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Dopamine receptors in a songbird brain

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ABSTRACT

Dopamine is a key neuromodulatory transmitter in the brain. It acts through dopamine receptors to affect changes in neural activity, gene expression, and behavior. In songbirds, dopamine is released into the striatal song nucleus Area X and the levels depend on social contexts of undirected and directed singing. This differential release is associated with differential expression of activity-dependent genes, such as egr1 (avian zenk), which in mammalian brain are modulated by dopamine receptors. Here, we cloned from zebra finch brain cDNAs of all avian dopamine receptors: the D1 (D1A, D1B, D1D) and D2 (D2, D3, D4) families. Comparative sequence analyses of predicted proteins revealed expected phylogenetic relationships, where the D1 family exists as single exon and the D2 family exists as spliced exon genes. In both zebra finch and chicken, the D1A, D1B, and D2 receptors were highly expressed in the striatum, the D1D and D3 throughout the pallium and within the mesopallium, respectively, and the D4 mainly in the cerebellum. Further, within the zebra finch, all receptors, except D4, showed differential expression in song nuclei relative to the surrounding regions, and developmentally regulated expression that decreased for most receptors during the sensory acquisition and sensorimotor phases of song learning. Within Area X, half of the cells expressed both D1A and D2 receptors, and higher proportion of the D1A only containing neurons expressed egr1 during undirected but not during directed singing. Our findings are consistent with hypotheses that dopamine receptors may be involved in song development and social context-dependent behaviors.

INTRODUCTION

The neurotransmitter dopamine plays an important role in motor control, modulation of motivated behaviors, and formation of memories (for review see Wise, 2004). Midbrain dopaminergic neurons (**Fig. 1,** orange) send profuse projections to the striatum and sparser projections to the pallium (**Fig. 1,** purple and green), including to the mammalian cortex (Kitt and Brauth, 1986; Durstewitz et al., 1999). When dopamine is released from pre-synaptic terminals of dopaminergic neurons, the dopamine binds to diverse sets of post-synaptic dopamine receptors that are classified into two families: D1 and D2. Generally, D1 receptors activate and D2 receptors inhibit neural activity, adenylate cyclase, and downstream signaling pathways (Stoof and Kebabian, 1984; Gerfen, 2000). The D1 family includes D1A (also called D1), D1B (also called D5), D1C, and D1D receptors; the D2 family includes D2, D3, and D4 receptors (Bunzow et al., 1988; Dearry et al., 1990; Sokoloff et al., 1990; Sunahara et al., 1990; Zhou et al., 1990; Grandy et al., 1991; Martens et al., 1991; Sunahara et al., 1991; Van Tol et al., 1991; Macrae and Brenner, 1995; Cardinaud et al., 1997; Hirano et al., 1998). D1C is not found in eutherian mammals and D1D is thought to be avian specific (Demchyshyn et al., 1995).

In birds, autoradiographic ligand binding and kinetic studies revealed that proteins of both receptor families are expressed in the brain (Richfield et al., 1987; Dietl and Palacios, 1988; Casto and Ball, 1994; Ball et al., 1995; Demchyshyn et al., 1995; Schnabel and Braun, 1996; Stewart et al., 1996; Schnabel et al., 1997; Kostal et al., 1999; Kubikova et al., 2009). However, it is not possible from these studies to determine expression of the specific receptor types. Specific expression of several receptors as determined by *in-situ* hybridizations revealed that the mRNAs of the D1A and D1B receptors are abundant in the chicken striatum, and D1B is also enriched in the mesopallium (Sun and Reiner, 2000); terminology from Reiner et al. (2004a).

The mRNA expression for the D2 receptor has been characterized in the turkey brain (Schnell et al., 1999), but its distribution did not correspond with the D2 ligand protein binding distribution known in birds (Richfield et al., 1987; Dietl and Palacios, 1988; Schnabel and Braun, 1996; Stewart et al., 1996; Kostal et al., 1999). The mRNA distribution was restricted to several pallial areas (mesopallium and caudal nidopallium) but the ligand binding distribution was mainly in the striatum, as it is in the mammalian brain (Mansour et al., 1990; Weiner et al., 1991). Expression of the D1D, D3, and D4 receptors has not been characterized in the avian brain.

In songbirds, multiple researchers hypothesized that the dopaminergic system is involved in the modulation of social context-dependent song production and song learning (Jarvis et al., 1998; Hessler and Doupe, 1999; Ding and Perkel, 2002; Ding and Perkel, 2004; Gale and Perkel, 2005; Sasaki et al., 2006; Schroeder and Riters, 2006; Yanagihara and Hessler, 2006; Hara et al., 2007; Heimovics and Riters, 2008; Heimovics et. al., 2009). Song production and learning are controlled by a network of interconnected vocal nuclei, also called song nuclei, distributed into two main pathways: 1) an anterior vocal pathway that forms a loop between LMAN in the pallium, Area X in the striatum, and DLM in the thalamus, and that is necessary for song learning and juvenile subsong production; and 2) a posterior vocal pathway that forms a projection from HVC to RA to brainstem motor neurons, and that is necessary for song production (**Fig. 1;** Nottebohm et al., 1976; Bottjer, 1987; Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Nordeen and Nordeen, 1993; Aronov et al., 2008). When songbirds sing, the song nuclei show increased expression of immediate early genes (IEGs), such as egr1 (Jarvis and Nottebohm, 1997; Wada et al., 2006); egr1 expression in the mammalian brain is modulated by dopamine receptors (Gerfen, 2000). The singing-driven egr1 expression occurs throughout the song system nuclei when zebra finches produce undirected song, but is low in the lateral part of the anterior pathway song nuclei and in the motor output nucleus RA when they produce directed song to another bird (Jarvis et al., 1998); undirected song is thought to be used for practice, whereas directed song is usually produced to a female during courtship (Sossinka et al., 1975; Jarvis et al., 1998; Kao et al., 2005; Ölveczky et al., 2005; Kao and Brainard, 2006). An opposite result of differential down-regulation in different social context occurs for the FoxP2 gene (Teramitsu and White, 2006).

We proposed that the social-context modulation of egr1 expression is controlled by dopaminergic input from the ventral tegmental area (VTA) and substanstia nigra pars compacta (SNc) onto presumed dopamine receptors in Area X (Jarvis et al., 1998). In this regard, the VTA-SNc has higher rates of increased neural activity, egr1 gene expression, and dopamine release into lateral Area X (LArea X) during directed relative to undirected singing (Sasaki et al., 2006; Yanagihara and Hessler, 2006; Hara et al., 2007). Further, although the VTA-SNc is not required for the social context differences in LArea X, it is required for high levels of singing-induced egr1 induction in LArea X and for the social context differences in LMAN and RA (Hara et al., 2007). These findings are consistent with the known presence of high levels of dopamine, its synthetic enzyme tyrosine hydroxylase, and D1-like ligand receptor binding in Area X (Lewis, 1981; Barclay and Harding, 1988; Sakaguchi and Saito, 1989; Barclay et al., 1992; Bottjer, 1993; Casto and Ball, 1994; Soha et al., 1996; Harding et al., 1998; Appeltants et al., 2000). They are also consistent with pharmacological studies that show that D1-like and D2-like receptors in zebra finch Area X display excitatory and inhibitory physiological responses similar to D1-like and D2-like receptors in mammalian brain (Ding and Perkel, 2002; Ding and Perkel, 2004; Gale and Perkel, 2005). However, nothing is known about the specific dopamine receptors nor their possible developmental regulation in the song nuclei.

To characterize dopamine receptors in the avian brain and address hypotheses on social context-dependent gene regulation in songbirds, we cloned zebra finch cDNAs of the D1A, D1B, avian specific D1D, D2, D3, and D4 receptors, characterized their protein coding sequences and brain distribution profiles in zebra finches and chickens, their expression in song nuclei, expression during song development, and receptor neuron types that show differential gene regulation during singing in different social contexts in zebra finches.

Anatomical Abbreviations

A	arcopallium
aDLM	anterior nucleus of DLM
AN	anterior nidopallium
Area X	Area X of the striatum
ASt	anterior striatum
APH	area parahippocampalis,
Cb	cerebellum
CMM	caudomedial mesopallium
CO	optic chiasma
B	basolateralis
DLA	dorsolateral anterior nucleus of the thalamus
DLM	dorsal lateral nucleus of dorsomedial thalamus
DNH	dorsal nucleus of the hyperpallium
DM	dorsal medial nucleus of the midbrain
DMA	dorsomedial anterior nucleus of the thalamus
DMP	dorsomedial posterior nucleus of the thalamus
E	entopallium
GCt	mesencephalic central gray
GP	globus pallidus
H	hyperpallium
Hp	hippocampus
HVC	nucleus HVC, a letter based name
IH	interstitial layer of the hyperpallium
IPC	nucleus isthmi, pars parvocellularis
LArea X	lateral Area X
LMAN	lateral magnocellular nucleus of anterior nidopallium
L2	Field L2
M	mesopallium
MArea X	medial Area X
MD	dorsal mesopallium
MLd	dorsal part of the lateral mesencephalic nucleus
MMAN	medial magnocellular nucleus of anterior nidopallium
MSt	medial striatum
MV	ventral mesopallium
N	nidopallium
NCM	caudal medial nidopallium
NIf	nucleus interface of the nidopallium
NIII	occulomotor nerve III
nXIIts	tracheosyringeal part of the hypoglossal nerve
PT	Pretectal nucleus
RA	robust nucleus of the arcopallium
RAm	nucleus retroambiguus
Rot	nucleus rotundus
SNc	substantia nigra, pars compacta
St	striatum
SVZ	subventricular zone
TeO	optic tectum
Uva	nucleus uvaeformis
V	ventricle
VP	ventral pallidum
VTA	ventral tegmental area

MATERIALS AND METHODS

Animals

We used 33 zebra finches (*Taeniopygia guttata*) and three chickens for this study. The zebra finches were adult males (more than 120 days old; n=13 total), adult females (n=3), and juvenile males (n=17). The chickens were adult males. The breakdown of experimental groups is described in the behavior section. All animal protocols were approved by the Duke University Institutional Animal Care and Use Committee.

