Localization of glutamatergic, GABAergic, and cholinergic neurons in the brain of the African cichlid fish, Astatotilapia burtoni

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Title: Localization of Glutamatergic, GABAergic, and Cholinergic Neurons in the Brain of the African Cichlid fish, Astatotilapia burtoni

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ABSTRACT

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 non-overlapping 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.
INTRODUCTION

The central nervous system 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 non-overlapping 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 (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; Lariviere et al., 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 non-mammalian 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; Maruska and Fernald, 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; Maruska et al., 2013b; 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 (12hr light:12hr 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 1x phosphate-buffered saline (1xPBS), rinsed in 1xPBS, 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 \[ \text{GSI} = \frac{(GM/BM) \times 100} \]. Brains were then embedded in OCT media (TissueTek, Sakura), sectioned in the transverse plane on a cryostat (Leica CM1850 or Cryostar NX50) at 20-µm, and collected onto 2 alternate sets of charged slides (VWR Superfrost plus). 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 paper, 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, 2015b; a). Our recent bioinformatic analyses also identified gad3 in A. burtoni, but in situ hybridization and 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), 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 min, 40 cycles of: (95°C for 15 sec, 55°C for 15 sec, 72°C for 1 min), and 72°C for 1 min. Purified PCR products (MinElute PCR kit, Qiagen) were then used as the template in the transcription reaction to incorporate digoxigenin (DIG)-labeled (DIG-labeling mix, Roche) 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 anti-sense 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, 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) and allowed to dry for 30-45 min. Sections were incubated at room temperature in the following RNAse-free solutions: 1xPBS (3x5min), 4% PFA (20 min), 1xPBS (2x5min), proteinase K (10µg/ml final conc. in proteinase K buffer; 50mM Tris-HCl pH 7.5, 5mM EDTA pH 8.0) (10min), 1xPBS (10min), 4% PFA (15min), 1xPBS (2x5min), milliQ water (1-2min), 0.1M triethanolamine-HCl pH 8.0 with 0.25% acetic anhydride (10min), and 1xPBS (5min). Slides were then incubated in pre-warmed hybridization buffer (without probe; 50% formamide, 5xSSC, 0.1% tween-20, 0.1% CHAPS, 5mM EDTA, 1mg/ml torula RNA) inside sealed humidified chambers in a hybridization oven at 60-65°C for 3hrs. 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: 2x SSC:formamide with 0.1% tween-20 (2x30min), 2x SSC:Maleate Buffer (MABT; 100mM maleic acid pH 7.2, 150mM NaCl, 0.1% tween-20) (2x15min), and MABT (2x10min). Slides were then washed in MABT at room temperature (2x10min), incubated in MABT with 2% bovine serum albumin (BSA) to block non-specific binding (3hrs), and incubated with
alkaline-phosphatase-conjugated anti-DIG Fab fragments (1:5000 dilution; Roche; RRID:AB_514497) overnight at 4°C in a sealed humidified chamber. Slides were then rinsed with MABT (3x30min) and incubated in alkaline phosphatase buffer (2x5min) 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 1-8hrs depending on the probe. Slides were then rinsed in 1xPBS (3x5min) to stop the reaction, fixed in 4% PFA (10min), washed in 1xPBS (3x5min) and coverslipped with aquamount media (Thermo-Scientific).

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 anti-sense probes, but with the T3 initiation sequence on the forward primer) were applied to one set of slides and run simultaneously with anti-sense 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 (see 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).

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 ventro-lateral 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) (Fig. 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 (Fig. 2J2; 3C). The secondary gustatory (SGn) and secondary visceral (SVn) nuclei also show *vglut1*-labeled cells (Fig. 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 (Fig. 2E2-G2;
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) (Fig. 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 (Fig. 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 (Dc) part (Fig. 2B2-C2). In contrast, no vglut1 expression is observed in any of the subpallial parts of the ventral telencephalon (Fig. 2B2-D2; 4D-I). Expression of vglut1 is also absent from the olfactory bulbs (Fig. 2A2; 4I).

