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RESEARCH ARTICLE

Expression of *tachykinin3* and related reproductive markers in the brain of the African cichlid fish *Astatotilapia burtoni*

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ABSTRACT

Neurokinin B, encoded by the *tachykinin3* gene, plays a crucial role in regulating reproduction in mammals via KNDy neurons and interaction with GnRH. Previous work in teleost fishes has focused on hypothalamic *tac3* expression for its role in reproduction, but detailed studies on extra-hypothalamic *tac3* expression are limited. Here, we identified two *tac3* genes in the social African cichlid fish *Astatotilapia burtoni*, only one of which produces a functional protein containing the signature tachykinin motif. In situ hybridization for *tac3a* mRNA identified cell populations throughout the brain. Numerous *tac3a* cells lie in several thalamic and hypothalamic nuclei, including periventricular nucleus of posterior tuberculum, lateral tuberal nucleus (NLT), and nucleus of the lateral recess (NRL). Scattered *tac3*-expressing cells are also present in telencephalic parts, such as ventral (Vv) and supracomissural (Vs) part of ventral telencephalon. In contrast to other teleosts, *tac3* expression was absent from the pituitary. Using double-fluorescent staining, we localized *tac3a*-expressing cells in relation to GnRH and kisspeptin cells. Although no GnRH-*tac3a* colabeled cells were observed, dense GnRH fibers surround and potentially synapse with *tac3a* cells in the preoptic area. Only minimal (<5%) colabeling of *tac3a* was observed in *kiss2* cells. Despite *tac3a* expression in many nodes of the mesolimbic reward system, it was absent from tyrosine hydroxylase (TH)-expressing cells, but *tac3a* cells were located in areas with dense TH fibers. The presence of *tac3a*-expressing cells throughout the brain, including in socially relevant brain regions, suggest more diverse functions beyond regulation of reproductive physiology that may be conserved across vertebrates.

KEYWORDS

endocrinology, GnRH, hormones, kisspeptin, reproduction, RRID:AB_143165, RRID:AB_2750561, RRID:AB_390204, RRID:AB_514497, RRID:AB_514504, RRID:SCR_000667, RRID:SCR_003070, RRID:SCR_014199, RRID:SCR_014329, tachykinin3, teleost

ABBREVIATIONS: 4v, fourth ventricle; ac, anterior commissure; ATn, anterior tuberal nucleus; CCeG, granular layer of corpus cerebelli; CCeM, molecular layer of corpus cerebelli; CCeP, Purkinje cell layer of corpus cerebelli; CG, central gray; CP, central posterior thalamic nucleus; CZ, central zone of tectum; Dc, central part of dorsal telencephalon; Dc-1, Dc, subdivision 1; Dc-3, Dc, subdivision 3; Dc-4, Dc, subdivision 4; Dc-5, Dc, subdivision 5; Dd, dorsal part of dorsal telencephalon; Dd-d, Dd, dorsal subdivision; Dd-v, Dd, ventral subdivision; Dl, lateral part of dorsal telencephalon; Dl-d, dorsal zone of Dl; Dl-g, granular zone of Dl; Dl-v2, ventral zone of Dl, subdivision 2; Dm, medial part of dorsal telencephalon; Dm-1, Dm, subdivision 1; Dm-2r, Dm, rostral subdivision 2; Dm-3, Dm, subdivision 3; Dp, posterior part of dorsal telencephalon; DWZ, deep white zone of tectum; Dx, unassigned subdivision of dorsal telencephalon; E, entopeduncular nucleus; Gn, glomerular nucleus; hc, horizontal commissure; IP, interpeduncular nucleus; lfb, lateral forebrain bundle; LT, lateral thalamic nucleus; NC, nucleus corticalis; NDILI, diffuse nucleus of the inferior lobe; nGMp, magnocellular preoptic nucleus, gigantocellular division; nHd, dorsal habenular nucleus; nHv, ventral habenular nucleus; NLT, lateral tuberal nucleus; NLTd, NLT, dorsal part; NLTi, NLT, intermediate part; NLTm, NLT, medial part; NLTv, NLT, ventral part; nMLF, nucleus of medial longitudinal fasciculus; nMMp, magnocellular preoptic nucleus, magnocellular division; NP, paracommissural nucleus; nPMp, magnocellular preoptic nucleus, parvocellular division; nPPa, parvocellular preoptic nucleus, anterior part; nPPp, parvocellular preoptic nucleus, posterior part; NPT, posterior tuberal nucleus; NRL, nucleus of the lateral recess; NRP, nucleus of the posterior recess; NT, nucleus taenia; oc, optic chiasm; ON, optic nerve; PAG, periaqueductal gray; pc, posterior commissure; PGl, lateral preglomerular nucleus; PGm, medial preglomerular nucleus; PGZ, periventricular gray zone of tectum; POA, preoptic area; PVO, paraventricular organ; Rs, superior reticular nucleus; SGn, secondary gustatory nucleus; smn, spinal motor neurons; SR, superior raphe nucleus; SVn, secondary general visceral (sensory) nucleus; SWGZ, superficial gray and white zone of tectum; TGN, tertiary gustatory nucleus; TL, torus longitudinalis; TLa, nucleus of the torus lateralis; TPp, periventricular nucleus of posterior tuberculum; TS, torus semicircularis; VAO, ventral accessory optic nucleus; Vc, central part of ventral telencephalon; VCeG, granular layer of valvula cerebelli; Vd, dorsal part of ventral telencephalon; Vd-c, Vd, caudal subdivision; Vde, descending tract of trigeminal nerve; Vd-r, Vd, rostral subdivision; Vi, intermediate part of ventral telencephalon; Vl, lateral part of ventral telencephalon; VL, vagal lobe; VMn, ventromedial thalamic nucleus; VOT, ventral optic tract; Vp, postcommissural part of ventral telencephalon; Vs, supracommissural part of ventral telencephalon; Vs-m, Vs, medial subdivision; Vv, ventral part of ventral telencephalon; Vv-r, Vv, rostral subdivision.

1 | INTRODUCTION

Reproduction is arguably the most important event in an animal's life. To be successful, reproduction requires the tight coordination of an animal's behavior and physiology. Together, these are regulated by the complex integration of both peripheral and central endocrine systems. Gonadotropin-releasing hormone 1, GnRH1, is considered the most important reproductive neuropeptide because it regulates pituitary function that leads to gonadal activity and steroidogenesis. Other neuropeptides, such as kisspeptins and neurokinin B (NKB), regulate GnRH1 output (Navarro et al., 2010; Ramaswamy et al., 2010) such that disrupting kisspeptin or NKB signaling inhibits the GnRH-induced luteinizing hormone pulse necessary for ovulation in females (reviewed in Rance, Krajewski, Smith, Cholanian, and Dacks [2010]). Hypogonadism, which is characterized by impaired gametogenesis, infertility, and postponed or inhibited sexual maturation, is found in humans with mutations in the genes encoding NKB (*tachykinin3*, *tac3*) or its receptors (de Roux et al., 2003; Rance et al., 2010; Topaloglu et al., 2009; Young et al., 2010). In mammals, NKB acts in a kisspeptin-dependent manner via autocrine and paracrine signaling. KNDy neurons, so called because they coexpress kisspeptin, NKB, and dynorphin, are found in the mammalian arcuate nucleus, and this is where the central regulation of reproduction occurs (Burke, Letts, Krajewski, & Rance, 2006; Goodman et al., 2007; Navarro et al., 2009). Although this regulation is well-documented in mammals, the importance of interactions between GnRH, kisspeptin, and NKB may differ in taxa with diverse reproductive strategies.

Similar to mammals, hypothalamic NKB cells in teleost fishes are studied for their potential role in reproduction. Zebrafish (*Danio rerio*) have two forms of the *tac3* gene (*tac3a*, *tac3b*) expressed throughout the brain, and expression of each gene increases with sexual maturation (Biran, Palevitch, Ben-Dor, & Levavi-Sivan, 2012; Ogawa et al., 2012). Goldfish (*Carassius auratus*) have varying levels of *tac3a* and *tac3b* in the hypothalamus depending on reproductive state (Qi et al., 2015), and in Nile tilapia (*Oreochromis niloticus*), only a single *tac3* gene was found, which is expressed in the gonadotropic cells of the pituitary (Biran et al., 2014). Detailed neuroanatomical surveys of *tac3* expression in the brain currently exist for only zebrafish and goldfish. Zebrafish have restricted expression of *tac3a* and *tac3b* throughout the brain (<10 regions for each transcript) (Ogawa et al., 2012), but a study in goldfish found ubiquitous and identical expression of *tac3a* and *tac3b* throughout the entire brain (Qi et al., 2015), casting doubt on probe specificity and/or accuracy of mapping. As such, a detailed neuroanatomical study on *tac3* expression in the brain of a more recently evolved fish is needed. Because of the colocalization of *tac3* in gonadotroph cells, Biran et al. (2014) propose that NKB may function at the level of the pituitary instead of the hypothalamus in Nile tilapia. This is further supported by the lack of colocalization of either of the *tac3*s with kisspeptin in zebrafish (Ogawa et al., 2012). Fishes, however, are the largest group of vertebrates, exhibiting high diversity in reproductive and parental care strategies that may be regulated by NKB and related signaling molecules. We analyze *tac3* distribution in the brain of a mouthbrooding fish, which exhibits extreme reproductive and maternal care behaviors. During spawning, females take their eggs into their buccal cavity where they are fertilized by the males. After this, she holds them for ~2 weeks, during which times she does not eat. *Tac3* is primarily studied in fishes with canonical spawning behaviors with little to no maternal care behaviors. Because neuropeptide systems and their functions vary greatly across teleosts, it is important to determine the relationship of *tac3* cells to other relevant signaling systems such as GnRH and kisspeptins in various fishes. These comparative analyses can identify which aspects may be species-specific adaptations and which may be conserved.

While there is considerable research highlighting the importance of hypothalamic NKB neurons for reproduction, we know little about NKB neuron function in other neural circuits, especially outside of the hypothalamus. Across taxa, NKB cells are also found in socially relevant regions of the brain, including the lateral septum, amygdala, hippocampus, and nucleus accumbens (Chawla, Gutierrez, Young, McMullen, & Rance, 1997; Marksteiner, Sperk, & Krause, 1992; Warden & Young, 1988). For example, NKB expression overlaps closely with the mesolimbic reward system. In mammals, central administration of NKB receptor agonists leads to increased extracellular dopamine levels (Marco et al., 1998), and dopaminergic input from the substantia nigra/ventral tegmental area regulates *tac3* mRNA in the caudate/putamen of rats (Burgunder & Young, 1989). Since NKB and DA cells lie in close proximity to each other, DA regulates NKB expression, and NKB affects dopaminergic cell output, it is also important to investigate the relationship of DA and NKB in other taxa.