Cloning of dopamine receptors and identification of full-length coding sequences

We attempted to clone cDNAs of dopamine receptors from zebra finch brain mRNA using degenerate primers for D1 and D2 family receptors. We chose primers that would amplify regions with sequences specific to a given receptor, to prevent cross-hybridization among receptors in the *in-situ* hybridizations. For the D1 family (D1A, D1B, and D1D), we chose conserved regions from human, rat, chicken, and/or xenopus sequences in Genbank (Sugamori et al., 1994; Demchyshyn et al., 1995; Sun and Reiner, 2000). For the D2 family (D2, D3, and D4), we chose conserved regions from human, mouse, rat, bovine, and/or xenopus sequences in Genbank. For D4, we tried four different degenerate primer combinations, but they did not work (not shown). Instead, at the completion of our study, we used sequences of a partially sequenced D4 receptor in the recently released zebra finch genome (UCSC browser and NCBI Accession number XM_002196640; Warren et al, in preparation) and of a full-length great tit songbird (*Parus major*) cDNA sequence (Accession number DQ006802; Fidler et al., 2007) to generate non-degenerate primers against the putative full-length coding DNA sequence of the zebra cDNA. The sequences of the degenerate (for D1A, D1B, D1D, D2, and D3) and non-generate (for D4) forward and reverse primers are in Table 1. RT-PCR was performed on total RNA from adult zebra finch brains with the appropriate primer pairs. PCR products were examined on 1.5% agarose gels and if predicted sized-bands were present, they were cut from the gel and cloned into the pGEMTeasy plasmid (Promega, Madison, WI). These plasmids were transformed into XL-1 blue E. coli supercompitent cells (Stragene, La Jolla, CA) and colonies with ampicillin (100ug/ml) resistance were selected on agarose plates. From the colonies, DNA minipreps were performed. Inserts were 5'- and 3'-end sequenced and DNA sequences analyzed with BLASTN (DNA) and BLASTX (protein) GenBank searches to confirm cloning. All sequences have been deposited into GenBank (Accession numbers in Table 1).

We used our partial dopamine receptor cDNA sequences to identify and predict the full-length coding sequences from the recently assembled sequenced zebra finch genome (assembled by the Genome Sequencing Center at the University of Washington St Louis and The UCSC Genome Browser http://genome.ucsc.edu/cgi-bin/hgGateway?db=taeGut1). The zebra finch cDNA fragments were BLAST searched against the zebra finch genome. The genomic region containing the hit sequence with the highest score was then scanned using GeneScan (Burge and Karlin, 1998; http://genes.mit.edu/GENSCAN.html) to generate predicted amino-acid coding regions. We then performed quality control analyses, i.e. support by ESTs and homologies to other species, to generate curated protein coding sequences. While we were preparing this paper for submission, the ENSEMBLE group generated machine predicted and annotated protein sequences from the zebra finch genome (ENSEMBLE set 54; Warren et al, in preparation) and we compared their dopamine receptor predictions (Accession numbers XP_002196692.1, XP_002193417.1, XP_002189224.1, XP_002191647.1, XP_002189176.1, XP_002196676.1 for D1A, D1B, D1D aka D1C, D2 variant 1, D3, and D4, respectively) to our cDNAs and curated predicted proteins (Accession numbers BR000842, BR000843, BR000844, BR000845 for D2 variant 1, BR000846, and BR000846, respectively). We created alignments across and within species using the European Bioinformatics Institute version of ClustalW (Higgins and Sharp, 1988; http://www.ebi.ac.uk/Tools/clustalw2/index.html), Dialign (Morgenstern 2004), or Genetyx (Zenetikkusu, Inc) software. We also used the nexus formatted text output of these programs to generate phylogenetic trees with the online interactive tree of life (iTOL) software (http://itol.embl.de/index.shtml). To generate alignment summary diagrams, we used MicroSoft Powerpoint and Word. To verify and identify transmembrane domains we used the TMHMM program at the Center for Biological Sequences (http://www.cbs.dtu.dk/ services/TMHMM/).

Behavior

Zebra finch males were housed overnight in sound isolation boxes. On the next morning, they were observed by audio and videotaped for at least 1 hour. Males that did not sing were taken as silent controls for the brain distribution study in adults (n=3) and for developmental expression analyses in juveniles (n=3 each age group of 15, 35, 45, and 75 days old, n=5 of 25 days old). Adult females (n=3) were treated in the same manner. For chickens, animals were taken while in their home cage. The animals were then sacrificed, brains were quickly dissected, frozen in OCT Compound (Sakura) in block molds, stored at -80°C, and used for single label *in-situ* hybridizations.

For the social-context singing behavior study, adult male zebra finches were treated in a similar manner, except that males that sang at least 20 song bouts of undirected (n=6) or directed (n=4) song in 1 hour were taken. This amount of singing is known to induce high levels of egr1 protein expression in Area X and other song nuclei (Jarvis et al., 1998; Kubikova et al., 2007). A song bout is defined as a bout of singing, separated by at least 2 s of silence from another bout of song. For the undirected song group, birds were kept in the sound isolation chamber alone. For the directed song group, a female was placed in the cage before the lights were turned on. The female was separated from the male by a cage-wall barrier, which encourages more directed singing due to lack of tactile contact with the female. After the 1 hour sessions, the males were perfused with 4% paraformaldehyde, their brains were dissected, postfixed in the same solution, immersed in 20% and then in 30% sucrose, frozen in OCT compound in block molds, stored at -80°C, and used for double and triple labeling experiments. The brains were perfused first rather than fresh frozen, because fixation before freezing generates better morphology for cell identifications in double and triple labeling experiments.

Single, double, and triple label *in-situ* hybridization and immunocytochemistry

Single label radioactive *in-situ* hybridizations (RISH) were done as previously described (Wada et al., 2004; Wada et al., 2006). Fresh frozen sections (12µm) were cut on a cryostat throughout the entire brain: left hemisphere in the sagittal plane and the right hemisphere in the coronal plane. Sections were mounted onto silanated glass slides and stored at -80^{o}C. Corresponding sections of all birds were fixed in 4% paraformaldehyde in PBS (pH 7.4) and hybridized at 65^{o}C with antisense ^{35}S-UTP labeled riboprobes made from the zebra finch dopamine receptor cDNAs of this study. T7 and SP6 RNA polymerases were used to make the antisense and sense riboprobes, respectively. The hybridized sections were first exposed to X-ray film (Biomax MR, Kodak) for 1-4 days, then dipped into autoradiographic emulsion (NTB2, Kodak), incubated for 2 to 4 weeks at 4°C, processed with Kodak developer (D-19) and fixer, and Nissl-stained with cresyl-violet acetate solution (Sigma). Films and slides with the same receptors were developed at the same time.

To perform double labeling *in-situ* hybridization experiments with D1A and D2 receptors, a ^{35}S-UTP labeled riboprobe was used for RISH for one dopamine receptor mRNA and a DIG-labeled riboprobe was used for fluorescent in-situ hybridization (FISH) for the other dopamine receptor mRNA. Fixed frozen thick sections (30µm) were cut in the sagittal plane on a cryostat and free-floated in PBS; we found that free-floating sections gave stronger signals for double label detection than slide mounted sections. To test for possible bias of the label, riboprobe types (^{35}S and DIG) were switched for the receptor types (D2 and D1A, respectively) in a second experiment. The DIG-labeled riboprobes were prepared from 0.15 µg of the selected cDNA, 4 µl of 5X RNA transcription buffer, 2 µl of 10X DIG labeling mix (Roche), 1 µl of RNase inhibitor, 1 µl of RNA polymerase, and DEPC water (20 µl total volume). The reaction mixture was incubated for 2 hours at 37^{o}C. Then 30 µl of DEPC water was added and the riboprobe was precipitated. Both riboprobes were simultaneously added to the same hybridization solution as that used for single label *in-situ* hybridizations (Wada et al., 2004) to obtain final concentrations of 10^7 cpm ^{35}S probe and 1.5-3 µg of DIG probe per 1 ml of hybridization solution. The riboprobes in solution were denatured for 6 min at 90^{o}C. The free-floating sections were washed 2x10 min in PBS, then 2x15 min in 0.75% glycine in PBS, 20 min in 0.3% TritonX-100 in PBS, rinsed 2x5 min in PBS, acetylated 10 min in 0.25% acetic anhydride and 0.03% trietanolamine solution, rinsed 3x5 min in PBS, and prehybridized for 45 min at 60°C and then 15 min at 70°C in the hybridization solution with the two riboprobes. Hybridization was done at 60°C for 16-18h; this is 5°C lower than for the single label *in-situ*, which helped to preserve tissue morphology and proteins for the immunocytochemistry. The next day, the sections were washed 2x5 min in 2xSSPE buffer, 30 min at 60°C in 2xSSPE buffer containing 50% formamide, 2x15 min at 60°C in 0.1xSSPE buffer, cooled for 5 min at R.T. in 0.1X SSPE buffer, and rinsed in PBS. Then endogenous peroxidase activity was quenched in 1% H_2O_2 for 20 min, the sections were washed 2x5 min in PBS and 1x5 min in TBS buffer (0.09 % NaCl, 0.11% Tris, pH 7.4), and non-specific binding was blocked for 30 min in TNB blocking buffer (1% blocking agent from TSA kit [Invitrogen] in TBS). The sections were then incubated with the primary antibody anti-DIG-HRP (Roche, 1:100 in TNB) at 4°C overnight.

