) at room temperature, and then developed with nitro-blue tetrazolium/5-bromo-4-chloro-3′-indolyphosphate (NBT/BCIP) substrate (Roche) at 37°C in the dark for 1–8 hours depending on the probe. Slides were then rinsed in 1×PBS (3 × 5 minutes) to stop the reaction, fixed in 4% PFA (10 minutes), washed in 1×PBS (3 × 5 minutes), and coverslipped with aquamount media (Thermo-Scientific, Pittsburgh, PA).

To test for probe specificity, several additional male brains were sectioned at 20 μm in the transverse plane and collected onto alternate sets of slides. Sense control probes for each gene of interest (generated as described above for antisense probes, but with the T3 initiation sequence on the forward primer) were applied to one set of slides and run simultaneously with antisense probes applied to another set of alternate slides. None of these sense controls showed any labeling in the brain (Fig. 1A–L).

Imaging and analysis

To map the distribution of glutamatergic, GABAergic, and cholinergic cells in the brain, slides of stained sections were visualized on a Nikon Eclipse Ni microscope and photographs were taken with a color digital camera (Nikon DS-Fi2) controlled by Nikon NIS-Elements software (RRID:SCR_014329). Chromogenic-reacted sections were viewed in both brightfield and phase contrast to facilitate visualization of neuroanatomical landmarks and brain nuclei in relation to DIG-labeled cells. Localization patterns were not examined in the pituitary gland. Images were sharpened and adjusted for contrast, brightness, and levels as needed in Photoshop CS6 (Adobe Systems, San Jose, CA; RRID:SCR_014199). In some cases, distracting artifacts were also removed from micrographs with the Photoshop clone tool. To facilitate identification of neuroanatomical structures and brain nuclei, we used a cresyl violet-stained *A. burtoni* reference brain (serial transverse 20-μm sections) and generated an annotated brain atlas resource (Fig. 2). The following references were used to create the *A. burtoni* atlas (Fernald and Shelton, 1985; Wullimann et al., 1996; Munoz-Cueto et al., 2001; Burmeister et al., 2009; Munchrath and Hofmann, 2010; Maruska et al., 2012; Dewan and Tricas, 2014).

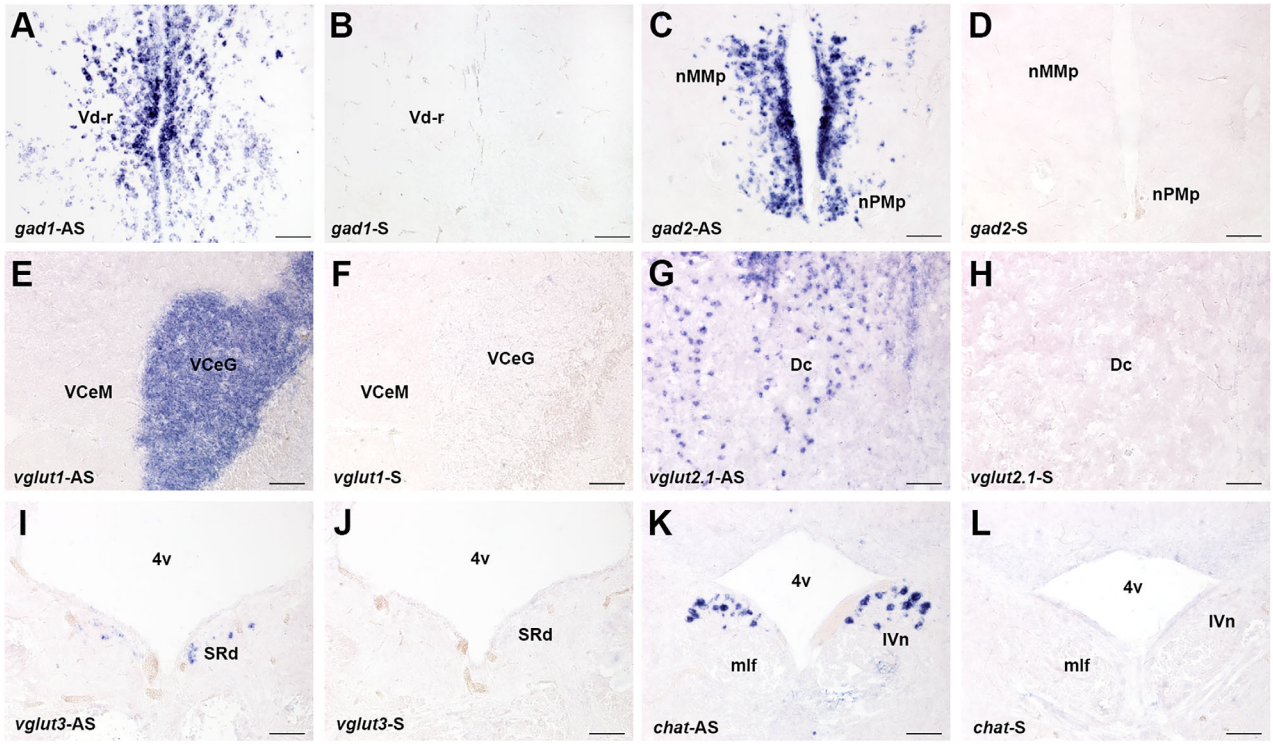

Figure 1. Representative examples of chromogenic in situ hybridization staining in the brain of *Astatotilapia burtoni* to illustrate probe specificity. Antisense (AS) and sense (S) control probes are shown for *gad1* (**A,B**), *gad2* (**C,D**), *vglut1* (**E,F**), *vglut2.1* (**G,H**), *vglut3* (**I,J**), and *chat* (**K,L**). Brightfield photomicrographs of antisense (A,C,E,G,I,K) and sense (B,D,F,H,J,L) probes for each marker were taken on alternate adjacent 20-μm transverse sections from the same brain that were run simultaneously in the same ISH experiment. Sense controls did not show any positive labeling for any of the candidate gene transcripts. See list for abbreviations. Scale bars = 50 μm. [Color figure can be viewed at wileyonlinelibrary.com]

RESULTS

Distribution of vesicular glutamate transporters (*vgluts*)

vglut1

Cells expressing *vglut1* are distributed in distinct nuclei that span from the rostral hindbrain to the telencephalon (Figs. 2A2–M2; 3; 4). No *vglut1* staining is observed in the rostral spinal cord or caudal hindbrain. A small group of *vglut1* cells (4–5 per section) lies in the ventrolateral hindbrain in the region of the intermediate reticular formation (Fig. 2K2). Expression of *vglut1* is most prominent in the granular layers of the corpus cerebelli (CCeG) and valvula cerebelli (VCeG), eminentia granularis (EG), periventricular granular cell mass of the caudal lobe (PG), and torus longitudinalis (TL) (Figs. 2F2–K2; 3A,B,D,F,G). Some scattered *vglut1* cells lie within the molecular layer of the corpus cerebelli (CCeM), but only in caudal sections (Fig. 2K2). The anterior octaval nucleus (AON) contains *vglut1* cells in the region where nVIII enters the brain (Figs. 2J2; 3C). The secondary gustatory (SGn) and secondary visceral (SVn) nuclei also show *vglut1*-labeled cells (Figs. 2H2; 3E). Some faint scattered cells are found throughout the periventricular gray zone (PGZ) of the tectum (Fig. 2D2–H2). Most of the preglomerular nuclei show clear *vglut1* staining, including the medial (PGm), lateral (PGl), and commissural (PGc) nuclei (Figs. 2E2–G2; 3G,H,J,K). Scattered weak *vglut1*-stained cells are also found in more rostral sections just lateral to the PGm/PGl, which is likely within the tertiary gustatory nucleus (TGN) (Fig. 3H,K). *vglut1* cells also lie within the prethalamic nucleus (PN). The medial part of the diffuse nucleus of the inferior lobe (NDILm) also contains some scattered cells along the region that borders the nucleus of the posterior recess (NRP) (Fig. 3I). Cells with *vglut1* label also occur in the habenula, with much darker staining in the dorsal nucleus (nHd) and weak to no staining in the ventral nucleus (nHv) (Figs. 2E2; 3H,L). Cells expressing *vglut1* are also found within the parvocellular superficial pretectal nucleus (PSP) (Fig. 2D2). Expression of *vglut1* is absent from other areas such as the preoptic area, thalamus, torus semicircularis, tegmentum, and other mesencephalic and diencephalic regions (see Fig. 2).

In the telencephalon, *vglut1* expression shows distinct patterns primarily within parts of the pallial dorsal telencephalon (Figs. 2A2–D2; 4A–I). Cells are found in the dorsal (Dd), lateral (Dl), and medial (Dm) (label is absent from Dm-2r) parts, but label is absent from the central

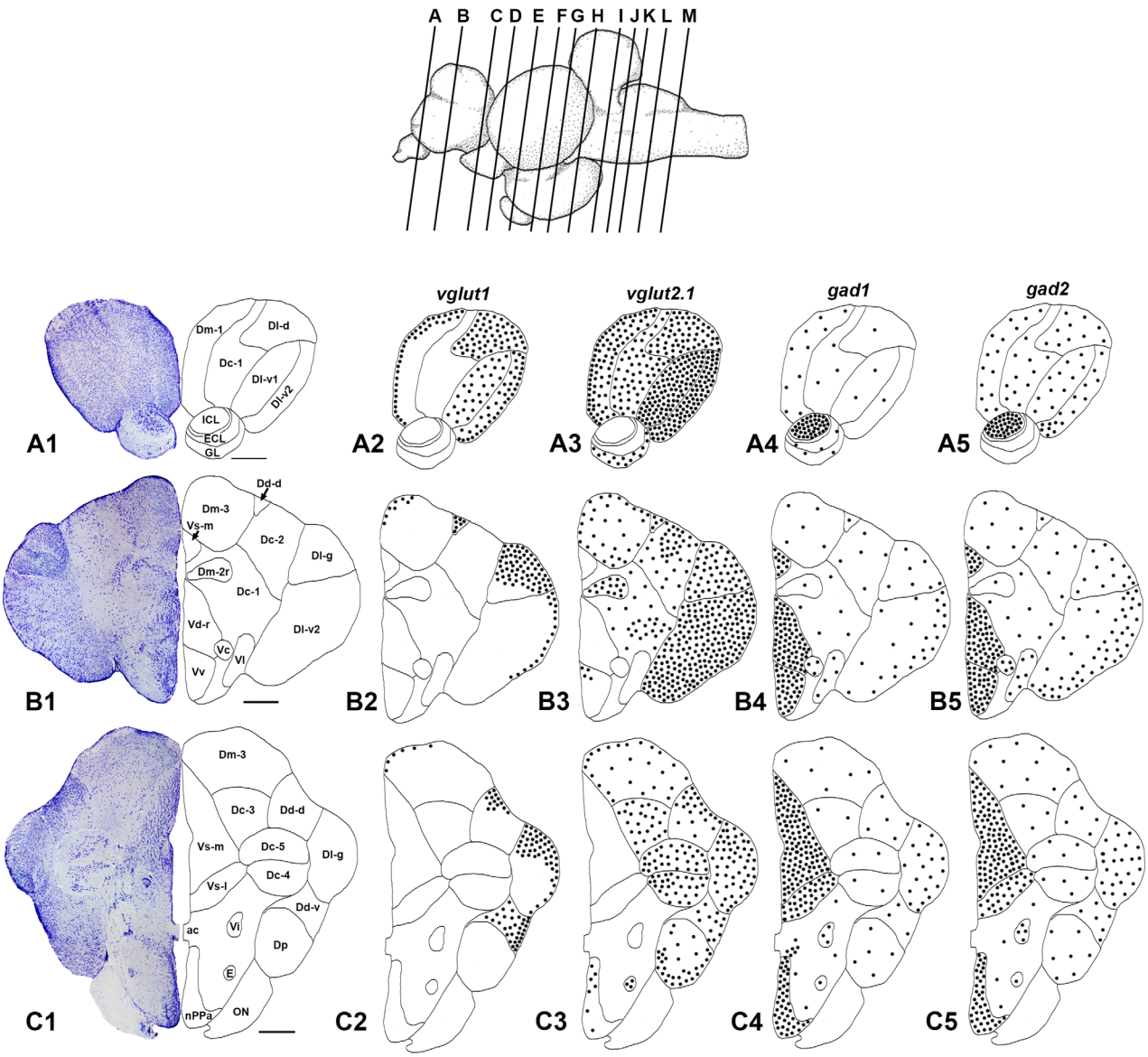

Figure 2. Localization of *vglut1, vglut2.1, gad1*, and *gad2*-expressing cells in the brain of *Astatotilapia burtoni*. Representative transverse sections are shown from rostral (A) to caudal (M). Left column in each row (**A1–M1**) shows a transverse section stained with cresyl violet (left side) and a traced mirror image with nuclei and other neuroanatomical structures labeled (right side). Localization of cells (dots) expressing *vglut1* (**A2–M2**), *vglut2.1* (**A3–M3**), *gad1* (**A4–M4**) and *gad2* (**A5–M5**) are shown on a traced image of the right side of each transverse section. Lateral view of the brain at top shows the approximate location of each section. See list for abbreviations. Scale bars = 250 μm. [Color figure can be viewed at wileyonlinelibrary.com]

(Dc) part (Fig. 2B2–C2). In contrast, no *vglut1* expression is observed in any of the subpallial parts of the ventral telencephalon (Figs. 2B2–D2; 4D–I). Expression of *vglut1* is also absent from the olfactory bulbs (Figs. 2A2; 4I).

vglut2.1

Expression of *vglut2.1* in the brain is the most widespread of the three *vgluts* examined (Figs. 2A3–M3; 5). Some scattered *vglut2.1* cells lie in the rostral spinal cord. In the hindbrain, *vglut2.1* cells form a cellular lamina at the lateral edge of the sensory vagal lobe (VL) and scattered cells also lie within the neuropil of VL (Fig. 2L3–M3) and the facial lobe. *vglut2.1*-expressing cells are also scattered within many octavolateralis nuclei including the medial (MON), anterior (AON), magnocellular (MgON), tangential (TON), and descending (DON) nuclei, as well as the dorsal (SOd) and ventral (SOv) secondary octaval nuclei (Figs. 2J3–K3; 5A). The nuclei of the reticular formation (Rs, Rm, Ri) contain *vglut2.1*-expressing cells throughout the hindbrain, and both the SGn and SVn contain cells with *vglut2.1* expression (Figs. 2H3–M3; 5B,C). The isthmal nucleus (NI) shows

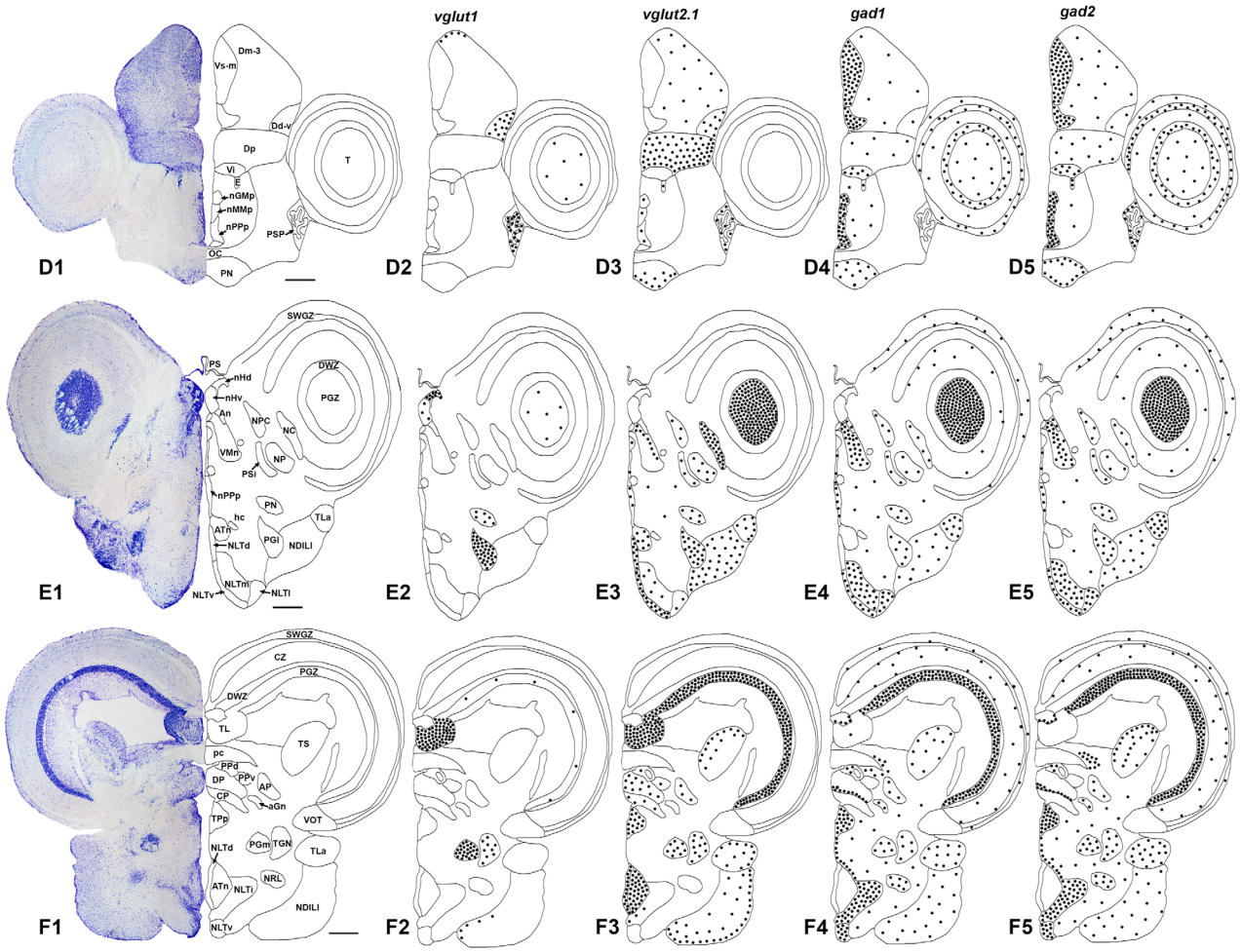

Figure 2. (Continued).

distinct *vglut2.1*-positive cells on the dorsal and medial aspects of this nucleus (Figs. 2H3; 5C). No *vglut2.1* expression is observed in either the corpus or valvula cerebelli structures. Cells expressing *vglut2.1* are predominant throughout the torus semicircularis (TS), particularly the central nucleus (TSc), as well as in the paratoral tegmental nucleus (PTT), anterior (NLVa) and central (NLVc) parts of the lateral valvular nucleus, and the nucleus of medial longitudinal fasciculus (nMLF) (Figs. 2F3–H3; 5C,D). *vglut2.1* cells also sit in the granule cell region of TL, and are abundant in the PGZ of the tectum (Figs. 2E3–H3; 5C,D). The glomerular nucleus (Gn), as well as the preglomerular nuclei (PGm, PGc, PGl) contains distinct *vglut2.1* expression (Figs. 2D3–G3; 5C–G). The PGm also shows both *vglut2.1* and *vglut1* expression (Fig. 9C,D). The lateral thalamic nucleus (LT), prethalamic nucleus (PN), nucleus corticalis (NC), central pretectal nucleus (NPC), intermediate division of the superficial pretectal nucleus (PSi), nucleus pretectalis (NP), PSP, paraventricular organ (PVO), TGN, and torus lateralis (TLa) all contain *vglut2.1*-expressing cells (Figs. 2D3–G3; 5C–F). Expression in corpus mammillare (CM) is also consistent, but weaker than the glomerular and preglomerular nuclei in this same area (Fig. 5D). Labeled *vglut2.1* cells lie within most of the thalamic nuclei, including nucleus of the thalamic eminence (nTE), central posterior thalamic nucleus (CP), dorsal posterior thalamic nucleus (DP), ventromedial thalamic nucleus (VMn), and anterior thalamic nucleus (An), as well as within the periventricular nucleus of the posterior tuberculum (TPp) (Figs. 2E3–F3; 5E).