vglut2.1: Expression of vglut2.1 in the brain is the most widespread of the three vgluts examined (Fig. 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 (Fig. 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 (Fig. 2H3-M3; 5B,C). The isthmal nucleus (NI) shows distinct vglut2.1-positive cells on the dorsal and medial aspects of this nucleus (Fig. 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) (Fig. 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 (Fig. 2E3-H3; 5C,D). The glomerular nucleus (Gn), as well as the preglomerular nuclei (PGm, PGc, PGl) contains distinct vglut2.1 expression (Fig. 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 (Fig. 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) (Fig. 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 NDILI (Fig. 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) (Fig. 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). In the telencephalon, vglut2.1-expressing cells are more widely
distributed than that of vglut1 (Fig. 2; 9A,B), and are abundant in pallial regions including
divisions of Dm, Dl, Dd, Dc, and Dp (Fig. 2A3-D3; 5H-I). In contrast, vglut2.1 staining is
primarily absent from parts of the subpallial ventral telencephalon (e.g., Vs, Vc, VI, Vd, Vp),
with the exception of some scattered cells observed in Vv (Fig. 2B3-D3; 5H). This
absence of vglut2.1 staining in subpallial regions is also similar to vglut1 (Fig. 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) (Fig. 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
co-expressed in serotonergic cells. In these same sections, a small population of vglut3-expressing cells is found lateral and ventral to the SR (arrows on 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).

Distribution of Glutamate Decarboxylases (gads)

gad1: Cells expressing gad1 are abundant from the hindbrain to the olfactory bulbs of A. burtoni (Fig. 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 (Fig. 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 (Fig. 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, MgON, AON, TON). All nuclei of the reticular formation (Rs, Rm, Ri) also contain prominent gad1-expressing cells (Fig. 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) (Fig. 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 (Fig. 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) (Fig. 2F4-H4; 7C,D). In the glomerular nucleus, gad1-expressing cells lie primarily surrounding or along its lateral border (Fig. 2G4; 7D).

In the diencephalon, gad1-expressing cells are abundant in hypothalamic regions such as subdivisions of NLT and NRL (Fig. 2E4-G4; 7E,F). gad1 appears absent from CM and PGc along the midline, but some scattered cells lie within PGl and PN (Fig. 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., NDILI, NDILm, NCIL, NMIL) (Fig. 2E4-H4; 7D,E). The ATn is noticeably devoid of gad1 expression (Fig. 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 (Fig. 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).

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 (Fig. 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 (Fig. 2B4-D4; 7J,K). In contrast, gad1 cells are only scattered within the pallial dorsal telencephalon such as
Dm, Dc, DI, Dp, as well as NT (Fig. 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) (Fig. 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* (Fig. 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 (Fig. 2H5-M5; 8A,B). Scattered *gad2*-expressing cells also exist throughout octavolateralis nuclei (MON, DON, MgON, TON, AON) (Fig. 2J5-L5; 8A). Nuclei of the reticular formation (Rs, Rm, Ri) also contain *gad2* cells throughout the hindbrain (Fig. 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 (Fig. 2F5-K5; 8B-D). Cells are also found throughout the eminentia granularis (EG) (Fig. 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 (Fig. 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 (Fig. 2F5-H5; 8C-D). In the Gn, *gad2*-expressing cells 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, NLLt, NLTv), NRL, and in and around NRP (Fig. 2E5-G5; 8C,F,G). While *gad2* appears absent from CM and PGc, scattered cells lie within PGl and PN (Fig. 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

\textit{gad1} (Fig. 2E5-H5; 8C,F). Similar to \textit{gad1}, the ATn is noticeably devoid of \textit{gad2}
expression, but the regions surrounding the ATn are GABAergic (Fig. 2F5; 8F; 9G,H). VTn
also contains scattered \textit{gad2} cells. The TPp shows dense \textit{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 (Fig. 2E5-F5; 8E,F), which is
also similar to \textit{gad1} (Fig. 9G,H). All preoptic nuclei show dense \textit{gad2} expression (Fig.
2C5-E5; 8I), and cells also lie in PSP (Fig. 2D5; 8I).

In the telencephalon, \textit{gad2}-expressing cells are densely stained and abundant
within all parts of the subpallial ventral telencephalon including Vv, Vd, Vc, Vi, Vs, and Vp
(Fig. 2B5-D5; 8I-K). In contrast, \textit{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) (Fig. 2A5-
D5; 8I,K,L), but are more abundant than \textit{gad1}-expressing cells in these same regions
(Fig. 9E,F). In the olfactory bulbs, \textit{gad2} expression is dense within the ICL, but primarily
absent from the ECL and GL (Fig. 2A5; 8L).

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

Representative adjacent sections shown in Figure 10 highlight some of the non-
overlapping distribution patterns between glutamatergic (**\textit{vglut2.1**)**- and GABAergic (**\textit{gad2**)**-
expressing cells throughout the cichlid brain. In the olfactory bulbs, \textit{gad} expression is
predominant in the ICL, while this region lacks \textit{vglut2.1} cells, but the outer layers show the
opposite pattern with mostly \textit{vglut2.1} expression and minimal \textit{gad} expression (Fig. 10A,B).