To perform triple labeling experiments for D1A, D2, and egr1, the same procedure was applied, but a polyclonal rabbit anti-egr1 antibody (Santa Cruz Biotechnology, cat. # sc-189, 1:200) was added at the same time as the anti-DIG-HRP antibody. The antibody was raised against a 19 amino acid peptide of the carboxy-terminus of the human egr-1 (sequence STGLSDMTATFSPRTIEIC). The specificity of a similar antibody raised against mouse egr1 which reciprocally cross reacts with human egr1 protein (Santa Cruz Biotechnology) was determined in zebra finches and canaries (Mello and Ribeiro 1998), where a single activity-induced protein in Western Blots is seen in songbird brain extracts. Preabsorbtion with the peptide to which the mouse antibody was raised completely eliminates the nuclear staining in tissue sections (Mello and Ribeiro, 1998). We BLAST searched the human peptide against Genbank, and found that it recognizes only egr1 sequences at high identity (>80%), including a stretch of 16 amino acids of the zebra finch peptide sequence at 87% identity (LSDINSAFSPRTIEIC), derived from the zebra finch genome and cDNA egr1 sequences. This human egr1 antibody has been used in many published investigations of egr-1 immunoreactivity in the avian brain. After 3x3 min washes in TBS-T buffer, D1A or D2 mRNAs were visualized using a TSA kit with the fluorophore Alexa 488 or Alexa 594 (Invitrogen). The sections were washed 3x3 min in TBS-T buffer and the egr1 protein (i.e. antibody) was detected by 2 hours of incubation with a goat anti-rabbit IgG conjugated to Alexa 488 or Alexa 594 (Invitrogen, 1:200). The Alexa colors were alternated for mRNA and protein. The sections were washed 3x3 min in TBS, mounted on slides, rinsed in deionized H_2O, and dried. Then they were dipped in autoradiographic emulsion (NTB-2, Kodak), exposed for 3 days, and developed. They were coversliped with Vectashield solution containing DAPI (Vector Laboratories).

Quantification and statistics

For single label in-situ hybridizations, we quantified dopamine receptor expression levels from the X-ray films. X-ray film brain gene expression images were digitally scanned from a dissecting microscope connected to a SPOT III CCD camera with SPOT Advanced imaging software (Diagnostic Instruments, Inc.). Scion Image (NIH) was used to measure the mean pixel intensities in the brain areas of interest. The whole song nucleus or surrounding area was selected using the Freehand selection tool in the Scion Image software. For HVC and RA, we chose HVC_{shelf} and RA_{cup} as the surrounding areas, respectively, for two reasons: 1) they are of the interest as auditory regions (Jarvis and Nottebohm, 1997; Mello et al 1998), and 2) their dopamine receptor gene expression was representative of the brain regions surrounding the respective song nuclei HVC and RA. HVC_{shelf} is the area ventrally adjacent to HVC and RA_{cup} is on the ventral anterior side of RA. Since the borders of these regions are not well defined, we measured the pixel density in smaller areas within these regions. The mean pixel density was calculated using the Analyse-Measure tool of the Scion Image. The pixel density of the film immediately adjacent to the measured areas in the brain was quantified and subtracted from the pixel density in each song nucleus and the area surrounding each song nucleus. Three counts per brain region were taken consistently from three adjacent sections per animal.

For double and triple labeling experiments, images from Area X were acquired at 40X magnification on a fluorescent microscope (Leica). D1A and D2 mRNA double labeling (^{35}S and DIG) was assessed in a 100x100 μm field in the center of the image (n=1226 DAPI^{+} cells from n=7 animals; average # of counted cells/bird = 190.4±25.3). D1A and D2 mRNA with egr1 protein triple labeling was assessed in a 290x220 μm field (n=747 total cells or average of 124.5±29.5 egr1^{+} cells/undirected singing bird and 198 total cells or average of 49.5±13.2 egr1^{+} cells/directed singing bird). We included a larger field of view for the triple labeling to increase cell sample size, due to the lower density of egr1 labeled cells relative to dopamine receptors labeled cells. The cells were considered labeled if the fluorescent signal surrounded (for dopamine receptors) or overlapped (for egr1) DAPI labeled nuclei. In the case of RISH, the cells were considered labeled if there were at least five silver grains in the emulsion above the cell within a distance of two times the DAPI-defined radius of the nucleus (i.e. above DAPI stained nucleus plus one radius). Further, to confirm that the numbers of D1A and/or D2 labeled cells using RISH in single and double/triple labeling experiments were the same, we counted the number of labeled cells in the single labeling experiment using the same method, but at 63X magnification. Nissl staining instead of DAPI was used to localize cells in the single labeling experiment. Three sections per animal were used and the values were averaged for each animal. In the single labeled sections we quantified the number of D1A^{+} and D2^{+} cells also in anterior striatum (ASt) and the number of D1B^{+} cells in LArea X and ASt.

For statistical analyses of the adult expression study (**Fig. 11**), all receptor types and brain areas were compared in paired t-test. The null hypothesis was that the ratio of expression in a song nucleus to its surrounding area is 1 - that is, no different. To avoid finding significant differences found by chance in multiple paired t-test, we used Bonferroni correction. The receptor types and song nuclei were selected as independent variables. In the developmental study (**Fig. 13**) we compared the expression of all receptors for each brain area or each song nucleus as a ratio with its surrounding region in separate ANOVAs, due to the many tests needed for multiple time points. To avoid significant differences found by chance in multiple ANOVAs, we used Bonferroni correction. The null hypothesis was that the expression for the individual receptor in each brain area does not change during development. The ANOVAs were followed by Fisher's protected least significant difference (Fisher's PLSD) post hoc tests. The independent variable was the receptor type. To assess a possible bias of radioactive vs DIG probes for D1A and D2 receptors we compared the numbers of labeled cells in reverse probe experiments for the same animals using a paired t-test.

Figure preparation

The photomicrographs were adjusted using Adobe Photoshop CS3 software. The levels function was used to spread grey values or colors within the visible spectrum. Color images were further color balanced so that the color of all sections are similar.

RESULTS

Comparative analyses of zebra finch dopamine receptor sequences

We successfully cloned cDNAs of all six dopamine receptors from the zebra finch brain: D1A, D1B, D1D, D2, D3, and D4 (**Fig. 2,** top lines of each panel). Using these cDNA sequences, Genescan protein prediction software, and comparisons with other species, we identified the full-length coding regions of these dopamine receptors in the zebra finch genome (**Fig. 2;** detailed protein alignments in **Fig. S1**). Consistent with findings in non-aves (Sunahara et al., 1990; Callier et al., 2003), we found that the zebra finch D1 family receptors exists as single exon genes, whereas the D2 family exists as spliced exons (**Fig. 2**). All receptors had the expected 7 transmembrane domains (TM) of G-protein coupled receptors (**Fig. S1**).

Cross-species comparisons revealed relatively high protein sequence homologies of the zebra finch receptors with chicken (80-96%) and human (64-80%). The D1A receptor had the highest levels of homologies, where relative to birds, humans only had a short 4a.a. deletion in the 3rd cytoplasmatic loop between TM domains 6 and 7 (**Fig. 2A; Fig. S1A**). The D1B receptor had less homology mainly due to a different and shorter sequence in humans in the 2nd extracellular loop between TM domains 4 and 5 (**Fig. 2B; Fig. S1B**). The zebra finch D1D receptor only had high homology (80%) to the chicken D1D receptor. Relative to zebra finch, the chicken had a small deletion in the 2nd extracellular loop and a longer carboxy terminal (**Fig. 2C; Fig S1C**). Some D1D sequence hits to chicken in the NCBI database were machine annotated as D1C (Accession #s NP_001136143 and FJ432666.1). However, we found that these chicken protein sequences and our homologous zebra finch D1D did not have sufficiently higher identities to non-avian D1C sequences (frog and fish) than they had to D1A and D1B sequences of these same species (BLAST hits of the zebra finch D1D were 61%, 60%, and 57% to Xenopus D1C, D1B, and D1A, respectively; accession #s P42291, P42290, and P42289 [Sugamori et al., 1994]). In addition, when we BLAST searched each zebra finch receptor against the zebra finch genome (UCSC browser) at a stringency that allowed cross-detection of D1-like or D2-like receptor genes within and sometimes across families, we were unable to find any other dopamine receptor than the six identified in this study. These findings suggest that the D1C annotations for chicken are incorrect and support the conclusion that D1D is unique to birds (Demchyshyn et al., 1995). They further suggest that D1C may not be found in birds.

Relative to the D1 family, the cross-species comparisons revealed that the D2 family had lower homologies, which appeared to be due to splicing differences within and across species. We identified two splice variants of the D2 receptor in our cDNA products. The longest cDNA variant, which we call variant 1, matched with 100% identity the protein we predicted from the finch genome sequence (Accession # BR000845) and to variant 1 predicted by ENSEMBLE (XP_002191647; **Fig. S2A**); it showed the closest cross-species homologies to chicken D2 variant 1 (90%) and human D2 variant 'long' (79%; **Fig. 2D; Fig. S1D**). Our other zebra finch variant, which we call variant 5, had a deletion within the 3rd cytoplasmic loop between TM domains 5 and 6 (**Fig. S2A**). Our D2 variant 5 was not found among the three additional zebra finch D2 variants 2, 3, and 4 predicted by ENSEMBLE, but all of these predicted variants had different deletions within the same 3rd cytoplasmic loop (**Fig. S2A**). We noted that this loop appeared to be alternatively spliced in cDNAs of chicken and humans (and other mammals), but these other species' variants also did not match our zebra finch variant 5. This suggests that alternative variants of the 3rd cytoplasmic loop are relatively unique across distantly related species or that the homologous splice variants have not yet been identified across species. The zebra finch D3 receptor had lower levels of homologies to chicken and human D3 (88% and 66%), due mainly to variation also within the 3rd cytoplasmic loop (**Fig. 2E; Fig S1E**). Relative to our zebra finch cDNA and our predicted D3 protein sequence (BR000846), the predicted chicken D3 had two deletions at the ends of the 3rd cytoplasmic loop, whereas the human had an alternative but shorter sequence within the loop (**Fig. 2E; Fig. S1E**). The D4 receptor also differed within the 3rd cytoplasmic loop of all three species, where the zebra finch and chicken had similar lengths, but alternative sequences, and humans had an alternative sequence that was nearly double the length of birds **(Fig. 2F; Fig. S1F).** These findings indicate that for the D2 receptor family, the 3rd cytoplasmic loop between the 5th and 6th transmembrane domains can undergo large evolutionary changes.

Phylogram analysis confirmed species and gene relationships, where the D1 receptors (D1A, D1B, and D1D) clustered closer among each other across species and as a family and the D2 receptors (D2, D3, and D4) among each other across species and as a family (**Fig. 3**). This analysis further revealed: 1) that the D1A and D1B receptors are more closely related to each other than they are to the avian specific D1D receptor; 2) that the D1 receptor family (D1A, D1B, and D1D) genes are more closely related to each other (shorter branch lengths) than the D2 family (D2, D3, and D4) are to each other (longer branch lengths; **Fig. 3**), in part due to the large differences in the 3rd cytoplasmic loop in the D2 family; and 3) that among the D2 family, D2 and D3 are more closely related to each other than they are to D4 (**Fig. 3**).