In the hypothalamus, the central nucleus of the inferior lobe (NCIL) contains large distinct *vglut2.1* cells, cells lie scattered within NDILm, and cells line the lateral edge of the brain within NDILl (Figs. 2E3–H3; 5C–F). *vglut2.1*-expressing cells are abundant in anterior tuberal nucleus (ATn), ventral tuberal nucleus (VTn), and exist at varying densities within the medial, dorsal, intermediate, lateral, and ventral parts of the lateral tuberal nucleus (NLTm, NLTd, NLTi, NLTl, NLTv) (Figs. 2E3–H3; 5F–G). Cells appear absent, however, from both NRP and NRL. Labeled *vglut2.1* cells lie in the entopeduncular nucleus (E), and only sparse scattered cells are found throughout most preoptic nuclei (e.g., nPPa, nMMp, nPMp) (Fig. 2C3–D3).

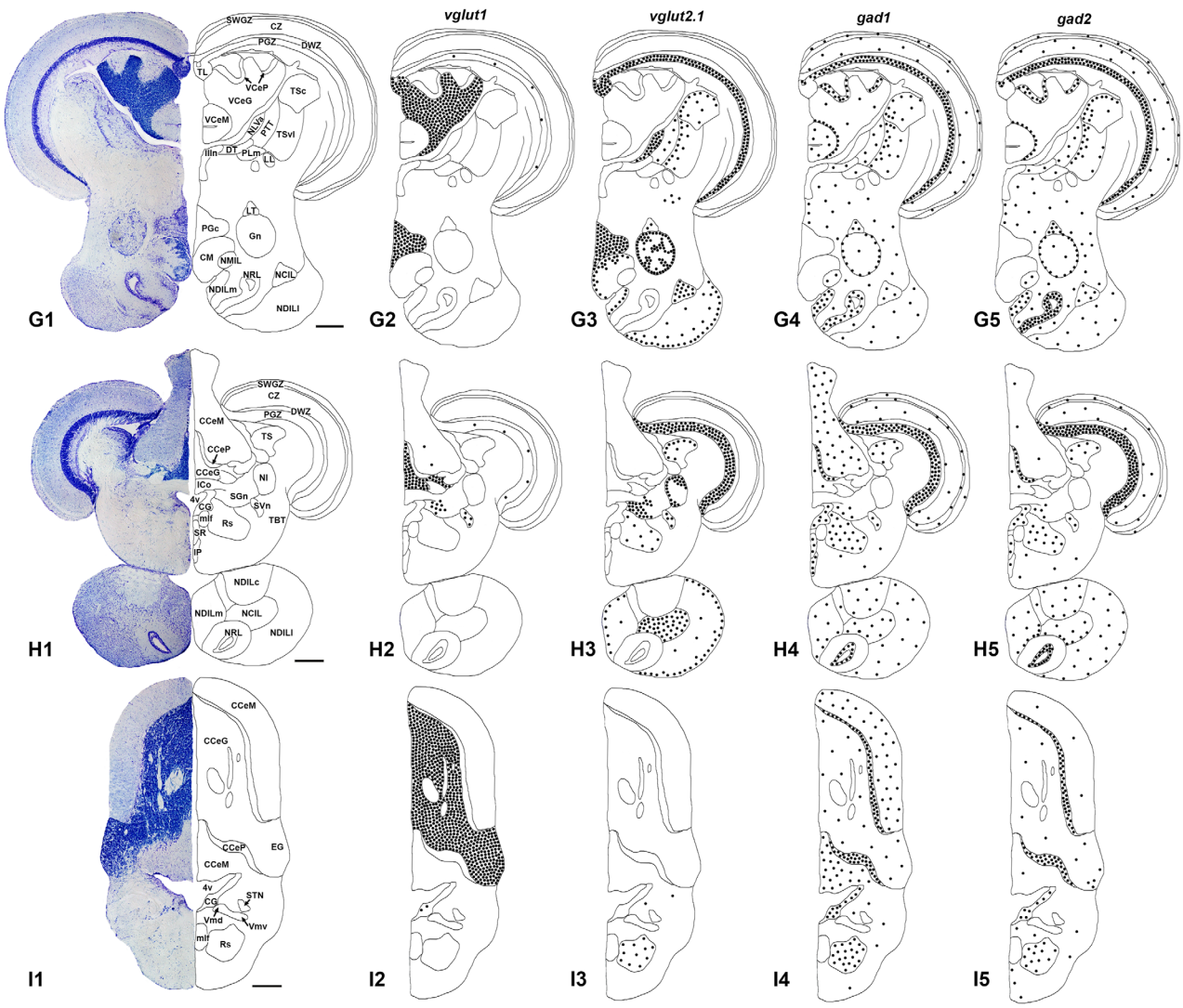

Figure 2. (Continued).

In the telencephalon, *vglut2.1*-expressing cells are more widely distributed than that of *vglut1* (Figs. 2; 9A,B), and are abundant in pallial regions including divisions of Dm, Dl, Dd, Dc, and Dp (Figs. 2A3–D3; 5H–I). In contrast, *vglut2.1* staining is primarily absent from parts of the subpallial ventral telencephalon (e.g., Vs, Vc, Vl, Vd, Vp), with the exception of some scattered cells observed in Vv (Figs. 2B3–D3; 5H). This absence of *vglut2.1* staining in subpallial regions is also similar to *vglut1* (Figs. 2; 9A,B). In the olfactory bulbs, *vglut2.1*-expressing cells are present in the outer glomerular layer (GL), but notably absent from the internal cellular layer (ICL) (Figs. 2A3; 5I).

vglut3

Neurons expressing *vglut3* show the most restricted distribution pattern of the *vgluts*, with cells only found in the hindbrain and hypothalamus of *A. burtoni* (Fig. 6). In the caudal hindbrain, *vglut3*-expressing neurons lie ventrally in the inferior olive (IO) (Fig. 6A). In more rostral hindbrain sections, *vglut3* cells lie in both the dorsal region of the superior raphe nucleus (SRd) beneath the 4th ventricle, and in the medial region of SR (SRm) along the midline (Fig. 6B). These same regions contain serotonergic neurons in this species (Loveland et al., 2014), but double-label studies are needed to determine whether *vglut3* is coexpressed in serotonergic cells. In these same sections, a small population of *vglut3*-expressing cells is found lateral and ventral to the SR (arrows in Fig. 6B). *vglut3* staining also exists at the medial tip of the NRL surrounding the lateral recess in the region of NRP (Fig. 6C). In more rostral sections, *vglut3*-expressing cells are abundant in the intermediate region of NLT (Fig. 6D).

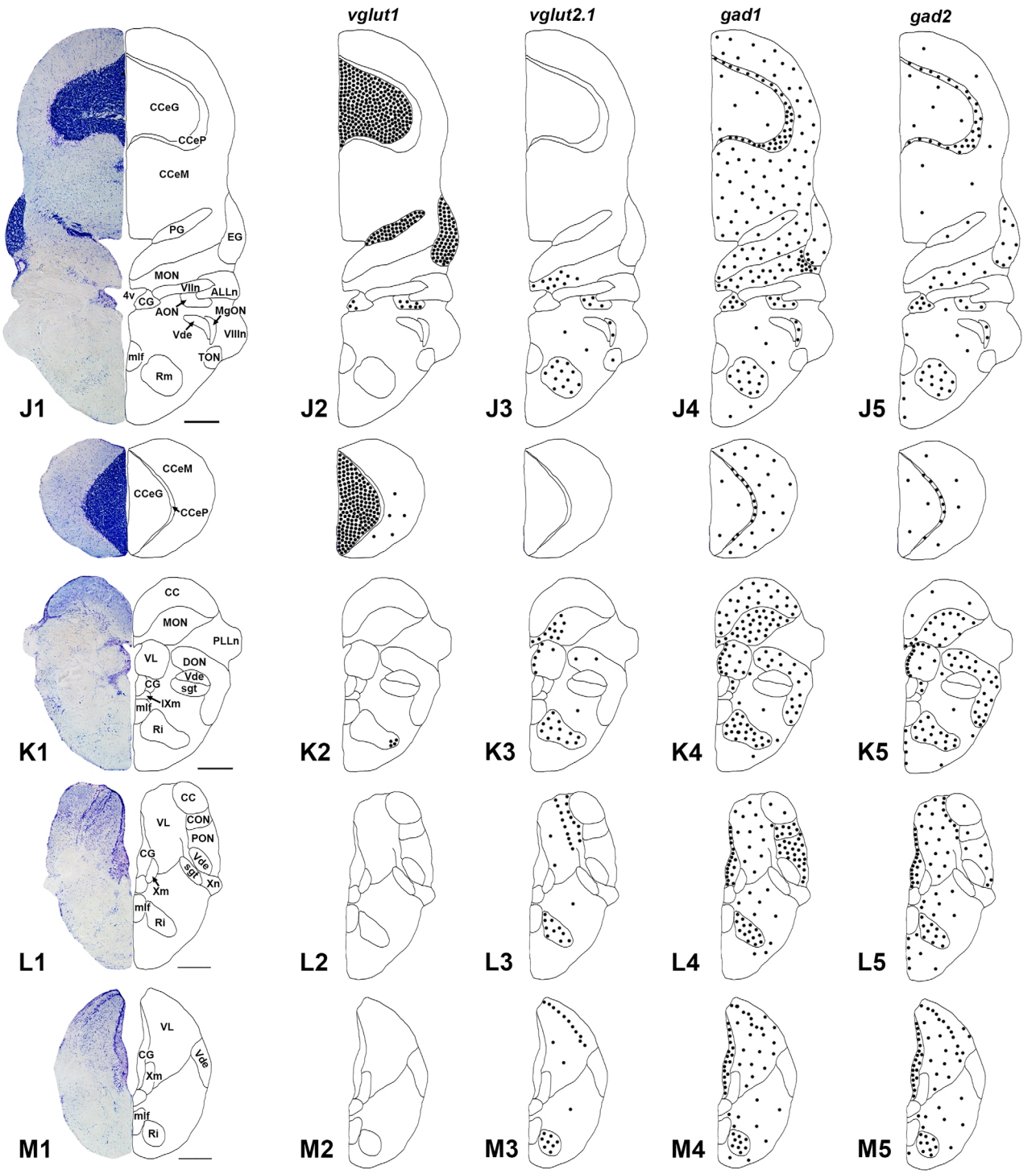

Figure 2. (Continued).

Distribution of glutamate decarboxylases (*gads*)

gad1

Cells expressing *gad1* are abundant from the hindbrain to the olfactory bulbs of *A. burtoni* (Figs. 2A4–M4; 7). Cells with *gad1* expression are scattered within the vagal lobe, and within the central gray zone that borders the fourth ventricle throughout the hindbrain (Figs. 2K4–M4; 7A–C). The octavolateralis nuclei (PON, CON, MON, DON, MgON, TON, AON), as well as the cerebellar crest (CC), contains *gad1*-expressing cells throughout (Figs. 2J4–L4; 7A,B). Qualitatively, *gad1*-expressing cells appear more abundant in mechanosensory processing nuclei (PON, CON, MON) compared to acoustic and vestibular nuclei (DON,

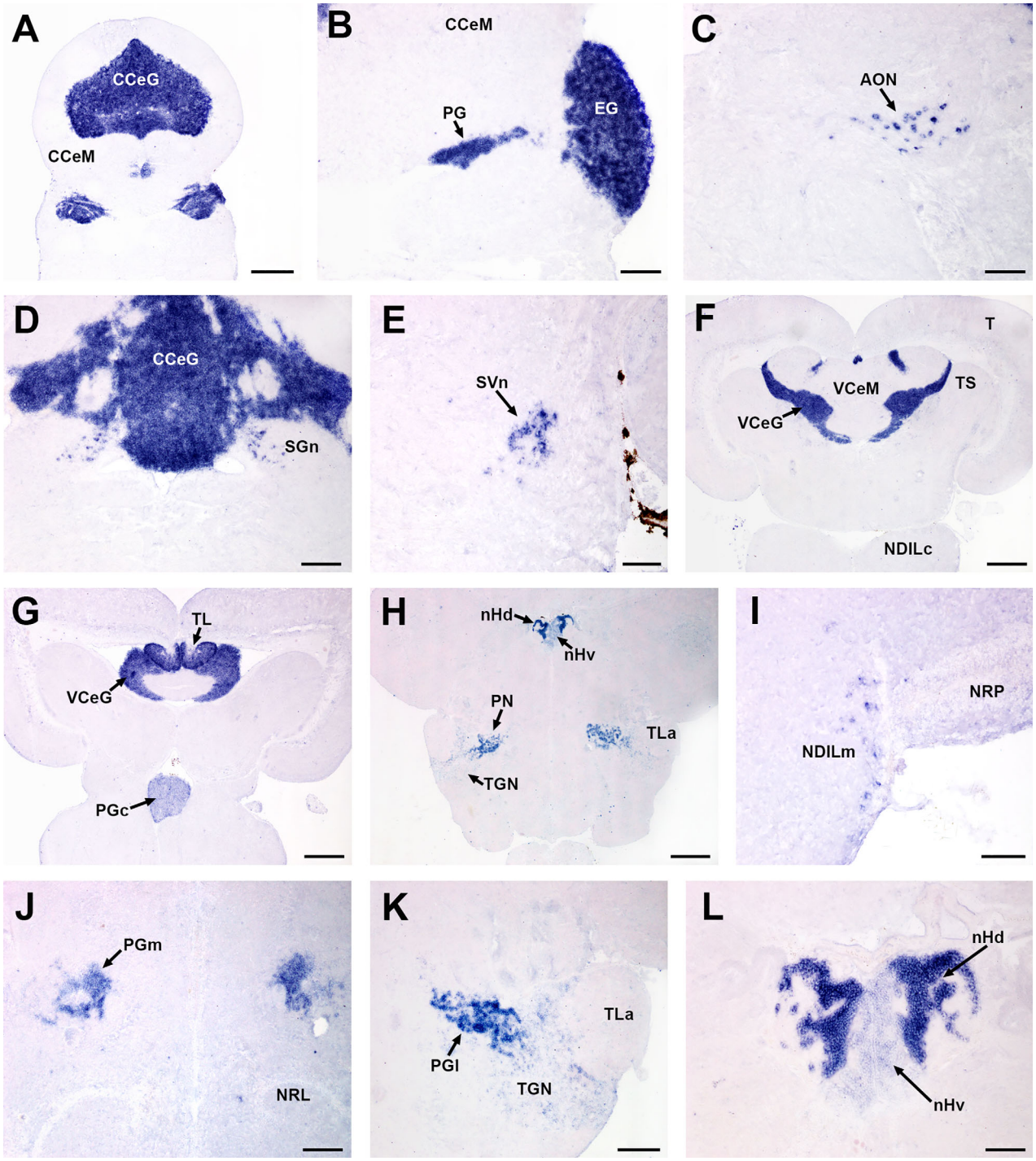

Figure 3. Representative brightfield photomicrographs of vesicular glutamate transporter 1 (*vglut1*)-expressing cells in the rhombencephalon, mesencephalon, and diencephalon of *Astatotilapia burtoni*. **A**: In the caudal cerebellum, *vglut1* labeling is prominent in the granular layer of the corpus cerebelli (CCeG). **B**: Intense *vglut1* label is also found in the EG and PG. **C**: The AON contains *vglut1*-expressing cells in the region of nVIII entry. **D**: In the rostral CCeG region, *vglut1* cells are found lateral to the fourth ventricle within the SGn. **E**: The SVn also contains *vglut1*-expressing cells. **F**: *vglut1* expression is abundant within the granular layer of the valvula cerebelli (VCeG). **G**: *vglut1* expression in the VCeG, TL and PGc. **H**: The PN, TGN, and habenula contain *vglut1*-expressing cells. **I**: *vglut1* cells lie along the medial edge of NDILm in the region of NRP. **J**: *vglut1* cells in PGm. **K**: *vglut1* expression in PGl and TGN. **L**: In the habenula, the dorsal nucleus shows intense *vglut1* expression while the ventral nucleus has faint expression. Photomicrographs were taken from 20-μm transverse sections. See list for abbreviations. Scale bars = 250 μm in A,F,G,H; 100 μm in B,D,J,K; 50 μm in C,E,I,L. [Color figure can be viewed at wileyonlinelibrary.com]