Most tetrapods have at least two forms of GnRH (GnRH1 and GnRH2; Conlon, Collin, Chiang, Sower, & Vaudry, 1993; King, Dufour, Fontaine, & Millar, 1990; Lovejoy et al., 1992; Mongiat et al., 2006; Rissman, Alones, Craig-Veit, & Millam, 1995), but fishes have an additional form, GnRH3 (Gothilf et al., 1996; Parhar, Soga, & Sakuma, 1998; Soga, Ogawa, Millar, Sakuma, & Parhar, 2005; White, Kasten, Bond, Adelman, & Fernald, 1995; Yamamoto et al., 1995). Across taxa, GnRH1 neurons are located in the preoptico-hypothalamic area and project to the pituitary (either directly as in fishes or via the hypophyseal portal system as in mammals) to regulate gonadotropin release (Amano et al., 1991; Grober, Fox, Laughlin, & Bass, 1994; Maney, Richardson, & Wingfield, 1997). GnRH2 neurons in the midbrain tegmentum project extensively throughout the brain (González-Martínez et al., 2002; Muske, King, Moore, & Millar, 1994; Yamamoto et al., 1995). GnRH3 neurons, located in ganglia of the terminal nerve, project to the telencephalon, retina, olfactory epithelium, and more diffusely throughout the brain (Chiba, Sohn, & Honma, 1996; Grens, Greenwood, & Fernald, 2005; Parhar & Iwata, 1994; Yamamoto et al., 1995). Each form of GnRH is implicated in different behaviors and physiological processes (Amano, Urano, & Aida, 1997; Chen & Fernald, 2008; Maruska & Tricas, 2011), so it is important to examine NKB/*tac3* expression in relation to all three GnRH forms.

Due to a genome duplication, teleost fishes have a paralogous form of the kisspeptin encoding-gene (*kiss1* and *kiss2*; [Ogawa & Parhar, 2013]). In teleosts examined to date, *kiss1* and *kiss2* have distinct neuroanatomical distribution (Servili et al., 2011), with *kiss1* located primarily in the habenula and *kiss2* expressed in a few hypothalamic nuclei, including the preoptic area, NRL, and NLT (Kanda, Karigo, & Oka, 2012; Kitahashi, Ogawa, & Parhar, 2009; Mitani, Kanda, Akazome, Zempo, & Oka, 2010; Servili et al., 2011). Interestingly, *kiss1*

expression in the habenula was lost in some fishes, including African cichlids, as well as in the western and African clawed frog (*Xenopus tropicalis* and *Xenopus laevis*) and some mammals (Lee et al., 2009; Lehman, Merkley, Coolen, & Goodman, 2010). The gene encoding NKB, *tachykinin3*, has multiple forms in some fishes, like zebrafish and goldfish (Biran et al., 2012; Ogawa et al., 2012; Qi et al., 2015), but more recently evolved fishes lost the *tac3b* gene (Biran et al., 2014; H. Chen et al., 2018). A bioinformatics study of the genomes of several African cichlids failed to identify a *tac3b* gene (Biran et al., 2014); however, a recent study of *Astatotilapia burtoni* prohormones identified a potential *tac3b* gene (Hu et al., 2016), which has yet to be further investigated. Since studies have been performed in three cyprinid fishes with known *tac3a* and *tac3b* genes, it is important to also examine whether *tac3b* exists in percomorph fishes. If the proposed *tac3b* gene does not produce a functional peptide or exist in the brain, is it possible that *tac3a* has expanded or altered its expression patterns to make up for the lack of *tac3b*?

We identified two *tac3* genes from the highly social African cichlid fish *Astatotilapia burtoni*. Aspects of both the GnRH and kisspeptin systems in this species are previously described (Chen & Fernald, 2006; Flanagan et al., 2007; Grone, Maruska, Korzan, & Fernald, 2010; Robison et al., 2001; White & Fernald, 1998a, 1998b), as well as a suite of other neuroendocrine signaling molecules (Maruska & Fernald, 2018). Both the female reproductive cycle and male dominance hierarchy in *A. burtoni* are associated with changes in the GnRH and kisspeptin systems (Foran & Bass, 1999; Greenwood & Fernald, 2004; Grone et al., 2010; Grone, Carpenter, Lee, Maruska, & Fernald, 2012; Maruska & Fernald, 2013; Soma, Francis, Wingfield, & Fernald, 1996), with important consequences for reproductive behaviors and physiology. As their reproductive behaviors and endocrine systems are well studied, *A. burtoni* makes an excellent candidate for a comprehensive study on *tac3*. Using in situ hybridization (ISH), we localize *tac3a* expressing cells throughout the cichlid brain. We also performed double-label staining with *tac3a* and GnRH or *kiss2* and describe the potential interaction between the two systems. Finally, we examine the distribution of *tac3a* cells in relation to dopaminergic cell populations. Together, this study provides a comprehensive and high neuroanatomical resolution overview of the *tac3* system in a social fish.

2 | MATERIALS AND METHODS

2.1 | Animals

Adult *Astatotilapia burtoni* were laboratory-bred from a wild-caught stock from Lake Tanganyika, Africa, maintained in an environment that mimicked natural conditions (pH = 7.6–8.0; 28–30°C; 12L:12D diurnal cycle), and fed cichlid flakes (AquaDine, Healdsburg, CA). Community aquaria (114 L) contained gravel substrate on the bottom and at least one terra cotta pot to serve as a spawning territory. 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. All animal care and collection were approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA.

2.2 | Phylogenetic analyses

Neurokinin B and its related proteins are produced by the *tachykinin3* gene (called *tachykinin2* in rodents). Throughout the text, we use NKB and NKB-RP (NKB related protein) when referring to the proteins produced by *tachykinin3* genes. If only a single *tachykinin3* gene is present, *tac3* is used, but *tac3a* and *tac3b* are specified if more than one gene has been identified. In some instances, *tac3* is used when generally referring to both genes in *A. burtoni*. Two *tac3* genes are identified in the *A. burtoni* genome. The *tac3a* gene closely resembled *tac3* genes previously identified in other African cichlids (Biran et al., 2014). In addition, *tac3b* was identified based on the scaffold location produced from a bioinformatics study of *A. burtoni* pituitary prohormones (Hu et al., 2016). The location identified in that study yielded a sequence that partially resembled the *tac3b* sequence of other fishes.

Phylogenetic analysis of previously identified *tac3* mRNA sequences was performed in Mega7 Software (RRID:SCR_000667). Sequences were translated and aligned by ClustalW. Both neighbor-joining and maximum-likelihood trees were generated using a bootstrap value of 500 and produced similar results. The *tac3* mRNA sequences in Table 1 were collected from GenBank and used in the analysis.

2.3 | Macro brain dissections and PCR

To determine which regions of the brain express *tac3a* and *tac3b*, we macrodissected brains from a dominant male, subordinate male, and gravid female. Brains were carefully removed from the head with ~5 mm of spinal cord attached. Pituitary and olfactory bulbs were first removed and collected. Next, the telencephalon was separated just rostral to the optic nerve using a surgical blade to carefully ensure that the preoptic area remained with the hypothalamus. The spinal cord was severed just proximal to the medulla, and the cerebellum was separated at the most ventral portion of the stalk. Finally, the hypothalamus was separated just below the tectum, and the "hindbrain" portion was separated just caudal to the tectum so that it included raphe nuclei and the reticular formation but the rostral portion of the PAG/CG remained in the tectum/thalamus portion. Because fish do not have a true cerebral aqueduct, the term periaqueductal gray is incorrect, and this region should instead be referred to as the central gray. Since both terms have been used previously in *A. burtoni*, and because of the significance of the PAG nomenclature (i.e., as a node of the social behavior network), we refer to this region as the PAG/CG, which includes a group of larger cells in the mesencephalon (previously referred to as the PAG) and smaller cells lining the fourth ventricle in the rhombencephalon (previously referred to as the CG). It is possible that the hypothalamus portion collected also includes some thalamic nuclei.

Macrodissected tissue was homogenized, RNA was isolated using RNeasy Plus Micro kits (Qiagen; Germantown, MD) following manufacturer's protocol, and RNA was reverse transcribed to cDNA using iScript (Biorad, Hercules, CA). cDNA, Platinum PCR SuperMix (ThermoFisher), water, and gene-specific primers (*tac3a*, forward:

CAACTTCAGATCAGACCATAGG, reverse: CACTTTCATAAGCCA GGTACA, amplicon: 408 bp; *tac3b*, forward: GCTCTGATGCAAAGG GAAA, reverse: TGAAACCGCCTCCTCTT, amplicon: 373 bp) were combined for PCR (95°C for 1 min; 45 cycles of 95°C for 15 s, 55°C for 15 s, 72°C for 1 min; 72°C for 1 min). About 2 μl of PCR product and 1 μl of loading dye were loaded into each well, and run on a 1% agarose gel at 110 V for 15 min. The product was visualized on a Biorad ChemiDoc and verified to be the appropriate size in relation to the ladder. Each fish was run separately and verified to have the same expression pattern. In addition, all samples were run with *actin* as a positive control (forward: AAGATGAAATCGCCGCACT, reverse: GGGTACTTCAGGGTCAGGATA, amplicon: 205 bp). Both negative controls (no RT enzyme in RT-PCR, no cDNA template in PCR) showed no bands in any reactions. PCR products (whole brain cDNA with gene-specific primers) were sequenced (Eurofin Genomics; Louisville, KY) for verification of primer specificity and amplification of each correct target gene.

2.4 | Brain tissue collection and preparation for ISH and IHC

A total of 12 fish were collected, including males and females of various reproductive states (N = 3 of dominant males, subordinate males, ovulated females, and mouthbrooding females). All animals were used for chromogenic ISH staining, but subordinate males were not used for fluorescent double label staining due to an overall reduction in *tac3a* staining. Animals were quickly netted from their home aquaria, measured for standard length (SL; 44.700 ± 2.040 mm) and body mass (BM; 2.105 ± 0.252 g), and killed via rapid cervical transection. Brains were exposed, fixed in 4% paraformaldehyde (PFA) in 1× phosphate buffered saline (1xPBS) overnight, rinsed twice in 1xPBS for 8–12 hr each, and cryoprotected in 30% sucrose prepared in 1xPBS for >12 hr but no longer than 1 week. Immediately prior to sectioning, brains were removed from the head and mounted in Optimal Cutting Temperature (OCT; Tissue-Tek, Torrance, CA) media before sectioning at 20 μm on a cryostat (Thermo Scientific HM525 NX). Sections were collected onto alternate sets of superfrost plus microslides (VWR, Chicago, IL), air dried flat at room temperature (RT) for 48 hr, and stored at −80°C in RNase free conditions until further processing.