We performed similar analyses on the cloned partial cDNA sequences to address whether these receptors could cross hybridize to each other in *in-situ* hybridization experiments. We found that the cDNA fragments of each gene were less than 67% identical to the predicted full length transcripts of any of the other receptors, except for the D1D cDNA fragment against the D1B transcript (74%, **Table 2).** However, none of them met our threshold of ~85% identity necessary to cross-hybridize under our in-situ hybridization conditions (Mello et al., 1997; Wada et al., 2004; Wada et al., 2006; and unpublished observations). Thus, as intended in our PCR cloning design, the cloned cDNA fragments we have obtained should detect expression patterns specific for each dopamine receptor.

Dopamine receptor expression patterns in the avian brain

Within the zebra finch telencephalon, each receptor had a unique expression pattern: D1A, D1B, and D2 (variant 1) were highly enriched in the striatum (a subpallial structure), D1D and D3 were enriched in different subdivisions of the pallium (sagittals in **Fig. 4**; frontals in **Fig. 5**), and D4 was barely expressed in the brain, except for the cerebellum (**Fig. 6**).

Within the subpallium, high D1A, D1B, and D2 mRNA levels were also present in the intrapeduncular nucleus (INP; **Fig. 4E**) and within the medial part of the septum (**Figs. 5F and 7F-H**). This is consistent with the hypothesis that the INP and medial septum are developmentally related to the striatum (Reiner et al., 2004a). Isolated cells with high levels of the D1A, D1B, D2, and D3 receptors were found in the pallidum (GP and VP; **Figs. 4E-F, 5E-F**), the homologue of the mammalian globus pallidus and ventral pallidum.

Within the pallium, D1A receptors were lowest in sensory thalamic recipient zones: L2 for auditory input, entopallium (E) for visual input, and basorostralis (B) for somatosensory input (**Figs. 4, 5E-F and 7I**). D1B was higher in the caudomedial part of the arcopallium (**Figs. 4C** and **5I-K**) and throughout the mesopallium (**Figs. 4** and **5A-F**). The D1A and D1B receptors were high locally around the DNH nucleus in the hyperpallium (**Figs. 4B-E and 5D**); DNH shows activation during night vision in migratory songbirds (Mouritsen et al., 2005). D1D showed gradients of expression, including within the nidopallium culminating in high levels in B but low levels in E and L2 (**Figs. 4, 5** and **7J**) and within the intermediate arcopallium around the song nucleus RA (**Figs. 4D, 5J**). All D1 receptor subtypes were higher in the parahipocampal area (APH) (**Fig. 7A-C**). The D2 receptor had a gradient of expression in the dorsal and ventral mesopallium, with the higher expression levels at the lamina between the two halves of the mesopallium. The D3 receptor expression was mainly restricted to the mesopallium (**Figs. 4 and 5**), the intermediate arcopallium (**Fig. 4E-F**), in a band of cells in the subventricular zone between the ventrical and hippocampus (**Figs. 4A-C and 7E**), and within the dorsal hippocampus (**Fig. 7E**).

Within the brainstem, five (D1A, D1B, D2, D3, and D4) of the six receptors were expressed with specific patterns (**Figs. 4-7**); the exception was D1D, which appeared to have little if any expression in the brainstem. D1A and D1B were enriched within dorsal thalamic nuclei, whereas D3 was enriched in their shell regions (**Figs. 4A and 5H**), which receive feedback projections from the telencephalon (Mello et al., 1998). Four of the receptors (D1A, D2, D3, and D4) were expressed in midbrain regions, such as the optic tectum (**Figs. 4D-F, 5H-K, and 6**). Notable differences were within the midbrain dopamine synthesizing cell groups, the VTA-SNc and GCt, respectively. In these cell groups, D2 was highly expressed, D1B was low, and the other receptors appeared undetectable (**Figs. 4A-B, 5I-J, and 7K-L**).

Within the cerebellum, all receptors were expressed, but each had a specific pattern. All receptors were expressed in the granular (inner) layer, from low to high levels in the following order: D1A<D1B<D2<D1D<D3<D4 (Fig. 8A-F). In addition, D1A and D1B were expressed in complementary patterns in the inner and outer halves, respectively, of the molecular (outer) layer (**Fig. 8G-H**). D3 showed high expression in many of the Purkinje neurons and in the deep cerebellar nuclei (**Fig. 8K-L**).

For all brain regions studied, there were no noticeable expression differences between D2 receptor splice variants 1 (**Figs. 4, 5, and 7**) and 5 (data not shown). For D3, we only tested the cDNA variant 1, as the other predicted variants by ENSEMBLE have not been identified among our cDNA clones. There were no predicted variants for D4 as of to date.

Using the zebra finch cDNAs as probes, we also performed a general expression analysis of these dopamine receptors on adult male chicken brains (**Fig. 9**). We found similar expression profiles to the zebra finch brain, but with some exceptions (compare **Figs. 4** and **9**). The most notable exception was expected, this being the absence of telencephalic song nuclei with differential expression (see below) in the chicken, a vocal non-learner (Nottebohm and Nottebohm, 1971). Other differences were that in the zebra finch, D1B is enriched at much higher levels in the striatum relative to the enriched expression in the mesopallium (**Fig. 4**), but in the chicken it is enriched at similar levels in the striatum and mesopallium (**Fig. 9B**), as previously reported (Sun and Reiner, 2000; Reiner et al., 2004a); D2 in the zebra finch is expressed at a detectable low, but enriched level in the mesopallium relative to the remaining pallium (**Fig. 4**), but this enrichment is not seen in the chicken (**Fig. 9D**); D3 in the zebra finch is evenly enriched throughout the mesopallium (**Fig. 4**), but in the chicken it is unevenly enriched with gradients of higher expression towards the boundaries of the dorsal and ventral mesopallium with the hyperpallium and nidopallium, respectively (**Fig. 9E**). The D1A, D1D, and D4 patterns are much more similar between zebra finch and chicken (**Figs. 4, 6, and 9A,C,F**).

Dopamine receptor expression in song nuclei

Similar to the specialized expression of glutamate neurotransmitter receptors in song nuclei (Wada et al., 2004), at least two or more of the dopamine receptors showed differential expression in the song nuclei relative to the surrounding brain subdivisions. For the pallial song nuclei HVC and RA, four of the five dopamine receptors showed differential expression, where both nuclei had higher D1B, D2, and D3 expression and lower D1D expression relative to the surrounding nidopallium (HVC$_{\text{shelf}}$) and arcopallium (RA$_{\text{cup}}$), respectively (**Figs. 10A-B** and **11**).

However, the differential expression in RA for D1D and D2 was much less than that seen for HVC; for RA, we compared expression only with the surrounding intermediate arcopallium, because other subdivisions within the arcopallium showed differential expression relative to each other and RA is located within the intermediate arcopallium (Reiner et al., 2004b). The differential D2 expression in both HVC and RA was due to high expression in isolated cells (**Fig. 10A-B**), indicating that differential expression could be cell type specific in these nuclei, such as in HVC's RA-projecting neurons, X-projecting neurons, or interneurons (Dutar et al., 1998; Kubota and Taniguchi, 1998; Mooney, 2000). LMAN showed generally low expression levels for all receptors, of which D1A and D1D were significantly lower than in the surrounding anterior nidopallium (AN; **Figs. 10C** and **11**). For the D2 receptor, however, similar to HVC and RA, there were isolated cells with higher expression levels in LMAN (**Fig. 8C**), but were not numerous enough to be detectable as differentially expressed in the X-ray film quantifications. In Area X, all three striatally enriched receptors (D1A, D1B and D2) were significantly expressed at higher levels above the surrounding anterior striatum (ASt; **Fig. 10C** and **11**). D1D and D3 were low in Area X, similar to the surrounding striatum. The D4 receptor showed no differential expression in any of the telencephalic song nuclei (**Fig. 11**), consistent with its barely detectable expression throughout the telencephalon (**Fig. 6**).

Within the thalamus, the anterior portion of DLM (aDLM), which we hypothesize to be the song nucleus part of DLM (Wada et al., 2004); Horita, Oka, Jarvis, Wada, in preparation), showed low to no detectable expression of dopamine receptors (**Fig. 10D**); there was lower D1A and D1B expression relative to the surrounding dorsal thalamus (**Fig. 10D**). In adult females, song nuclei are atrophied except for LMAN (Nottebohm and Arnold, 1976; Nixdorf-Bergweiler, 2001), and there was a lower expression of D1D in "LMAN" relative to the surrounding AN (n=3 females, not shown). We did not note differential expression in any other areas of female brains where the song nuclei are located in males.

In summary, HVC, RA and Area X expressed one or more dopamine receptors, which were higher than the surrounding brain subdivisions. In contrast, LMAN and aDLM had low to no detectable expression of dopamine receptors, which in some cases were lower than the surrounding brain subdivisions. The D1D receptor was low if not absent in all of these nuclei relative to the rest of the telencephalon, and D4 was low or absent in both the song nuclei and surrounding brain areas.

Dopamine receptor expression in song nuclei during juvenile development

To quantify potential developmental differences in song nuclei, we performed two types of measurements: 1) analyses of absolute expression levels, which allowed comparisons among song nuclei; and 2) analyses of relative expression levels to each immediate surrounding brain subdivision, which allowed assessment of specialized expression in song nuclei. Most dopamine receptors showed high absolute expression levels in song nuclei early in juvenile development (PHD15), followed by significant decreases in the pallial song nuclei during the sensory acquisition (PHD25-35), subsong (PHD35-45), plastic song (PHD75), to adult (>PHD90) crystallized song phases. Specifically, D1A, D1B and D3 were higher in HVC during juvenile development and decreased in adulthood (**Figs. 12A-B** and **13A**) while in the surrounding $\text{HVC}_{\text{shelf}}$ they initially increased, then remained stable during vocal development and finally decreased in adulthood (**Fig. 13B**). Despite the decreases in both HVC and $\text{HVC}_{\text{shelf}}$, D1B and D3 expression still remained higher in HVC than in the surrounding nidopallium (i.e. $\text{HVC}_{\text{shelf}}$) in adults (**Figs. 11** and **13C**). Within RA, a similar developmental trajectory of decreased expression was found (**Figs. 12C** and **13D**), except that D1A was not significantly higher than surrounding intermediate arcopallium (RA_{cup}) at all of the developmental ages studied (**Fig. 13F**). Within LMAN, all receptors, except D2, were slightly higher in absolute levels in early juvenile development than in adults (**Fig. 13G**). The surrounding AN showed some increases, followed by decreases also for most receptors (**Fig. 13H**). However, relative to the surrounding AN, only D1A showed an abrupt change in LMAN - a sharp decrease between PHD15-25 (**Fig. 13I**). D4 in juvenile animals was still expressed at low levels throughout the telencephalon with no apparent differences in song nuclei (**Fig. 6**).