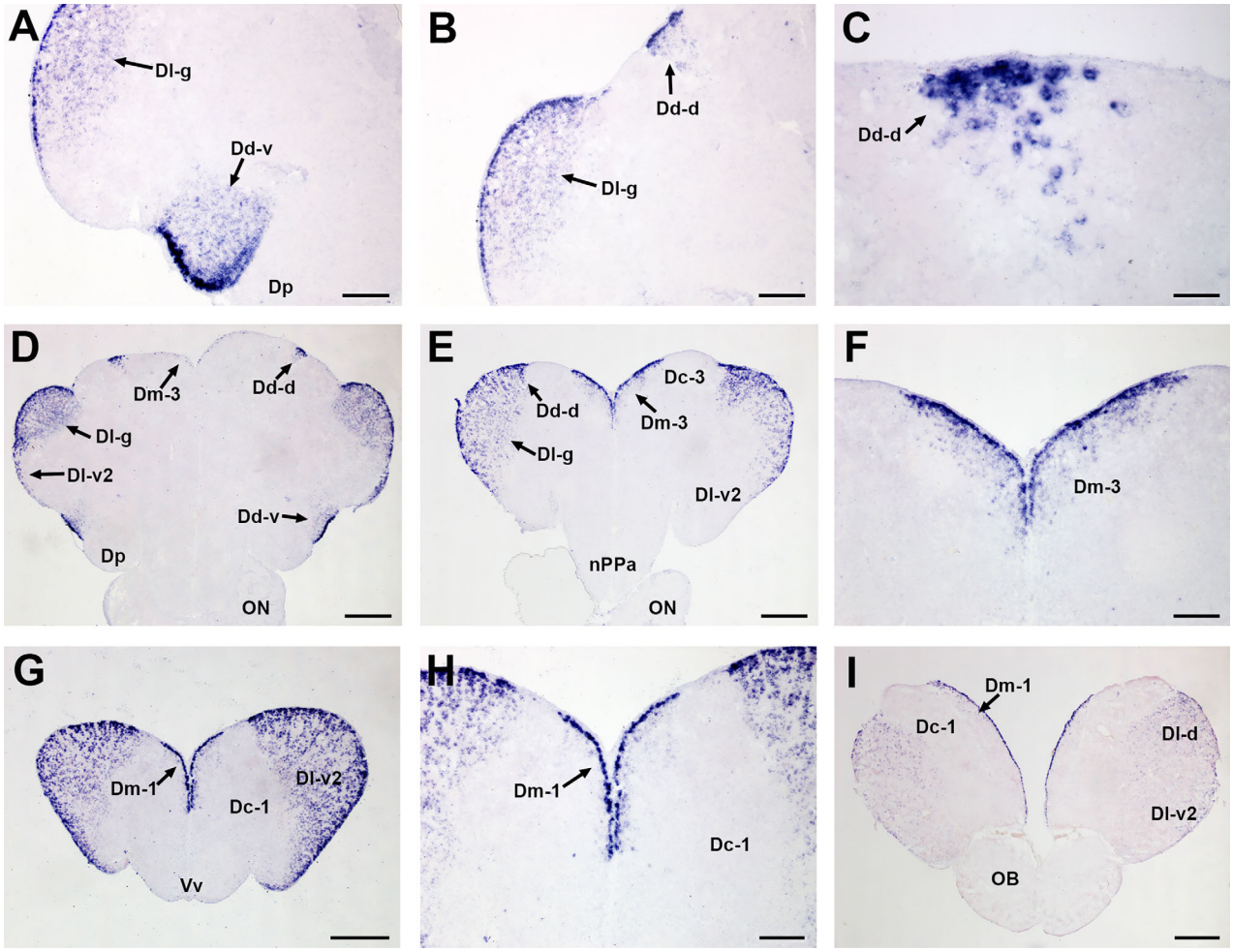

Figure 4. Representative brightfield photomicrographs of vesicular glutamate transporter 1 (*vglut1*)-expressing cells in the telencephalon of *Astatotilapia burtoni*. **A**: In the caudal telencephalon, *vglut1* cells lie in the ventral region of Dd above Dp. **B**: *vglut1* cells in Dl-g and Dd-d. **C**: Higher magnification of *vglut1* cells in Dd-d. **D**: *vglut1*-expressing cells in Dl regions, Dd-d and Dd-v, as well as scattered cells dorsally in Dm-3. **E**: *vglut1* cells extend rostrally in regions of Dl, Dd, and Dm regions along the midline. Note the absence of *vglut1* in Dc regions, ventral telencephalic regions, and the preoptic area. **F**: Higher magnification of *vglut1* cells found dorsally along the midline in Dm-3. **G**: In more rostral sections, abundant *vglut1* expression is found in the Dl-v2, and along the dorsal midline in Dm-1, but is absent in Dc-1. **H**: Higher magnification of Dm-1 region showing *vglut1*-expressing cells along the midline and absence in Dc region. **I**: In the most rostral telencephalon, *vglut1* is evident along the midline in Dm-1, and within lateral Dl regions, but is absent in the olfactory bulbs (OB). Photomicrographs were taken from 20-μm transverse sections and are depicted from the caudal (A) to rostral (I) telencephalon. See list for abbreviations. Scale bars = 250 μm in D,E,G,I; 100 μm in A,B,F,H; 25 μm in C. [Color figure can be viewed at wileyonlinelibrary.com]

MgON, AON, TON). All nuclei of the reticular formation (Rs, Rm, Ri) also contain prominent *gad1*-expressing cells (Figs. 2H4–M4; 7A,C), as well as the interpeduncular nucleus (IP) along the ventral midline (Fig. 7C). In cerebellar structures, *gad1*-expressing cells lie primarily in the Purkinje cell layer of the corpus cerebelli (CCeP) and valvula cerebelli (VCeP), but scattered cells are also found within both the granular (CCeG, VCeG) and molecular layers (CCeM, VCeM) (Figs. 2F4–K4; 7C,D). Notably, *gad1*-expressing cells in the molecular layer are more numerous than *gad2*-expressing cells in these same areas. Scattered cells also exist throughout the eminentia granularis (EG) (Fig. I2–J2), and within the torus longitudinalis (Fig. 2F4–G4). In the tectum, dense *gad1* staining is found in the PGZ, with scattered cells also present in the central zone (CZ) and more superficial zones (Figs. 2D4–H4; 7C,D,G). Many tegmental nuclei contain scattered *gad1* cells, and the TS also shows prominent *gad1* expression but cells appear more abundant within the ventrolateral nucleus (TSvl) (Figs. 2F4–H4; 7C,D). In the glomerular nucleus, *gad1*-expressing cells lie primarily surrounding or along its lateral border (Figs. 2G4; 7D).

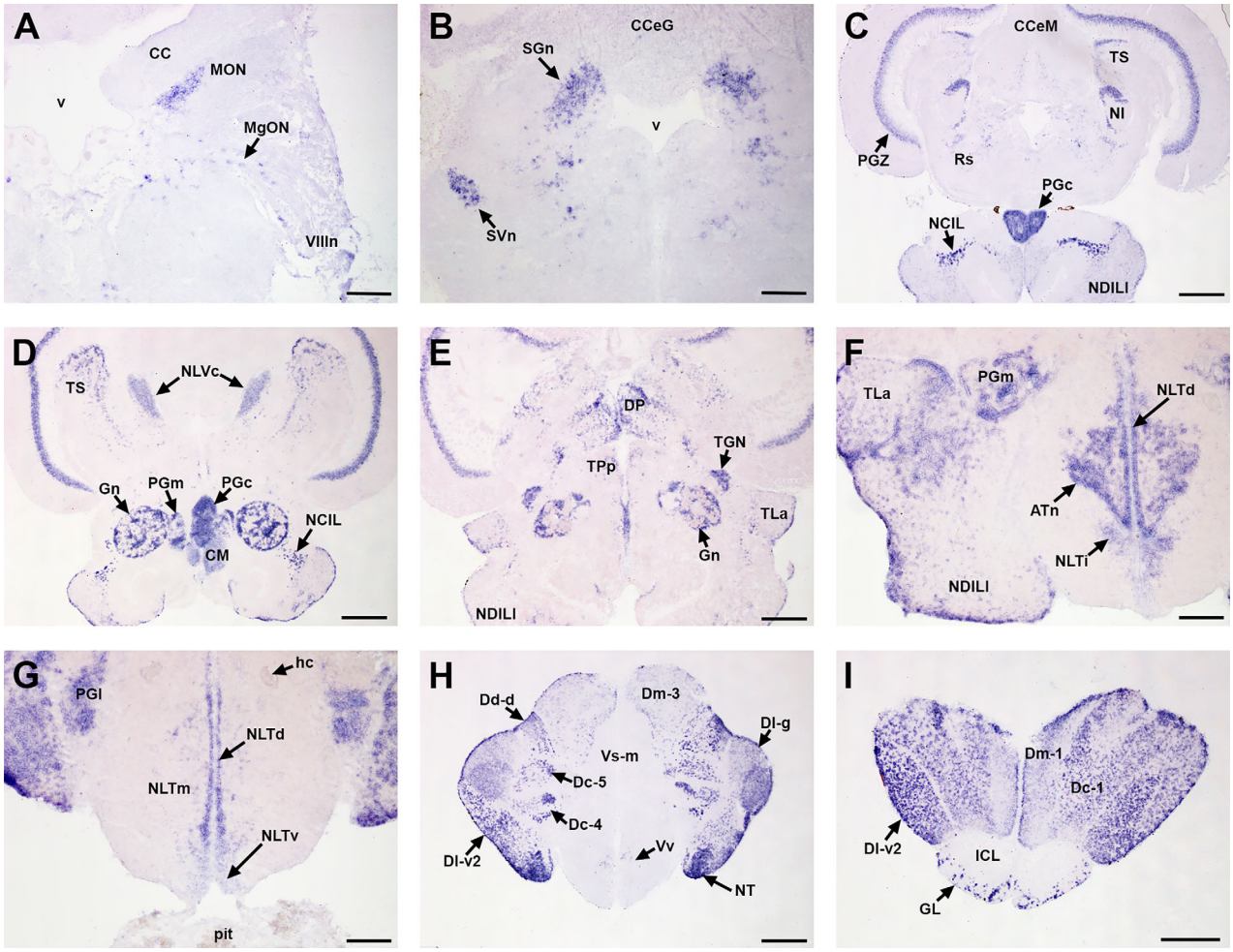

Figure 5. Representative brightfield photomicrographs of vesicular glutamate transporter 2.1 (*vglut2.1*)-expressing cells in the brain of *A. burtoni*. **A**: In the hindbrain, *vglut2.1* cells lie in several octavolateralis nuclei including the MON and MgON. **B**: *vglut2.1*-expressing cells are found adjacent to the fourth ventricle in the SGn, and more ventrolaterally in the SVn. **C**: *vglut2.1*-expressing cells are abundant in the PGZ of the tectum, scattered throughout the torus semicircularis (TS), and found in the dorsal and medial portions of the isthmal nucleus (NI). The PGc showed intense *vglut2.1* labeling, and cells lie in several hypothalamic nuclei including large cells in NCIL, and scattered cells throughout NDILl and NDILm. **D**: The glomerular (Gn), preglomerular (PGm, PGc), and CM nuclei contain *vglut2.1*-expressing cells. The NLV also shows dense *vglut2.1* labeling. **E**: Several thalamic nuclei and nuclei of the posterior tuberculum also contain *vglut2.1* cells. **F**: *vglut2.1*-expression predominates the anterior tuberal nucleus (ATn). **G**: *vglut2.1*-expressing cells also lie within several nuclei of the NLT. **H**: In the telencephalon, *vglut2.1*-expressing cells are abundant within all dorsal pallial nuclei (Dm, Dd, Dl, Dc, Dp), but noticeably absent from most ventral subpallial nuclei (Vs, Vd, Vp, Vc, Vl), with the exception of a few scattered cells in Vv. **I**: In the olfactory bulbs, *vglut2.1* expression is seen in the glomerular layer (GL), but is absent from the ICL. Photomicrographs were taken from 20-μm transverse sections and are depicted from caudal (A) to rostral (I). See list for abbreviations. Scale bars = 250 μm in C,D,E,H,I; 100 μm in A,B,F,G. [Color figure can be viewed at wileyonlinelibrary.com]

In the diencephalon, *gad1*-expressing cells are abundant in hypothalamic regions such as subdivisions of NLT and NRL (Figs. 2E4–G4; 7E,F). *gad1* appears absent from CM and PGc along the midline, but some scattered cells lie within PGl and PN (Figs. 2D4–F4; 7D). No *gad1* (or *gad2*) expression exists in the habenula (Fig. 2E4,E5). Scattered *gad1*-expressing cells are also found in several nuclei of the inferior lobe of the hypothalamus (e.g., NDILl, NDILm, NCIL, NMIL) (Figs. 2E4–H4; 7D,E). The ATn is noticeably devoid of *gad1* expression (Figs. 2F4; 7F), but VTn contains some scattered *gad1* cells. The TPp shows dense *gad1* expression, and expression is also seen in PVO, dorsal periventricular pretectal nucleus (PPd), and several other pretectal and thalamic nuclei (e.g., An, In, VMn, IC), but appears absent from both CP and DP (Figs. 2E4–F4; 7F,G,H). The intercalated nucleus (IC) is identified here as a band of *gad1* (and *gad2*)-expressing cells between the *gad1/gad2*-lacking DP and CP, as described in the zebrafish (Mueller and Guo, 2009).

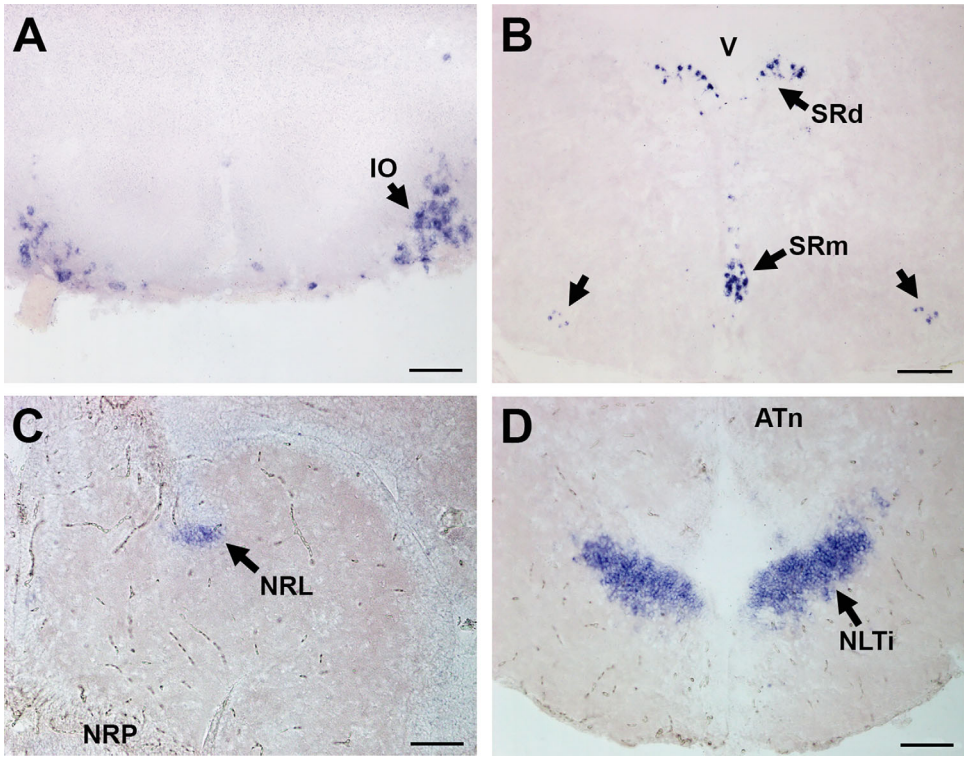

Figure 6. Representative brightfield photomicrographs of vesicular glutamate transporter 3 (*vglut3*)-expressing cells in the brain of *Astatotilapia burtoni*. **A**: In the hindbrain, *vglut3*-expressing cells lie in the inferior olive (IO). **B**: The superior raphe nucleus also contains *vglut3*-expressing cells in both dorsal (SRd) and medial (SRm) divisions. Several labeled cells (arrows) are also consistently located ventrolateral to the SR in the same sections. **C**: In the hypothalamus, a small group of *vglut3*-expressing cells is found at the medial tip of the NRL in the region of the NRP. **D**: *vglut3*-labeled cells are also observed in the intermediate part of NLT. Photomicrographs were taken from 20-μm transverse sections. See list for abbreviations. Scale bars = 50 μm in A,C,D; 100 μm in B. [Color figure can be viewed at wileyonlinelibrary.com]

Cells labeled for *gad1* also lie in the rostral thalamic region described as the reticular thalamic nucleus in zebrafish (Mueller and Guo, 2009; Mueller, 2012). All preoptic nuclei show dense *gad1* expression (Figs. 2C4–E4; 7I,J), primarily concentrated along the midline, with some scattered cells labeled more laterally within the POA neuropil.