2.5 | Preparation of DIG-labeled riboprobes and ISH

To localize *tac3* to specific regions of the brain, we used chromogenic ISH with gene-specific digoxigenin (DIG)-labeled riboprobes for the *A. burtoni tac3a* and *tac3b* genes. Primers were carefully designed to ensure specificity to each form of *tac3* (same primers used above for PCR). Riboprobes were generated using previously described methods (Butler & Maruska, 2016; Grone & Maruska, 2015a, 2015b). Briefly, probe templates were amplified from whole brain *A. burtoni* cDNA with gene-specific primers (Life Technologies, Bethesda, MD). DIG-labeled nucleotides (DIG RNA-labeling mix, Roche, Nutley, NJ) were then incorporated into the purified PCR product. Purified probes (GE Illustra Probe Quant G-50 microcolumns) were diluted and stored at −20°C until use. Sense control probes were generated by adding the T3 recognition sequence (AATTAACCCTCACTAAAGGG) to the sense primer and generated as described above for antisense probes.

ISH was done following previously published protocols (Butler & Maruska, 2016; Grone & Maruska, 2015a, 2015b). Slides of cryosectioned brains were brought to room temperature and outlined with a hydrophobic barrier (Immedgepen; Vector Laboratories, Burlingame, CA). Slides were then treated with 1xPBS (3 × 5 min), 4% PFA (20 min), 1xPBS (2 × 5 min), proteinase K solution (10 min), 1xPBS, 4% PFA (15 min), 1xPBS (2 × 5 min), milliQ water (3 min), 0.1 M triethanolamine-HCl pH = 8.0 with acetic anhydride (10 min), and 1xPBS (5 min). Slides were then incubated for 3 hr in prehybridization buffer at 60–65°C before probe solution (probe in hybridization buffer) was added to each slide, covered with hybrislips (Life Technologies, Carlsbad, CA), and incubated overnight at 60–65°C for ~18 hr. Following DIG probe incubation the slides were washed at 60–65°C in prewarmed 2× saline sodium citrate (SSC):50% formamide (2 × 30 min), 1:1 mixture of 2× SSC: maleate buffer (MABT; 2 × 15 min) and MABT (2 × 10 min). Slides were then washed at room

TABLE 1 Sequences used for phylogenetic analysis of *Tac3* systems in fishes

Common name	Latin name	*tac3*	*tac3a*	*tac3b*
Antarctic toothfish	*Dissostichus mawsoni*	BK008104.1		
Atlantic cod	*Gadus morhua*	BK008107.1		
Atlantic salmon	*Salmo salar*		BK008102.1	BK008103.1
Channel catfish	*Ictalurus punctatus*	BK008101.1		
Fathead minnow	*Pimephales promelas*	BK008100.1		
Goldfish	*Carassius auratus*		KF177342.1	KF177343.1
Grass carp	*Ctenopharyngodon idella*		JN105351.1	KJ577570.1
Grass rockfish	*Sebastes rastrelliger*	BK008105.1		
Stripped seabass	*Morone saxatilis*	KT361626.1		
Medaka	*Oryzias latipes*	NM_001278903.1		
Nile tilapia	*Oreochromis niloticus*		KF471673.1	XR_269234.2
Orange spotted grouper	*Epinephelus coioides*	KT152147.1		
Rainbow smelt	*Osmerus mordax*	BK008111.1		
Starry flounder	*Platichthys stellatus*	KP742474.1		
Zebrafish	*Danio rerio*		NM_001256389.1	NM_001256390.1

temperature with MABT (2 × 10 min) and nonspecific binding was blocked with MABT containing 2% BSA for 3 hr. Following blocking, slides were incubated with anti-DIG antibody (Roche; RRID:AB_514497) diluted 1:5000 in blocking solution (MABT +2% BSA) at 4°C overnight, flat, in a humidified chamber. Following incubation, the slides were rinsed in MABT (3 × 30 min) at room temperature, washed in alkaline phosphatase (AP) buffer (2 × 5 min), and developed in NBT/BCIP (Roche) solution at 37°C for 4 hr for *tac3a* and 24 hr for *tac3b*. After development, slides were washed in PBS (3 × 5 min), fixed in 4% PFA for 10 min, washed in PBS (3 × 5 min), coverslipped with aqueous mounting media (Aquamount, Lerner Laboratories, Pittsburgh, PA), and allowed to dry on a flat surface overnight. To verify probe specificity, alternate sets of cryosectioned brains were stained simultaneously with the sense and antisense probes. Sense controls did not produce any labeling in the brain (Figure 1).

2.6 | Double fluorescent ISH and ISH–IHC

Several sets of slides were double labeled for *tac3a* mRNA and either *kisspeptin 2* mRNA (*kiss2*; KM115576.1, forward: TTGCGCTCTCATTGC TATC, reverse: CTGTCGCCGTTTGACTTT, amplicon size: 408 bp) or GnRH protein. To map the distribution of *tac3* cells in relation to dopaminergic cells, several sets of slides were also double labeled for *tac3* and tyrosine hydroxylase (TH, rate-limiting enzyme in dopamine synthesis; Millipore AB152). Double fluorescent ISH–immunohistochemistry was performed by first staining slides for *tac3a* mRNA using the chromogenic ISH protocol described above. On the final day of the ISH, slides were rinsed in MABT (3 × 10 min) and developed using SigmaFast Red instead of NBT/BCIP solution. After stopping the reaction with 1xPBS washes (4 × 10 min) at RT in the dark, nonspecific binding was blocked by incubating slides in 1xPBS containing 5% normal goat serum, 0.2% BSA, and 0.3% Triton-X for 2 hr. After blocking, slides were incubated in either GnRH 635.5 antibody (1:2,000) or TH antibody (1:5,000) at 4°C for 12–16 hr. Slides were washed in 1xPBS (3 × 10 min), incubated in Alexa Fluor 488 goat antirabbit antibody (1:400) for 2 hr in 1xPBS and 10% NGS, and washed in 1xPBS (3 × 10 min). Slides were coverslipped and cell nuclei counterstained with DAPI Fluorogel II (Electron Microscopy Services, Hatfield, PA).

Double fluorescent ISH was performed as described in Grone and Maruska (2015a, 2015b). The chromogenic ISH protocol was used until hybridization, at which time both probes (fluorescein-labeled *tac3a* probe and DIG-labeled *kiss2* probe) were applied in equal concentrations and incubated at 60°C overnight in sealed, humidified chambers. Slides were then washed in 2xSSC (2 × 30 min) at 60°C, TNT (Tris-NaCl-tween; 2 × 10 min) at RT, TNT with 0.3% H_2O_2 (10 min), and TNT (3 × 5 min). To block nonspecific binding, slides were incubated for 1.5 hr in 0.5% blocking reagent (Roche) dissolved in TNT. All subsequent steps were performed in the dark. After incubation with Anti Fluorescein-POD Fab fragments (Roche, Basel, Switzerland; 1:250; RRID:AB_514504) overnight at 4°C, slides were rinsed in TNT buffer (4 × 10 min). *Tac3a* development was done by incubating slides in Alexa Fluor 488 tyramide for 3 hr. After washing with TNT (2 × 5 min), quenching with 0.3% H_2O_2 in TNT (10 min) and TNT washes (2 × 5 min), nonspecific binding was again blocked by incubating slides for 1 hr at RT with 0.5% blocking solution dissolved in TNT. Slides were then incubated with Anti-DIG Fab fragments (Roche; 1:5,000) for 1.5 hr at RT, washes with TNT (2 × 5 min), and *kiss2* staining was developed by incubating slides in SigmaFast Red for 1 hr at 37°C. Reactions were stopped by incubating slides in TNT (3 × 5 min), rinsed with milliQ water (5 min) and coverslipped and counterstained with DAPI Fluorogel II.

2.7 | Antibody characterization

Both primary antibodies (TH and GnRH) were previously used and validated in *A. burtoni* or closely related cichlid fishes. We used Alexa Fluor 488 goat anti-rabbit (Thermo Scientific, Waltham, MA, A11008; RRID:AB_143165) secondary antibody in both TH and GnRH staining. The polyclonal TH antibody (Millipore, Burlington, MA, AB152; RRID: AB_390204; prepared in rabbit) was previously validated for use in *A. burtoni* via western blot staining (O'connell, Fontenot, & Hofmann, 2011). Using this TH antibody, bands representing TH1 and TH2 were present at the predicted size. TH staining in our study is also similar to that reported previously for *A. burtoni* (O'Connell, Fontenot, & Hofmann, 2013) and in other fishes (Kawabata, Hiraki, Takeuchi, & Okubo, 2012; Rink & Wullimann, 2002).