In the telencephalon, \textit{gad} expression is abundant in subpallial parts of the ventral
telencephalon, while \textit{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 (VIIIn), abducens (VIn), trigeminal (Vn), trochlear (IVn), and oculomotor (IIIIn). 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 rostro-caudal 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; Fig. 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., \textit{vglut2.1} in Vv) of glutamatergic cells (Fig. 12A-D). The preoptic area contains dense GABAergic expression along with some scattered \textit{vglut2.1} and \textit{chat}-expressing cells (Fig. 12A,C,D). The VTn contains both GABAergic and glutamatergic markers, but the ATn expresses only \textit{vglut2.1} with a clear absence of GABAergic staining within the nucleus and prominent staining surrounding it (Fig. 12A,D,E). The TPa contains both glutamatergic and GABAergic cells, and the PAG expresses primarily GABAergic markers (Fig. 12A,E,F). Cells expressing \textit{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, \textit{Astatotilapia burtoni}, and represents one of the most complete descriptions to date of brain localization patterns using these multiple neurotransmitter markers in any teleost fish. Our results also demonstrate largely non-overlapping 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 brain regions in other teleost fishes to facilitate a better understanding of neural circuitry and nuclei functions across species.

\textit{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 \textit{vgluts} 1-3 in the brain of any fish species. Cells expressing the glutamatergic markers \textit{vgluts} 1-3 showed different distribution patterns within the brain of \textit{A. burtoni}, with the most widespread expression observed for \textit{vglut2.1}, and more restricted expression of \textit{vglut1} and \textit{vglut3}. While there is little information on the differential distribution of the three \textit{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 non-overlapping 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 \textit{vgluts} 1-3 throughout the cichlid brain provides putative additional levels of control over excitatory synapses.

In mammals, \textit{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 \textit{A. burtoni}, \textit{vglut1} is expressed in subdivisions of DI (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 \textit{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 co-expressed 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 co-expression 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 heterogenous 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, $vglut2.1$ 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 co-expression. 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 co-release of glutamate and GABA from the same synaptic vesicles (Zimmermann et al., 2015). Since this co-release from VGLUT3-expressing GABAergic synapses likely has postsynaptic effects that differ from classical GABAergic synapses, there may be sub-populations 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 co-temporal transmitter release with glutamate as a fast excitatory transmitter and other transmitters like serotonin acting as a post-excitatory “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 co-expresses 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 to 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* were also largely overlapping in *A. burtoni*, and in most vertebrates, both *gad* genes are co-expressed 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 VI, *A. burtoni* telencephalic chat cells are found in Vv (but not in VI), 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 paper (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 sub-populations 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.
CONFLICT OF INTEREST STATEMENT

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 manuscript. Designed experiments: KPM, JMB, KEF, DTP. Performed experiments, collected and analyzed data: KPM, JMB, KEF, DTP. Wrote and edited the manuscript: KPM, JMB, KEF, DTP. Provided funding, equipment, reagents and supplies: KPM.

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Abbreviations

4v  fourth ventricle
ac  anterior commissure
aGn  anterior glomerular nucleus
ALLn  anterior lateral line nerve
An  anterior thalamic 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 octaval 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
DI  lateral part of the dorsal telencephalon
DI-d  dorsal part of lateral part of the dorsal telencephalon
DI-g  granular zone of lateral part of the dorsal telencephalon
DI-v1 ventral part of the lateral part of the dorsal telencephalon, subdivision 1
DI-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  internal cell layer of olfactory bulb
ICo  isthmic commissure
Illn  oculomotor nucleus
IO  inferior olive
IP  interpeduncular nucleus
IVn  trochlear nerve nucleus
IXn  glossopharyngeal nerve nucleus
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
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 preptectalis
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 perilemniscal nucleus
PN  prethalamic nucleus
POA  preoptic area
PON  posterior octaval 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  paratalor 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
VIIIln octaval nerve (cranial nerve VIII)
VIIln facial nerve (cranial nerve VII)
VInc abducens nerve (cranial nerve VI) nucleus, caudal part
VI 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)
LITERATURE CITED


Filippi A, Mueller T, Driever W. 2014. vglut2 and gad expression reveal distinct patterns of dual GABAergic versus glutamatergic cotransmitter phenotypes of dopaminergic


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(7):1125-1143.