In the telencephalon, *gad1*-expressing cells are abundant within all parts of the subpallial ventral telencephalon including Vv, Vd, Vc, Vl, Vs, and Vp (Figs. 2B4–D4; 7J,K). In contrast, *gad1* cells are only scattered within the pallial dorsal telencephalon such as Dm, Dc, Dl, Dp, as well as NT (Figs. 2A4–D4; 7J,K). In the olfactory bulbs, *gad1* expression is dense within the ICL, and some scattered cells are found within other layers of the olfactory bulbs (ECL, GL) (Figs. 2A4; 7L).

gad2

Cells expressing *gad2* are also abundant from the spinal cord to the olfactory bulbs of *A. burtoni* in a pattern that largely overlaps that of *gad1* (Figs. 2A5–M5; 8; 9). Cells expressing *gad2* are scattered within the vagal lobe, and within the central gray zone that borders the fourth ventricle throughout the hindbrain (Figs. 2H5–M5; 8A,B). Scattered *gad2*–expressing cells also exist throughout octavolateralis nuclei (MON, DON, MgON, TON, AON) (Figs. 2J5–L5; 8A). Nuclei of the reticular formation (Rs, Rm, Ri) also contain *gad2* cells throughout the hindbrain (Figs. 2H5–M5; 8A,B). In cerebellar structures, *gad2*-expressing cells primarily lie in the Purkinje cell layer of the corpus cerebelli and valvula cerebelli, but scattered cells are also found within the granular and molecular layers (Figs. 2F5–K5; 8B–D). Cells are also found throughout the eminentia granularis (EG) (Figs. 2I5–J5; 8B), and in the torus longitudinalis (Fig. 2F5–G5). In the tectum, dense *gad2* stain is found in the PGZ, with scattered cells also present in the CZ and more superficial zones (Figs. 2D5–H5; 8H), which is similar to *gad1*. The TS also shows scattered *gad2* expression throughout the TSc and TSvl nuclei, and many tegmental nuclei contain scattered *gad2* cells (Figs. 2F5–H5; 8C,D). In the Gn, *gad2*-expressing cells

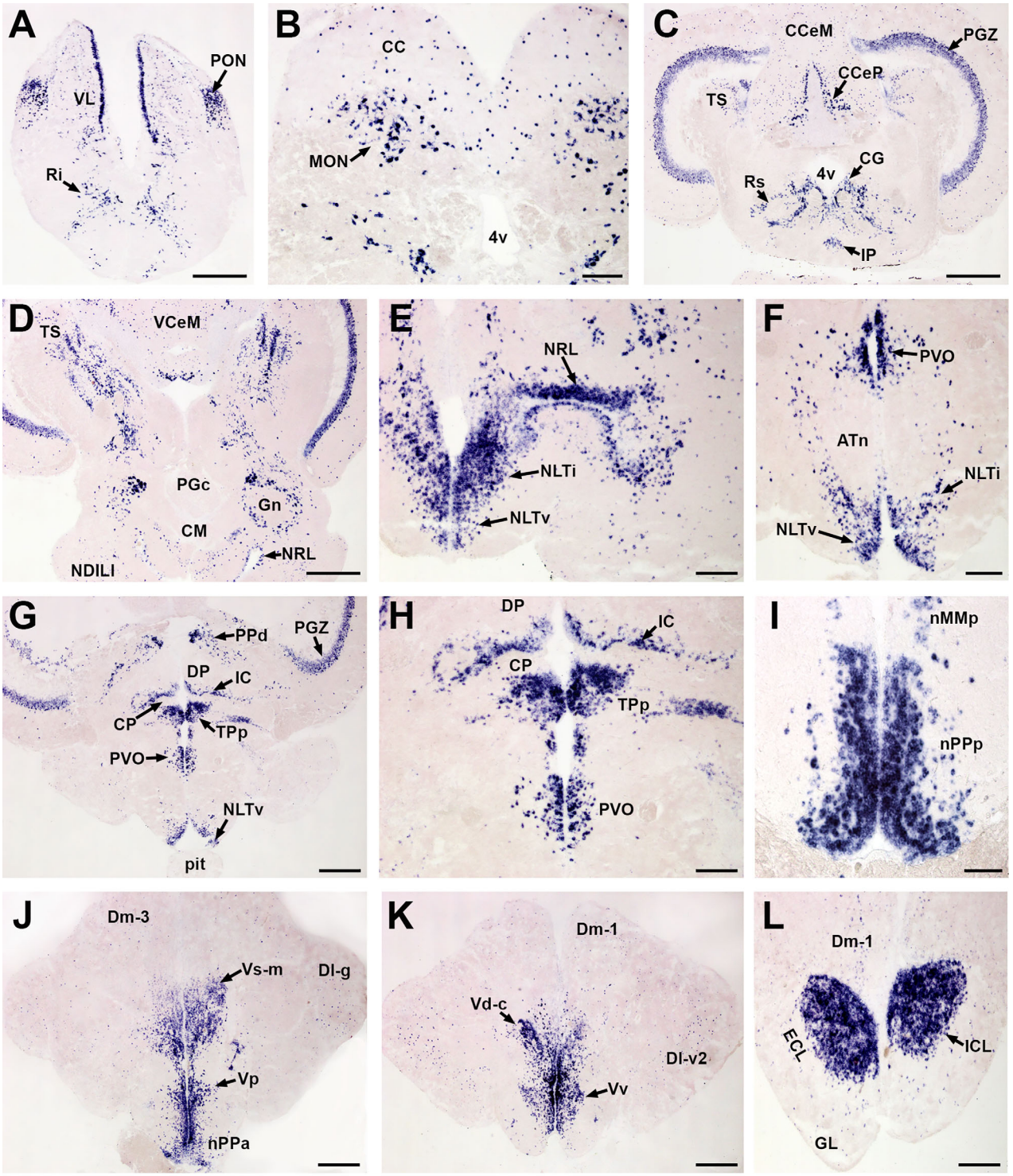

Figure 7. Representative brightfield photomicrographs of glutamate decarboxylase 1 (*gad1*)-expressing cells in the brain of *Astatotilapia burtoni*. **A**: *gad1* cells are abundant in the hindbrain within regions of the vagal lobe, posterior octavolateralis nucleus (PON), and nuclei of the reticular formation. **B**: *gad1*-expressing cells in the medial octavolateralis nucleus (MON) and cerebellar crest (CC). **C**: *gad1*-labeled cells are abundant in the PGZ of the tectum, Purkinje (CCeP) and molecular (CCeM) layers of the corpus cerebelli, and nuclei of the reticular formation. **D**: In midbrain regions, *gad1*-expressing cells are abundant in the torus semicircularis (TS), tegmental nuclei, and surround the glomerular nucleus (Gn), but are absent along the midline within PGc and CM. **E**: Dense *gad1* labeling is found in hypothalamic regions of the nucleus of the lateral recess (NRL) and lateral tuberal nucleus (NLT). **F**: *gad1* label is present in several NLT regions and PVO, but is absent from ATn. **G**: *gad1*-expressing cells are abundant in distinct diencephalic nuclei along the midline. **H**: Higher magnification of dense *gad1* expression in thalamic, posterior tuberculum, and paraventricular organ nuclei. **I**: *gad1*-expressing cells are abundant throughout the preoptic nuclei. **J**: Dense *gad1* labeling is found in ventral subpallial telencephalic nuclei (e.g., Vd-c, Vp, Vd, Vv), but only scattered cells are labeled in dorsal pallial telencephalic nuclei (e.g., Dm, Dl, Dp). **K**: *gad1*-expressing cells are abundant in Vd-c and Vv. **L**: Dense *gad1* label is found in the inner cellular layer (ICL) of the olfactory bulbs (OB), with scattered cells in the other OB layers. Photomicrographs were taken from 20-μm transverse sections and are depicted from caudal (A) to rostral (L). See list for abbreviations. Scale bars = 250 μm in A,C,D,G,J,K; 100 μm in B,E,F,H,L; 50 μm in I. [Color figure can be viewed at wileyonlinelibrary.com]

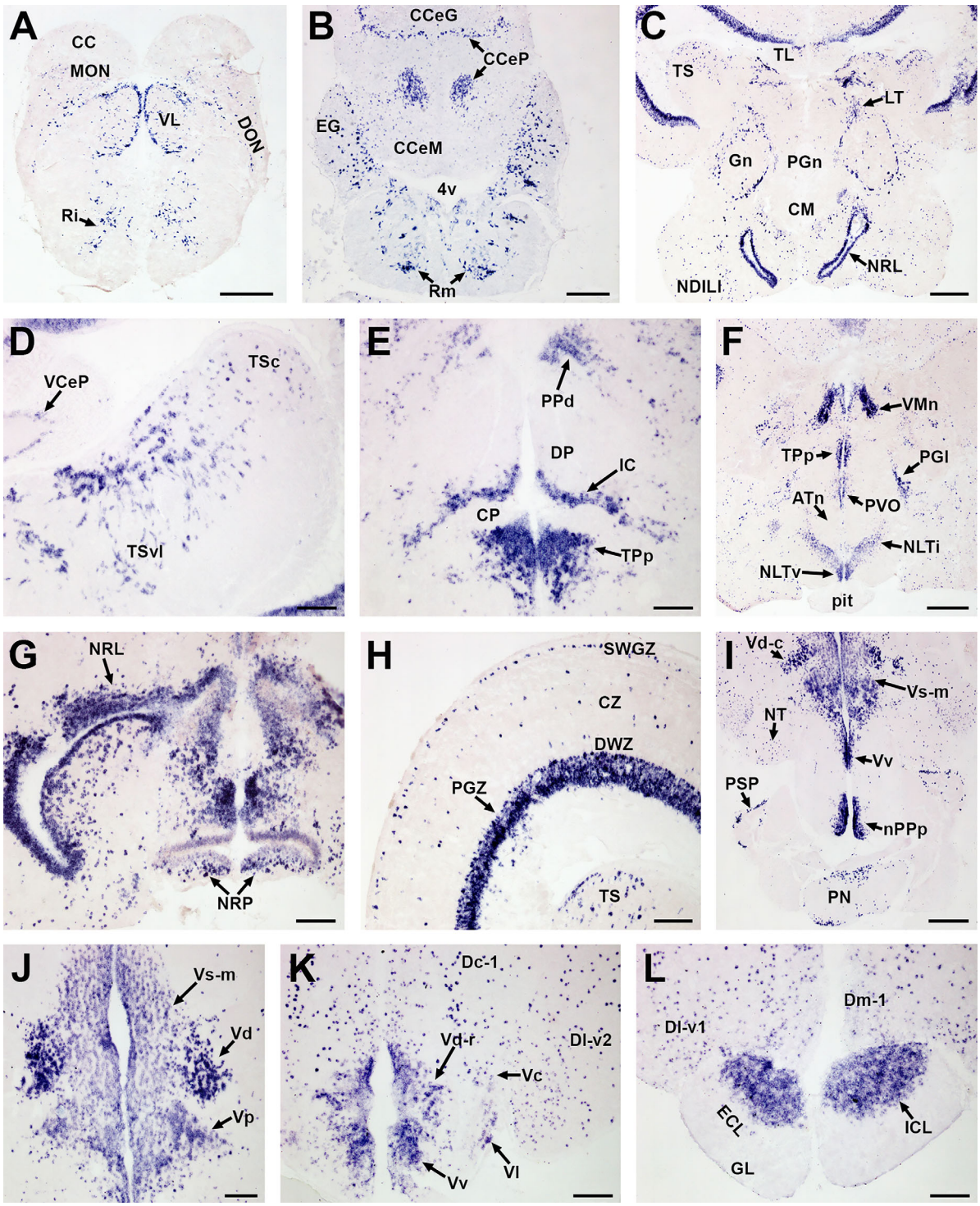

Figure 8. Representative brightfield photomicrographs of glutamate decarboxylase 2 (*gad2*)-expressing cells in the brain of *Astatotilapia burtoni*. **A**: *gad2* cells in the hindbrain within the vagal lobe, medial and descending octaval nuclei and reticular formation. **B**: In the corpus cerebelli, Purkinje cells show strong *gad2* labeling, while scattered *gad2* cells are also found in the granular (CCeG) and molecular (CCeM) layers. **C**: *gad2* labeling is abundant in several mesencephalic (e.g., TS, LT) and diencephalic (e.g., NRL, NDIL) regions, but is notably absent from PGc, CM, and more central regions of the Gn. **D**: *gad2*-expressing cells in the torus semicircularis are found in both TSc and TSvl regions. **E**: *gad2*-expressing cells are also found in PPd, IC, and TPp, but are largely absent from DP and CP. **F**: *gad2* labeling is abundant in several NLT nuclei, the PVO and TPp, but is absent from ATn. **G**: The NRL and NRP contain abundant *gad2*-expressing cells. **H**: In the tectum, dense *gad2* staining is found within the PGZ, and scattered *gad2*-labeled cells are found within the CZ and SWGZ. **I**: *gad2*-expressing cells are abundant in preoptic nuclei and parts of the ventral telencephalon. **J**: Dense *gad2* label is found in Vs-m, Vd-c, and Vp. **K**: *gad2*-expressing cells are found in the subpallial ventral telencephalic regions of Vd, Vc, Vl, and Vv. In contrast to ventral telencephalic nuclei, more scattered *gad2*-expressing cells lie throughout the dorsal telencephalic regions (e.g., Dm, Dl, Dd, Dc). **L**: In the olfactory bulbs, dense *gad2* stain is found in the inner cellular layer. Photomicrographs were taken from 20-μm transverse sections and are depicted from caudal (A) to rostral (L). See list for abbreviations. Scale bars = 250 μm in A–C,F,I; 100 μm in D,E,G,H,J,K,L. [Color figure can be viewed at wileyonlinelibrary.com]

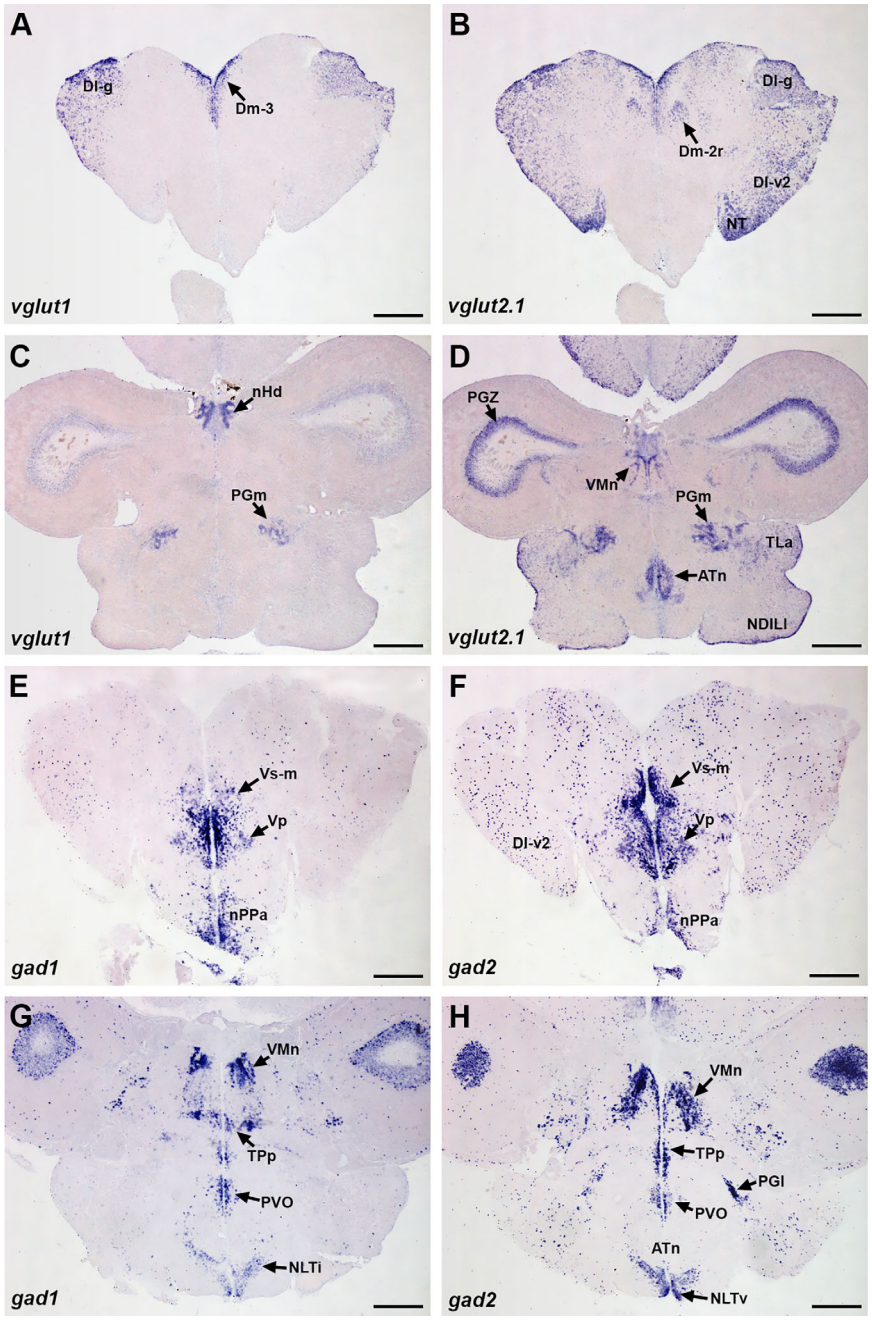

Figure 9. Representative brightfield photomicrographs of adjacent sections comparing different glutamatergic (*vglut1, vglut2.1*) and GABAergic (*gad1, gad2*) markers in the brain of *Astatotilapia burtoni*. **A,B**: Adjacent transverse sections through the anterior preoptic area and caudal telencephalon showing label for *vglut1* (A) and *vglut2.1* (B). **C,D**: Adjacent transverse sections through the thalamic region showing *vglut1* (C) and *vglut2.1* (D) expression. **E,F**: Adjacent transverse sections through the anterior preoptic area and caudal telencephalon show mostly overlapping distributions for *gad1* (E) and *gad2* (F). **G,H**: Adjacent transverse sections through the thalamic and hypothalamic region show labeling for *gad1* (G) and *gad2* (H). Photomicrographs were taken from 20-μm transverse sections. See list for abbreviations. Scale bars = 250 μm. [Color figure can be viewed at wileyonlinelibrary.com]

surround this nucleus in rostral sections (Fig. 8C), but lie within the Gn in more caudal sections (Fig. 2G5).