The primary GnRH antibody was gifted from I.S. Parhar (originally from L. Jennes from Univ. Kentucky; RRID:AB_2750561) and was previously used and validated in a variety of fishes, including cichlids (Parhar, Pfaff, & Schwanzel-Fukuda, 1996; Soga et al., 2005). The polyclonal (prepared in rabbits) mammalian GnRH 635.5 antibody (also referred to as LR-1) is a broad-based "promiscuous" antibody generated against the conserved region of the C-terminal, is directed against QHWSYGLRPG, and recognizes all three forms of teleost GnRH

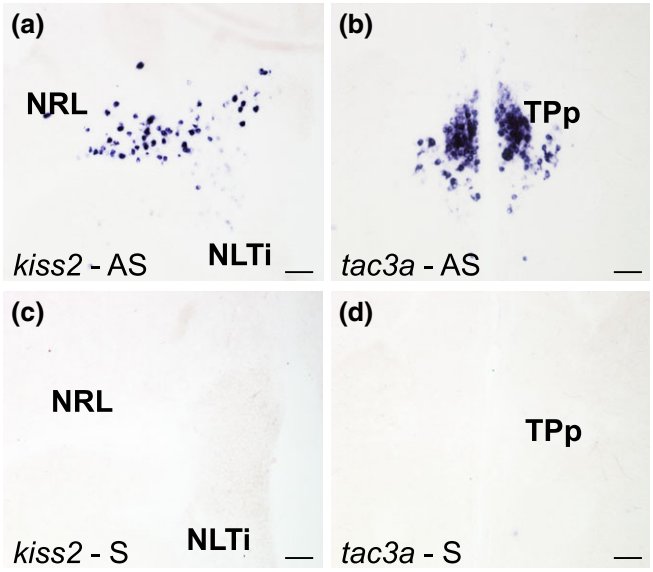

FIGURE 1 Representative examples of chromogenic in situ hybridization staining in coronal brain sections of *Astatotilapia burtoni* to illustrate probe specificity. Antisense (AS) and sense (S) control probes were run simultaneously for both *kiss2* (a, b) and *tac3a* (c, d). scale bars = 50 μm. See list for abbreviations [Color figure can be viewed at wileyonlinelibrary.com]

(GnRH1, GnRH2, and GnRH3). GnRH staining observed in *A. burtoni* was consistent with that observed in other teleosts using this and various GnRH antibodies or mRNA probes (González-Martínez et al., 2002; Gothilf et al., 1996; Grober et al., 1994; Maruska & Tricas, 2007; Parhar et al., 1998). Furthermore, staining of GnRH1 cells in the preoptic area is identical to that shown previously in *A. burtoni* by IHC, ISH, and GnRH1 transgenic animals (Ma, Juntti, Hu, Huguenard, & Fernald, 2015; White et al., 1995; White & Fernald, 1993).

2.8 | Imaging and analysis

Distribution of *tac3* cells is based on consensus from brains in all animals. Stained slides were visualized on a Nikon Eclipse Ni microscope controlled by Nikon Elements software (RRID:SCR_014329). Chromogenic-reacted sections were viewed with a color digital camera (Nikon DS-Fi2) in both brightfield and phase contrast to facilitate identification of neuroanatomical markers and visualize cytoarchitecture based on an *A. burtoni* brain atlas and relevant papers (Fernald & Shelton, 1985; Maruska, Butler, Field, & Porter, 2017). Fluorescent slides were viewed and photographed with a monochrome digital camera (Nikon DS QiMc) using appropriate wavelength filters. For each section, images were adjusted for contrast, brightness, and levels as needed, pseudocolored, and merged in ImageJ (imagej.nih.gov/ij/; RRID:SCR_003070). Distracting artifacts (e.g., endothelial cell fluorescence) was removed using the clone tool in Photoshop (Adobe Systems, San Jose, CA; RRID: SCR_014199) for visualization purposes.

3 | RESULTS

3.1 | Identification and phylogenetic analysis of *tac3* genes

Two *tac3* sequences were identified in GenBank based on other known sequences and bioinformatics and proteomic study of the *A. burtoni* pituitary (see methods for details). The *tac3a* sequence shared high sequence similarity with other teleosts and had 100% similarity to other African cichlids (Figure 2). As previously reported for some African cichlids, the predicted *A. burtoni* NKBa peptide contains an isoleucine instead of a valine in the signature motif. While the identified *tac3b* sequence shared partial sequence similarity, it does not appear to yield two functional proteins (NKB and the NKB-related protein). Part of the gene appears to encode an NKB-related protein highly similar to other teleosts, but the portion encoding NKBb does not produce a peptide with a signature tachykinin motif (Figure 2c).

3.2 | Distribution of *tac3a*

Within the brain, *tac3a* amplified via PCR in the macrodissected telencephalon, hypothalamus, tectum/thalamus, cerebellum, hindbrain, and spinal cord but not in the olfactory bulbs or pituitary (Figure 3). In contrast to the widespread expression of *tac3a*, *tac3b* amplified weakly only in the thalamus/tectum and hypothalamus, but nowhere else. Despite this weak amplification via PCR, multiple DIG-probes for *tac3b* failed to stain any cells in the brain. ISH was, however, used to further localize *tac3a*-expressing cell populations in the brain.

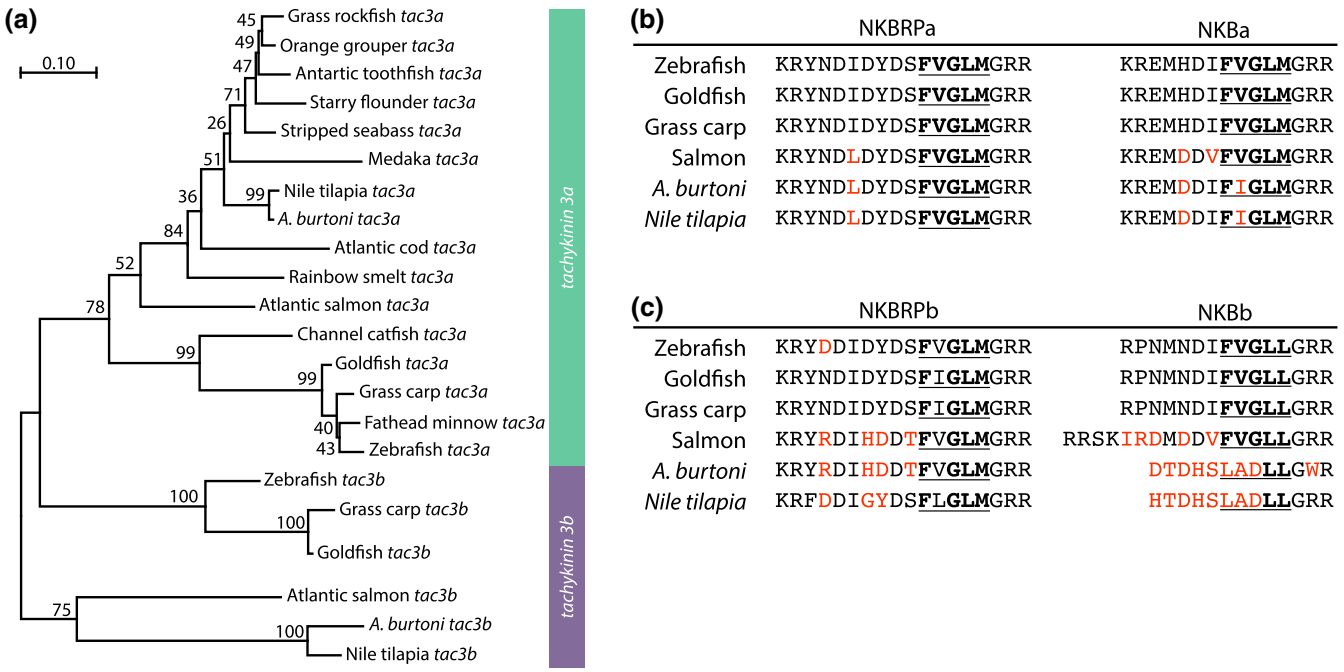

FIGURE 2 Phylogenetic analysis of *tac3* gene sequences in teleosts. An unrooted phylogenetic tree of *tac3* sequences (a) was generated using maximum-likelihood based on alignments via ClustalW (b, c). Bootstrap values based on 500 replicates are indicated at nodes and the substitution rate per residue is indicated by the scale bar in (a). All *tac3a* and *tac3b* genes group together based on form, and within in each group, African cichlid genes cluster together. Mature NKB and NKB-related peptide (NKBRP) contain a signature tachykinin motif (underlined/bolded). However, the *tac3b* gene does not appear to make an NKBb protein containing the signature motif (c). Letters in red in (b, c) indicate divergence from conserved teleost sequences. GenBank accession numbers can be found in Table 1 [Color figure can be viewed at wileyonlinelibrary.com]

Cells expressing *tac3a* are distributed in regions spanning from the hindbrain through the telencephalon (Figures 4 and 5). In the rhombencephalon, scattered *tac3a*-expressing cells lie in the vagal lobe (VL) close to the periaqueductal gray/central gray (PAG/CG; Figure 4a, see methods section 2.3 for details on nomenclature). Scattered *tac3a*-expressing cells are also in the superior reticular nucleus. There are no *tac3a*-expressing cells in the cerebellum (corpus or valvula regions). In the mesencephalon, t*ac3a*-expressing cells lie along the fourth ventricle (4v) in the PAG/CG, and along the midline in the superior raphe (SR) and interpeduncular (IP) nuclei (Figures 4b and 5a, b). A few large *tac3a* cells lie in the PAG/CG (Figures 4c and 5e,f). In the diencephalon, abundant *tac3a*-expressing cells extend through the medial, but not lateral portions of the nucleus of the lateral recess (NRL; Figures 4c–e and 5c,d). *Tac3a* cells are found in all subdivisions of the lateral tuberal nucleus (NLT; Figures 4d–g and 5g–i). A group of *tac3a*-expressing cells is located in the intermediate part of the NLT (NLTi) extending toward the NRL. Along the midline, lightly stained *tac3a* cells are in the dorsal part of the NLT, and more densely packed *tac3a*-expressing cells lie in the ventral NLT (NLTv). The medial NLT (NLTm) contains *tac3a*-expressing cells along its border with the anterior tuberal nucleus (ATn), but no cells are within the ATn. A few darkly stained *tac3a*-expressing cells are found in the ventral portion of the posterior tuberal nucleus (NPT) close to the NLTi (Figures 4d and 5c).

The most prominent *tac3a* staining is found in the periventricular nucleus of the posterior tuberculum (TPp; Figures 4e,f and 5g–i), with some scattered but densely stained cells just ventral to TPp in the paraventricular organ (PVO; Figures 4f and 5h,i). More lateral to the TPp, scattered *tac3a*-expressing cells lie near the lateral preglomerular nucleus (PGl) and ventral accessory optic nucleus (VAO; Figures 4f and 5h). Although distinct cells were not observed, *tac3a* staining appeared "fuzzy" in the ventral habenular nucleus (Figure 4g; but no staining was observed in sense control tissue), possibly due to the small, tightly packed neurons with little cytoplasm.

Scattered *tac3a*-expressing cells are distributed in all subdivisions of the preoptic area (POA; Figures 4g–j and 5j) except the magnocellular preoptic nucleus, gigantocellular division (nGMp). The most abundant *tac3a*-expressing POA cells lie in the magnocellular preoptic nucleus, magnocellular division (nMMp) with scattered cells also in the magnocellular preoptic nucleus, parvocellular division (nPMp) and in both the anterior and posterior parts of the parvocellular preoptic nucleus (nPPa; nPPp).