Within Area X, of the three striatally expressed receptors, D1A and D1B showed initial increases followed by decreases in absolute expression levels during juvenile development whereas D2 showed a gradual increase into adulthood (**Fig. 13J**). A similar result was found for the surrounding striatum (**Fig. 13K**). However, relative to the surrounding striatum, all three receptors showed gradual increases in Area X (**Figs. 12D-F** and **13L**). The increased differential expression of D1A and D2 in Area X was already present at the sensory-motor phase of song learning (between days 25 and 35 for D1A; 25 and 45 for D2, $p<0.05$) and increased further thereafter. The D1B differential increase occurred between the late plastic song phase, day 75 and adulthood (**Figs. 12D-F** and **13L**). These findings suggest that D1A and D1B expression levels in the striatum surrounding Area X decreased more than they decreased in Area X, resulting in net higher levels in Area X in adulthood.

In summary, expression of most dopamine receptors in song nuclei and the surrounding brain subdivisions decreases during development. However, the decreases in relative expression are not uniform, such that expression in song nuclei can change independently of expression in the surrounding brain subdivisions.

Co-localization of D1 and D2 receptors

Next we investigated whether D1A and D2 receptors exist in the same or in distinct cells of Area X. We chose to examine D1A and D2, as there has been a long-standing issue for the mammalian striatum (for review see Callier et al., 2003) and recently for avian Area X (Ding and Perkel, 2002) as to whether these two receptors are expressed in overlapping or distinct sets of striatal neurons. In double labeling in-situ hybridization experiments using one radioactive (RISH; D1A in **Fig. 14A$_1$,** black silver grains) and one fluorescent (FISH; D2 in **Fig. 14A$_2$,** red) probe, we found that in Area X at least 95% (**Fig. 14C,** total of silent group) of the cells (as detected by DAPI nuclear stain, **Fig. 14A$_3$,** blue) expressed either D1A or D2 receptors (i.e. only 4.4 ± 0.7% [average ± SEM] were DAPI$^+$ without D1A or D2 label). More than half of the cells expressed both receptors simultaneously, i.e. were double-labeled (**Fig. 14C,** D1A$^+$/D2$^+$/DAPI$^+$). Of the single labeled cells, the majority (28.1 ± 2.7% of the total cells) expressed the D1A receptor, whereas the minority (15.1 ± 1.9%) expressed the D2 receptor (**Fig. 14C** and **14C,** D1A$^+$/DAPI$^+$ and D2$^+$/DAPI$^+$, respectively). When including all cells (single and double labeled) the majority still expressed D1A (80.5% ± 1.8, average ± SEM) relative to D2 (67.6% ± 2.4). This differential expression persisted also after alternating the probes (RISH vs. FISH, **Fig. 14D**), and thus these results were not influenced by the label combination used.

Since we noted that the contrast in the number of silver grains in labeled versus unlabeled cells was stronger in single label *in situ* hybridizations (compare **Fig. 14A$_1$** and **14B$_2$** with **Fig. S3A** and **S3B** for D1A and D2, respectively), we counted the average numbers of D1A- and D2-containing cells also there. We found that 77.8% ± 2.4 (average ± SEM) of all Nissl stained cells expressed the D1A receptor and 70.2% ± 2.0 of all cells expressed the D2 receptor. These averages were not significantly different from the numbers of D1A (80.5 ± 1.8; p=0.36, t-test) and D2 (67.6% ± 2.4; p=0.44) DAPI expressing cells quantified in the double labeling experiment. Since the averages in single label *in situ* hybridizations add up to 148.1%, these results indicate that there must be cells (at least 48.1%) that express both D1A and D2. Thus, the fluorescent+radioactive double label and radioactive single label *in situ* hybridizations yield similar conclusions.

Further we wanted to determine whether the numbers of $D1A^+$ and $D2^+$ cells were similar or different in the surrounding ASt as in Area X. We found that in ASt caudal-ventral adjacent to Area X 72.7 ± 1.3% of all Nissl stained cells expressed the D1A receptor and 66.4 ± 2.0% of all cells expressed the D2 receptor. This distribution in ASt did not differ significantly from the distribution in Area X (p=0.26 for both receptors, t-test). However, the average number of labeled cells/63X field was higher in Area X (average of 143.0 ± 9.4 for D1A and 131.1 ± 8.0 for D2) than in ASt (103.3 ± 8.8 for D1A and 101.3 ± 6.8 for D2; p<0.05 for both, t-test). This result suggests that there is a higher density of cells (labeled and unlabelled) in Area X, which may explain in part its specialized expression.

In addition, we counted the numbers of $D1B^+$ Nissl stained cells in Area X and ASt. Remarkably, we found that D1B was expressed in 84.6 ± 1.5% of the Nissl stained cells in Area X and 84.3 ± 1.8% in ASt. Similarly as for D1A and D2 receptors, the average number of labeled cells/63X field was higher in Area X (141 ± 4.9) than in ASt (105.7 ± 8.8; p<0.05, t-test). Since the averages of D1B and D2 single labeled cells add to 152.2%, these results predict that at least 52.2% cells in Area X would express both of these receptors.

Social context, singing-induced egr1, and dopamine receptor co-localization

In mammals, D1 receptors up-regulate egr1 expression whereas D2 receptors inhibit its expression (Gerfen et al., 1995). Given this knowledge, we hypothesized that in songbirds during undirected singing, the low levels of dopamine released by VTA-SNc axons onto Area X neurons would preferentially activate D1 receptors and thereby increase egr1 expression to high levels in Area X, whereas during directed singing, the high levels of dopamine would preferentially activate D2 receptors and thereby dampen the singing driven increase of egr1 expression in Area X (Sasaki et al., 2006; Hara et al., 2007). If this were true, one would expect a differential distribution of egr1 expression in D1 and D2 containing neurons during singing in different social contexts (egr1 is expressed only in neurons in an adult brain – Kubikova et al., 2007; Man et al., 2007). To address this hypothesis, we analyzed triple labeling expression to co-localize D1A (**Fig. 14B$_1$,** green) and D2 (**Fig. 14B$_2$,** white silver grains) receptor mRNA with singing-induced egr1 protein (**Fig. 14B$_3$,** red; merged in **Fig. 14B$_4$**) in lateral Area X (LArea X), the portion of Area X that shows social context-dependent gene regulation (Jarvis et al., 1998).

We found that after undirected singing, the majority (86.4 ± 2.3%, average ± SEM) of the egr1$^+$ neurons were D1A$^+$ and D1A$^+$/D2$^+$ (**Figs. 14B$_4$** and **14C,** D1A$^+$/egr1$^+$, D1A$^+$/D2$^+$/egr1$^+$); a small minority were D2$^+$ only (8.1 ± 1.3%, **Fig. 14C,** D2$^+$/egr1$^+$) or did not express either dopamine receptor (5.4 ± 1.2%, not shown in graph). After directed singing, the average number of egr1$^+$ neurons was less than after undirected singing (33 ± 11.5 for directed vs. 93.4 ± 9.0 for undirected egr1$^+$ cells per section, average ± SEM), as expected (Jarvis et al., 1998). The distribution of egr1 expression among D1A$^+$, D2$^+$, and D1A$^+$/D2$^+$ neurons during directed singing was similar to that seen during undirected singing (**Fig. 14C**). However, when we compared the distribution of D1A and D2 labeled cells co-localized with egr1$^+$ to the distribution of all D1A and D2 labeled cells (co-localized with DAPI), we found significant differences across singing contexts. During undirected singing, there was a significant 148% over-representation of D1A$^+$ neurons (**Fig. 14C,** D1A$^+$/egr1$^+$) and a 54% under-representation of D2$^+$ neurons with egr1 expression (**Fig. 14C**, D2$^+$/egr1$^+$). During directed singing, there was a significant 52% under-representation of D2$^+$ neurons with egr1 expression (**Fig. 14C**, D2$^+$/egr1$^+$). In summary, these findings suggest that during undirected singing egr1 is preferentially expressed in D1A neurons and preferentially not expressed in D2 neurons, and during directed singing egr1 is preferentially not expressed in D2 neurons.

DISSCUSSION

The dopaminergic system in vertebrates has been studied for its function in multiple behaviors such as motor activity, cognition, motivation, reward, learning (including learned vocal communication), sleep, and mood (Salamone, 1992; Pace-Schott and Hobson, 2002; Wise, 2004; Doupe et al., 2005; Panksepp, 2005; Berridge, 2007; Schultz, 2007). However, there has been little knowledge about the function of dopamine receptors in the central nervous systems of birds. In this study we cloned cDNAs of all six dopamine receptors, D1A, D1B, D1D, D2, D3, and D4 from the avian brain. We determined their phylogenetic relationships, brain distributions in adult zebra finches (a songbird) and chickens (galliformes), in juvenile zebra finches during different song learning phases, and in zebra finches during singing in different social context. Below we discuss the evolution of dopamine receptors, their localization, their significance for vocal learning, and implications for social-context dependent singing behavior.

Evolution of dopamine receptors in birds

Our sequence analyses suggest that as in mammals (Callier et al., 2003) the D1 family of receptors in birds exists as single exon genes and the D2 family exists as spliced multiple exons (see Demchyshyn et al., 1995 for chicken). This finding and the fact that the D1 and D2 receptor protein sequences are more related to other monoamine receptors (serotonin, adrenergic, and trace amine receptors) than they are to each other supports the conclusion that the ancestral D1 and D2 receptors diverged from duplications of a seven transmembrane receptor, had two separate evolutionary histories, and then converged on their ability to bind dopamine independently (Callier et al., 2003). After convergence, further gene duplication apparently led to the different receptor subtypes of each family among vertebrates. It is believed that D1C was subsequently lost in mammals and D1D subsequently lost in most vertebrates except birds, or that D1D evolved independently in birds (Demchyshyn et al., 1995; Callier et al., 2003; Le Crom et al., 2004). Our sequence analyses suggest a distant relationship of avian D1D to D1A and D1B, and thus the theory of D1D being lost in other vertebrates (Callier et al., 2003) is the one most supported by our findings**;** the alternative is that D1D duplicated from one of the other D1 receptors (e.g. D1B) in the ancestor of all extant birds and then evolved faster than the other D1 receptors.