Cells expressing *gad2* are abundant in hypothalamic regions such as NLT nuclei (NLTi, NLTm, NLTl, NLTv), NRL, and in and around NRP (Figs. 2E5–G5; 8C,F,G). While *gad2* appears absent from CM and PGc, scattered cells lie within PGl and PN (Figs. 2D5–E5; 8C,F). Scattered cells are also found in several nuclei of the inferior lobe of the hypothalamus (e.g., NDILl, NDILm, NCIL, NMIL) in a pattern that largely overlaps that of

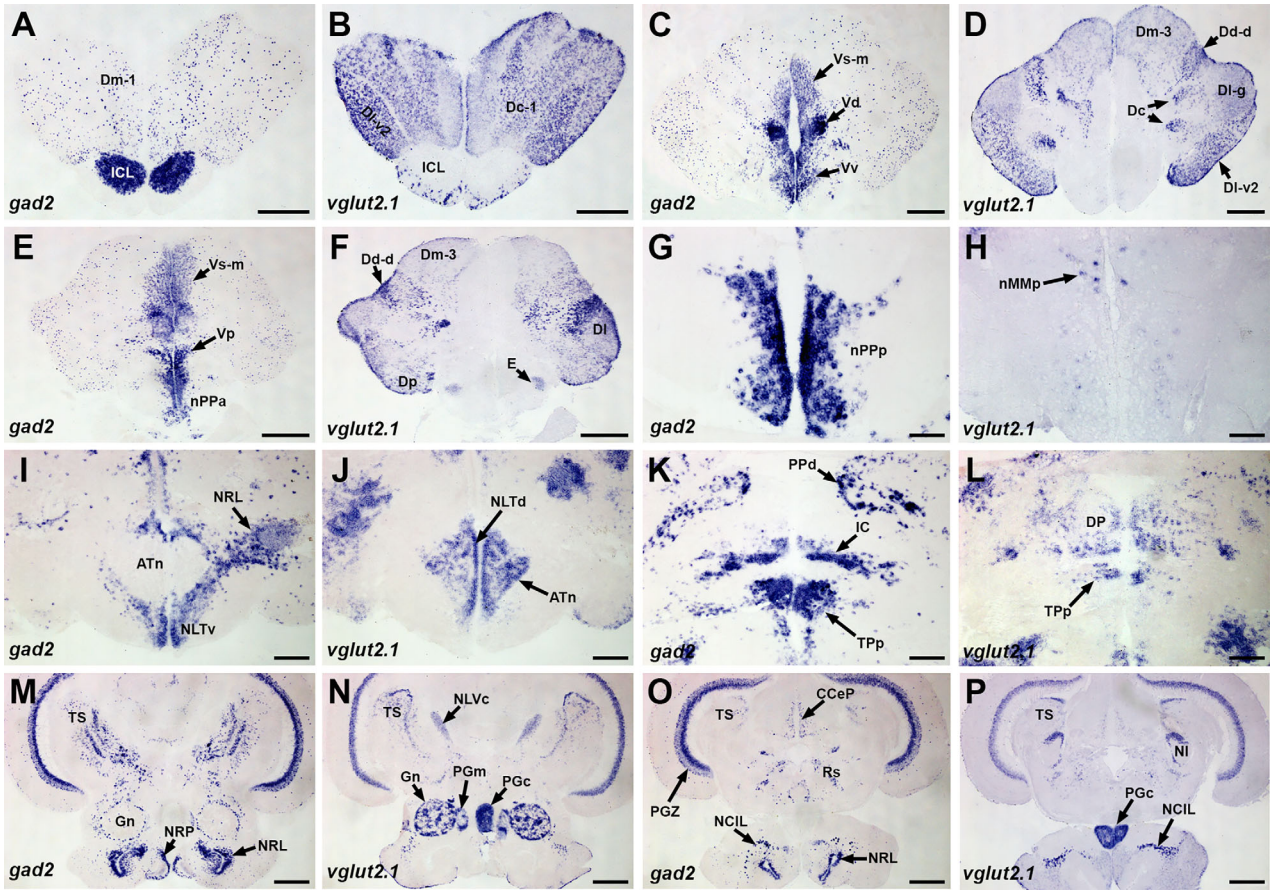

Figure 10. Representative brightfield photomicrographs of adjacent sections comparing GABAergic (*gad2*) and glutamatergic (*vglut2.1*) regions in the brain of *Astatotilapia burtoni*. Dense GABAergic staining is prevalent in the ICL of the olfactory bulbs, subpallial ventral telencephalic regions, and the preoptic area (**A,C,E,G**), while *vglut2.1* labeling is reduced or absent in these same areas (**B,D,F,H**). In contrast, *vglut2.1* staining is abundant in pallial regions of the dorsal telencephalon (B,D,F), while *gad1/2* is reduced or absent (A,C,E). *vglut2.1*-expressing cells lie in the ATn and NLTd (**J**), while these regions are devoid of *gad1/2* labeling (**I**). In the thalamus, *gad2* and *vglut2.1* also label adjacent but primarily nonoverlapping nuclei (**K,L**). While both GABAergic and glutamatergic cells are expressed in similar regions of the tectum (e.g., PGZ) and reticular formation nuclei (e.g., Rs, Ri, Rm), other mesencephalic and rhombencephalic regions such as Gn, TS, NI, and several preglomerular nuclei show primarily nonoverlapping distribution patterns (**M–P**). Each pair of photomicrographs was taken from 20-μm adjacent transverse sections labeled for *gad2* (A,C,E,G,I,K,M,O) and *vglut2.1* (B,D,F,H,J,L,N,P) from rostral (A,B) to caudal (O,P). See list for abbreviations. Scale bars = 250 μm in A–F,M–P; 100 μm in I–L; 50 μm in G–H. [Color figure can be viewed at wileyonlinelibrary.com]

gad1 (Figs. 2E5–H5; 8C,F). Similar to *gad1*, the ATn is noticeably devoid of *gad2* expression, but the regions surrounding the ATn are GABAergic (Figs. 2F5; 8F; 9G,H). VTn also contains scattered *gad2* cells. The TPp shows dense *gad2* expression, and expression is also seen in the diencephalic PVO, the pretectal PPd, and several thalamic nuclei (e.g., VMn), but appears absent from both CP and DP (Figs. 2E5–F5; 8E,F), which is also similar to *gad1* (Fig. 9G,H). All preoptic nuclei show dense *gad2* expression (Figs. 2C5–E5; 8I), and cells also lie in PSP (Figs. 2D5; 8I).

In the telencephalon, *gad2*-expressing cells are densely stained and abundant within all parts of the subpallial ventral telencephalon including Vv, Vd, Vc, Vl, Vs, and Vp (Figs. 2B5–D5; 8I–K). In contrast, *gad2* cells are only scattered within the parts of the pallial dorsal telencephalon such as Dm, Dc, Dl, Dp, as well as nucleus taenia (NT) (Figs. 2A5–D5; 8I,K,L), but are more abundant than *gad1*-expressing cells in these same regions (Fig. 9E,F). In the olfactory bulbs, *gad2* expression is dense within the ICL, but primarily absent from the ECL and GL (Figs. 2A5; 8L).

Summary of glutamatergic (*vglut*)- and GABAergic (*gad*)-expressing nuclei

Representative adjacent sections shown in Figure 10 highlight some of the nonoverlapping distribution patterns between glutamatergic (*vglut2.1*)- and GABAergic

(*gad2*)-expressing cells throughout the cichlid brain. In the olfactory bulbs, *gad* expression is predominant in the ICL, while this region lacks *vglut2.1* cells, but the outer layers show the opposite pattern, with mostly *vglut2.1* expression and minimal *gad* expression (Fig. 10A,B). In the telencephalon, *gad* expression is abundant in subpallial parts of the ventral telencephalon, while *vglut2.1* expression is essentially absent from these same regions (Fig. 10C–F). In contrast, the pallial parts of the dorsal telencephalon show the opposite staining pattern, with abundant *vglut2.1* expression, and only scattered *gad*-expressing cells (Fig. 10C–F). The preoptic area is also largely GABAergic, with only few scattered *vglut2.1*-expressing cells throughout (Fig. 10G,H). The ATn and NLTd contain only *vglut2.1*-expressing cells, but the surrounding regions including the NRL and NLTv are GABAergic (Fig. 10I,J). In the thalamus, some nuclei seem to contain both GABAergic and glutamatergic cells to some extent, but there may also be subpopulations within these nuclei that requires further analyses. The GABAergic intercalated nucleus separates the thalamic DP and CP, which both contain some *vglut2.1*-expressing cells but are devoid of *gad* expression (Fig. 10K,L). The TPp also appears to contain subpopulations of cells that express both *gads* and *vglut2.1*, although double-labeling experiments are needed to determine whether they are expressed in the same or adjacent cells (Fig. 10K,L). The glomerular nucleus was another region with distinct *vglut2.1*-expressing cells within it, and GABAergic cells surrounding the border (Fig. 10M,N). The torus semicircularis contains both *gad*- and *vglut2.1*-expressing cells, but *vglut2.1* expression appears more concentrated in the TSc, while *gad* is more abundant in the TSvl (Fig. 10M,N). GABAergic and glutamatergic cells are expressed in similar zones of the tectum (e.g., PGZ) and in reticular formation nuclei (e.g., Rs, Ri, Rm), but several other diencephalic, mesencephalic, and rhombencephalic regions such as NRL, several preglomerular nuclei, NI, NLV, and the cerebellum show primarily non-overlapping distribution patterns (Fig. 10M-P).

Distribution of choline acetyltransferase (*chat*)

chat

chat-expressing neuron populations are predominantly located in caudal nuclei of the mesencephalon and rhombencephalon (Fig. 11). Expression of *chat* is found in the giant spinal motor neurons of the rostral spinal cord, and scattered cells lie in these same sections dorsal to the central canal (Fig. 11A). Large *chat*-expressing neurons are found within nuclei of all motor cranial nerves (Fig. 11B–D,G,H): vagal (Xm), glossopharyngeal (IXn), facial (VIIn), abducens (VIn), trigeminal (Vn), trochlear (IVn), and oculomotor (IIIn). The octavolateralis efferent nucleus (OEN) and inferior and superior nuclei of the reticular formation also contain large *chat*-expressing cells (Fig. 11E,G). Labeled *chat* cells also exist within the descending octaval nucleus in the region near the descending trigeminal tract and lateral to the vagal lobe (Fig. 11C). Cells expressing *chat* mRNA are also abundant in the caudal region of NLV, the SGn, and the rostral tegmental nucleus (RTN) (Fig. 11F,G,I).

Scattered but abundant *chat*-expressing cells also lie in the PGZ of the tectum throughout its rostrocaudal extent (Fig. 11I,J). In the diencephalon, a small population of *chat*-expressing cells is found laterally in the region of PGI (Fig. 11K), and a few small cells are observed in the nPPp and nPPa of the preoptic area. In the telencephalon, the only *chat*-expressing neurons exist in the subpallial Vv along the midline ventricle (Fig. 11L).

Summary of glutamatergic, GABAergic and cholinergic cells within SDMN nuclei

Localization of glutamatergic, GABAergic, and cholinergic neurons within each putative SDMN nucleus of the cichlid brain is summarized in Table 2 and Figure 12. The pallial SDMN nuclei (Dm, Dl) predominantly express glutamatergic markers (*vglut1, vglut2.1*), with a more scattered distribution of GABAergic (*gad1, gad2*)-expressing cells throughout parts of the dorsal telencephalon (Table 2; Figs. 2; 10; 12A-D). In contrast, the subpallial SDMN nuclei (Vs/Vp, Vv, Vd, Vc) show the opposite pattern, with dense GABAergic marker expression and either absence (in Vs/Vp, Vd, Vc) or only limited scattered expression (e.g., *vglut2.1* in Vv) of glutamatergic cells (Fig. 12A–D). The preoptic area contains dense GABAergic expression along with some scattered *vglut2.1* and *chat*-expressing cells (Fig. 12A,C,D). The VTn contains both GABAergic and glutamatergic markers, but the ATn expresses only *vglut2.1* with a clear absence of GABAergic staining within the nucleus and prominent staining surrounding it (Fig. 12A,D,E). The TPp contains both glutamatergic and GABAergic cells, and the PAG expresses primarily GABAergic markers (Fig. 12A,E,F). Cells expressing *vglut3* are not found in any SDMN regions (Table 2).

DISCUSSION

This study provides an overview of the distribution of glutamatergic, GABAergic, and cholinergic cells in the brain of the emerging model cichlid fish, *Astatotilapia burtoni*, and represents one of the most complete descriptions to date of brain localization patterns using

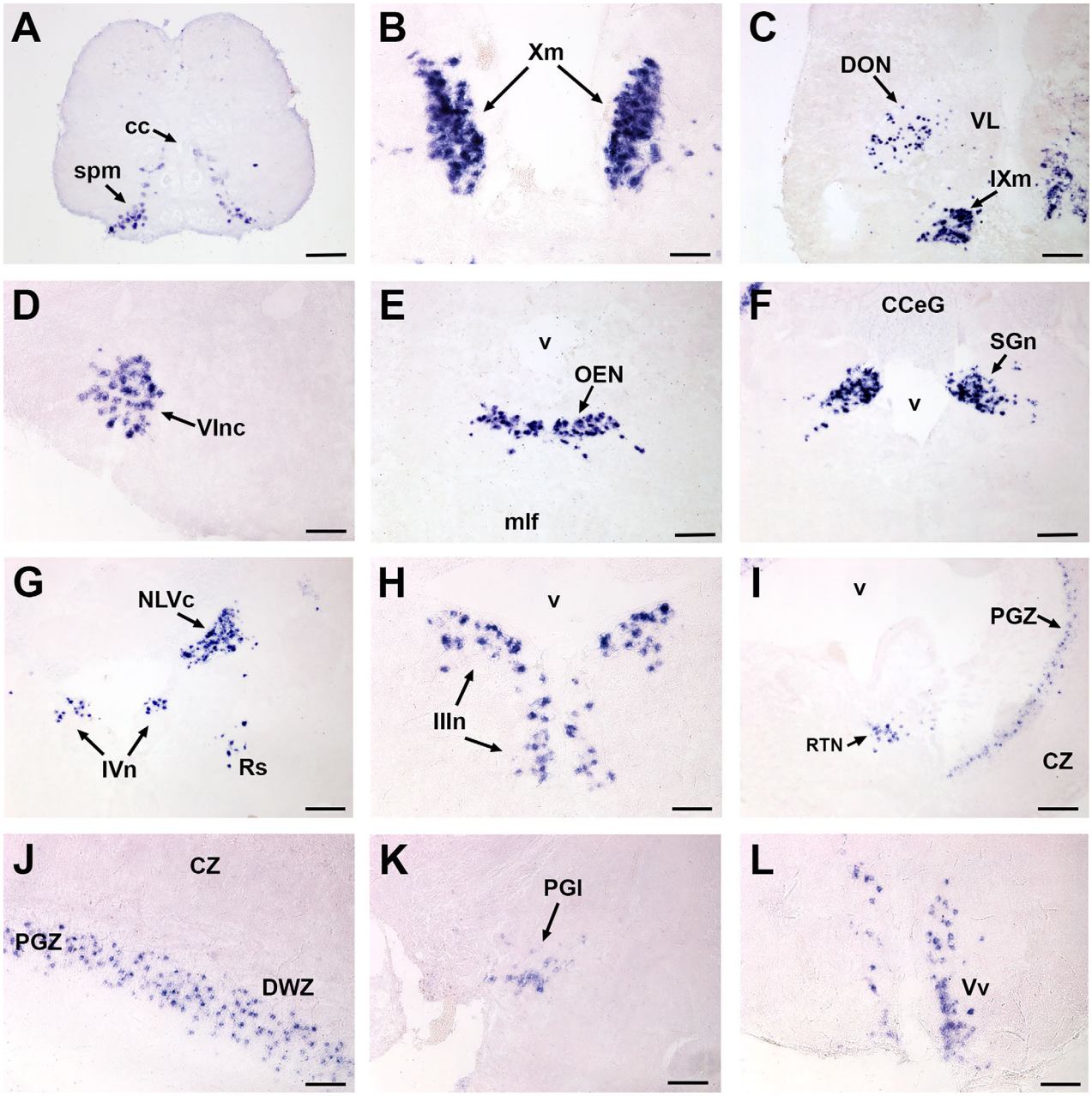

Figure 11. Representative brightfield photomicrographs of choline acetyltransferase (*chat*)-expressing cells in the brain of *Astatotilapia burtoni*. **A**: In the rostral spinal cord, *chat* expression is found ventrally in large spinal motor neurons (spm). **B**: The vagal motor nucleus (Xm) contains intense *chat* label. **C**: The glossopharyngeal motor nucleus (IXm) and descending octaval nucleus (DON) located lateral to the vagal lobe (VL) contain numerous *chat*-expressing cells. **D**: *chat* labeling in the nucleus of the abducens nerve, caudal division (VInc). **E**: Large *chat*-expressing cells in the octavolateralis efferent nucleus (OEN). **F**: *chat* expression in the secondary gustatory nucleus (SGn). **G**: *chat*-expressing cells in the trochlear nucleus (IVn), caudal region of the lateral valvular nucleus (NLVc), and superior reticular formation nucleus (Rs). **H**: *chat* expression in the oculomotor nucleus (IIIn). **I**: *chat*-expressing cells in the putative rostral tegmental nucleus (RTN). **J**: *chat*-expressing cells are abundant in the periventricular gray zone (PGZ) of the tectum. **K**: The lateral zone of preglomerular nucleus (PGl) contains scattered *chat*-labeled cells. **L**: *chat*-expressing cells are found along the ventricle within the ventral part of the ventral telencephalon (Vv). Photomicrographs were taken from 20-μm transverse sections and are depicted from caudal to rostral (A–L). See list for abbreviations. Scale bars = 50 μm in B,D,H,J,K,L; 100 μm in A,C,E,F,G,I. [Color figure can be viewed at wileyonlinelibrary.com]

these multiple neurotransmitter markers in any teleost fish. Our results also demonstrate largely nonoverlapping distributions of GABAergic and glutamatergic cells in many brain regions, including those of the conserved social decision-making network. This pattern suggests that these markers can be used to help identify specific

TABLE 2.

