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Neurochemical profiling of dopaminergic neurons in the forebrain of a cichlid fish, *Astatotilapia burtoni*☆

Lauren A. O'Connell [a,b,1,2], Miles R. Fontenot [b,1], Hans A. Hofmann [a,b,c,*]

[a] Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, TX 78705, USA
[b] Section of Integrative Biology, University of Texas at Austin, Austin, TX 78705, USA
[c] Institute for Neuroscience, University of Texas at Austin, Austin, TX 78705, USA

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ABSTRACT

Across vertebrates, the mesolimbic reward system is a highly conserved neural network that serves to evaluate the salience of environmental stimuli, with dopamine as the neurotransmitter most relevant to its function. Although brain regions in the dopaminergic reward system have been well characterized in mammals, homologizing these brain areas with structures in teleosts has been controversial, especially for the mesencephalo-diencephalic dopaminergic cell populations. Here we examine the neurochemical profile of five dopaminergic cell groups (Vc, POA, PPr, TPp, pTn) in the model cichlid *Astatotilapia burtoni* to better understand putative homology relationships between teleosts and mammals. We characterized in the adult brain the expression patterns of three genes (*etv5*, *nr4a2*, and *pitx3*) that either specify dopaminergic cell fate or maintain dopaminergic cell populations. We then determined whether these genes are expressed in dopaminergic cells. We find many striking similarities in these gene expression profiles between dopaminergic cell populations in teleosts and their putative mammalian homologs. Our results suggest that many of these dopaminergic cell groups are indeed evolutionarily ancient and conserved across vertebrates.

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1. Introduction

Dopamine is an evolutionarily ancient neurotransmitter present in many eukaryotes and serves as a neuromodulator of many behavioral processes, such as learning and memory (Wise, 2004; Hyman et al., 2006), social behavior (Young et al., 2011; O'Connell and Hofmann, 2011a), and the selection of motor programs (Joshua et al., 2009; Vidal-Gadea et al., 2011). The functional contributions of various dopaminergic cell groups to behavioral patterns are well studied in laboratory rodents due to their established utility for understanding nervous system disorders (Koob and Volkow, 2010; Lodge and Grace, 2011; Plowman and Kleim, 2011). However, the evolutionary relationships of dopaminergic cell groups across vertebrates are not well understood as their location significantly varies across vertebrate lineages (Wullimann and Mueller, 2004; Butler and Hodos, 1996;

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Abbreviations: aTn, anterior tuberal nucleus; CP, central posterior thalamic nucleus; D, dorsal (pallial) part of the telencephalon; Dc, central part of D; Dc-2, subdivision of Dc; Dd, dorsal part of D; Dl, lateral part of D; Dld, dorsal region of Dl; Dlg, granular region of Dl; Dlv, ventral region of Dl; Dlvv, ventral zone of Dlv; Dm, medial part of D; Dm-1,3, subdivisions of Dm; Dm2c, caudal part of Dm-2; Dn, diffuse nucleus of the inferior lobe; Dp, posterior part of D; E, entopeduncular nucleus; Gn, glomerular nucleus; H, habenula; HC, horizontal commissure; LHn, lateral hypothalamic nucleus; LR, lateral recess; LT, longitudinal torus; mPGn, medial preglomerular nucleus; nLT, nucleus of the lateral torus; OB, olfactory bulb; OPT, optic tract; OT, optic tectum; PN, prethalamic nucleus; POA, preoptic area; PPd, dorsal periventricular pretectal nucleus; PPr, rostral periventricular pretectal nucleus; pTn, posterior tuberal nucleus; PVO, paraventricular organ; ST, semicircular torus; TPp, periventricular posterior tuberculum; V, ventral (subpallial) division of the telencephalon; Vc, central part of V; Vd, dorsal nucleus of V; Vdc, caudal region of Vd; VH, ventral hypothalamus; Vi, intermediate part of V; VM, ventromedial thalamic nucleus; Vp, postcommissural nucleus of V; Vs, supracommissural nucleus of V; Vsl, lateral region of Vs; Vsm, medial region of Vs; vTn, ventral tuberal nucleus; Vv, ventral part of V.

* Corresponding author at: Section of Integrative Biology, The University of Texas at Austin, 1 University Station - C0930, Austin, TX 78712, USA. Tel.: +1 512 475 6754; fax: +1 512 471 3878.
E-mail address: hans@utexas.edu (H.A. Hofmann).

[1] These authors contributed equally to this work.
[2] Current address: FAS Center for Systems Biology, Harvard University, Cambridge, MA 02138, USA.

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Yamamoto and Vernier, 2011; O'Connell and Hofmann, 2011b, 2012). This is of particular relevance as non-mammalian model systems are becoming increasingly useful for studying the role of dopamine and other biogenic amines, especially with regard to questions that are not very tractable in rodent model systems, such as functional analyses of social networks (Winberg et al., 1997) and vocal communication (Sasaki et al., 2006; Huang and Hessler, 2008; Heimovics et al., 2009). Here we examine the neurochemical profiles of several teleost dopaminergic cell groups in order to better understand their putative homology relationships with mammalian dopaminergic cell groups.

The substantia nigra (SNc) and ventral tegmental area (VTA) are particularly well studied in mammals due to their central roles in regulating motor and behavioral decision-making, respectively (Mogenson et al., 1980; Berridge and Robinson, 1998; Platt, 2002; Sugrue et al., 2005; Balleine et al., 2007). The SNc regulates motor output and its dopaminergic cell population has been studied in much detail due to its role in motor deficits in disease phenotypes such as Parkinson's disease (Fearnley and Lees, 1991; Shulman et al., 2011; Wirdefeldt et al., 2011). On the other hand, the VTA is involved in risk-taking and reward evaluation (Schultz, 1998; Tobler et al., 2005; Preuschoff and Bossaerts, 2007) as well as the processing of nociception (Sotres-Bayón et al., 2001). In mammals, both of these cell groups are located in the mesencephalon and also extend into the basal diencephalon, have a common developmental origin, and thus have remarkably similar neurochemical and gene expression profiles (Grimm et al., 2004; Chung et al., 2005). In contrast, dopaminergic cell groups are located throughout the forebrain and hindbrain in teleosts, but not in the midbrain, which makes establishing functionally similar cell groups between the mammalian mesencephalic dopaminergic neurons and dopaminergic cell groups in teleosts exceedingly difficult (Wullimann and Mueller, 2004; O'Connell and Hofmann, 2011b; Yamamoto and Vernier, 2011). Nevertheless, several research groups have recently made significant strides toward elucidating the functionally analogous dopaminergic cell groups in teleosts and mammals (Yamamoto and Vernier, 2011; Schweitzer et al., 2011). Yet despite this progress it is still unclear which cell groups in the teleost brain are functionally analogous to the mammalian SNn and VTA. To investigate the putative relationships between mammalian and teleost dopaminergic cell populations, we examined the expression profiles of three transcription factors (*etv5*, *pitx3*, and *nr4a2*) involved in differentiation and maintenance of dopaminergic cells.