In the telencephalon, the most prominent staining is located in the medial subdivision of the supracommissural part of the ventral telencephalon (Vs-m; Figures 4i,j and 5k). Scattered cells also populate the granular zone of the lateral part of the dorsal telencephalon (Dl-g; Figures 4j,k and 5l). A few lightly stained cells are also scattered along the midline in the medial part of the dorsal telencephalon, subdivision 1 (Dm-1; Figures 4k,l and 5n) and in the area between the rostral portion of the dorsal part of the ventral telencephalon, rostral subdivision (Vd-r) and rostral portion of the ventral part of the ventral telencephalon (Vv-r; Figures 4k,l and 5m). There are no *tac3a*-expressing cells in the olfactory bulbs.

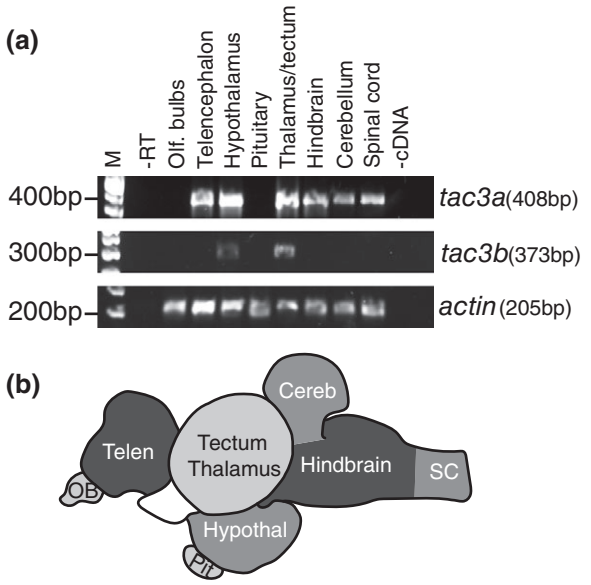

FIGURE 3 Expression of *tac3a* and *tac3b* in macrodissected brain regions of *A. burtoni*. Representative expression is shown by PCR from reverse transcribed cDNA and gel electrophoresis. (a) *Tac3a* is expressed in the telencephalon, hypothalamus, thalamus/tectum, hindbrain, cerebellum, and spinal cord. *Tac3b* only amplified in the hypothalamus and thalamus/tectum. Neither *tac3a* nor *tac3b* amplified in the pituitary or olfactory bulbs. No band was observed in any negative controls, and *actin* amplified in all tissue samples as a positive control. Base pair (bp) numbers to the left are size of the indicated ladder band while bp numbers following each gene on the right represent the product size. The first column (M) depicts the marker ladder. Brain regions were macrodissected as depicted in (b)

3.3 | Dual fluorescent labeling of *tac3a* with GnRH and *kiss2*

To test whether *tac3a* was coexpressed in the same cells or brain regions as GnRH or *kiss2* neurons, we performed double-label experiments. Cells expressing *kiss2* lie along the dorsal edge of the NRL and in the medial portion of the NRL extending toward the NLTi (Figure 6a). In the NRL, *tac3a* and *kiss2*-expressing cells are regionally distinct, with *kiss2* cells located in the dorsomedial portion of the NRL, and *tac3a*-expressing cells predominantly on the ventral edge of NRL. In the NLTi (but possibly an extension of the NRL), *kiss2* and *tac3a* cells are intermingled (Figure 6b,c). Although a few scattered colabeled cells are observed (Figure 6b), this comprises less than 5% of the overall *kiss2* population in the NLTi. Since such a small percentage of cells are colabeled, and because other *tac3a* and *kiss2* cells overlap, it is possible that these do not represent true coexpression but are rather overlapping cells of similar shape and size.

GnRH neurons and *tac3a* cells are both located in the same region of the POA, but no colabeling is observed (Figure 6d). In addition, these are GnRH1 cells in this region and are much larger than *tac3a* cells (soma area; GnRH: 291.302 ± 46.956 μm^2; *tac3a*: 50.416 ± 9.483 μm^2; N = 4 cells each from three animals of various reproductive states). GnRH-containing varicose fibers appear to project to and surround *tac3a*-expressing cells in the POA, although direct synaptic connections

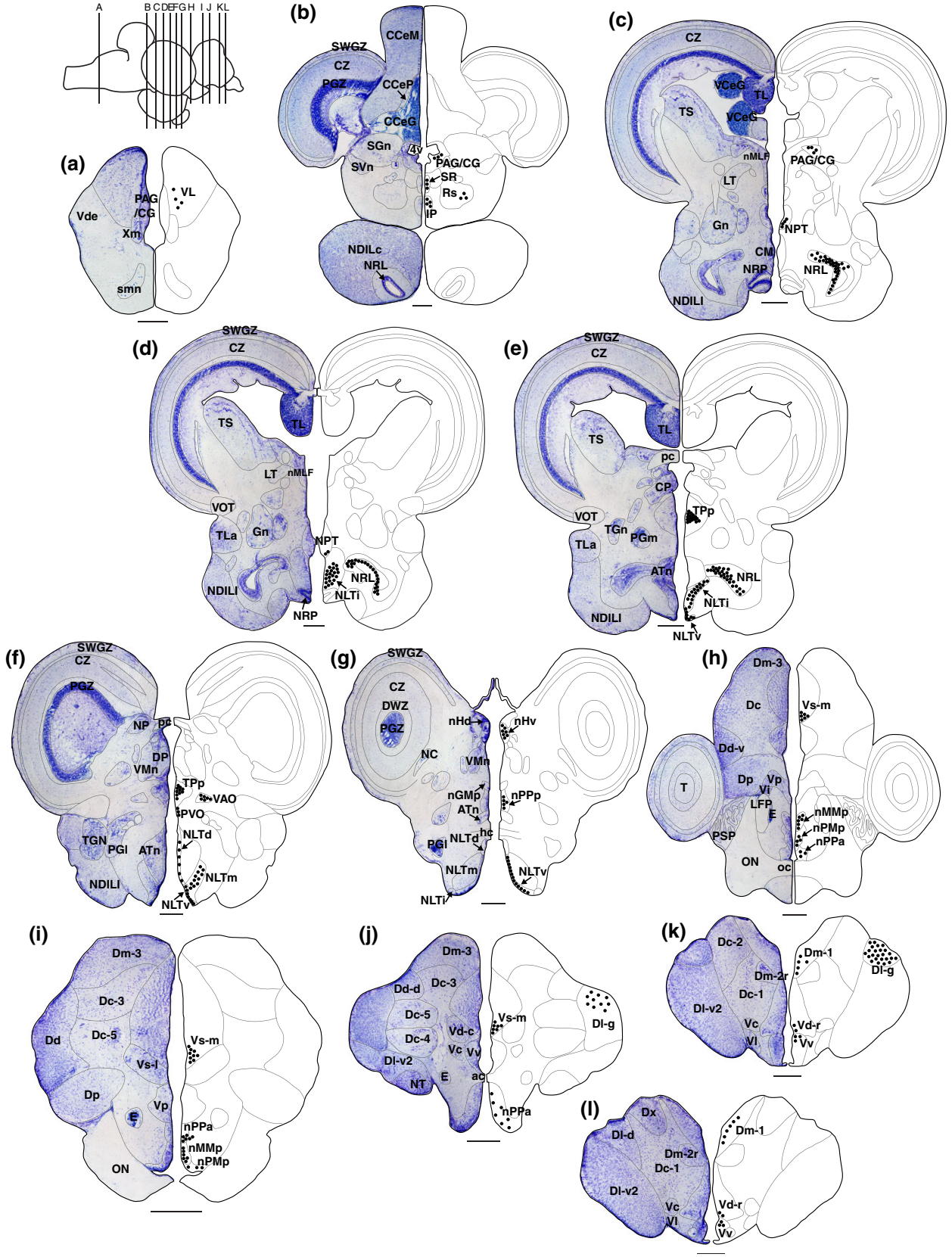

FIGURE 4 Localization of *tac3a*-expressing cells in the brain of *Astatotilapia burtoni*. Approximate locations of transverse sections shown caudal (a) to rostral (l) are depicted in inset. Left side of each panel depicts a cresyl violet stained brain section with regions outlined. In the right side of each panel, localization of *tac3a*-expressing cells (dots) is shown. Scale bar under each panel represents 250 μm. See list for abbreviations [Color figure can be viewed at wileyonlinelibrary.com]

were not determined (Figure 6e,f). There is no proximity of *tac3a*-expressing cells to GnRH2 or GnRH3 cells.

3.4 | Dual fluorescent labeling of *tac3a* and TH

To examine the relationship between *tac3a* cells and the dopaminergic system, we co-labeled with tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis. TH fibers and cells are found in close proximity to *tac3a*-expressing cells in the diencephalon. In the TPp, which is known for its dopaminergic cell population and has the highest expression of *tac3a* in the brain, the two cell groups were adjacent to each other, but distinct, with a few scattered cells intermingled at the border but no colabeling within the same cells (Figure 6g). Throughout the TPp, TH-labeled cells appeared more dorsal and lateral to *tac3a* cells (Figure 6g,h). A collection of TH fibers was observed projecting to the *tac3a*-labeled cell group in the nHv, but no contact between TH fibers and *tac3a* cells was observed (Figure 6i). TH-labeled cells also occur in several regions of the POA, as well as extensive fibers, but no colabeling of TH and *tac3a* in cell bodies was observed (Figure 6j,k). Similarly, in the telencephalon, TH cells are located in the Vc in close proximity to *tac3a*-labeled cells of Vv/Vd but are not coregionalized. Although TH cells are located near *tac3a* cells throughout the brain, the two appear to be distinct, such that no *tac3a*-expressing cells also expressed TH. Possible connections between the two systems may exist due to the dense TH fibers throughout the brain, or potentially via *tac3a*-cells projecting to the nearby TH cells.

4 | DISCUSSION

We identified two *tachykinin3* genes in the social African cichlid fish *Astatotilapia burtoni*, but only *tac3a* appears to produce a peptide with the tachykinin signature motif. We localized *tac3a*-expressing cells throughout the brain, and described expression patterns in relation to GnRH, *kiss2*-expressing cells, and the dopamine system, providing one of the most comprehensive and detailed localization studies of *tachykinin3* and its relation to other relevant signaling molecules in the teleost brain.