Localization of GABAergic, Glutamatergic, and Cholinergic Markers Within Nuclei of the Social Decision-Making Network (SDMN) of *Astatotilapia burtoni*

SDMN nucleus	*gad1*	*gad2*	*vglut1*	*vglut2.1*	*vglut3*	*chat*	Putative mammalian homolog
Dm	+	+	++	+++	–	–	Pallial amygdala
Dl	+	+	+++	+++	–	–	Medial pallium/hippocampus
Vv	+++	+++	–	+	–	++	Septum
Vd	+++	+++	–	–	–	–	Striatum/basal ganglia/nucleus accumbens
Vc	++	+++	–	–	–	–	Striatum
Vs/Vp	+++	+++	–	–	–	–	Basal/central/extended amygdala
POA	+++	+++	–	+	–	+	Preoptic area
VTn	++	++	–	++	–	–	Anterior hypothalamus
ATn	–	–	–	+++	–	–	Ventromedial hypothalamus
TPp	+++	+++	–	++	–	–	Ventral tegmental area
PAG	++	++	–	–	–	–	Periaqueductal gray

–, absent; +, low density; ++, moderate density; +++, high density. See list for abbreviations. Note that putative mammalian homologs are only "in part" for many nuclei and are based on consensus from the following references (Wullimann and Mueller, 2004; Forlano and Bass, 2011; O'Connell and Hofmann, 2011; Ganz et al., 2012; Maximino et al., 2012; Demski, 2013; Goodson and Kingsbury, 2013; Ganz et al., 2014).

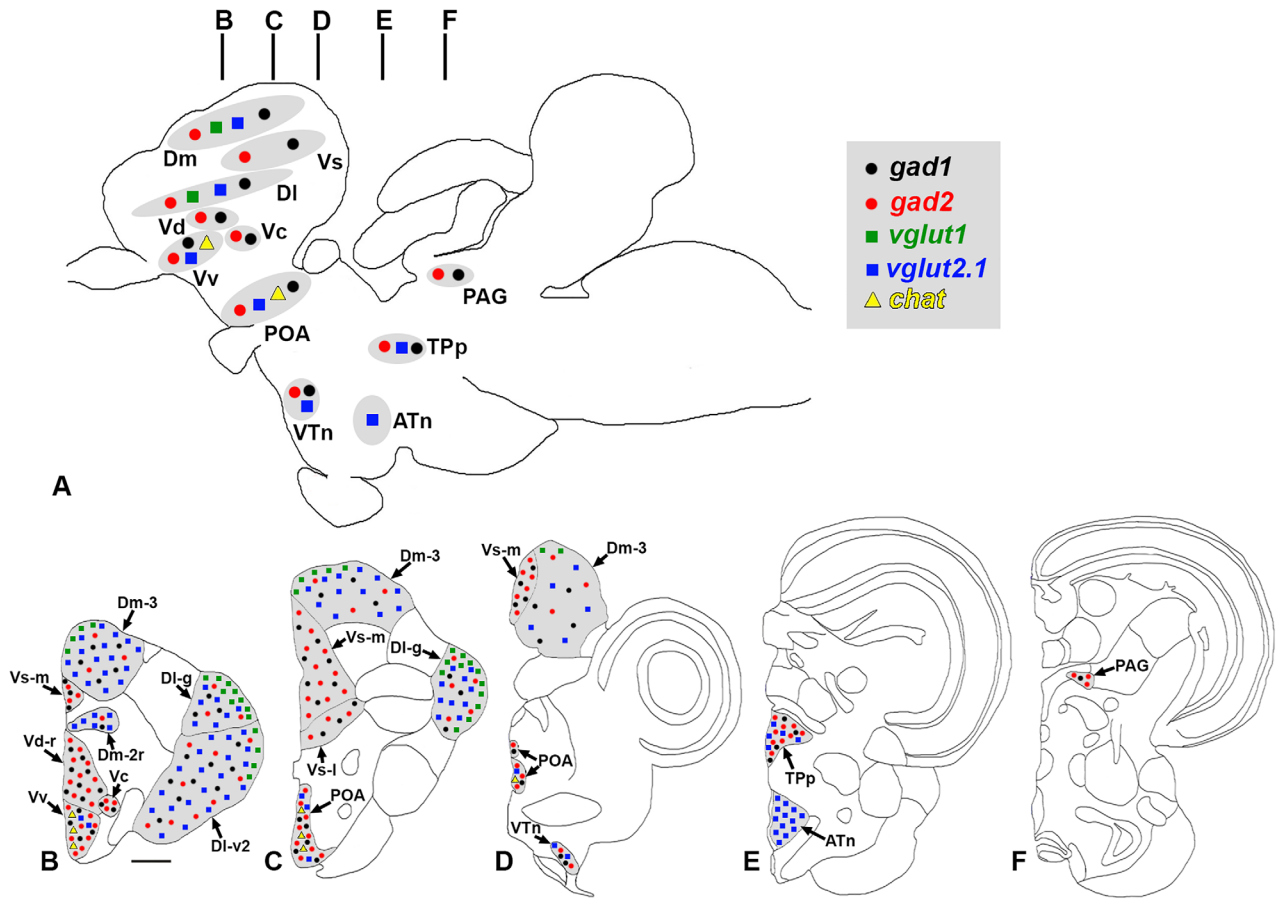

Figure 12. Summary distribution patterns of GABAergic (*gad1, gad2*), glutamatergic (*vglut1, vglut2.1*), and cholinergic (*chat*)-expressing cells within nuclei of the social decision making network (SDMN) of *Astatotilapia burtoni*. **A**: Sagittal view of the brain with the locations of each SDMN nucleus indicated by gray ovals. SDMN nuclei are positioned to minimize overlap for visualization purposes and therefore locations are only approximate. Colored symbols represent presence and absence of each marker within each nucleus. **B–F**: Representations of transverse sections containing SDMN regions (gray) from rostral (B) to caudal (F). Right half of brain is shown and the location of each transverse section is indicated in A. Symbols indicate the relative density of labeled cells for *gad1* (black dots), *gad2* (red dots), *vglut1* (green squares), *vglut2.1* (blue squares), and *chat* (yellow triangles). See list for abbreviations. Scale bar = 250 μm.

brain regions in other teleost fishes to facilitate a better understanding of neural circuitry and nuclei functions across species.

Distribution of glutamatergic cells

Glutamate is the most abundant excitatory transmitter in the nervous system of vertebrates, and packaging of glutamate by vGluts into synaptic vesicles for release is crucial for function of neural circuits and essential for life (Takamori, 2006; Liguz-Lecznar and Skangiel-Kramska, 2007; Wallen-Mackenzie et al., 2010; El Mestikawy et al., 2011). Here we provide the first complete description of the distribution of *vgluts 1–3* in the brain of any fish species. Cells expressing the glutamatergic markers *vgluts 1–3* showed different distribution patterns within the brain of *A. burtoni*, with the most widespread expression observed for *vglut2.1*, and more restricted expression of *vglut1* and *vglut3*. While there is little information on the differential distribution of the three *vgluts* in other teleosts for comparison, this overall pattern shows similarities to that of mammals (Kaneko et al., 2002; Gras et al., 2005; Vigneault et al., 2015), suggesting it is relatively well-conserved in vertebrates. While all three vGluts facilitate transport of cytoplasmic glutamate into synaptic vesicles, their complementary but largely nonoverlapping distribution patterns in the brain, as well as their distinct subcellular localization, helps define subsets of excitatory glutamatergic neurons in the vertebrate CNS (Kaneko and Fujiyama, 2002; Gras et al., 2005; Takamori, 2006; Liguz-Lecznar and Skangiel-Kramska, 2007). Thus, differential expression of *vgluts 1-3* throughout the cichlid brain provides putative additional levels of control over excitatory synapses.

In mammals, *VGLUT1* is predominantly expressed in forebrain regions such as the neocortex, piriform and entorhinal cortex, striatum, amygdala, and hippocampus (Liguz-Lecznar and Skangiel-Kramska, 2007). Similarly, in *A. burtoni, vglut1* is expressed in subdivisions of Dl (homologous in part to the medial pallium, hippocampus) and other dorsal telencephalon regions (e.g., Dm, Dd) that may be homologous to pallial regions of the mammalian brain (Ganz et al., 2014). There is evidence in mammals that VGLUT1 in these regions is associated with emotional and behavioral functions. For example, cognitive impairment in mice was observed after selective reduction of *VGLUT1* in the hippocampus (King et al., 2014), and mice deficient in *VGLUT1* show social memory deficits and schizophrenia-like behavioral abnormalities (Inta et al., 2012). Further, antidepressants were shown to increase *VGLUT1* mRNA expression in the hippocampus and cortical regions of mammals (Moutsimilli et al., 2005), suggesting it may be a useful marker for antidepressant activity. Thus, our localization of *vglut1*-expressing cells in similar regions of the teleost brain suggest the roles of this subpopulation of glutamatergic neurons in behaviors relevant to social interactions such as anxiety, sensory perception, and learning and memory may extend to fishes as well.

There were also prominent *vglut1*-expressing cells along the ventricular midline and outer brain surface of the dorsal telencephalon in *A. burtoni* (e.g., Dm, Dl, Dd). These are the same areas that serve as cell proliferation zones and contain glial cells in *A. burtoni* and other teleosts (Forlano et al., 2001; Zupanc and Sirbulescu, 2011; Maruska et al., 2012), and is similar to the "pattern D-type" pallial distribution of transcription regulators shown in the zebrafish brain (Diotel et al., 2015). Thus, it is also possible that some of the *vglut1*-expressing cells in the *A. burtoni* forebrain contribute to neurogenesis, cell differentiation, or are glial cells. Exocytosis of glutamate from glial cells can modify synaptic transmission across vertebrates, and VGLUT1 is expressed in astrocytes in the hippocampus, striatum, and frontal cortex of mammals (Ormel et al., 2012). Future studies are needed, however, to test whether *vglut1* serves any role in gliotransmission or neurogenic niches in fishes.

In *A. burtoni, vglut1*-expressing cells were also abundant in the granular layer of both the corpus and valvula cerebelli, which is consistent with that found in zebrafish (Hamling et al., 2015) and mammals (Hashimoto and Hibi, 2012). Specifically, VGLUT1 is a presynaptic marker for the parallel fiber to Purkinje cell synapse that originates from the granule cells and is conserved in the vertebrate cerebellum.

Cells expressing *vglut2.1* showed the most widespread distribution pattern in *A. burtoni*, and were localized to regions that spanned from the olfactory bulb to the hindbrain. While this pattern shows some similarities to that seen in mammals and birds (Ni et al., 1995; Gras et al., 2005; Islam and Atoji, 2008), there is only limited information on *vglut2* expression in the teleost brain for comparison (Higashijima et al., 2004; Filippi et al., 2014). In zebrafish, *vglut2*-expression (mixed riboprobes for *vglut2a/vglut2b*) was found in the posterior tuberculum, where it was coexpressed in dopaminergic neurons detected via tyrosine hydroxylase (rate-limiting enzyme in catecholamine synthesis used as a dopaminergic marker) immunoreactivity (Filippi et al., 2014). The periventricular region of the posterior tuberculum (TPp) of teleost fishes is thought to be homologous in part to the ventral tegmental area (VTA) of mammals, and is part of the mesolimbic reward system and the SDMN (O'Connell and Hofmann, 2011), although there is some disagreement about this homology (Tay et al., 2011; Yamamoto and Vernier, 2011; Goodson and Kingsbury, 2013). In

mammals, this region is characterized by dopaminergic neurons, GAD-expressing neurons, and VGLUT2-expressing neurons, with evidence for coexpression of some of these transmitters in the same cells (Morales and Root, 2014). Conditional knockout of *VGLUT2*-expressing dopamine neurons in the mouse VTA also caused memory deficits due to disruption of projections from these neurons to the hippocampus (Nordenankar et al., 2015). We also demonstrate that the TPp of the cichlid contains *gad1/gad2*-expressing cells and *vglut2.1*-expressing cells, and a previous study showed that this region contains dopaminergic tyrosine hydroxylase-immunoreactive neurons (O'Connell et al., 2011). It is possible, therefore, that the TPp is similar to the VTA of mammals in that it is a heterogeneous region with subpopulations of neurons involved in diverse functions such as motivation, reward processing, learning and memory, and sensory function. Functional studies on the TPp in fishes, however, are lacking.

The abundant and widespread distribution of *vglut2.1* indicates it is the main vesicular transporter for the majority of glutamatergic synapses throughout the cichlid brain, as described in other vertebrates (Liguz-Lecznar and Skangiel-Kramska, 2007; Atoji, 2011; Vigneault et al., 2015). In mammals, however, while *VGLUT1* expression is abundant in rostral brain regions such as the cerebral cortex and hippocampus, *VGLUT2* expression is most abundant in more caudal diencephalic and brainstem regions such as the thalamus, hypothalamus, amygdaloid nuclei, lower brainstem, and cerebellar nuclei (Fremeau et al., 2001; Liguz-Lecznar and Skangiel-Kramska, 2007). This pattern differs somewhat in the cichlid, where in addition to *vglut2.1* expression in diencephalic, mesencephalic, and rhombencephalic regions, this transporter was also abundant in rostral telencephalic regions in a pattern that resembles that of both *VGLUT1* and *VGLUT2* in mammals. For example, *vglut2.1* in the cichlid was abundant in pallial telencephalic nuclei and essentially absent from subpallial nuclei, a pattern that was opposite that of the GABAergic markers *gad1* and *gad2*. Similar abundant telencephalic *VGLUT2* expression was also seen in the avian pallium, with absence of *VGLUT2* in subpallial regions (Islam and Atoji, 2008; Atoji, 2011), suggesting that *vglut2* expression in fishes is more similar to birds than mammals. Additional studies in other vertebrate taxa (e.g., amphibians, reptiles) are needed, however, to understand the significance of these differences in VGLUT expression and function. Further, while the neurotransmitter phenotypes of this subpallium-pallium distinction are similar across vertebrates, the reason for a predominant glutamatergic pallium and GABAergic subpallium remains unknown.

In *A. burtoni, vglut3* showed restricted expression to the hypothalamus, raphe nucleus, and inferior olive. In the vertebrate brain, vGlut3 is found primarily in GABAergic, serotonergic and cholinergic neurons that do not release glutamate as their primary transmitter, where it may play a role in stimulating vesicular uptake of primary transmitters such as serotonin and acetylcholine into synaptic vesicles (i.e., vesicular synergy) (Fremeau et al., 2002; Schafer et al., 2002; Gras et al., 2008; Amilhon et al., 2010). This pattern of *vglut3* expression in putative GABAergic (NRL, NLT) and serotonergic (raphe) neurons was also seen here in the cichlid, although double-labeling studies are needed to confirm coexpression. *VGLUT3*-knockout mice also show increased anxiety-like behaviors, which are linked to altered serotonergic signaling (Amilhon et al., 2010). In mammalian GABAergic neurons that express *VGLUT3*, however, there is also evidence for corelease of glutamate and GABA from the same synaptic vesicles (Zimmermann et al., 2015). Since this corelease from *VGLUT3*-expressing GABAergic synapses likely has postsynaptic effects that differ from classical GABAergic synapses, there may be subpopulations of GABAergic neurons within individual hypothalamic nuclei of the cichlid. In mammals, *VGLUT3* is also found in the arcuate nucleus (putative homolog of NLT in fishes) and may play a role in metabolism and energy balance (Collin et al., 2003). Further, there is also evidence that *VGLUT3*-containing synapses provide cotemporal transmitter release with glutamate as a fast excitatory transmitter and other transmitters like serotonin acting as a postexcitatory "brake" (Schafer et al., 2002). This mechanism adds a layer of fine-tuning and may contribute selective and target-specific synapses to the neural circuitry that regulates rapidly changing motivational and behavioral states.

In *A. burtoni, vglut3*-expressing cells were also observed in the inferior olive (IO), a hindbrain nucleus involved in motor control. The axons of the inferior olivary neurons supply the climbing fibers to the cerebellum that synapse on proximal dendrites of Purkinje cells. Studies in mammals and zebrafish demonstrate that the IO contains *vglut2*-positive glutamatergic neurons (Hioki et al., 2003; Bae et al., 2009; Takeuchi et al., 2015). While *vglut3* was not detected in the brain of developing zebrafish, it is expressed in hair cells of the inner ear and mechanosensory neuromasts where it is required for synaptic transmission (Obholzer et al., 2008). Thus, *vglut3*-expressing cells in the IO of *A. burtoni* is consistent with the conserved glutamatergic nature of the vertebrate IO, but absence of *vglut2.1* in this region suggests there may be a subpopulation of IO neurons in the cichlid that coexpresses another transmitter type.