Much effort has been made to characterize the genomic contributions to dopamine neuron specification by searching for conserved motifs of dopamine pathway genes across species (Hobert et al., 2010; Fujimoto et al., 2011). Flames and Hobert (2009) first proposed a regulatory logic and conserved "DA motif" in *C. elegans*, which appeared to be conserved in mammals. Specifically, the "Ets-related" family of transcription factors was found to determine dopaminergic cell fate in *C. elegans* (*via ast-1*) and mouse olfactory dopamine neurons (*via etv1*). Following this regulatory logic, the Etv variant *etv5* expressed in the mammalian midbrain dopaminergic neurons (Gray et al., 2004) may be involved in regulating midbrain dopaminergic cell fate in vertebrates (although this may not be the case in *Mus musculus*; see Wang and Turner, 2010). The brain distribution of *etv5* has not been determined outside of rodents and thus it is unknown whether *etv5* is expressed in the SNc/VTA populations of other taxa.

In mammals, *nr4a2* (also known as *nurr1*) and *pitx3* are two important transcription factors that play crucial roles in the maintenance of midbrain dopaminergic neurons. Although not required for midbrain dopaminergic neuron development in mammals, *nr4a2* is essential for maintenance and transmitter synthesis and release (Smits et al., 2003; Kadkhodaei et al., 2009). Importantly, knockdown of *nr4a2* disrupts dopamine neuron maturation in zebrafish (*Danio rerio*; Luo et al., 2008). Nr4a2 directly regulates the expression of *pitx3* (Volpicelli et al., 2012), which is specifically required for terminal differentiation and maintenance of SNc neurons (Smidt et al., 1997, 2004) as *pitx3* knockout in mice results in ablation of dopaminergic SNc neurons, but VTA dopaminergic neurons are less affected (Smidt and Burbach, 2007). In comparison, the neural distribution, colocalization in dopaminergic cells, and functional relevance of *nr4a2* and *pitx3* are poorly understood in teleosts.

The cichlid family of fishes offers unique opportunities for comparative studies of the complex and plastic behavior patterns involved in behavioral decision-making and motor output, as rapid radiation of species with diverse social phenotypes allows comparison across closely related species (Hofmann, 2003; Kocher, 2004). Here we use the cichlid *Astatotilapia burtoni*, a model system in social neuroscience (Robinson et al., 2008), which is ideally suited for studying how the social environment influences phenotypic plasticity. A better understanding of the dopaminergic systems in this cichlid will allow us to not only gain insights into the neural substrates of social decision-making, but also provide an excellent model for determining how manipulations of specific dopaminergic cell populations alter social behavior, risk-taking, and motor output within ecologically relevant contexts.

We have previously described the distribution of dopamine-associated genes (tyrosine hydroxylase and dopamine D1 and D2 receptors) in *A. burtoni* (O'Connell et al., 2011), and here we expand on that work by examining the neurochemical profiles of five dopaminergic cell groups that are hypothesized to regulate behavior and motor output in teleosts (Schweitzer et al., 2011). Specifically, we examined dopaminergic populations in the central part of the ventral telencephalon (Vc), the preoptic area (POA), the rostral periventricular pretectal nucleus (PPr), the periventricular nucleus of the posterior tuberculum (TPp), and the posterior tuberal nucleus (pTn). Of particular interest are the TPp and pTn, which are candidate dopaminergic cell groups in teleosts that may be functionally analogous to the mammalian SNc/VTA or other mammalian diencephalic dopamine groups (Rink and Wullimann, 2001; Filippi et al., 2010; Yamamoto and Vernier, 2011; Tay et al., 2011). Importantly, the mammalian VTA and SNc do indeed extend into the basal diencephalon, up to the ventral part of the third prosomere, supporting their putative homology to the TPp in teleosts, which is also located mostly in the third prosomere (Wullimann and Rink, 2002). Our overall objective is to gain insight into which dopaminergic cell groups in the teleost forebrain may be neurochemically homologous to the mammalian midbrain dopaminergic cell groups by examining expression patterns of *etv5*, *nr4a2*, and *pitx3*, which encode transcription factors of known importance in dopaminergic cell specification or maintenance.

2. Methods

2.1. Animals

Male and female *A. burtoni* descended from a wild-caught stock population were kept in aquaria under conditions mimicking their natural environment as previously described (O'Connell et al., 2011). All work was carried out in compliance with the Institutional Animal Care and Use Committee at The University of Texas at Austin.

2.2. Cloning of A. burtoni etv5, nr4a2, and pitx3 cDNA

To obtain the *A. burtoni etv5*, *nr4a2*, and *pitx3* sequences, degenerate primers were designed using CODEHOP (http://blocks.fhcrc.org/codehop.html) based on the zebrafish protein sequences (GenBank accession numbers: Etv5, AAT68296; Nr4a2, NP_001106956.1; Pitx3, NP_991238.1) and homologous sequences from stickleback (*Gasterosteus aculeatus*), medaka (*Oryzias latipes*), and fugu (*Takifugu rubripes*) obtained using the UCSC Genome Browser (http://genome.ucsc.edu/).

Table 1
Primers using in cloning *A. burtoni* Etv5, N4r2a, and Pitx3.

Gene	Reaction	Forward (5′–3′)	Reverse (5′–3′)
Etv5	Outer PCR	GGTGCCCGACGARCARTTYGT	CGTCGGGGTCGCACACRAAYTTRTA
Etv5	Nested PCR	GCTGGAGGGCAAGGTGAARCARGAR	CGGGCCGGTTCTTCTGDATNCCCCA
Nr4a2	Outer PCR	GCC CTG CGT GCA GGC NCA RTA YGG	GGT GAA GCC GGG GAT CTT YTC NGC C
Nr4a2	Nested PCR	GGTGCCAGTACTGCCGGTTYCARAAR	GCCGGTCAGCAGGTCGTARAAYTGYT
Pitx3	Outer 3′RACE PCR	GGCTACCAAGAGCCTAGCAGCAAGC	Universal RACE Primer Mix (Clontech)
Pitx3	Nested 3′RACE PCR	TCAGCACTCAACTCGGTAGCAGTGAC	Nested RACE Primer (Clontech)
Pitx3	Probe PCR	CTGCAACGACAACTGGAAGA	AAGCCGACCTTTGACCTATG

A. burtoni Etv5 was isolated in a nested PCR approach using whole-brain cDNA as template, an annealing temperature of 52 °C and 40 rounds of amplification (see Table 1 for primer details). This initial PCR product was then used as template in a nested PCR using two nested primers with an annealing temperature of 73.6 °C and 30 rounds of amplification. This nested PCR resulted in a 213 bp fragment of *A. burtoni etv5*.