4.1 | Phylogenetic analysis

The teleost *tac3* gene produces two functional proteins: one corresponding to neurokinin B, and a neurokinin B related protein (NKBRP; sometimes called neurokinin F, NKF), each of which contain the signature tachykinin motif. One *A. burtoni tac3* gene (called *tac3a* throughout) produces NKB and NKBRP identical to that in Nile tilapia (Biran et al., 2014). While the NKBRP contains a leucine instead of an isoleucine in the sixth amino acid position, the signature motif (FVGLM) is consistent with all other teleosts. Similar to other African cichlids, the NKB protein produced by this gene in *A. burtoni* has an isoleucine in the second position of the motif instead of a valine. Whole genome duplications in teleosts resulted in multiple forms of some genes, and zebrafish, goldfish, and Atlantic salmon (*Salmo salar*) all have two *tac3* genes. Despite a second genome duplication in some fish, like goldfish, only two forms of tac3 have been found. We also found a second gene with a similar sequence to that of *tac3b*, first identified by a proteomic study (Hu et al., 2016) of the pituitary, but it does not appear that the *A. burtoni* or Nile tilapia *tac3b* gene produces a functional NKB peptide. Although it does not produce NKB protein, we did detect the potential *tac3b* gene in the brain using PCR. However, ISH using multiple *tac3b* probes failed to positively stain any cells. Chen et al. (2018) identified the same sequence of the Nile tilapia genome as a potential *tac3b* sequence, but were unable to obtain a cDNA sequence using GeneScan or reveal a potential *tac3b* sequence in the orange-spotted grouper (*Epinephelus coioides*) using the identified sequence. They, and others, have suggested that *tac3b* has become a pseudogene in more recently evolved fishes, like African cichlids (Biran et al., 2014; H. Chen et al., 2018). The sequence identified as *tac3b* does not possess any introns, further supporting it as a pseudogene. Thus, the identified *tac3b* gene likely does not reflect NKB expression in the brain of *A. burtoni*.

4.2 | Comparative expression of *tac3a* and *tac3b*

Tac3 expression in the brain was previously described in zebrafish (Biran et al., 2012; Ogawa et al., 2012), goldfish (Qi et al., 2015), striped bass (*Morone saxatilis*; Zmora, Wong, Stubblefield, Levavi-Sivan, & Zohar, 2017), and orange-spotted grouper (Chen et al., 2018; Table 2). The distribution of *tac3a* in *A. burtoni* appears to be more widely distributed than that observed in previously examined teleosts (but see Qi et al. [2015]). In grouper and striped bass, expression was limited to the diencephalon, but a thorough neuroanatomical description was not the focus of these studies, so additional smaller cell populations could have been overlooked. While Qi et al. (2015) found ubiquitous expression of both *tac3a* and *tac3b* throughout all regions of the goldfish brain, zebrafish and *A. burtoni* have more restricted expression. Our PCR indicated that *tac3a* was expressed in all major parts of the brain except the olfactory bulbs and pituitary. ISH confirmed *tac3a* mRNA in the telencephalon, tectum/thalamus, hypothalamus, hindbrain, and spinal cord, but failed to definitively stain any cells in the cerebellum. Due to high density of small cells with little cytoplasm, staining in this region often appears fuzzy. While hazy staining can be a result of background or nonspecific staining, no staining of this type was observed in the cerebellum of brains hybridized with the sense control probe. However, some animals appeared to have a light blue staining haze in the granular layer of the corpus cerebellum (GGeG), suggesting the possibility of low levels of *tac3a*. In addition to the diencephalon, we identified small *tac3a*-containing cell populations in the medial and lateral parts of the dorsal telencephalon. Furthermore, scattered *tac3a* cells were found in the intermediate area between the dorsal and ventral parts of the ventral telencephalon, and a larger cell group was found in the ventral portion of the Vs-l. Ogawa et al. (2012) documented *tac3b* staining in the Dl of the zebrafish brain, but no *tac3a* staining in the telencephalon (Biran et al., 2012; Ogawa et al., 2012). Thus, cyprinid fishes with both *tac3a* and *tac3b* have expression of each gene throughout the brain, including the telencephalon where only *tac3b* is expressed. In

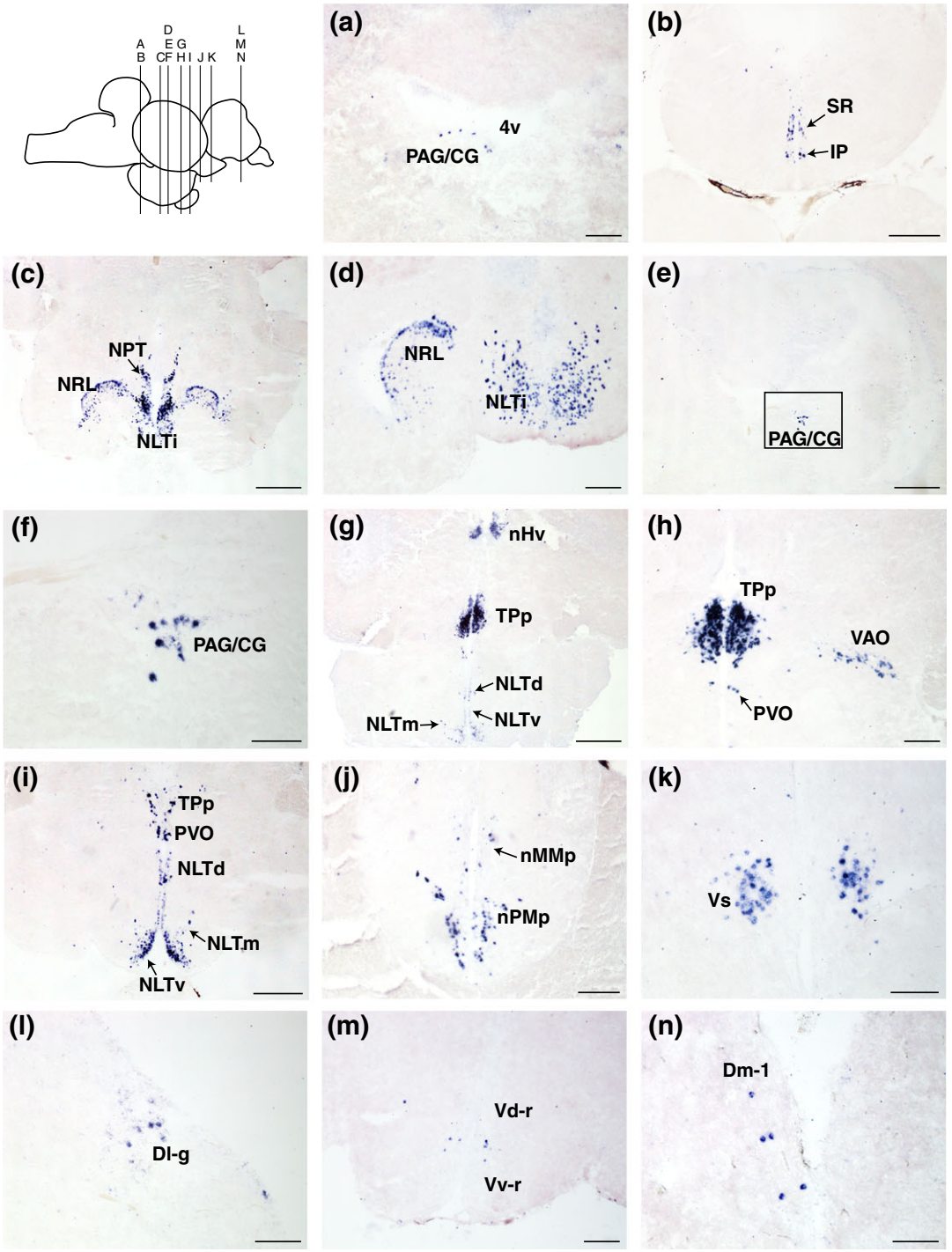

FIGURE 5 Representative photomicrographs of *tac3a* in situ hybridization staining throughout the *Astatotilapia burtoni* brain. Scattered *tac3a*-cells lie along the 4v in the central gray (a) *tac3a*-expressing cells are also found along the midline in the IP and SR of the rhombencephalon (b). *Tac3a* cells are located in several hypothalamic regions, including the NPT (c), NRL (c,d), and NLTi (c,d). Scattered *tac3a* cells were also found in the PAG/CG (e,f). In the diencephalon, *tac3a* expression is in neurons in the nHv (g), TPp (g–i), NLT (dorsal, ventral, and medial subdivisions; c,d,g–i), PVO (h,i), VAO (h), and in several preoptic area subdivisions, including the nMMp and nPMp (j). *tac3a*-expressing cells are also found in the telencephalon (k–n). A group of *tac3a* cells lie in the ventral portion of the Vs along the midline (k). Scattered, weakly stained *tac3a* cells are also found throughout the Dl-g (l). A small group of *tac3a* cells are found between the rostral portions of the Vv and Vd (m). Additional *tac3a* cells lie close to the midline in the Dm-1 region of the brain (n). See list for abbreviations. Schematic at top left represents approximate locations of staining. Photomicrographs were taken from 20 μm transverse sections. Scale bars = 250 μm in (b, c, e, g, i); 100 μm in (a, d, h, j, m); 50 μm in (f, k, l, n) [Color figure can be viewed at wileyonlinelibrary.com]

A. burtoni, a more recently evolved fish with only one copy of the *tac3* gene, its expression is similar to the combined distribution of *tac3a* and *tac3b* in zebrafish. This suggests that *tac3a* expression in percomorphs may have expanded to encompass and supplement for the loss of a functional *tac3b* gene. Because these telencephalic *tac3b* cells were only documented once previously and were not the focus of that study, the potential function of these extra-hypothalamic cells is unknown.