Our findings support the conclusion that birds do not have D1C (Demchyshyn et al., 1995), although this has been more recently questioned (Le Crom et al., 2004). Avian chicken sequences in the public databases (NCBI) have been annotated as D1C. However, we find that these sequences are 100% identical to chicken D1D (Demchyshyn et al., 1995) and share close identity to our zebra finch D1D clone. Further, we have not been able to find a separate D1C-like sequence in the currently sequenced zebra finch or chicken genomes (as determined by BLAT searches with xenopus D1C). This suggests that D1C may have been lost in both mammals and birds. Determining if it was lost before the ancestral split between birds and mammals requires determining whether D1C is present in non-avian reptiles. We have not been able to find D1C sequences in 454 high throughput cDNA sequences of crocodile brain (unpublished findings). We also could not find any other dopamine receptors in the current draft of the zebra finch genome than the six we describe here. Given these findings, we may have identified all dopamine receptors in the songbird, and they do not include D1C.

For the D2 receptor family, our analyses suggest that there are multiple spliced forms that affect the third cytoplasmic loop. This loop is important for intracellular signaling functions of dopamine receptors. The loop physically links the G-protein component of the receptor to cAMP, adenylate cyclase, and extracellular kinase (ERK) proteins, which in turn regulates downstream processes, including modulation of activity-dependent gene expression such as egr1 (Ilani et al., 2002; Takeuchi and Fukunaga, 2004). We have not seen a brain expression difference between the two D2 zebra finch cDNA splice variants 1 and 5 thus far discovered, indicating that the different functional proteins could work in the same brain regions, if not the same neurons. However, some differential expression of D2 splice variants of the third cytoplasmic loop have been found in mammals by RT-PCR, where the longer variant (variant 1) is expressed at slightly higher levels in the striatum and the shorter one at higher levels in the VTA-SNc (Guivarc'h et al., 1995; Khan et al., 1998). It is possible that these small differences are not easily detected by in-situ hybridization. In general, the variable regions of homology we found between zebra finch and human vs. chicken and human for the D2, D3, and D4 receptors suggest that the D2 family of receptors may be rapidly evolving, and that these changes occurred after galliformes and songbirds split from their common ancestor.

Brain expression profiles of dopamine receptors

The most salient finding of our expression analysis is that the more canonical and apparently ancestral dopamine receptors (D1A, D1B, and D2) are enriched in the striatum, which is innervated by VTA-SNc, whereas the more derived receptors (D1D and D3) are enriched in pallial regions, which is innervated by other midbrain dopaminergic and noradredergic neuron groups (e.g. GCt and LoC). These brain expression patterns appear to be representative of birds, as they were similar in zebra finch (except song nuclei) and chicken, species that are distantly related to each other (Hackett et al., 2008). The patterns we found are consistent with previous reports of D1A and D1B mRNA expression patterns in chicken (Sun and Reiner, 2000), D1-like and D2-like receptor ligand binding patterns in chickens, quails, and pigeons (Richfield et al., 1987; Dietl and Palacios, 1988; Ball et al., 1995; Schnabel and Braun, 1996; Stewart et al., 1996; Schnabel et al., 1997; Kostal et al., 1999), and D1-like receptor binding pattern in starling (Casto and Ball, 1994; Heimovics et. al., 2009). The later studies are the only previous distribution studies of dopamine receptors in a songbird. The most recent one (Heimovics et. al., 2009) found relationships between D1-like densities in midbrain dopaminergic neurons and breeding context. We found some species differences in the pallium between zebra finches and chickens, most notably in the mesopallium where D1B was higher, D2 lower, and D3 expressed in gradients in chicken relative to zebra finch. This suggests that for dopamine receptor gene regulation in the mesopallium, there could be cis-regulatory mutations between zebra finch and chicken to control species-specific differential expression.

In contrast to the conserved pattern that we found in zebra finch and chicken, there is a substantial discrepancy with the D2 mRNA pattern previously reported in turkey brain (Schnell et al., 1999). While we found that D2 mRNA was highest in the striatum of both zebra finch and chicken, Schnell et al (1999) found that it was nearly all pallial in turkey. To explain a pallial mRNA pattern that does not correlate with the D2 ligand binding pattern in birds, Schnell et al (1999) proposed that avian D2 mRNA may be transported to the striatum, synthesized into protein, and then the mRNA rapidly degraded in the striatum. We believe that this hypothesis is unlikely in light of our findings, as it would mean that D2 mRNA synthesis, transport, and degradation are all very different in two very closely related species, chicken and turkey, but not different in two distantly related species, chicken and zebra finch. Thus, we wondered if the turkey result represented differential expression of D2 splice variants; but we find that their turkey cDNA probe (Accession # AF056201) is the homologue to the chicken D2 cDNA variant 1 (96%, # EU313425 by Zu and Hang) and our zebra finch D2 cDNA variant 1 (86%; **Fig S2B**). Thus, we believe that either the turkey is different than other avian species or the difference between studies is due to an unknown technical factor. In either case, our results suggest that D2 is highly expressed in the avian striatum across species as in mammals.

The avian dopamine receptor patterns we found are consistent with the new view of avian brain organization and homologies with mammals and other vertebrates (Reiner et al., 2004a; Jarvis et al., 2005). As in birds, the D1A, D1B and D2 receptors are most abundant in the mammalian striatum and proposed fish striatum, relative to much lower levels of isolated expression in pallial areas (Meador-Woodruff et al., 1991; Weiner et al., 1991; Rappaport et al., 1993; Choi et al., 1995; Kapsimali et al., 2000; Hurd et al., 2001). Further, as in birds, the D2 receptor in mammals is specifically enriched in the VTA-SNc (Vallone et al., 2000; Callier et al., 2003). As in birds, the D3 receptor in mammals is expressed at lower levels in the brain, with expression mainly restricted to pallial regions, including a sparse distribution in the hippocampus and higher expression in layers V-VI of anterior cortical regions (possibly analogous cell type to avian arcopallium projection neurons; Jarvis et al., 2005), and in Purkinje neurons of the cerebellum (Bouthenet et al., 1991; Landwehrmeyer et al., 1993a; Landwehrmeyer et al., 1993b; Diaz et al., 1997). Although there is high D3 (and D1B) in the avian mesopallium, there is no agreement on a homologous structure or cell group in mammals (Jarvis et al., 2005). For the D4 receptor, large discrepancies exist as to where it is expressed in the mammalian brain (Noain et al., 2006); these discrepancies are thought to be due to use of non-specific antibodies and ligands. Using a sensitive BAC transgene in front of the D4 promoter, similar to zebra finch, D4 was found to be low to absent throughout most of the mouse telencephalon (Noain et al., 2006). However, high expression was found in discrete isolated cells of the deep layers of the prefrontal cortex and of the ventral pallidum (Noain et al., 2006). Unlike in the zebra finch brain, no D4 transgene expression was found in the mouse cerebellum, although prior studies reported D4 protein expression in the rat cerebellum using antibodies (Barili et al., 2000). In both mammals and chicken, D4 is expressed at high levels in the retina (Rohrer and Stell, 1995; Zawilska et al., 2003).

The low forebrain expression levels of the D4 receptor are intriguing given prior findings of its role in novelty behavior and attention deficit hyperactivity disorder (ADHD). Deletions, inserts, and single nucleotide polymorphisms of the D4 receptor gene are all associated with variations in novelty seeking behavior, ADHD, and other personality traits in the great tit (a songbird), humans, vervet monkeys, and horses (Kluger et al., 2002; Momozawa et al., 2005; Bailey et al., 2007; Fidler et al., 2007). A subset of humans with ADHD and an associated repetitive sequence mutation in their D4 receptor protein sequence have relatively smaller volumes of prefrontal cortex and cerebellar cortex. Since ADHD, novelty seeking, and other behaviors presumably involve the telencephalon, it is possible that low, but barely detectable levels of the D4 receptor in the telencephalon lead to robust functions upon binding dopamine.

The different dopamine receptors have different binding affinities for dopamine and different response after binding dopamine. The D2 receptors have the highest binding affinities, followed by different D1 affinities in the order D3>D4>D2>D1D>D1B>D1A; each receptor also has different agonist-dependent desensitization, e.g. D1A>D1B (Demchyshyn et al., 1995; Kostal et al., 1999; Vallone et al., 2000; Callier et al., 2003; Kubikova et al., 2009). The pharmacological binding profile of the dopamine receptors to various agonists and antagonists is very similar among the avian, mammalian, and other vertebrate species (Demchyshyn et al., 1995; Kubikova et al., 2009). This suggests that after dopamine release and binding, specific brain areas and neuron types will have functional differences depending on densities of the individual receptor types.