The *A. burtoni nr4a2* sequence was obtained using nested touchdown PCR with an annealing temperature of 58 °C that decreased by 2 °C every 3 cycles for 9 cycles and then continued 30 more cycles at 50 °C. This same touchdown protocol was used in a nested PCR reaction that resulted in a 296 bp fragment.

To clone *A. burtoni pitx3*, we designed a primer in a highly conserved 5′ region of the zebrafish *pitx3* (GenBank accession no. 402974) that differed from the *pitx1* and *pitx2* to use in 3′ RACE-PCR (Rapid Amplification of cDNA Ends-PCR; Clontech, Mountain View, CA) according to manufacturer's instructions. We generated a 950 bp fragment of *A. burtoni pitx3* using this method and used this sequence to design primers to isolate a suitable fragment for *in situ* hybridization probe generation. We then performed a PCR with an annealing temperature of 51 °C and 30 rounds of amplification, yielding a 200-base-pair product in the 3′ untranslated region of the *A. burtoni* Pitx3. We designed the *in situ* hybridization probe in this portion of the mRNA to avoid detecting *pitx1* and *pitx2*, which are very similar to *pitx3* in some coding regions.

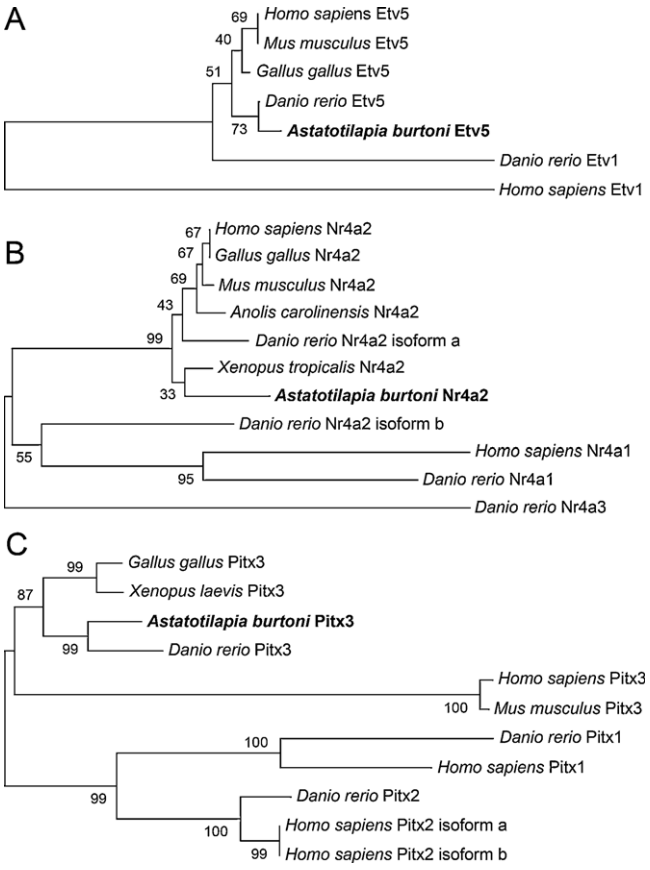

Fig. 1. Comparison of *A. burtoni* Etv5, Nr4a2, and Pitx3 genes with other vertebrates. Rooted neighbor-joining trees show that the *A. burtoni* Etv5 (A), Nr4a2 (B), and Pitx3 (C) sequences (in bold) cluster with orthologous genes in other vertebrates and are distinctly different from paralogous sequences in the same gene family, which were used as outgroups. Numbers at branch-points indicate confidence from a bootstrap analysis of 5000 repetitions.

The *etv5*, *nr4a2*, and *pitx3* target fragments were cloned into a pCRII-TOPO vector (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The partial mRNA sequences, which present the consensus of three sequenced clones for each gene, have been submitted to GenBank (*etv5*, GU145551.1; *nr4a2*, GU145552.1; *pitx3*, GU145553.1). Since determining the sequence for these genes, the draft genome of *A. burtoni* has been released. We used NCBI BLAST to search for paralogs of *etv5*, *nr4a2*, and *pitx3* in the *A. burtoni* genome and found no evidence of paralogs. This suggests that the additional *nr4a2* paralog that likely arose from a duplication event early on in the teleost lineage, and is present in zebrafish and medaka (*O. latipes*), has been lost in cichlids, similar to the situation found in tetraodontiforms (*Fugu rubripes* and *Tetraodon viridis*) (Blin et al., 2008).

2.3. Phylogenetic analyses of etv5, nr4a2, and pitx3

The partial mRNA sequences obtained for *A. burtoni etv5*, *nr4a2*, and *pitx3* were translated into protein sequence and compared to Etv5, Nr4a2, and Pitx3 protein sequences of multiple species (Fig. 1). Using Mega 4 (http://www.megasoftware.net/mega4/mega.html), we aligned the sequences with ClustalW and generated rooted nearest neighbor-joining gene trees with 5000 bootstrap repetitions. Genbank accession numbers and species information used in analysis are as follows: *H. sapiens* Pitx3: NP_005020.1; *H. sapiens* Pitx1: AAH03685.1; *H. sapiens* Pitx2 isoform a: NP_001191328.1; *H. sapiens* Pitx2 isoform b: NP_001191327.1; *M. musculus* Pitx3: AAB87380.1; *G. gallus* Pitx3: XP_421631.2; *X. laevis* Pitx3: NP_001079212.1; *D. rerio* Pitx3: AAT68296.1; *D. rerio* Pitx2: NP_571050.1; *D. rerio* Pitx1: NP_001035436.3; *H. sapiens* Nr4a2: NP_006177.1; *H. sapiens* Nr4a1: CAG32985.1; *M. musculus* Nr4a2: NP_001132981.1; *G. gallus* Nr4a2: XP_422166.2; *X. tropicalis* Nr4a2: NP_001093678.1; *D. rerio* Nr4a2a: ABV31068.1; *D. rerio* Nr4a2b: NP_001002406.1; *D. rerio* Nr4a1: NP_001002173.1; *D. rerio* Nr2a3: NP_001166100.1; *H. sapiens* Etv5: NP_004445.1; *H. sapiens* Etv1: CAG47050.1; *M. musculus* Etv5: NP_076283.2; *G. gallus* Etv5: XP_422651; *D. rerio* Etv5: NP_001119933.1; *D. rerio* Etv1: NP_001025353.1.