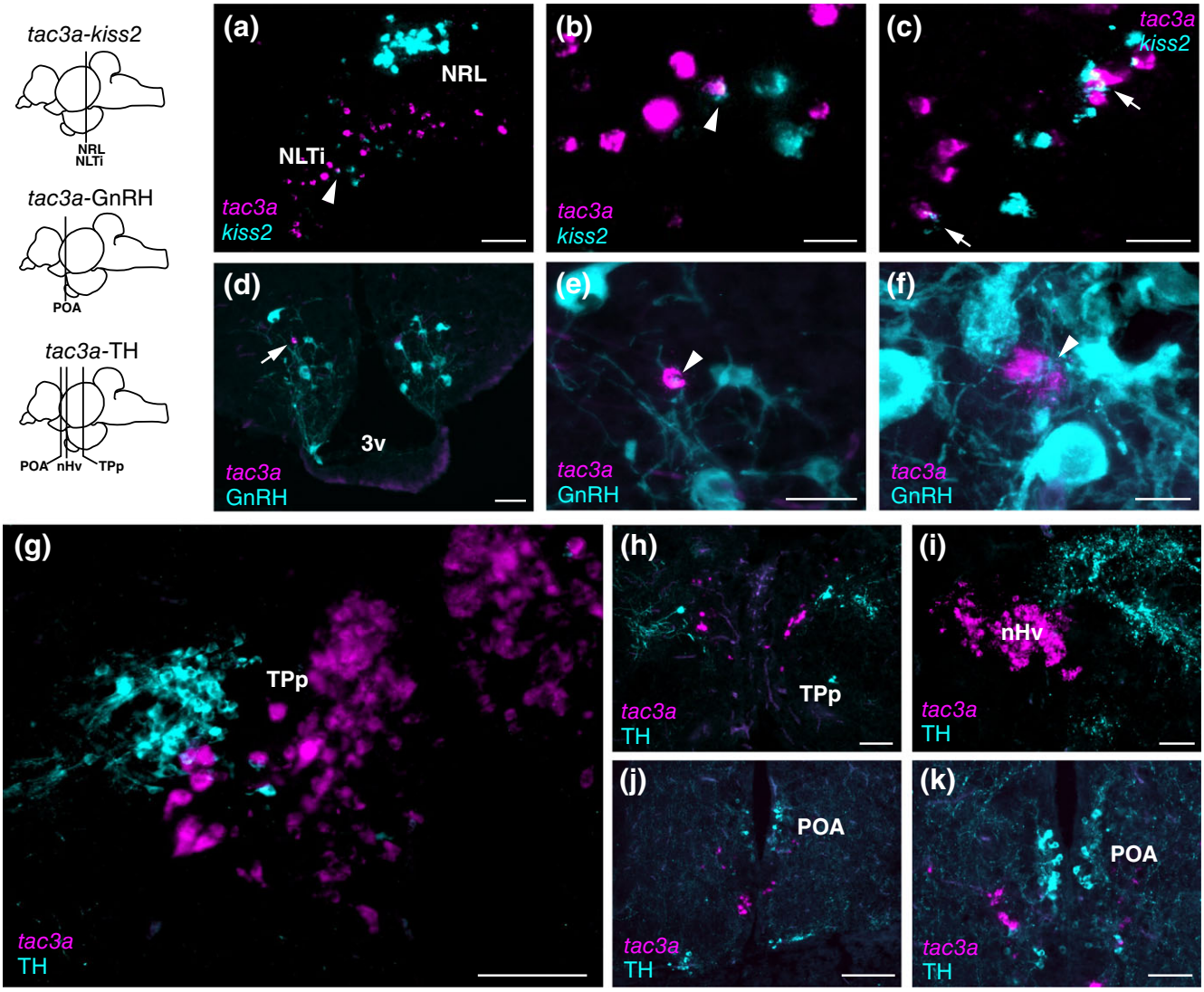

FIGURE 6 Dual-fluorescence staining for *tac3a*, *kiss2*, GnRH, and TH in the *A. burtoni* brain. *Kiss2*-expressing cells were only located in the NRL/NLTi area. In the NRL, *kiss2* (blue) and *tac3a* (magenta) cells are spatially distinct, with *kiss2*-expressing cells on the dorsal border of NRL and *tac3a* cells on the ventral border (a). In the NLTi area, *kiss2* and *tac3a* cells are intermingled with scarce double-labeled cells (arrowhead in a, b). Several instances of overlapping *kiss2* and *tac3a* cells were observed (arrows in c), but cells appear different shapes and thus are likely not single, colabeled cells. Large GnRH1 cells and abundant fibers (blue) are found in the preoptic area (d). *Tac3a*-expressing cells are fewer and smaller in size than GnRH1 cells. Arrow in (d) points to a *tac3a*-labeled cell. Although no colabeled cells are observed, GnRH fibers appear in close proximity to *tac3a* cells (e,f). Arrowheads indicate GnRH varicosities near *tac3a* cells in (e, f). Representative photomicrographs showing distinct spatial distribution of *tac3a* and TH cells in the TPp (g, h), habenula (i), and preoptic area (j, k). TH cells (blue) are more lateral to most *tac3a* cells (magenta), with some comingling but no colabeled cells in the TPp (g, h). In the habenula, dense *tac3a* staining is observed in the nHV and dense TH fibers are observed extending toward this region but there are no TH positive cells (i). In the preoptic area, TH cells are primarily located along the midline, but most *tac3a* cells are located more lateral (j, k). Dense TH fibers are found throughout the POA (k). Schematics at left represent approximate locations of staining. Photomicrographs were taken from 20 μm transverse sections. Scale bars = 100 μm in (h, j); 50 μm in (a, c, d, g, i, k); 25 μm in (e); 10 μm in (b, f) [Color figure can be viewed at wileyonlinelibrary.com]

4.3 | Expression and potential functions of *tac3a* in relation to GnRH and kisspeptin

Most *tac3*-expressing cells lie in the hypothalamus of fishes. The zebrafish hypothalamus has 20-fold higher *tac3a* expression compared to other brain areas (Zhou et al., 2012). In orange-spotted grouper, *tac3* expression is restricted to the diencephalon, and within the hypothalamus, no *tac3* mRNA was detected in the preoptic area. With this exception, the teleosts examined to date have several regions where *tac3* is consistently expressed: preoptic area, habenula, NLT, TPp, PVO, NRL, and NPT. Because this is where most *tac3a* cells are located, and they have close proximity to sex steroid receptor-, GnRH-, and kisspeptin-expressing cells (Table 3), the majority of research has focused on the functional role of NKB in the hypothalamus, including the regulation of gonadotropes and kisspeptins.

In the *A. burtoni* preoptic area, *tac3a* cells are in close proximity to GnRH1 cells, but not GnRH2 or GnRH3 cells. In striped bass, NKB-expressing neurons are not innervated by GnRH fibers, but NKB fibers are close to GnRH neurons in the nPPa (but they do not express the NKB receptor, NK3R (Zmora et al. (2017). In several

TABLE 2 Expression of *tac3a* cells in the teleost brain

	Astatotilapia burtoni	*Carassius auratus*[1]	*Danio rerio* [2,3]	*Epinephelus coioides* [4]	*Morone saxatilis* [5]
Telencephalon					
Dl	+	+	–	–	–
Dm	+	+	–	–	–
Vd	+	+	–	–	–
Vv	+	+	–	–	–
Vs	+	+	–	–	–
Diencephalon					
POA	+	+	+	–	+
NLT	+	+	+	+	+
ATn	–	–	–	–	+
TPp	+	+	+	+	–
PVO	+	+	+	+	–
VAO	+	–	+	–	–
NRL	+	+	+	+	+
NPT	+	+	+	–	–
VMn	–	+	–	+	–
nHv	+	+	+	+	+
Mesencephalon					
PAG/CG	+	nd	+	–	–
SR	+	nd	–	–	–
IP	+	nd	–	–	–
Rhombencephalon					
PAG/CG	+	nd	+	–	–
Rs	+	nd	–	–	–
VL	+	nd	–	–	–
Pituitary	–	+	+	+	+

Summary of *tac3a* neuroanatomical distribution in 5 teleost fishes based on (1) Qi et al., 2015; (2) Ogawa et al., 2012; (3) Biran et al., 2012; (4) Chen et al., 2018; and 5. Zmora et al., 2017. + indicates *tac3a-expressing cells* observed, while – indicates the absence of positive staining. nd in the goldfish mesencephalon and rhombencephalon are because authors did not discuss or show images of these regions. All regions with positive staining in *A. burtoni*, *D. rerio*, *E. coides*, and *M. saxatilis* are listed, but not all regions with reported *tac3a* staining in *C. auratus* are listed due to its widespread expression.

teleosts, *tac3a*/NKB cells, likely from the POA, also project to the pituitary. This NKB released to the pituitary is thought to modulate LH and FSH production. Neurokinin B administration influences GnRH and gonadotropin production, and intraperitoneal injections of NKB resulted in higher circulating levels of LH in zebrafish (Biran et al., 2012) and higher LH and FSH levels in Nile tilapia (Biran et al., 2014). Injecting the NKB analog senktide into the brain of ewes resulted in higher levels of LH (Billings et al., 2010), and senktide in the presence of estradiol increased LH levels in female rats (Navarro et al., 2009). In ewes, NKB acting via NK3Rs in the retrochiasmatic area also results in LH increase similar to that seen with the preovulatory LH surge (Billings et al., 2010). Primary Nile tilapia pituitary cells in culture incubated with tilapia NKB produced LH and FSH at similar levels to when they were administered sGnRH (salmon form of GnRH; Biran et al., 2014). Furthermore, Biran et al. (2014) found gonadotropic cells also expressed *tac3a* and its receptors. Together, this led them to suggest that the *tac3* system regulates gonadotrophs via autocrine and paracrine signaling within the pituitary. However, others have suggested that NKB works through independent pathways (through the brain and pituitary) to regulate reproduction in fishes (Zmora et al., 2017).

Despite *tac3* expression in the pituitary of tilapia (Biran et al., 2014), zebrafish (Biran et al., 2012; Ogawa et al., 2012), goldfish (Qi et al., 2015), grass carp (Hu, He, Ko, Lin, & Wong, 2014), and orange spotted grouper (Chen et al., 2018), we did not detect *tac3a* mRNA in the *A. burtoni* pituitary via PCR or ISH. Similarly, a recent study of pituitary peptides detected *tachykinin1* (substance P) and *tachykinin4* (hemokinin) but no *tac3* (NKB) product by mass spectrometry of *A. burtoni* pituitaries (Hu et al., 2016), further suggesting that tac3/NKB is absent from the *A. burtoni* pituitary despite its presence in the pituitary of the closely related Nile tilapia (Biran et al., 2014). It is possible that expression levels were too low for detection, however other studies have found pituitary levels of *tac3* to be similar to that found in the hypothalamus (Biran et al., 2012; Qi et al., 2015). Alternatively, rapid translation of mRNA into protein could account for the absence of detectable mRNA levels. This is an important difference between *A. burtoni* and tilapia, two very closely related cichlid fishes with similar reproductive and maternal care systems. Although we did not find *tac3a* expression in the pituitary, we did find scattered *tac3a* cells in the same region of the preoptic area (POA) that contains the GnRH1 cells known to project to the pituitary (Bushnik & Fernald, 1995). We did not, however, observe any cells colabeled for *tac3a* and GnRH. While we could not visualize NKB fibers, only *tac3a*-expressing cell bodies, dense GnRH fibers surround *tac3a*-positive cells with varicosities on or near these cells,