A long-standing issue about dopamine receptors in the mammalian brain is whether D1 and D2 receptors are expressed in the same striatal neurons or in different ones. Some studies report segregation into distinct neuronal populations (Gerfen et al., 1990; Le Moine and Bloch, 1995) while other studies report co-expression in a moderate number to nearly all striatal spiny neurons (Ariano et al., 1997; Aizman et al., 2000; Lee et al., 2004). Differences between studies could be due to differences in techniques, between receptor subtypes examined (D1A vs D1B) or species differences. In songbirds, a previous study using pharmacological manipulations suggested that there is considerable (qualitative) overlap of D1-like and D2-like receptor physiological responses in the same neurons of Area X, but also some segregation, where the D1-like receptors mediated enhancement of excitability at a hyperpolarized state and the D2-like receptors mediated its reduction (Ding and Perkel, 2002). Consistent with these findings, we show that in LArea X, about half of the cells express mRNAs for both D1A and D2 receptors. Other cells contained either D1A or D2 receptors, but the number of D1A only cells was twice as high as the number of D2 only cells. We believe a similar distribution will apply to the remaining striatum, as we did not visibly note gross differences in the distribution of double labeling outside of LArea X and the proportions of single labeled cells in ASt was similar to LArea X. It is not clear, however, whether the mRNA proportions are translated one-to-one into receptor protein densities. The ligand binding studies do not help answer this question, as there is no consensus on whether there is more D1-like receptors (Schnabel and Braun, 1996; Kleitz et al., 2009), comparable amount of D1-like and D2-like receptors (Stewart et al., 1996; Kostal et al., 1999) or more D2-like receptors (Richfield et al., 1987; Stewart et al., 1996) in the avian striatum. With these ligand studies, it is not possible to determine the receptor types. It is possible that some proportion of cells not only contain D1A$^+$/D2$^+$ expressing neurons, but D1B$^+$/D2$^+$ expression neurons, as suggested from quantifications of our single labeling *in situ* hybridizations. Although we have not been able to find another study that examined whether D1B and D2 are co-expressed in the same neurons in intact brain, in transfected cells these receptors are able to form heterooligomers (So et al., 2009).

Possible functional consequences for vocal learning

We found that during all stages of song development, there is a large overlap in expression of genes belonging to the D1 and D2 receptor families in three song nuclei (HVC, RA, and LAreaX), but with differential abundance that presumably results in different functional dopamine responses of each song nucleus. In LMAN there are relatively low levels of dopamine receptors regardless of the developmental stage. This finding suggests a lesser function of dopamine in LMAN.

The developmental changes of dopamine receptor levels in song nuclei roughly correspond with their dopamine and dopamine turnover levels, and inversely correlate with tyrosine hydroxylase immunoreactivity levels (Soha et al., 1996; Harding et al., 1998); tryrosine hydroxylase is the rate limiting enzyme for dopamine synthesis. Unlike the receptors levels determined in this study, dopamine levels are low in song nuclei in young ~20 day old birds, increase to higher levels between 35-55 days old, but thereafter as the birds become adults the dopamine levels, like the receptors, decrease (Harding et al., 1998). Inversely to the receptors, the density of tyrosine hydroxylase fibers in song nuclei gradually increases relative to the surrounding brain regions throughout juvenile development (Soha et al., 1996). For the receptors, we further found that some changes (increase or decrease) in song nuclei are co-regulated with changes in the surrounding brain subdivisions, but not by as much. Other changes appear to be specific to the song nuclei; these are the decreases of D1A, D1B, and D3 receptors in HVC, and D1B and D3 in RA. Taken together, these findings suggest that although there is decreased dopamine receptor expression and dopamine levels in song nuclei during development, the decrease is not as strong as in the surrounding brain areas, and it is countered by increased tyrosine hydroxylase fiber innervation and thus presumably dopaminergic innervation from the midbrain.

The differential expression of dopamine receptors in song nuclei from juvenile development to adulthood could have important consequences on song learning and song production. The higher levels of D1B and D3 receptors in adult HVC and RA could lead to differential dopamine control of motor circuits for singing relative to motor circuits adjacent to song nuclei that control other behaviors (Feenders et al., 2008). The increases in D1A receptor levels in Area X between days 25 and 35, and in D2 receptor levels between days 25 and 45, could lead to higher activation of Area X neurons (D1A) in the sensory acquisition phase followed by counteraction of this response (D2) in the plastic subsong phase of song learning. This hypothesis is testable and perhaps plausible given the known role of dopamine in learning in mammals (Reynolds and Wickens, 2002; Schultz, 2007) and birds (Stewart et al., 1996; Rose and Stewart, 1999). In young chickens, 30 min after one trial passive avoidance training there is a large increase of D1-like but not D2-like receptor binding in the medial striatum [(Stewart et al., 1996); terminology adopted from (Reiner et al., 2004a)] and this is accompanied by increases in the size of the synaptic density in the same structure (Rose and Stewart, 1999). In songbirds, systemic administration of D1/D2 antagonist between post-hatch days 45-57 decreases directed singing in adult zebra finch (Harding, 2004), but the authors did not study song learning.

Dopamine receptors in Area X and singing related activation

A frequently asked question is the mechanism of social context-dependent egr1 induction in LArea X during singing, where there are high levels of both egr1 mRNA and protein during undirected singing and low levels during directed singing (Jarvis et al., 1998; Kubikova et al., 2007). Plausible candidates for this modulation are the dopamine receptors since dopamine is present in LArea X at high levels during directed singing and at low levels during undirected singing (Sasaki et al., 2006), and since dopamine is released from the midbrain cell groups VTA and SNc (Lewis, 1981; Gale and Perkel, 2006; Castelino et al., 2007; Hara et al., 2007; Gale et al., 2008; Person et al., 2008) which also exhibit social context-dependent differences in activity and gene regulation (Yanagihara and Hessler, 2006; Hara et al., 2007).

Our results suggest that during undirected singing, egr1 expression in LArea X occurs mainly in neurons containing D1A receptors with or without D2 receptors, and less so in neurons that contain only D2 receptors. Such an effect is consistent with findings in the mammalian striatum, where when acting alone, activation of D1-like receptors increases and activation of D2-like receptors decreases egr1 expression. Our findings are also consistent with electrophysiological actions of both receptors in birds and mammals, where D1-like receptors excite and D2-like receptors inhibit evoked activity in mammalian striatum and in Area X neurons (Ding and Perkel, 2002). The smaller number of egr1$^+$ neurons after directed singing could be a consequence of a dominant function of D2 receptors in D1A$^+$/D2$^+$ cells with high dopamine levels. However, it has been found that synergistic activation of D1-like and D2-like receptors in the same neurons can lead to even higher increases in egr1 expression than the activation of D1-like receptors alone (Keefe and Gerfen, 1995; Wirtshafter, 2007). If this were to occur in intact brain, than an alternative mechanisms could be responsible for the social context differences, such as the affinity state of D2 receptors and the amount of dopamine release (Richfield et al., 1989). Lastly, other systems might play a role in modulating social context-dependent egr1 expression. For example, depletion of noradrenergic input leads to increased egr1 expression in Area X after directed singing (Castelino and Ball, 2005) and dopamine has been shown to bind to alpha-2-adrengeric receptors in Area X (Cornil et al., 2008), suggesting the involvement of the noradrenergic system in the regulation of social context-dependent activation of Area X.

In summary, this study sets a basis for deciphering dopamine function in the avian brain, deciphering differential dopamine activation of different avian brain subdivisions, and regulation of dopamine in song nuclei during song learning and social-context dependent singing.

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FIGURE LEGENDS

Fig. 1. Diagram of the avian brain highlighting pallial, striatal, and pallidal telencephalic areas and the song system of songbirds. Black arrows: posterior vocal pathway. White arrows: anterior vocal pathway. Dashed arrows: connections between the two pathways. Orange arrows: Dopaminergic (GCt and VTA-SNc) input into the song nuclei. Abbreviations are in the anatomical abbreviations list.

Fig. 2. General structure and sequence comparisons of the six zebra finch dopamine receptors relative the homologous receptors in chicken and human. **A-F:** For each receptor, shown is the size and position of the cloned zebra finch cDNA fragment (top solid blue lines) aligned to the predicted exon coding (blue open bars) and intron non-coding (black lines) sequence of the zebra finch genome. * is a stop codon. The D1 genes have one coding exon each, D2 has 8, D3 has 6, and D4 has 4. Below the chromosomal sequences are comparisons of the genome-predicted or mRNA-derived protein coding sequences between zebra finch (turquoise), chicken (green), and human (orange bars). The % values indicate percent identities between zebra finch and chicken or zebra finch and human. The lighter blue 5' end of the zebra finch D1B protein indicates that this region has not been sequenced yet in the genome, but is expected to be present due to the sequence found in the chicken and human genomes. The D4 zebra finch cDNA clone spans the full-length coding region, but the accession number refers to 687bp of cDNA sequence obtained to date; the remaining sequence is inferred from the zebra finch genome. White bars represent deletions (del); darker color bars represent non-homologous alternate sequences (alt seq). The predicted zebra finch proteins are those that we generated and curated with GENESCAN and the UCSC genome browser. We further curated the predicted ENSEMBLE chicken D3 protein

sequence, as it had 300 more 5' a.a. than all other D3 proteins in the database, which we believe was a computational error. Detailed protein alignments are shown in **Fig. S1**. CL3 – cytoplasmatic loop 3; EL2 – extracellular loop 2.

Fig. 3. Phylogenetic analyses of dopamine receptors in the zebra finch, chicken, and human. Shown is a phylogram generated using the full-length protein coding sequences (**Fig. S1**), dialign alignments (http://bibiserv.techfak.uni-bielefeld.de/dialign/submission.html), and the iTOL tree generating software (http://itol.embl.de/). For D2 and D3, variant 1 sequences were used. Branch lengths represent evolutionary time separating gene relationships (longer branch, more time). The D1 family has shorter branch lengths indicating that they are probably more closely related than the D2 family. All receptor types show closer homologies to each other across species than they do to other receptor types within species.

Fig. 4. Expression profiles of dopamine receptor types in sagittal series from adult male zebra finch brain. **A-F:** Rows showing medial to lateral series across two pages of this report with the respective drawings on the left. Columns are labeled on the top for each receptor (D4 pattern is shown separately in Fig. 6). The images were taken under darkfield microscopy. White silver grains, dopamine receptor mRNA expression; red, cresyl violet stain. Rostral is right, dorsal is up. Scale bar, 1 mm. The sequences of the cDNA probes used are in Genbank (Accession #s AB372107, AB372108, AB372109, AB3490795, AB327111, for D1A, D1B, D1D, D2 transcript variant 1, and D3 respectively; Table 1).

Fig. 5. Expression profiles of dopamine receptor types in frontal series of sections of one brain hemisphere of an adult male zebra finch. **A-K:** Rows showing rostral to caudal series with the respective drawings on the left. Columns are labeled on the top for each receptor. The images were taken under darkfield microscopy. White silver grains, dopamine receptor mRNA expression; red, cresyl violet stain. Dorsal is up, medial is right. Scale bar, 1 mm.