2.4. In situ hybridization (ISH)

Males ($n = 3$) and females ($n = 4$) were killed and their brains rapidly dissected, frozen in Optimal Cutting Temperature (O.C.T.) compound (Tissue-Tek, Fisher Scientific, Pittsburgh, PA), and stored at −80 °C. Brains were then cryo-sectioned at 25 μm into four series and thaw-mounted onto Super-Frost Plus slides (Erie Scientific Co., Portsmouth, NH) that were stored at −80 °C for less than 4 weeks until processing for ISH as previously described in Munchrath and Hofmann (2010) and O'Connell et al. (2011). Riboprobes were reverse-transcribed in the presence of fluorescein-labeled UTP (Roche, Indianapolis, IN) using a T7/SP6 Maxiscript *in vitro* transcription kit (Ambion, Austin, TX) to produce antisense or sense probes that were fluorescein (FITC) labeled. The template used to make each riboprobe was 213 bp for *etv5*, 296 bp for *nr42a* and 200 bp for *pitx3*. One series of brain sections were used to detect each gene.

2.5. Double-labeling using dual fluorescent ISH and immunohistochemistry (IHC)

To determine if *etv5*, *nr4a2*, and *pitx3* are expressed in dopamine neurons, we co-localized *etv5*, *nr4a2*, and *pitx3* mRNA with tyrosine hydroxylase (TH) protein using dual fluorescent ISH and IHC. *A. burtoni* males ($n = 3$) and females ($n = 4$) were killed and their brains rapidly dissected and fixed in 4% PFA in 1× PBS (pH 7.4) at 4 °C for 2 h. Brains were then washed in 1× PBS and cryo-protected in 30% sucrose in 1× PBS overnight at 4 °C before embedding in O.C.T. and storing at −80 °C. Brains were then cryo-sectioned at 25 μm into four series and thaw-mounted onto Super-Frost Plus slides (Erie Scientific Co., Portsmouth, NH) that were stored at −80 °C until processing for ISH. FITC-labeled riboprobes were hybridized to the tissue as described above. After an overnight hybridization, cover slips were removed in 65 °C 5× SSC, and then washed in 0.2× SSC three times for 20 min each at 65 °C. Slides were equilibrated in TN buffer (100 mM Tris–HCl pH 7.4, 150 mM NaCl) for 5 min and then blocked in TNB (0.5% blocking reagent [Perkin Elmer, Waltham, MA] in TN buffer) for 30 min. Slides were then incubated with anti-FITC-POD (Roche, Indianapolis, IN, 1:500 in TNB) for 30 min, washed in TNT (0.05% Tween20 in TN buffer) three times for 5 min each, and then incubated in FITC-Tyramide (Perkin Elmer) for 10 min. Slides were then washed in TNT for 5 min and then immediately processed for IHC detection of TH as previously described (O'Connell et al., 2011). Briefly, after the last wash of ISH, slides were rinsed twice in PBS and incubated in

3% hydrogen peroxide in PBS for 20 min, and blocked in normal goat serum. The sections were then incubated at room temperature overnight in 1:500 primary antibody (rabbit anti-TH; Millipore, Bedford, MA; catalog no. AB152). This antibody has been previously shown to bind specifically to *A. burtoni* TH1 and TH2 proteins (O'Connell et al., 2011). Sections were then rinsed twice in PBS and then incubated in 1:200 goat anti rabbit Cy3 (Jackson Immunoresearch, West Grove, PA) in a 2% NGS and 0.3% Triton X-100 in PBS solution for 2 h. Slides were then rinsed twice in PBS and cover-slipped with DAPI hardset fluorescent mounting media (Vector Laboratories, Burlingame, CA). For control sections, all procedures were the same, except that primary antibody was omitted or sense riboprobe was used in place of the anti-sense probes. Both these controls showed no signal.

2.6. Photomicroscopy

Brightfield and fluorescent optics were used to visualize ISH and IHC staining throughout the brain at low (5×) and high (20×) magnification. Photographs were taken with a digital camera (AxioCam MRc; Zeiss) attached to a Zeiss AxioImager.A1 AX10 microscope using the AxioVision (Zeiss) image acquisition and processing software. Images were compiled and brightness and contrast enhanced in Adobe Photoshop CS5.

3. Results

Here we describe the brain distribution of several transcription factor genes that are implicated in dopaminergic cell speciation or maintenance. We also determined whether these genes are expressed in dopaminergic cell populations by colocalizing their mRNA with an antibody targeting tyrosine hydroxylase (TH), which catalyzes the rate-limiting step in catecholamine synthesis