Distribution of GABAergic cells

In *A. burtoni, gad1*- and *gad2*-expressing cells were abundant throughout the brain in a distribution pattern that is in general agreement with that reported for other fish species that used techniques of in situ hybridization to detect *gad* genes (Anglade et al., 1999; Martyniuk et al., 2007; Trabucchi et al., 2008; Mueller and Guo, 2009) as well as immunohistochemistry to detect GABA itself (Martinoli et al., 1990; Medina et al., 1994).

Our findings of dense GAD staining in the subpallium, but scarce staining in the pallium, are also consistent with studies in zebrafish (Mueller and Guo, 2009; Mueller et al., 2011) as well as some tetrapods (Barale et al., 1996; Katarova et al., 2000), suggesting that *gad1/gad2* can be used as a subpallial marker to facilitate identification of septal and striatal nuclei in other teleosts.

The distribution of *gad1* and *gad2* was also largely overlapping in *A. burtoni*, and in most vertebrates, both *gad* genes are coexpressed in most GABAergic neurons (Esclapez et al., 1994; Trabucchi et al., 2008). Studies in mammals show that both GAD isoforms can synthesize GABA, but GAD1 appears to preferentially synthesize cytoplasmic GABA for metabolic purposes, while GAD2 primarily regulates the vesicular pool for release (Soghomonian and Martin, 1998). Thus, the level of expression of each GAD isoform can show regional and cellular differences that allow more flexibility in GABA-mediated neurotransmission (Feldblum et al., 1993; Soghomonian and Martin, 1998).

The anterior tuberal nucleus (ATn) in *A. burtoni* was noticeably devoid of GABAergic cells, but *gad1/gad2*-expressing cells were found surrounding this nucleus, which is similar to other fishes (Anglade et al., 1999; Mueller and Guo, 2009). The ATn, in part, is thought to be the putative teleostean homolog of the mammalian ventromedial hypothalamus (VMH), a distinction based on location, expression of sex-steroid receptors, and connections to the POA and telencephalon (Folgueira et al., 2004; Forlano et al., 2005; Goodson, 2005; O'Connell and Hofmann, 2011). Since the VMH of mammals is also surrounded by GAD-expressing cells with an absence of cells inside the nucleus (Tobet et al., 1999), this similar distribution pattern in fishes provides further support for the teleost ATn as a partial VMH homolog. While the mammalian VMH does not contain GAD-expressing cells, it does contain GABAergic fibers (partially from connections with other *gad1/gad2*-expressing SDMN nuclei) and GABA-A and GABA-B receptors, and several VMH-mediated functions are altered by manipulations of the GABAergic system (Ogawa et al., 1991; Tobet et al., 1999; Chan et al., 2013). The VMH is implicated in many diverse homeostatic and behavioral functions in mammals, including social behaviors such as aggression and mating (Canteras et al., 1994; Gao and Horvath, 2008; Falkner and Lin, 2014).

Distribution of cholinergic cells

Many cholinergic cell groups found in the cichlid brain are highly conserved across all vertebrates (e.g., cranial and spinal motor nuclei, isthmal nucleus, SGn, tegmentum) (Rodriguez-Moldes et al., 2002). Further, the overall distribution of *chat*-expressing cells in *A. burtoni* is similar to that described in other teleost fishes (e.g., zebrafish, minnow, midshipman, trout, goldfish) (Ekström, 1987; Brantley and Bass, 1988; Perez et al., 2000; Rodriguez-Moldes et al., 2002; Clemente et al., 2004; Mueller et al., 2004; Giraldez-Perez et al., 2009). In contrast to zebrafish, trout, and minnow (Ekström, 1987; Perez et al., 2000; Mueller et al., 2004), however, in which telencephalic ChAT-expressing cells are found in the Vl, *A. burtoni* telencephalic *chat* cells are found in Vv (but not in Vl), a distribution that is similar to that described in Vv of midshipman fish (Brantley and Bass, 1998). These species-specific differences are not surprising, given the diversity of fishes, but further studies are needed to determine any functional significance. Nevertheless, the cholinergic neurons in these nuclei of the fish subpallium are likely homologous to cholinergic septal neuron populations in tetrapods and represent a well-conserved cell group found in fishes, amphibians, reptiles, birds, and mammals (Rodriguez-Moldes et al., 2002). These cholinergic populations in septal nuclei are implicated in diverse functions including learning and memory, attention, arousal, and cognitive behavioral decisions (Mufson et al., 2003; Lin et al., 2015).

The only other forebrain nucleus that contained a few scattered *chat*-expressing cells in *A. burtoni* was the parvocellular preoptic area. These cholinergic cells are also found in other teleosts (Ekström, 1987; Perez et al., 2000; Mueller et al., 2004), as well as tetrapods, representing another well-conserved cell group (Rodriguez-Moldes et al., 2002). There is evidence in mammals that cholinergic circuits in the preoptic area control male and female sexual behaviors (Floody et al., 2011; Floody, 2014), as well as influence activity of the reproductive axis via inputs to gonadotropin-releasing hormone neurons (Turi et al., 2008). Thus, cholinergic neurons in the cichlid preoptic area may also play roles in mediating both social behavior and physiological changes related to reproduction and transitions between subordinate and dominant status, which occur

rapidly in this species (Maruska and Fernald, 2014; Maruska, 2015).

The habenular-interpeduncular pathway connects limbic areas of the forebrain and midbrain and is also a well-conserved cholinergic system across vertebrates. However, demonstration of *chat*-expressing cells in the habenula of different fish species is variable. Similar to the minnow and midshipman fish (Ekström, 1987; Brantley and Bass, 1988), *chat*-expressing neurons were absent from the habenula of *A. burtoni*, but zebrafish (Mueller et al., 2004), goldfish (Villani et al., 1994), and trout (Perez et al., 2000) do contain habenular cholinergic cells, which is similar to that seen in higher vertebrates (Contestabile et al., 1987). In many fishes, however, the bilaterally paired habenular nuclei show left–right asymmetries, and a recent study demonstrated that the dorsal habenular nucleus in zebrafish contains a discrete *chat*-expressing subnucleus characterized by a duplicated cholinergic gene locus (Hong et al., 2013). The discovery of additional cholinergic genes that show left–right lateralization in the zebrafish dorsal habenular nucleus suggests that this may also be the case in other teleosts in which cholinergic cells remain undetected in the habenula, including *A. burtoni*.

Summary of neurotransmitter markers within SDMN nuclei

Here we provide the most complete description to date of the localization patterns of glutamatergic, GABAergic, and cholinergic cells in the brain of a single teleost fish species. While we use the teleost terminology to discuss current putative homologies for SDMN nuclei in this article (Table 2), readers are reminded that many homologies between teleosts and other vertebrates are still controversial and require future studies. With this caveat in mind, many SDMN nuclei in the cichlid express multiple transmitter markers, but most show a predominance of either GABAergic (Vv, Vc, Vd, Vs/Vp, POA, PAG) or glutamatergic expression (Dm, Dl, ATn), with only the TPp and VTn showing a more mixed expression pattern. It is also important to note, however, that most brain nuclei contain heterogeneous subpopulations of neurons that express different neurotransmitters as well as other modulatory compounds such as neuropeptides, biogenic amines, and neurosteroids that can influence neural circuit function on different temporal scales. Cholinergic neurons were sparse within SDMN regions themselves, but their role as output motor neurons to the skeletal muscles are equally important for the ultimate expression of context-appropriate social behaviors. While identifying the locations of these neurotransmitter markers within SDMN and other relevant processing regions is an important first step, examining the distribution of different receptor types within these regions and the connections with other nuclei are also needed to better understand the inputs and outputs relevant to behavioral outcomes. Although not examined here, there is some evidence in mammals for differential expression levels of neurotransmitters in some SDMN regions related to social context or status that warrants further investigation across taxa (Jasnow and Huhman, 2001; Choi et al., 2006). We also propose that while the SDMN represents a solid framework for examining the neural control of social decisions in an evolutionary context, we should also take a broader approach towards understanding how context-appropriate behaviors are produced by including analyses of sensory and motor processing circuits.

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CONFLICT OF INTEREST

The authors have no known or potential conflicts of interest.

ROLE OF AUTHORS

All authors had full access to all of the data, take responsibility for the integrity of the data analysis, and approved the final article. Designed experiments: KPM, JMB, KEF, DTP. Performed experiments, collected and analyzed data: KPM, JMB, KEF, DTP. Wrote and edited the article: KPM, JMB, KEF, DTP. Provided funding, equipment, reagents, and supplies: KPM.

LITERATURE CITED

Amilhon B, Lepicard E, Renoir T, Mongeau R, Popa D, Poirel O, Miot S, Gras C, Gardier AM, Gallego J, Hamon M, Lanfumey L, Gasnier B, Giros B, El Mestikawy S. 2010. VGLUT3 (vesicular glutamate transporter type 3) contribution to the regulation of serotonergic transmission and anxiety. J Neurosci 30:2198–2210.

Anglade I, Mazurais D, Douard V, Le Jossic-Corcos C, Mananos EL, Michel D, Kah O. 1999. Distribution of glutamic acid decarboxylase mRNA in the forebrain of the rainbow trout as studied by in situ hybridization. J Comp Neurol 410:277–289.

Atoji Y. 2011. Immunohistochemical localization of vesicular glutamate transporter 2 (vGluT2) in the central nervous system of the pigeon (Columba livia). J Comp Neurol 519:2887–2905.

Bae YK, Kani S, Shimizu T, Tanabe K, Nojima H, Kimura Y, Higashijima S, Hibi M. 2009. Anatomy of zebrafish cerebellum and screen for mutations affecting its development. Dev Biol 330:406–426.

Barale E, Fasolo A, Girardi E, Artero C, Franzoni MF. 1996. Immunohistochemical investigation of gamma-aminobutyric acid ontogeny and transient expression in

the central nervous system of Xenopus laevis tadpoles. J Comp Neurol 368:285–294.
Ben-Ari Y, Khalilov I, Kahle KT, Cherubini E. 2012. The GABA excitatory/inhibitory shift in brain maturation and neurological disorders. Neuroscientist 18:467–486.
Bosma PT, Blazquez M, Collins MA, Bishop JD, Drouin G, Priede IG, Docherty K, Trudeau VL. 1999. Multiplicity of glutamic acid decarboxylases (GAD) in vertebrates: molecular phylogeny and evidence for a new GAD paralog. Mol Biol Evol 16:397–404.
Braida D, Ponzoni L, Martucci R, Sparatore F, Gotti C, Sala M. 2014. Role of neuronal nicotinic acetylcholine receptors (nAChRs) on learning and memory in zebrafish. Psychopharmacology 231:1975–1985.
Brantley RK, Bass AH. 1988. Cholinergic neurons in the brain of a teleost fish (Porichthys notatus) located with a monoclonal antibody to choline acetyltransferase. J Comp Neurol 275:87–105.
Brawand D, Wagner CE, Li YI, Malinsky M, Keller I, Fan S, Simakov O, Ng AY, Lim ZW, Bezault E, Turner-Maier J, Johnson J, Alcazar R, Noh HJ, Russell P, Aken B, Alfoldi J, Amemiya C, Azzouzi N, Baroiller JF, Barloy-Hubler F, Berlin A, Bloomquist R, Carleton KL, Conte MA, D'Cotta H, Eshel O, Gaffney L, Galibert F, Gante HF, Gnerre S, Greuter L, Guyon R, Haddad NS, Haerty W, Harris RM, Hofmann HA, Hourlier T, Hulata G, Jaffe DB, Lara M, Lee AP, MacCallum I, Mwaiko S, Nikaido M, Nishihara H, Ozouf-Costaz C, Penman DJ, Przybylski D, Rakotomanga M, Renn SC, Ribeiro FJ, Ron M, Salzburger W, Sanchez-Pulido L, Santos ME, Searle S, Sharpe T, Swofford R, Tan FJ, Williams L, Young S, Yin S, Okada N, Kocher TD, Miska EA, Lander ES, Venkatesh B, Fernald RD, Meyer A, Ponting CP, Streelman JT, Lindblad-Toh K, Seehausen O, Di Palma F. 2014. The genomic substrate for adaptive radiation in African cichlid fish. Nature 513:375–381.
Burmeister SS, Munshi RG, Fernald RD. 2009. Cytoarchitecture of a cichlid fish telencephalon. Brain Behav Evol 74: 110–120.
Canteras NS, Simerly RB, Swanson LW. 1994. Organization of projections from the ventromedial nucleus of the hypothalamus: a Phaseolus vulgaris-leucoagglutinin study in the rat. J Comp Neurol 348:41–79.
Chan O, Paranjape SA, Horblitt A, Zhu W, Sherwin RS. 2013. Lactate-induced release of GABA in the ventromedial hypothalamus contributes to counterregulatory failure in recurrent hypoglycemia and diabetes. Diabetes 62: 4239–4246.
Clemente D, Porteros A, Weruaga E, Alonso JR, Arenzana FJ, Aijon J, Arevalo R. 2004. Cholinergic elements in the zebrafish central nervous system: Histochemical and immunohistochemical analysis. J Comp Neurol 474:75–107.
Collin M, Backberg M, Ovesjo ML, Fisone G, Edwards RH, Fujiyama F, Meister B. 2003. Plasma membrane and vesicular glutamate transporter mRNAs/proteins in hypothalamic neurons that regulate body weight. Eur J Neurosci 18:1265–1278.
Contestabile A, Villani L, Fasolo A, Franzoni MF, Gribaudo L, Oktedalen O, Fonnum F. 1987. Topography of cholinergic and substance P pathways in the habenulo-interpeduncular system of the rat. An immunocytochemical and microchemical approach. Neuroscience 21:253–270.
Dewan AK, Tricas TC. 2014. Cytoarchitecture of the telencephalon in the coral reef multiband butterflyfish (*Chaetodon multicinctus* : Perciformes). Brain Behav Evol 84: 31–50.
Diotel N, Rodriguez Viales R, Armant O, Marz M, Ferg M, Rastegar S, Strahle U. 2015. Comprehensive expression map of transcription regulators in the adult zebrafish telencephalon reveals distinct neurogenic niches. J Comp Neurol 523:1202–1221.
Eckenstein F, Thoenen H. 1983. Cholinergic neurons in the rat cerebral cortex demonstrated by immunohistochemical localization of choline acetyltransferase. Neurosci Lett 36:211–215.
Egozi Y, Kloog Y, Sokolovsky M. 1986. Acetylcholine rhythm in the preoptic area of the rat hypothalamus is synchronized with the estrous cycle. Brain Res 383:310–313.
Ekström P. 1987. Distribution of choline acetyltransferase-immunoreactive neurons in the brain of a cyprinid teleost (*Phoxinus phoxinus* L.). J Comp Neurol 256:494–515.
El Mestikawy S, Wallen-Mackenzie A, Fortin GM, Descarries L, Trudeau LE. 2011. From glutamate co-release to vesicular synergy: vesicular glutamate transporters. Nat Rev Neurosci 12:204–216.
Esclapez M, Tillakaratne NJ, Kaufman DL, Tobin AJ, Houser CR. 1994. Comparative localization of two forms of glutamic acid decarboxylase and their mRNAs in rat brain supports the concept of functional differences between the forms. J Neurosci 14(3 Pt 2):1834–1855.
Falkner AL, Lin D. 2014. Recent advances in understanding the role of the hypothalamic circuit during aggression. Front Syst Neurosci 8:168.
Feldblum S, Erlander MG, Tobin AJ. 1993. Different distributions of GAD65 and GAD67 mRNAs suggest that the two glutamate decarboxylases play distinctive functional roles. J Neurosci Res 34:689–706.
Fernald RD, Maruska KP. 2012. Social information changes the brain. Proc Natl Acad Sci U S A 109(Suppl 2):17194–17199.
Fernald RD, Shelton LC. 1985. The organization of the diencephalon and the pretectum in the cichlid fish, *Haplochromis burtoni*. J Comp Neurol 238:202–217.
Filippi A, Mueller T, Driever W. 2014. vglut2 and gad expression reveal distinct patterns of dual GABAergic versus glutamatergic cotransmitter phenotypes of dopaminergic and noradrenergic neurons in the zebrafish brain. J Comp Neurol 522:2019–2037.
Floody OR. 2014. Role of acetylcholine in control of sexual behavior of male and female mammals. Pharmacol Biochem Behav 120:50–56.
Floody OR, Katin MJ, Harrington LX, Schassburger RL. 2011. Cholinergic control of male mating behavior in hamsters: effects of central oxotremorine treatment. Pharmacol Biochem Behav 100:299–310.
Folgueira M, Anadon R, Yanez J. 2004. Experimental study of the connections of the telencephalon in the rainbow trout (*Oncorhynchus mykiss*). II: Dorsal area and preoptic region. J Comp Neurol 480:204–233.
Forlano PM, Deitcher DL, Myers DA, Bass AH. 2001. Anatomical distribution and cellular basis for high levels of aromatase activity in the brain of teleost fish: aromatase enzyme an mRNA expression identify glia as source. J Neurosci 21:8943–8955.
Forlano PM, Deitcher DL, Bass AH. 2005. Distribution of estrogen receptor alpha mRNA in the brain and inner ear of a vocal fish with comparisons to sites of aromatase expression. J Comp Neurol 483:91–113.
Fremeau RT Jr, Burman J, Qureshi T, Tran CH, Proctor J, Johnson J, Zhang H, Sulzer D, Copenhagen DR, Storm-Mathisen J, Reimer RJ, Chaudhry FA, Edwards RH. 2002. The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. Proc Natl Acad Sci U S A 99:14488–14493.
Fremeau RT Jr, Troyer MD, Pahner I, Nygaard GO, Tran CH, Reimer RJ, Bellocchio EE, Fortin D, Storm-Mathisen J, Edwards RH. 2001. The expression of vesicular