TABLE 3 Expression of GnRH, kisspeptin, and dopamine systems in *A. burtoni* brain

	tac3a	GnRH	GnRH receptors[1]	*kiss2*	Kiss receptors[2]	TH[3]	Dopamine receptors[3]
Telencephalon							
Dl	+	f	+	–	+	f	+
Dm	+	f	+	–	+	f	+
Vd	+	f	+	–	+	f	+
Vv	+	f	+	–	+	f	+
Vs	+	f	+	–	+	–	+
Diencephalon							
POA	+	+	+	–	+	+	+
NLT	+	f	+	+	+	f	+
TPp	+	f	+	–	+	+	+
PVO	+	f	+	–	+	+	+
VAO	+	f	+	–	–	f	+
NRL	+	f	+	+	+	–	+
NPT	+	f	+	–	+	+	+
nHv	+	f	+	–	+	–	+
Mesencephalon							
PAG/CG	+	f	nd	–	–	+	+
SR	+	f	nd	–	+	nd	nd
IP	+	f	nd	–	–	nd	nd
Rhombencephalon							
PAG/CG	+	f	nd	–	–	+	+
Rs	+	f	nd	–	+	nd	nd
VL	+	f	nd	–	+	nd	nd

GnRH description based on staining with the 635.5 antibody that stains all three forms of GnRH. Cells in POA represent GnRH1 cells, but the GnRH form expressed in fibers cannot be distinguished. Expression patterns based on personal observation and (1) (Chen & Fernald, 2006); (2) (Grone et al., 2010); (3) (O'connell et al., 2011). + indicates cellular staining, f indicates fibers, and – indicates no staining. nd indicates that region was not examined or described in referenced studies.

suggesting a potential interaction between the two systems. Although more research is still needed, we propose that the tac3/GnRH interaction, if it occurs at all, may occur at the level of the hypothalamus of *A. burtoni*, rather than at the pituitary.

The best studied NKB cells in the tetrapod brain are the KNDy (Kisspeptin-NKB-Dynorphin) neurons in the arcuate nucleus. All teleosts examined to date have dense Tac3 staining in the NLT, the putative arcuate homolog. In *A. burtoni*, *tac3a*-expressing cells exist in all subdivisions of the NLT. To test for colocalization with kisspeptin, we double-labeled for *kiss2* and *tac3a* mRNAs. *Kiss2* expression was restricted to the NRL/NLTi area and found nowhere else in the brain. Within the NRL, *tac3a* and *kiss2* have distinct expression patterns, but *kiss2* and *tac3a*-labeled cells were intermingled within the NLTi. A small percentage (<5%) of *kiss2*-positive cells also expressed *tac3a.* In striped bass, NKB cells in the NLT (labeled as ATn, but cells appear to be similar in location to NLTm/NLTi cells in the *A. burtoni* brain) "strongly innervate" *kiss2* neurons in the NRL, and these *kiss2* neurons in turn project more sparsely to *tac3*/NKB neurons in the NLT (Zmora et al., 2017). In zebrafish, kisspeptin and tac3 neurons have distinct neuroanatomical distributions in the brain (Ogawa et al., 2012). NKB and NKF injections lowered *kiss1* and *kiss2* mRNA levels in the striped bass. *Kiss1/kiss2/GnRH3* triple knockout zebrafish have normal onset of puberty and other reproductive measures but increased *tac3a* brain expression (Liu et al., 2017). While GnRH1 and kisspeptin cells are relatively restricted within the teleost brain, receptors are widely expressed, including in many regions with *tac3a*-expressing cells (Chen & Fernald, 2006; Flanagan et al., 2007; González-Martínez et al., 2004; Grone et al., 2010; Moncaut, Somoza, Power, & Canário, 2005; Okubo, Suetake, Usami, & Aida, 2000; Soga et al., 2005) but double labels are needed to see if *tac3* cells express these receptors. Further research is also needed to examine NKB-receptor expression patterns in relation to GnRH- and kisspeptin-expressing cells. Interestingly, recent research indicates that kisspeptin is not involved in regulating the HPG axis in medaka fish (Nakajo et al., 2018) or zebrafish (Tang et al., 2014). Kisspeptin knockout fish had normal reproductive measures (e.g., maturation, gonadotropin levels, GnRH neuronal firing rate), indicating that it is not essential for reproduction, at least in some fish species. As such, the functional role of kisspeptin, and the NKB regulation of kisspeptin deserves further attention.

4.4 | Comparative expression of *tac3*/NKB in socially relevant brain regions

Although the distribution of NKB cells is only described in a few mammalian species (Chawla et al., 1997; Lucas, Hurley, Krause, & Harlan, 1992; Marksteiner et al., 1992; Shughrue, Lane, & Merchenthaler, 1996; Warden & Young, 1988; Zhang & Harlan, 1994), it appears similar to that of *tac3a* described here in the *A. burtoni* brain. The *tac3a*-expressing cells in *A. burtoni* are found in regions that are putative homologs to those expressing NKB cells in tetrapods, including

amygdalar nuclei, BNST, hippocampus, nucleus accumbens, and septal nuclei. Although there is some variability in localization among studies and homologies are still not well-defined and are debated, most have found that *tac3*/NKB is expressed in socially relevant brain regions (Table 4). In *A. burtoni*, expression was found in many socially relevant nuclei, with the exception of Vc and ATn. This is consistent with the absence of NKB cells in the mammalian ventromedial hypothalamus (VMH, putative homolog in part of ATn). However, in rodents, NKB staining was observed in several striatal nuclei (putative homolog in part of Vc) (Marksteiner et al., 1992; Warden & Young, 1988). Because of overlap between the mesolimbic reward system and *tac3a* staining, we also double labeled for *tac3a* and TH, a dopaminergic cell marker. Although *tac3a* and TH were found in close proximity throughout the brain, no *tac3a*-TH colabeled cells were observed. However, dopamine receptors (primarily D2R) are found throughout the *A. burtoni* brain (O'Connell et al., 2013), including in all regions with *tac3a* expression. In the guinea pig NAcc, striatum, and hippocampus, NK3R agonists enhance extracellular DA levels (Marco et al., 1998), suggesting a functional overlap between the NKB and DA systems. Thus, more research is needed on the potential function of NKB outside of the traditional KNDy neurons and their role in reproduction.

Tac3a cells in *A. burtoni* are found in several regions of the telencephalon and as far back as the vagal lobe. Despite the existence of these cells in socially relevant brain regions in several species (Chawla et al., 1997; Ogawa et al., 2012; Qi et al., 2015; Zmora et al., 2017), we know little about them. Several studies have injected NK3R agonists or antagonists to investigate their effects on behaviors. For example, injections of the NK3R agonist senktide into the raphe nucleus inhibits food and water intake in male rats (Paris, Mitsushio, & Lorens, 1991). Tachykinin receptors, including NK3R, are proposed to modify taste sensitivity, especially to sodium-rich tastes (Ciccocioppo, Polidori, Pompei, De Caro, & Massi, 1994). Rodents injected with an NK3R agonist suppress their salt intake, even when chronically sodium deficient (Flynn, 2006; Smith & Flynn, 1994), and injections with selective NK3R agonists reduce alcohol consumption (Ciccocioppo, Panocka, Pompei, De Caro, & Massi, 1994). In addition, NKB and NK3R are involved in consolidation of fear memories, and knockdown of NKB cells in the central amygdala impairs this consolidation (Andero et al., 2016; Andero, Dias, & Ressler, 2014). To date, however, no studies have investigated the potential role of *tac3* in social behaviors in any taxa. In fishes, the only functional studies of *tac3* have focused on its role in regulating reproductive neuropeptides, such as GnRH, kisspeptin, and gonadotropins. However, the wide distribution of *tac3a* cells in the cichlid brain, particularly in socially–relevant processing regions, suggests it may be involved in physiology and behavior beyond regulation of the hypothalamic–pituitary–gonadal axis.

TABLE 4 Expression of *tac3*a/NKB in socially relevant nuclei of fish and rodents

Teleost region	Putative mammalian homology	*Astatotilapia burtoni*	Rats
Dm	Pallial amygdala	+	+
Dl	Medial pallium/hippocampus	+	+
Vv	Septum/striatum	+	+
Vd	Striatum/basal ganglia/ nucleus accumbens	+	+
Vc	Striatum	–	+
Vs/Vp	Basal/central/extended amygdala	+	+
POA	Preoptic area	+	+
nH	Habenula	+	+
VTn	Anterior hypothalamus	nd	+
ATn	Ventromedial hypothalamus	–	–
TPp	Ventral tegmental area	+	+/–
PAG/CG	Periaqueductal gray/ central gray	+	+
SR	Raphe	+	–

Expression pattern in rats based on Lucas et al. (1992), Marksteiner et al. (1992), Shughrue et al. (1996), Warden and Young (1988) and Zhang and Harlan (1994). + indicates positive staining, – no staining, and +/– different results between studies. nd in the VTn of *A. burtoni* is due to uncertainty about its location. Note that putative mammalian homologs are only "in part" for many nuclei and are based on consensus from the following references: Demski (2013), Ganz et al. (2012), Ganz et al. (2014), Goodson and Kingsbury (2013), Meek and Nieuwenhuys (1998), O'Connell and Hofmann (2011) and Wulliman, Rupp, and Reichert (2012).

5 | CONCLUSIONS

The African cichlid fish *Astatotilapia burtoni* is an excellent model system for future studies on the function of *tac3a* in the brain. Although previous studies found that hypothalamic *tac3* expression is mediated by reproductive state (Biran et al., 2014; Chen et al., 2018; Qi et al., 2015), further research should examine the impact of male social status on *tac3a* expression in the brain. Because expression is widely distributed, a fine-scale neuroanatomical approach should be taken to examine specific *tac3* cell-populations in the brain, not just on a macro level. For example, *tac3a* cells are found in close proximity to cells expressing appetite stimulating and inhibiting proteins (Porter, Roberts, & Maruska, 2017) and are located in regions of the brain implicated in parental care and feeding. Since *A. burtoni* is a maternal mouthbrooding fish, it would be interesting to compare levels of *tac3a* in these regions between feeding, reproductively active females and nonfeeding females engaged in maternal care. *Tac3a* expression is widely distributed throughout the *A. burtoni* brain, including in close proximity to the GnRH, kisspeptin, and dopamine systems. Thus, its expression in socially relevant brain regions warrants further research on the function of NKB cell populations in fishes.

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AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

The authors have no known or potential conflicts of interest.

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