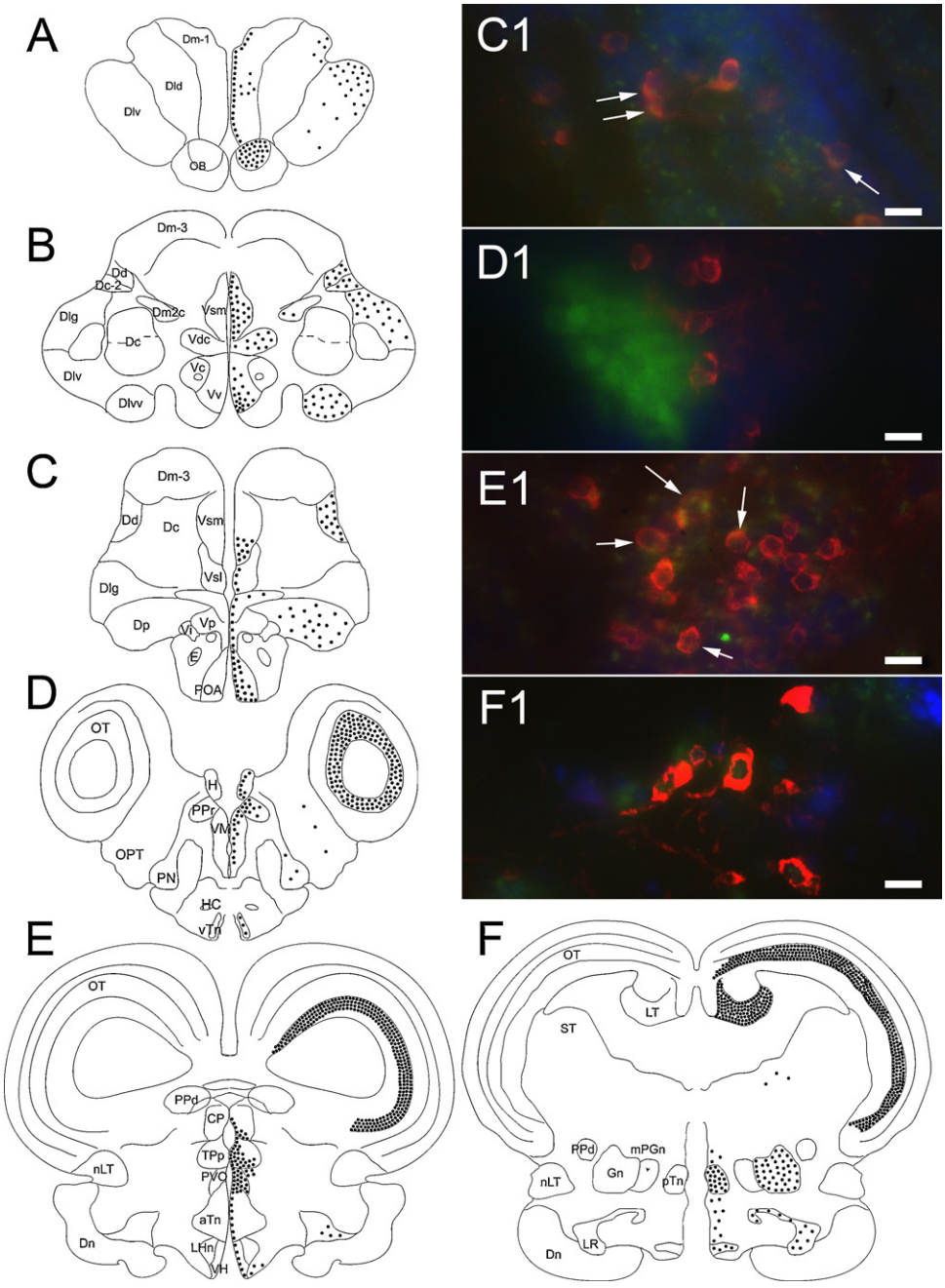

Fig. 2. Distribution of Etv5 in the *A. burtoni* forebrain and expression in dopaminergic cell groups. Representative sections of the *A. burtoni* forebrain are presented as the first image in each panel with nomenclature labeled on the left half and Etv5 mRNA expression patterns shown as dots on the right half. Representative micrographs show expression of Etv5 mRNA (green) and TH protein (red) in the preoptic area (POA, C1), rostral periventricular pretectal nucleus (PPr, D1), periventricular posterior tuberculum (TPp, E1), and posterior tuberal nucleus (pTn, F1). DAPI (blue) stain shows cell nuclei. All scale bars are 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(Levitt et al., 1965). Note that all TH-positive cell groups in the teleost forebrain are dopaminergic (Ma, 1994). The distributions shown here are representative of *A. burtoni* females and dominant and subordinate males, because there were no qualitative differences (in total presence or absence) between sexes or phenotypes.

3.1. Forebrain distribution of etv5 and co-localization with TH

Robust staining for *etv5* mRNA was seen in discrete cell bodies throughout the *A. burtoni* forebrain (Fig. 2). Expression of *etv5* is present in the olfactory bulb (OB), primarily in the granule layer. In the dorsal telencephalon (D), *etv5* is present along the medial part of D (Dm-1) and the dorsal and ventral subregions of the lateral part of D (Dlv, Dld). The dorsal part of D (Dd), the second subdivision of Dc (Dc-2), the granular region Dl (Dlg), the ventral zone of Dlv (Dlvv), and posterior part of D (Dp) show moderate *etv5* presence. A few cell bodies in the caudal part of Dm-2 (Dm-2c) also contain *etv5*. In the ventral telencephalon (V), *etv5* is present in the ventral part of V (Vv), the caudal region of the lateral part of V (Vdc), the lateral and medial regions of the supracommisural part of V (Vsl, Vsm), and the postcommisural nucleus of V (Vp).

The preoptic area (POA) is moderately rich in *etv5* expression in the parvocellular, magnocellular, and gigantocellular regions. *etv5* is also present along the medial line of the anterior tuberal nucleus (aTn), the ventromedial thalamic nucleus (VM), and the central