glutamate transporters defines two classes of excitatory synapse. Neuron 31:247–260.
Ganz J, Kroehne V, Freudenreich D, Machate A, Geffarth M, Braasch I, Kaslin J, Brand M. 2014. Subdivisions of the adult zebrafish pallium based on molecular marker analysis. F1000Research 3:308.
Gao Q, Horvath TL. 2008. Neuronal control of energy homeostasis. FEBS Lett 582:132–141.
Giraldez-Perez RM, Gaytan SP, Torres B, Pasaro R. 2009. Colocalization of nitric oxide synthase and choline acetyltransferase in the brain of the goldfish (Carassius auratus). J Chem Neuroanat 37:1–17.
Gold PE. 2003. Acetylcholine modulation of neural systems involved in learning and memory. Neurobiol Learn Mem 80:194–210.
Goodson JL. 2005. The vertebrate social behavior network: evolutionary themes and variations. Horm Behav 48:11–22.
Goodson JL, Kingsbury MA. 2013. What's in a name? Considerations of homologies and nomenclature for vertebrate social behavior networks. Horm Behav 64:103–112.
Gras C, Herzog E, Bellenchi GC, Bernard V, Ravassard P, Pohl M, Gasnier B, Giros B, El Mestikawy S. 2002. A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. J Neurosci 22:5442–5451.
Gras C, Vinatier J, Amilhon B, Guerci A, Christov C, Ravassard P, Giros B, El Mestikawy S. 2005. Developmentally regulated expression of VGLUT3 during early post-natal life. Neuropharmacology 49:901–911.
Gras C, Amilhon B, Lepicard EM, Poirel O, Vinatier J, Herbin M, Dumas S, Tzavara ET, Wade MR, Nomikos GG, Hanoun N, Saurini F, Kemel ML, Gasnier B, Giros B, El Mestikawy S. 2008. The vesicular glutamate transporter VGLUT3 synergizes striatal acetylcholine tone. Nat Neurosci 11:292–300.
Grone BP, Maruska KP. 2015a. Divergent evolution of two corticotropin-releasing hormone (CRH) genes in teleost fishes. Front Neurosci 9:365.
Grone BP, Maruska KP. 2015b. A second corticotropin-releasing hormone gene (CRH2) is conserved across vertebrate classes and expressed in the hindbrain of a basal neopterygian fish, the spotted gar (*Lepisosteus oculatus*). J Comp Neurol 523:1125–1143.
Grone BP, Maruska KP. 2016. Three distinct glutamate decarboxylase genes in vertebrates. Sci Rep 6:30507.
Gutierrez R, Romo-Parra H, Maqueda J, Vivar C, Ramirez M, Morales MA, Lamas M. 2003. Plasticity of the GABAergic phenotype of the "glutamatergic" granule cells of the rat dentate gyrus. J Neurosci 23:5594–5598.
Hales TG, Sanderson MJ, Charles AC. 1994. GABA has excitatory actions on GnRH-secreting immortalized hypothalamic (GT1-7) neurons. Neuroendocrinology 59:297–308.
Hamling KR, Tobias ZJ, Weissman TA. 2015. Mapping the development of cerebellar Purkinje cells in zebrafish. Dev Neurobiol 75:1174–1188.
Han SK, Abraham IM, Herbison AE. 2002. Effect of GABA on GnRH neurons switches from depolarization to hyperpolarization at puberty in the female mouse. Endocrinology 143:1459–1466.
Hashimoto M, Hibi M. 2012. Development and evolution of cerebellar neural circuits. Dev Growth Differ 54:373–389.
Higashijima S, Mandel G, Fetcho JR. 2004. Distribution of prospective glutamatergic, glycinergic, and GABAergic neurons in embryonic and larval zebrafish. J Comp Neurol 480:1–18.
Hioki H, Fujiyama F, Taki K, Tomioka R, Furuta T, Tamamaki N, Kaneko T. 2003. Differential distribution of vesicular glutamate transporters in the rat cerebellar cortex. Neuroscience 117:1–6.
Inta D, Vogt MA, Perreau-Lenz S, Schneider M, Pfeiffer N, Wojcik SM, Spanagel R, Gass P. 2012. Sensorimotor gating, working and social memory deficits in mice with reduced expression of the vesicular glutamate transporter VGLUT1. Behav Brain Res 228:328–332.
Islam MR, Atoji Y. 2008. Distribution of vesicular glutamate transporter 2 and glutamate receptor 1 mRNA in the central nervous system of the pigeon (*Columba livia*). J Comp Neurol 511:658–677.
Kaneko T, Fujiyama F. 2002. Complementary distribution of vesicular glutamate transporters in the central nervous system. Neurosci Res 42:243–250.
Kaneko T, Fujiyama F, Hioki H. 2002. Immunohistochemical localization of candidates for vesicular glutamate transporters in the rat brain. J Comp Neurol 444:39–62.
Katarova Z, Sekerkova G, Prodan S, Mugnaini E, Szabo G. 2000. Domain-restricted expression of two glutamic acid decarboxylase genes in midgestation mouse embryos. J Comp Neurol 424:607–627.
Kaufman DL, Houser CR, Tobin AJ. 1991. Two forms of the gamma-aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. J Neurochem 56:720–723.
Kimura H, McGeer PL, Peng JH, McGeer EG. 1981. The central cholinergic system studied by choline acetyltransferase immunohistochemistry in the cat. J Comp Neurol 200: 151–201.
King MV, Kurian N, Qin S, Papadopoulou N, Westerink BH, Cremers TI, Epping-Jordan MP, Le Poul E, Ray DE, Fone KC, Kendall DA, Marsden CA, Sharp TV. 2014. Lentiviral delivery of a vesicular glutamate transporter 1 (VGLUT1)-targeting short hairpin RNA vector into the mouse hippocampus impairs cognition. Neuropsychopharmacology 39:464–476.
Lariviere K, MacEachern L, Greco V, Majchrzak G, Chiu S, Drouin G, Trudeau VL. 2002. GAD(65) and GAD(67) isoforms of the glutamic acid decarboxylase gene originated before the divergence of cartilaginous fishes. Mol Biol Evol 19:2325–2329.
Lariviere K, Samia M, Lister A, Van Der Kraak G, Trudeau VL. 2005. Sex steroid regulation of brain glutamic acid decarboxylase (GAD) mRNA is season-dependent and sexually dimorphic in the goldfish Carassius auratus. Brain Res Mol Brain Res 141:1–9.
Lee CC, Sherman SM. 2009. Glutamatergic inhibition in sensory neocortex. Cereb Cortex 19:2281–2289.
Liguz-Lecznar M, Skangiel-Kramska J. 2007. Vesicular glutamate transporters (VGLUTs): The three musketeers of glutamatergic system. Acta Neurobiol Exp 67:207–218.
Lin SC, Brown RE, Hussain Shuler MG, Petersen CC, Kepecs A. 2015. Optogenetic dissection of the basal forebrain neuromodulatory control of cortical activation, plasticity, and cognition. J Neurosci 35:13896–13903.
Lopez JM, Perlado J, Morona R, Northcutt RG, Gonzalez A. 2013. Neuroanatomical organization of the cholinergic system in the central nervous system of a basal actinopterygian fish, the senegal bichir Polypterus senegalus. J Comp Neurol 521:24–49.
Loveland JL, Uy N, Maruska KP, Carpenter RE, Fernald RD. 2014. Social status differences regulate the serotonergic system of a cichlid fish, Astatotilapia burtoni. J Exp Biol 217(Pt 15):2680–2690.
Maqueda J, Ramirez M, Lamas M, Gutierrez R. 2003. Glutamic acid decarboxylase (GAD)67, but not GAD65, is constitutively expressed during development and transiently overexpressed by activity in the granule cells of the rat. Neurosci Lett 353:69–71.

Martinoli MG, Dubourg P, Geffard M, Calas A, Kah O. 1990. Distribution of GABA-immunoreactive neurons in the forebrain of the goldfish, Carassius auratus. Cell Tissue Res 260:77–84.

Martyniuk CJ, Awad R, Hurley R, Finger TE, Trudeau VL. 2007. Glutamic acid decarboxylase 65, 67, and GABA-transaminase mRNA expression and total enzyme activity in the goldfish (Carassius auratus) brain. Brain Res 1147: 154–166.

Maruska KP. 2015. Social transitions cause rapid behavioral and neuroendocrine changes. Integr Comp Biol 55:294–406.

Maruska KP, Fernald RD. 2013. Social regulation of male reproductive plasticity in an African cichlid fish. Integr Comp Biol 207:2–12.

Maruska KP, Fernald RD. 2014. Social regulation of gene expression in the African cichlid fish *Astatotilapia burtoni*. In: Canli T, editor. Handbook of molecular psychology. New York: Oxford University Press. p 52–78.

Maruska KP, Carpenter RE, Fernald RD. 2012. Characterization of cell proliferation throughout the brain of the African cichlid fish *Astatotilapia burtoni* and its regulation by social status. J Comp Neurol 520:3471–3491.

Maruska KP, Becker L, Neboori A, Fernald RD. 2013a. Social descent with territory loss causes rapid behavioral, endocrine, and transcriptional changes in the brain. J Exp Biol 216(Pt 19):3656–3666.

Maruska KP, Zhang A, Neboori A, Fernald RD. 2013b. Social opportunity causes rapid transcriptional changes in the social behavior network of the brain in an African cichlid fish. J Neuroendocrinol 25:145–157.

Medina M, Reperant J, Dufour S, Ward R, Le Belle N, Miceli D. 1994. The distribution of GABA-immunoreactive neurons in the brain of the silver eel (Anguilla anguilla L.). Anat Embryol (Berl) 189:25–39.

Morales M, Root DH. 2014. Glutamate neurons within the midbrain dopamine regions. Neuroscience 282C:60–68.

Moutsimilli L, Farley S, Dumas S, El Mestikawy S, Giros B, Tzavara ET. 2005. Selective cortical VGLUT1 increase as a marker for antidepressant activity. Neuropharmacology 49:890–900.

Mueller T. 2012. What is the thalamus in zebrafish? Front Neurosci 6:64.

Mueller T, Guo S. 2009. The distribution of GAD67-mRNA in the adult zebrafish (teleost) forebrain reveals a prosomeric pattern and suggests previously unidentified homologies to tetrapods. J Comp Neurol 516:553–568.

Mueller T, Vernier P, Wullimann MF. 2004. The adult central nervous cholinergic system of a neurogenetic model animal, the zebrafish Danio rerio. Brain Res 1011:156–169.

Mueller T, Dong Z, Berberoglu MA, Guo S. 2011. The dorsal pallium in zebrafish, *Danio rerio* (Cyprinidae, Teleostei). Brain Res 1381:95–105.

Mufson EJ, Ginsberg SD, Ikonomovic MD, DeKosky ST. 2003. Human cholinergic basal forebrain: chemoanatomy and neurologic dysfunction. J Chem Neuroanat 26:233–242.

Munchrath LA, Hofmann HA. 2010. Distribution of sex steroid hormone receptors in the brain of an African cichlid fish, *Astatotilapia burtoni*. J Comp Neurol 518:3302–3326.

Munoz-Cueto JA, Sarasquete C, Zohar AH, Kah O. 2001. An atlas of the brain of the gilthead seabream (*Sparus aurata*). College Park, MD: Maryland Sea Grant.

Ni B, Wu X, Yan GM, Wang J, Paul SM. 1995. Regional expression and cellular localization of the Na(+)-dependent inorganic phosphate cotransporter of rat brain. J Neurosci 15:5789–5799.

Nordenankar K, Smith-Anttila CJ, Schweizer N, Viereckel T, Birgner C, Mejia-Toiber J, Morales M, Leao RN, Wallen-Mackenzie A. 2015. Increased hippocampal excitability and impaired spatial memory function in mice lacking VGLUT2 selectively in neurons defined by tyrosine hydroxylase promoter activity. Brain Struct Funct 220:2171–2190.

Obholzer N, Wolfson S, Trapani JG, Mo W, Nechiporuk A, Busch-Nentwich E, Seiler C, Sidi S, Sollner C, Duncan RN, Boehland A, Nicolson T. 2008. Vesicular glutamate transporter 3 is required for synaptic transmission in zebrafish hair cells. J Neurosci 28:2110–2118.

O'Connell LA, Hofmann HA. 2011. The vertebrate mesolimbic reward system and social behavior network: A comparative synthesis. J Comp Neurol 519:3599–3639.

O'Connell LA, Hofmann HA. 2012. Evolution of a vertebrate social decision-making network. Science 336:1154–1157.

O'Connell LA, Fontenot MR, Hofmann HA. 2011. Characterization of the dopaminergic system in the brain of an African cichlid fish, Astatotilapia burtoni. J Comp Neurol 519:75–92.

O'Connell LA, Rigney MM, Dykstra DW, Hofmann HA. 2013. Neuroendocrine mechanisms underlying sensory integration of social signals. J Neuroendocrinol 25:644–654.

Ogawa S, Kow LM, Pfaff DW. 1991. Effects of GABA and related agents on the electrical activity of hypothalamic ventromedial nucleus neurons in vitro. Exp Brain Res 85:85–92.

Ormel L, Stensrud MJ, Bergersen LH, Gundersen V. 2012. VGLUT1 is localized in astrocytic processes in several brain regions. Glia 60:229–238.

Perez SE, Yanez J, Marin O, Anadon R, Gonzalez A, Rodriguez-Moldes I. 2000. Distribution of choline acetyltransferase (ChAT) immunoreactivity in the brain of the adult trout and tract-tracing observations on the connections of the nuclei of the isthmus. J Comp Neurol 428:450–474.

Picciotto MR, Higley MJ, Mineur YS. 2012. Acetylcholine as a neuromodulator: cholinergic signaling shapes nervous system function and behavior. Neuron 76:116–129.

Richardson SB, Hollander CS, D'Eletto R, Greenleaf PW, Thaw C. 1980. Acetylcholine inhibits the release of somatostatin from rat hypothalamus in vitro. Endocrinology 107: 122–129.

Rodriguez-Moldes I, Molist P, Adrio F, Pombal MA, Yanez SE, Mandado M, Marin O, Lopez JM, Gonzalez A, Anadon R. 2002. Organization of cholinergic systems in the brain of different fish groups: a comparative analysis. Brain Res Bull 57:331–334.

Schafer MK, Varoqui H, Defamie N, Weihe E, Erickson JD. 2002. Molecular cloning and functional identification of mouse vesicular glutamate transporter 3 and its expression in subsets of novel excitatory neurons. J Biol Chem 277:50734–50748.

Soghomonian JJ, Martin DL. 1998. Two isoforms of glutamate decarboxylase: why? Trends in pharmacological sciences 19:500–505.

Takamori S. 2006. VGLUTs: 'exciting' times for glutamatergic research? Neurosci Res 55:343–351.

Takeuchi M, Matsuda K, Yamaguchi S, Asakawa K, Miyasaka N, Lal P, Yoshihara Y, Koga A, Kawakami K, Shimizu T, Hibi M. 2015. Establishment of Gal4 transgenic zebrafish lines for analysis of development of cerebellar neural circuitry. Dev Biol 397:1–17.

Tay TL, Ronneberger O, Ryu S, Nitschke R, Driever W. 2011. Comprehensive catecholaminergic projectome analysis reveals single-neuron integration of zebrafish ascending and descending dopaminergic systems. Nat Commun 2: 171.

Tian N, Petersen C, Kash S, Baekkeskov S, Copenhagen D, Nicoll R. 1999. The role of the synthetic enzyme GAD65 in the control of neuronal gamma-aminobutyric acid release. Proc Natl Acad Sci U S A 96:12911–12916.

Tobet SA, Henderson RG, Whiting PJ, Sieghart W. 1999. Special relationship of gamma-aminobutyric acid to the ventromedial nucleus of the hypothalamus during embryonic development. J Comp Neurol 405:88–98.

Trabucchi M, Trudeau VL, Drouin G, Tostivint H, Ihrmann I, Vallarino M, Vaudry H. 2008. Molecular characterization and comparative localization of the mRNAs encoding two glutamic acid decarboxylases (GAD65 and GAD67) in the brain of the African lungfish, Protopterus annectens. J Comp Neurol 506:979–988.

Turi GF, Liposits Z, Hrabovszky E. 2008. Cholinergic afferents to gonadotropin-releasing hormone neurons of the rat. Neurochem Int 52:723–728.

Vigneault E, Poirel O, Riad M, Prud'homme J, Dumas S, Turecki G, Fasano C, Mechawar N, El Mestikawy S. 2015. Distribution of vesicular glutamate transporters in the human brain. Front Neuroanat 9:23.

Villani L, Guarnieri T, Zironi I. 1994. Choline acetyltransferase and NADPH-diaphorase localization in the goldfish habenulo-interpeduncular system. Neurosci Lett 173:67–70.

Wagner S, Castel M, Gainer H, Yarom Y. 1997. GABA in the mammalian suprachiasmatic nucleus and its role in diurnal rhythmicity. Nature 387:598–603.

Wallen-Mackenzie A, Wootz H, Englund H. 2010. Genetic inactivation of the vesicular glutamate transporter 2 (VGLUT2) in the mouse: what have we learnt about functional glutamatergic neurotransmission? Upsala J Med Sci 115:11–20.

Watanabe M, Fukuda A, Nabekura J. 2014. The role of GABA in the regulation of GnRH neurons. Front Neurosci 8: 387.

Wullimann MF, Mueller T. 2004. Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior. J Comp Neurol 475:143–162.

Wullimann MF, Rupp B, Reichert H. 1996. Neuroanatomy of the zebrafish brain: a topological atlas. Basel, Switzerland: Birkhauser.

Yamamoto K, Vernier P. 2011. The evolution of dopamine systems in chordates. Front Neuroanat 5:21.

Zimmermann J, Herman MA, Rosenmund C. 2015. Co-release of glutamate and GABA from single vesicles in GABAergic neurons exogenously expressing VGLUT3. Front Synap Neurosci 7:16.

Zupanc GK, Sirbulescu RF. 2011. Adult neurogenesis and neuronal regeneration in the central nervous system of teleost fish. Eur J Neurosci 34:917–929.