Grone BP, Maruska KP. 2016. Three distinct glutamate decarboxylase genes in vertebrates. Scientific reports.


FIGURE LEGENDS

Figure 1. Representative examples of chromogenic in situ hybridization staining in the brain of *Astatotilapia burtoni* to illustrate probe specificity. Anti-sense (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 anti-sense (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.

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 and see supplementary material for full *A. burtoni* brain atlas. Scale bars=250 µm.

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 PGm, NDILm 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 PGI 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.

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 DI regions, Dd-d and Dd-v, as well as scattered cells dorsally in Dm-3. E: vglut1 cells extend rostrally in regions of DI, 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 DI-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 DI 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.

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 ventro-laterally 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, VI), with the exception of a few scattered cells in Vv. I: In the olfactory bulks, 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.

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 ventro-lateral 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.

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.

**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, but is absent from ATn. **G:** The NRL, NRP, and PVO all 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 of and 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, VI, and Vv. In contrast to ventral telencephalic nuclei, more scattered *gad2*-expressing cells lie throughout the dorsal telencephalic regions (e.g., Dm, DI, 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, and 100 µm in D-E, G-H, J-L.
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.

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 non-overlapping 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 non-overlapping 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, and 50 µm in G-H.

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 glossopharyngeal motor nucleus (IXm), and superior reticular formation nucleus (Rs). H: *chat* expression in the oculomotor nucleus (IIn). I: *chat*-expressing cells in the caudal region of the lateral valvular nucleus (NLVc), and superior reticular formation nucleus (Rs). J: *chat*-expressing cells are abundant in the periventricular gray zone (PGZ) of the tegmentum. 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-H). See list for abbreviations. Scale bars= 50 µm in B, D, H, J, K, L; 100 µm in A, C, E, F, G, I.

**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 (A) 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.
### TABLE 1. Primer Pair Sequences Used to Generate Templates for the Synthesis of Gene-specific Riboprobes in *Astatotilapia burtoni*

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<th>Gene</th>
<th>Forward (5’→3’)</th>
<th>Reverse* (5’→3’)</th>
<th>Product size (bp)</th>
<th>Ascension Number</th>
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*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.*
TABLE 2. Localization of GABAergic, Glutamatergic, and Cholinergic Markers within Nuclei of the Social Decision Making Network (SDMN) of Astatotilapia burtoni

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<th>SDMN Nucleus</th>
<th>gad1</th>
<th>gad2</th>
<th>vglut1</th>
<th>vglut2.1</th>
<th>vglut3</th>
<th>chat</th>
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<td>+</td>
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<td>+++</td>
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<td>++</td>
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-, 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 1. Representative examples of chromogenic in situ hybridization staining in the brain of Astatotilapia burtoni to illustrate probe specificity. Anti-sense (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 anti-sense (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.

157x93mm (300 x 300 DPI)
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 and see supplementary material for full A. burtoni brain atlas. Scale bars=250 µm.

Fig. 2

304x254mm (300 x 300 DPI)
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Fig. 2

304x254mm (300 x 300 DPI)
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 PGe. H: The PGM, NDILm 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 PGI 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.
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 DI, 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.
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 ventro-laterally 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 PGZ showed intense vglut2.1 labeling, and cells lie in several hypothalamic nuclei including large cells in NCIL, and scattered cells throughout NDIL 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, Di, 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.
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 ventro-lateral 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.

Fig. 6

133x105mm (300 x 300 DPI)
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.

Fig. 7

203x254mm (300 x 300 DPI)
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 TPPp, but are largely absent from DP and CP. F: gad2 labeling is abundant in several NLT nuclei, but is absent from ATn. G: The NRL, NRP, and PVO all 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 of and 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, VI, 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, and 100 μm in D-E, G-H, J-L.

203x254mm (300 x 300 DPI)
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.

Fig. 9
125x186mm (300 x 300 DPI)
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 non-overlapping 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 non-overlapping 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, and 50 µm in G-H.

Fig. 10
188x133mm (300 x 300 DPI)
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 (IIIIn). 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 pregglomerular 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-

203x254mm (300 x 300 DPI)
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 (A) 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.

Fig. 12
174x125mm (300 x 300 DPI)
Using *in situ* hybridization in an African cichlid fish, the authors provide one of the most complete descriptions of localization patterns of glutamatergic (*vglut1, vglut2.1, vglut3*), GABAergic (*gad1, gad2*), and cholinergic (*chat*) neurons in the brain of any teleost fish. A summary of localization patterns within putative nuclei of the conserved social decision-making network (gray ovals) is also provided.