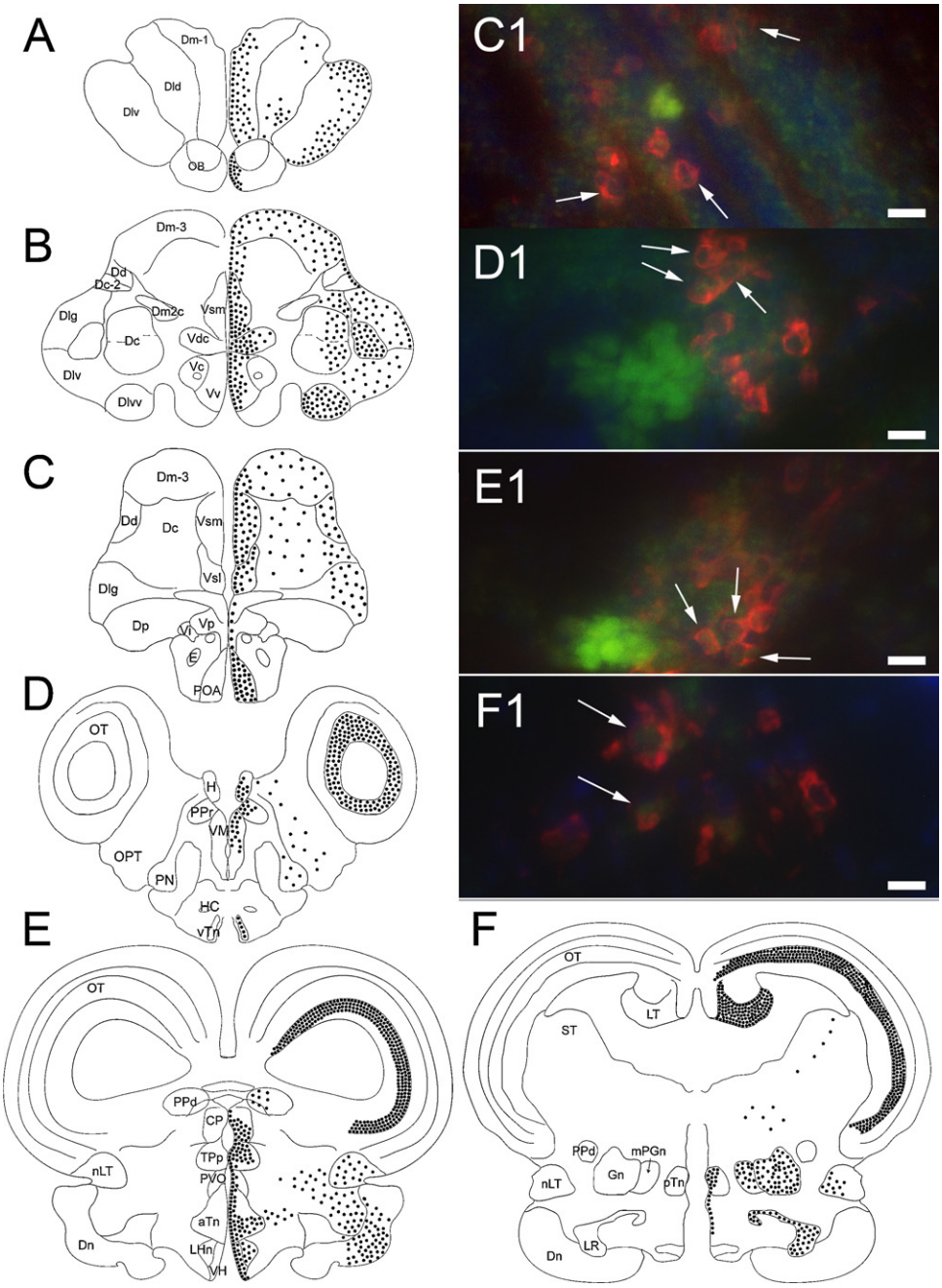

Fig. 3. Distribution of Nr4a2 in the *A. burtoni* forebrain and expression in dopaminergic cell groups. Representative sections of the *A. burtoni* forebrain are presented as the first image in each panel with nomenclature labeled on the left half and Nr4a2 mRNA expression patterns shown as dots on the right half. Representative micrographs show expression of Nr4a2 mRNA (green) and TH protein (red) in the preoptic area (POA, C1), rostral periventricular pretectal nucleus (PPr, D1), periventricular posterior tuberculum (TPp, E1), and posterior tuberal nucleus (pTn, F1). DAPI (blue) stain shows cell nuclei. All scale bars are 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

posterior thalamic nucleus (CP). The paraventricular organ (PVO), the posterior tuberculum (TPp), and the optic tectum (OT) also contain *etv5*. The dorsal periventricular pretectal nucleus (PPd) also contains a few *etv5* positive cells. More caudally, *etv5* expression extends caudally in both the TPp and OT. *etv5* is also expressed in the longitudinal torus (LT). Finally, moderate numbers of *etv5*-positive cell bodies were found in lateral recess (LR) and corpus glomerulosum pars rotunda (Gn).

We next determined whether *etv5* co-localized with any putative forebrain dopaminergic cell groups. We found co-localization of *etv5* with TH in the POA (Fig. 2C1), TPp (Fig. 2E1), and the pTn (Fig. 2F1). Although *etv5* is expressed in the PPr, we found no co-localization with TH in this region (Fig. 2D1).

3.2. Forebrain distribution of nr4a2 and co-localization with TH

Expression of *nr4a2* was generally much more abundant than *etv5* or *pitx3*. Robust staining for *nr4a2* was seen in discrete cell bodies throughout the telencephalon of *A. burtoni* (Fig. 3). *nr4a2* is present in the OB, primarily in the mitral layer. We find *nr4a2* expression along Dm-1 and the length of Dlv and Dld. Dd, Dc-2, Dlg, Dlvv, and Dc all contain *nr4a2*. *nr4a2* is also widely expressed throughout the ventral telencephalon. *nr4a2* was detected in Vv, throughout Vdc, and much of Vsm. In the caudal ventral telencephalon, *nr4a2* is present within Vp and Vsl.

We found strong *nr4a2* signal in the parvocellular, magnocellular, and gigantocellular POA. Caudal to the telencephalon, *nr4a2*

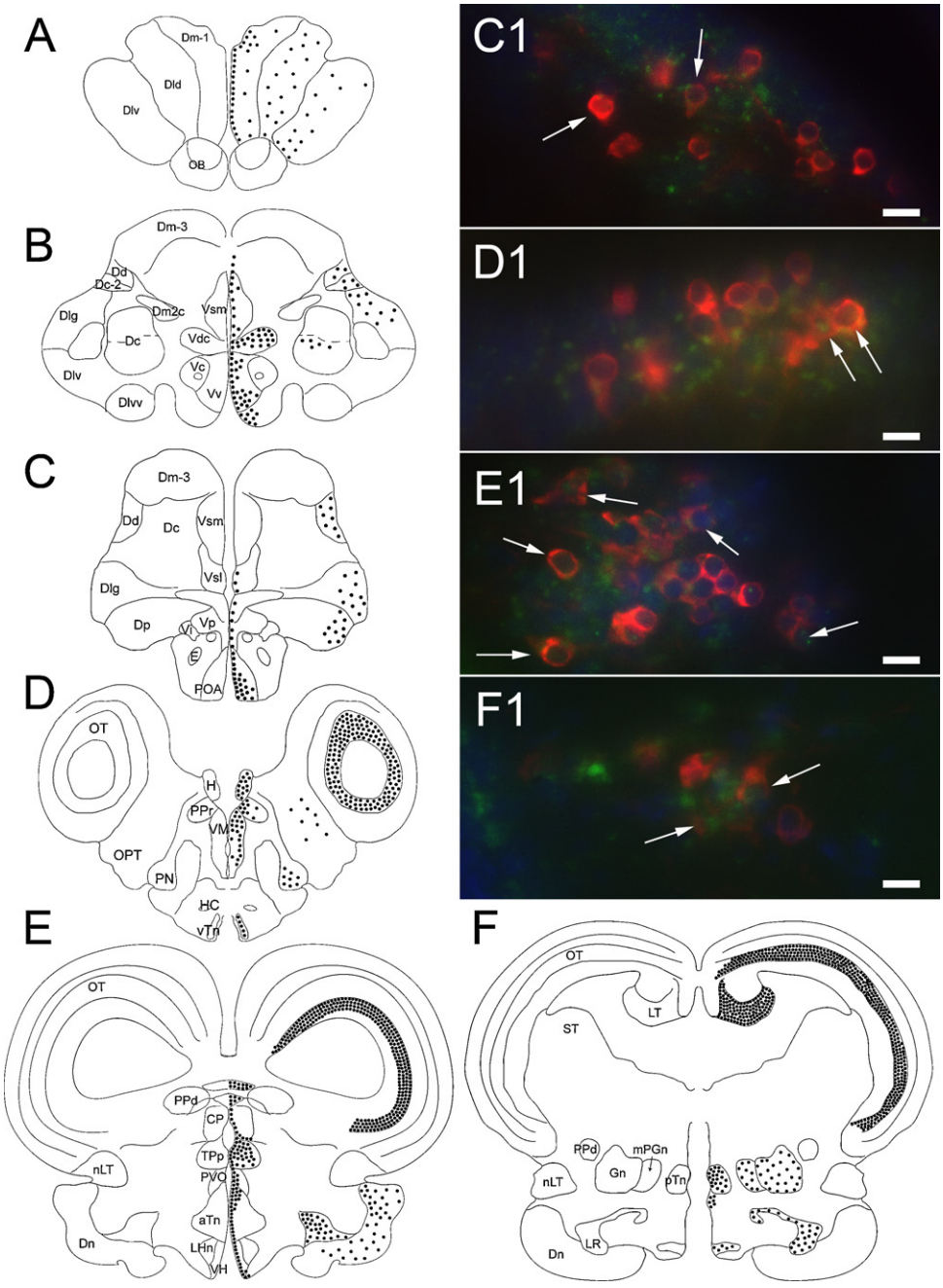

Fig. 4. Distribution of Pitx3 in the *A. burtoni* forebrain and expression in dopaminergic cell groups. Representative sections of the *A. burtoni* forebrain are presented as the first image in each panel with nomenclature labeled on the left half and Pitx3 mRNA expression patterns shown as dots on the right half. Representative micrographs show expression of Pitx3 mRNA (green) and TH protein (red) in the preoptic area (POA, C1), rostral periventricular pretectal nucleus (PPr, D1), periventricular posterior tuberculum (TPp, E1), and posterior tuberal nucleus (pTn, F1). DAPI (blue) stain shows cell nuclei. All scale bars are 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

is present along the PVO, aTn and VM. The CP, TPp, and OT show *nr4a2* expression. The PPd also contains some *nr4a2*. In contrast to *etv5* expression, *nr4a2* is found in the lateral hypothalamic nucleus (LHn), diffuse nucleus of the inferior lobe (Dn), and nucleus of the lateral torus (nLT). Finally, moderate numbers of *nr4a2*-positive cell bodies were found in the LR, Gn, and medial preglomerular nucleus (mPGn).

To determine whether *nr4a2* was expressed in putative dopaminergic neurons in the *A. burtoni* forebrain, we co-localized *nr4a2* with TH and found co-localization in the POA, PPr, TPp, and pTn (Fig. 3C1–F1).

3.3. Forebrain distribution of pitx3 and co-localization with TH

Expression of *pitx3* was generally less widespread than *nr4a2* expression. *pitx3* is expressed throughout the telencephalon of *A. burtoni* (Fig. 4). In contrast to *etv5* and *nr4a2*, *pitx3* expression is absent from the OB. In the dorsal telencephalon, *pitx3* is present along Dm-1 and the length of Dlv and Dld. Dd, Dc-2, Dlg, Dc, and Dp all show moderate *pitx3* expression. *pitx3* is widely expressed throughout the ventral telencephalon, including Vv, Vdc, Vsm, Vsl, and Vp.

pitx3 is expressed in the parvocellular, magnocellular, and gigantocellular POA. Caudal to the telencephalon, *pitx3* is expressed in the PVO, aTn, VH, and CP. The TPp and OT both contain *pitx3*. Much like the *nr4a2* expression, *pitx3* is found in Dn. High levels of *pitx3* are expressed in the LT. Finally, *pitx3* is expressed in the LR, Gn, and mPGn.

We next determined if *pitx3* was expressed in putative dopaminergic cells in the *A. burtoni* forebrain by co-localizing *pitx3* with TH protein. We found that *pitx3* is expressed in dopaminergic cells groups in the POA, PPr, TPp, and pTn (Fig. 4C1–F1).

4. Discussion

We have described the expression patterns in the forebrain of the model cichlid, *A. burtoni*, of three regulatory genes (*etv5*, *nr4a2*, and *pitx3*) important for mammalian midbrain dopamine cell group maturation and maintenance. We also determined whether *etv5*, *nr4a2*, and *pitx3* mRNAs were present in putative dopaminergic cell groups by co-localization with TH. Collectively, our results suggest *etv5*, *nr4a2*, and *pitx3* play important roles in dopamine neuron maturation and/or maintenance in diencephalic, but not telencephalic, dopamine cell groups of the teleost brain (Table 2).

Table 2
Summary of cichlid dopamine cell group neurochemistry.

DA group	Etv5		Nr4a2		Pitx3	
	Present	TH	Present	TH	Present	TH
Vc	–	–	–	–	–	–
POA	+	+	+	+	+	+
PPr	+	–	+	+	+	+
TPp	+	+	+	+	+	+
pTn	+	+	+	+	+	+

Summary of *A. burtoni* dopaminergic (DA) cell group neurochemistry. The presence or absence of Etv5, Nr4a2, and Pitx3 within a certain brain area (first column in each group) is noted with "+" or "–", respectively. Co-localization of a particular gene with TH (second column in each group) is indicated by is noted with "+".

4.1. Comparative neurochemistry of dopamine neurons in teleosts

Most of the work on specification of dopaminergic neurons in teleosts comes from zebrafish, and here we discuss this literature in relation to the distribution of *nr4a2* and *pitx3* in the cichlid brain as described in the present study. Although the expression of these genes is widely distributed throughout the teleost forebrain, their co-localization patterns with tyrosine hydroxylase suggest that they may play important roles in dopaminergic cell differentiation and/or maintenance. To our knowledge, the distribution of *etv5* expression has not previously been described in any teleost and thus our discussion with regard to teleosts will focus on *nr4a2* and *pitx3*.

The first thorough description of *nr4a2* expression in a teleost brain was provided by Kapsimali et al. (2001) in medaka. In both *A. burtoni* and medaka, *nr4a2* is expressed in the dorsal and ventral telencephalon, habenula, POA, and diencephalic regions with dopaminergic cells and/or fibers, such as the TPp, PTn, PPr, and optic tectum. There are some species differences in *nr4a2* expression, as we found *nr4a2* mRNA in Dc, the preglomerular nucleus, and the olfactory bulb. The olfactory bulb of mouse also expresses *nr4a2* (Zetterström et al., 1996; Saucedo-Cardenas et al., 1998). Similar to our findings, Kapsimali et al. (2001) also found that *nr4a2* co-localizes with TH in the POA, PPr, TPp, and PTn. Several studies in developing zebrafish have also reported co-localization of *nr4a2* with TH in the POA, PPr, TPp, and PTn, but not the telencephalic dopamine cell groups (Filippi et al., 2007; Blin et al., 2008). Morpholino knockdown studies in zebrafish have further tested the importance of *nr4a2* in dopamine cell maintenance, although the results do not show a clear pattern. Filippi et al. (2007) report that *nr4a2* knockdown decreases the number of TH-positive neurons in the POA and PPr, but not in the TPp. Moreover, these authors did not find evidence of *nr4a2* co-localization with TH in the TPp. However, others studies suggest that *nr4a2* is necessary for dopamine cell maintenance in not only the POA and PPr, but also in the telencephalon and TPp (Blin et al., 2008; Luo et al., 2008). Clearly, more studies are needed to better understand exactly what dopaminergic cell groups in the teleost brain are maintained by *nr4a2*.

Most of what is currently known about *pitx3* in the teleost brain comes from work in zebrafish, and compared to our results in cichlids, there are some striking species differences. During development, *pitx3* is strongly expressed in the zebrafish diencephalon (Shi et al., 2005). Filippi et al. (2007) found *pitx3* widely expressed near diencephalic dopamine neurons, but found no evidence for co-localization with any TH-positive cell groups. This is in contrast to our finding in *A. burtoni* that *pitx3* does co-localize with many diencephalic dopamine cell groups. Moreover, morpholino-mediated knockdown of *pitx3* did partially ablate TH-positive diencephalic neurons, although the authors argue that this was due to non-specific apoptosis (Filippi et al., 2007). As is the case with *nr4a2* discussed above, more work is necessary to determine what role *pitx3* plays in dopaminergic cell specification or maintenance in teleosts.

4.2. Contrasting teleost and mammalian homologies

Comparing dopaminergic cell populations between teleosts and mammals is extremely difficult, in part, due to the eversion of the telencephalon during development in teleosts, rather than an inversion as in all tetrapods (Northcutt and Braford, 1980; Nieuwenhuys and Meek, 1990). Moreover, the location of dopaminergic cell populations is extremely variable across vertebrates, much more so than the distribution of dopamine receptors (O'Connell and Hofmann, 2012). A particular challenge is that there are no midbrain dopaminergic neurons in teleosts and thus identifying the dopaminergic cell groups that may be functionally analogous to the mammalian SNc and VTA is difficult (Ekström et al., 1992; Smeets and González, 2000; O'Connell et al., 2011; Schweitzer et al., 2011; Yamamoto and Vernier, 2011).

However, due to the biomedical relevance of these cell groups for our understanding of a range of neurological disorders, progress toward identifying these functionally analogous cell groups is crucial, as teleosts provide tractable model systems for complex dopamine-associated disease phenotypes, such as motor deficits (Mok and Munro, 1998) or impaired social behavior phenotypes (Winberg and Nilsson, 1992; Guo, 2004; Buske and Gerlai, 2011, 2012).

The telencephalic dopamine cell group located in area Vc of teleosts has unknown homology to mammalian systems. Based mostly on neurochemical evidence, Vc (along with Vd) is hypothesized to be the teleost homolog of the mammalian striatum (Wullimann and Mueller, 2004; O'Connell and Hofmann, 2011b). Often overlooked in the early descriptions of the mammalian dopaminergic system, striatal neurons positive for dopaminergic cell markers, including TH, have since been found, although these populations are more prominent in primates compared to rodents (Betarbet et al., 1997). Unfortunately, no studies have examined the co-localization of gene transcripts associated with the differentiation or maintenance of dopaminergic cells in the mammalian striatum, even though this information would shed light on the ontogeny of these poorly understood dopaminergic cell populations.

With the finding that the *ets* family of transcription factors may specify different populations of dopaminergic cell groups in the mammalian brain, such as Etv1 specifying dopamine cell fate in the olfactory bulbs (Flames and Hobert, 2009), it was quickly suggested that Etv5, which is present in the mammalian SNc and VTA (Gray et al., 2004), may also play some role in differentiation of dopamine neurons. Our findings suggest this may also be the case in teleosts, as *etv5* co-localizes with TH-positive neurons in the TPp and PTn. However, a recent study in mouse suggests that midbrain dopaminergic cell specification may be independent of *etv5* (Wang and Turner, 2010). Obviously, more studies are needed in both mammals and teleosts to determine whether or not the invertebrate cis-regulatory motif and *ets* family of transcription factors that regulates dopamine specification in *C. elegans* also extends to a more general regulatory mechanism for vertebrate mesencephalic dopamine cell development.

Genetic studies dissecting the role of *nr4a2* in dopamine cell fate in both mammals and teleosts have led to more refined hypotheses regarding homologies of dopamine cell population across vertebrates. As is the case in teleosts, *nr4a2* is present in many regions throughout the fore- and midbrain of mammals (Saucedo-Cardenas and Conneely, 1996; Zetterström et al., 1996; Saucedo-Cardenas et al., 1998). In mouse, *nr4a2* coexpression with TH seems to be limited to the midbrain SNc and VTA (Bäckman et al., 1999), whereas relatively few POA dopaminergic neurons express *nr4a2*. We found that in *A. burtoni*, *nr4a2* also co-localizes with TH in a subset of dopaminergic cells in the POA and TPp. Knock-down of *nr4a2* leads to degeneration of midbrain dopamine neurons in mice (Wallén et al., 1999), suggesting that Nr4a2 is indeed critical for maintenance of both SNc and VTA neurons (Kadkhodaei et al., 2009). Similarly, in zebrafish, morpholino knockdown of *nr4a2b* results in failed development of dopamine neurons in the posterior tuberculum (Blin et al., 2008; Luo et al., 2008). However, morpholino knockdown of *nr4a2* in zebrafish also leads to reduction of dopamine neurons in the pretectum, POA, and telencephalon (Filippi et al., 2007; Blin et al., 2008; Luo et al., 2008). Given that the Nr4a2 is generally critical for midbrain dopaminergic cell groups in mammals, it is important to examine gene profiles that will allow us to distinguish the SNc-like and VTA-like neurons in the teleost brain.

There are many striking similarities, but also some differences, in the function and expression distribution of *pitx3* in teleosts and mammals. Most notably, *pitx3* expression is widespread in the teleost brain, whereas in mammals, where *pitx3* expression is confined to mesencephalic dopaminergic neurons (Smidt et al., 1997). Pitx3 is required for the development of SNc dopaminergic neurons, as mice lacking Pitx3 fail to develop SNc dopaminergic neurons, but the VTA dopamine neurons remain intact (Nunes et al., 2003). Interestingly, knockdown of *pitx3* in zebrafish development results in a partial ablation of diencephalic dopaminergic neurons (Filippi et al., 2007), although the authors argued that this was due to unrelated apoptosis. Given that *pitx3* is expressed in dopamine neurons in cichlids, but not zebrafish (Filippi et al., 2007), it may be fruitful for future studies to utilize morpholino technologies in cichlid development to better understand the evolutionary relationships of *pitx3* maintenance of dopaminergic neurons in teleosts and beyond.

5. Conclusions

Our results describing the expression of *nr4a2* and *pitx3* in putative dopaminergic neurons in the preoptic area (POA) and posterior tuberculum (TPp) of a teleost suggests a differentiation pathway similar to those in the mammalian POA and SNc/VTA. Such overlap in neurochemical profiles in the dopaminergic cells of mammals and teleosts may be indicative of field homology (Puelles and Medina, 2002) with dopaminergic cell groups originating in the third prosomere across vertebrates. Future work should attempt to study the expression and colocalization of *etv5*, *nr4a2*, *pitx3*, and genes associated with the differentiation or maintenance of dopamine cell groups in the same developmental stages in various species.

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