Fig. 6. Expression profile of the D4 dopamine receptor in the sagittal plane from male zebra finch brains during development and in adulthood. Images were taken from film autoradiograms and inverted. Only several sections are shown as there was not much differential expression of D4 receptor in the telencephalon. White, dopamine receptor mRNA expression. Dorsal is up, rostral is right. Scale bar, 1 mm. Genbank accession number of probe sequence is GQ359780 (Table 1).

Fig. 7. Higher power images of differential dopamine receptor subtype expression in specific zebra finch brain regions. **A-E:** the hippocampal formation; **F-H:** the septum; **I-J:** Field L2, NCM and CMM; **K-L:** the midbrain dopaminergic cell groups VTA and SNc. Arrowheads in A-E point to the ventricle. All sections are coronal, except for I and J which are sagittal. White silver grains, dopamine receptor mRNA expression; red, cresyl violet stain. Scale bars, 0.5 mm.

Fig. 8. Higher power images of differential dopamine receptor subtype expression in the cerebellum. The top row shows film autoradiogram images that were inverted, where expression can be seen in the granular layer for all receptors; the Nissl staining of the dense granular layer in the *in-situ* hybridizations masks the label. The bottom row shows Nissl stained images in

darkfield, where the differential expression (white silver grains) of several receptors (D1A, D1B, and D3) can be seen in the inner and outer halves of the molecular layer and in Purkinje cells, respectively (arrows). Scale bars, 0.25 mm.

Fig. 9. Expression profiles of dopamine receptor subtypes in adult chicken brain. Images were taken from film autoradiograms and inverted; white, mRNA signal. Two sagittal sections are shown per receptor; the distance from the midline is ~1.5 (top) and 3.5 mm (bottom). **A-C:** Upper rows show the D1 receptor family (D1A, D1B, and D1D). **D-F:** Lower rows show the D2 receptor family (D2, D3, and D4). The hybridizations were done with the zebra finch S^{35}-UTP labeled cRNA riboprobes using the same high stringency conditions as for the zebra finch in-situ hybridizations. Scale bar, 0.5 cm.

Fig. 10. Higher power images of differential expression of different dopamine receptor subtypes in the song nuclei HVC, RA, LMAN, LAreaX, and aDLM of an adult male zebra finch. Insets for the D2 receptor show higher power images of isolated labeled cells in pallial song nuclei (HVC, RA, LMAN); inset for LArea X is shown for comparison. All sections are sagittal except for the last row showing aDLM, which is coronal. White silver grains, dopamine receptor mRNA expression; red, cresyl violet stain. Scale bar, 0.5 mm (insets magnified 2.7 more times).

Fig. 11. Quantification of the expression levels of the dopamine receptors in the song nuclei HVC, RA, LMAN, and LAreaX relative to their surrounding brain regions of HVC_{shelf}, RA_{cup}, AN, and ASt in adult male zebra finches. Each bar represents mean ± SEM measured from film autoradiograms. Statistical analysis was done by paired t-test where the ratio was compared to a

ratio equal 1. Asterisks above or below bars represent p-values: * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Fig. 12. Images of differential dopamine receptor subtype expression in the song nuclei during development. **A**: D1A receptors in HVC; **B**: D3 in HVC; **C**: D3 in RA; **D-F**: D1A, D1B and D2 in LArea X and LMAN. The age of each bird in days is labeled in the top right corner of each image. All sections are sagittal. The images were taken from film autoradiograms. Black, mRNA signal. Scale bars, 0.5 mm.

Fig. 13. Quantification of dopamine receptor subtype expression in the song nuclei (**A, D, G, J**), surrounding areas (**B, E, H, K**), and their ratios (**C, F, I, L**) during development. Each point represents mean ± SEM. * $p<0.05$ and ** $p<0.01$, significant changes in densities during the whole of development assessed by ANOVA with Bonferroni correction. Fisher's PLSD post hoc tests revealed significant differences between the individual time points, but for the sake of clarity, in the figure are shown only the differences between 15 day old birds and adults as $$ $p<0.01$ and $$$ $p<0.001$, and differences between two adjacent time points (marked above the later one) as # $p<0.05$, ## $p<0.01$, and ### $p<0.001$.

Fig. 14. Images of D1A receptor, D2 receptor, and singing-driven egr1 co-localization in LArea X of the striatum. **A_1-A_4:** Co-localization of double labeled D1A and D2 cells. **A_1**, D1A receptor mRNA labeled with silver grains (black) in brightfield view using radioactive in situ hybridization (RISH); **A_2**, D2 receptor mRNA labeled red using fluorescent situ hybridization (FISH); **A_3**, cell nuclei labeled blue with DAPI; **A_4**, merged image of D1A, D2, and DAPI; the

D1A receptor signal is inverted and now silver grains are white. White arrows, $D1A^+/DAPI^+$ cells; red arrows, $D2^+/DAPI^+$ cells; yellow arrows, $D1A^+/D2^+/DAPI^+$ cells. **B_1-B_4:** Co-localization of double and triple labeled neurons with D1A, D2, and undirected singing-driven egr1 expression. **B_1,** D1A receptor mRNA in the cytoplasm labeled green using fluorescent in situ hybridization; **B_2**, D2 receptor mRNA in the cytoplasm labeled with silver grains (white) above cell nuclei labeled blue by DAPI using radioactive in situ hybridization (image taken in darkfield view); **B_3**, egr1 protein labeled red in the nucleus using immunocytochemistry. **B_4**, merged image showing overlap of the D1A, D2, and/or egr1. Green arrow, $D1A^+/egr1^+$ neuron; yellow arrow, $D1A^+/D2^+/egr1^+$ neuron. Scale bars, 10 μm. **C:** The proportion of cells that express only D1A, only D2, or D1A and D2 receptors. Open bars show the proportion of cells for each receptor type relative to all cells ($DAPI^+$, n=1226 across 7 birds); hatched bars show the proportions relative to all egr1 labeled neurons after singing (after undirected singing, n=747 $egr1^+$ cells total or 124.5±29.5 average $egr1^+$ cells per bird, n=6 birds; or after directed singing, n=198 $egr1^+$ cells total or 49.5±13.2 average of $egr1^+$ cells per bird, n=4 birds). *$p<0.05$, ANOVA followed by Fisher's PLSD post hoc test. **D:** Percentages of D1A and D2 expressing cells comparing radioactive (RISH) and fluorescent (FISH) in situ hybridizations that were alternated for the D1A and D2 receptors. No differences in RISH vs. FISH results were found ($p=0.86$ for D1A; $p<0.30$ for D2 t-test, n=6 animals, averaged over several sections). Regardless of probe combination, there is a higher percentage of D1A relative to D2 expressing cells (*** $p<0.001$, paired t-test between the percentages of D1A and D2 DAPI-labeled cells, single or double labeled, within LArea X of each animal).

Table 1. Accession Numbers and Primers. Listed are the cloned dopamine receptor cDNA gene transcripts (Gene Tr), their accession numbers (No), the forward and reverse primers used to clone them, and the clone lengths. The primers for D1B and D1D as well for D2 v1, D2 v5, and D3 receptors were identical.

Table 2. Cross dopamine receptor cDNA Sequence Identities. Shown are the percent sequence identities of each cloned dopamine cDNA fragment (D1A, D1B, D1D, D2, D3) and full-length cDNA (D4) to the full-length transcript of all the dopamine receptors. Since the full-length transcript is expected to be expressed in cells, the identities indicate cross in-situ hybridization potential of each receptor cDNA fragment to the full-length transcript of the other five receptors. All cross-cDNA identities are below our 85% cut off.

Fig. S1. Comparative protein sequence analyses of zebra finch dopamine receptor subtypes relative to chickens and humans. **A-F:** Receptor alignments for D1A, D1B, D1D, D2, D3, and D4, respectively. The protein coding sequences were aligned with clustalW http://www.ebi.ac.uk/Tools/clustalw2/ and then manually edited to correct errors in alignments. Text color coding: Red, small+hydrophobic, including aromatic (-Y) amino acids; Blue, acidic a.a.; Magenta, basic a.a.; Green, hydroxyl+amine+basic (-Q) a.a.. Receptor domains were predicted with TMHMM software (http://www.cbs.dtu.dk/services/TMHMM/) based on the zebra finch sequences. The exact predictions can slightly differ for different species, based on sequence differences. TM, transmembrane domain (boxed regions); CL, cytoplasmic loop; EL, extracellular loop. Outside and inside domains are the amino- and carboxy-terminal regions, respectively, that are positioned outside and inside of the cell, respectively. Note that the TM domains are mainly hydrophobic. For D1B (B), the first ~23 a.a. of the zebra finch protein has not yet been sequenced from the zebra finch genome or cloned as a cDNA, and thus the sequence shown is partial. For D1D (C), the chicken sequence shown is the one annotated by some sources as D1C. For D2 (D), variant 1 for birds and the variant long of humans are aligned. For D3 (E), the prediction algorithms generated a longer protein at the amino terminal end in chicken than that supported by avian EST evidence and homologies to zebra finch and other vertebrate species (our analysis). Thus, we truncated the chicken sequence at the start site for zebra finch. The closest human D3 variant (variant 1) to the zebra finch protein was aligned. For D4, the zebra finch sequence between the arrows was determined from the cDNA clone of this study (part of which has not yet been sequenced yet in the genome), whereas the remaining sequence was determined from the genome. Accession numbers of the clones used are shown in Fig. 2.

Fig. S2. Zebra finch D2 receptor variant alignments. **A:** Protein sequence alignments of cDNA supported and genomic predicted protein sequences of D2 splice variants. The cDNA inferred protein variants 1 and 5 (D2v1 and D2v5) were cloned in this study. The variants 3-4 (D2v1-D2v4) were predicted by ENSEMBLE and are in NCBI Genbank. Color-coding and labels follow the format described in the legend of Fig. S1. Note the splice variations in the 3rd cytoplasmic loop (CL3). **B:** Alignments of the zebra finch D2 variant 1 used for in situ hybridizations in this study with chicken D2 variant 1 and the turkey D2 long variant used by Schnell et al (1999).

Fig. S3. Images from single label radioactive in-situ hybridization showing **A:** D1A and **B:** D2 receptor mRNA (silver grains in emulsion; black dots) above Nissl labeled cells (grey) in Area X of the striatum in zebra finch. Arrows, labeled cells; arrow heads, non-labeled cells. Scale bar, 10 μm.

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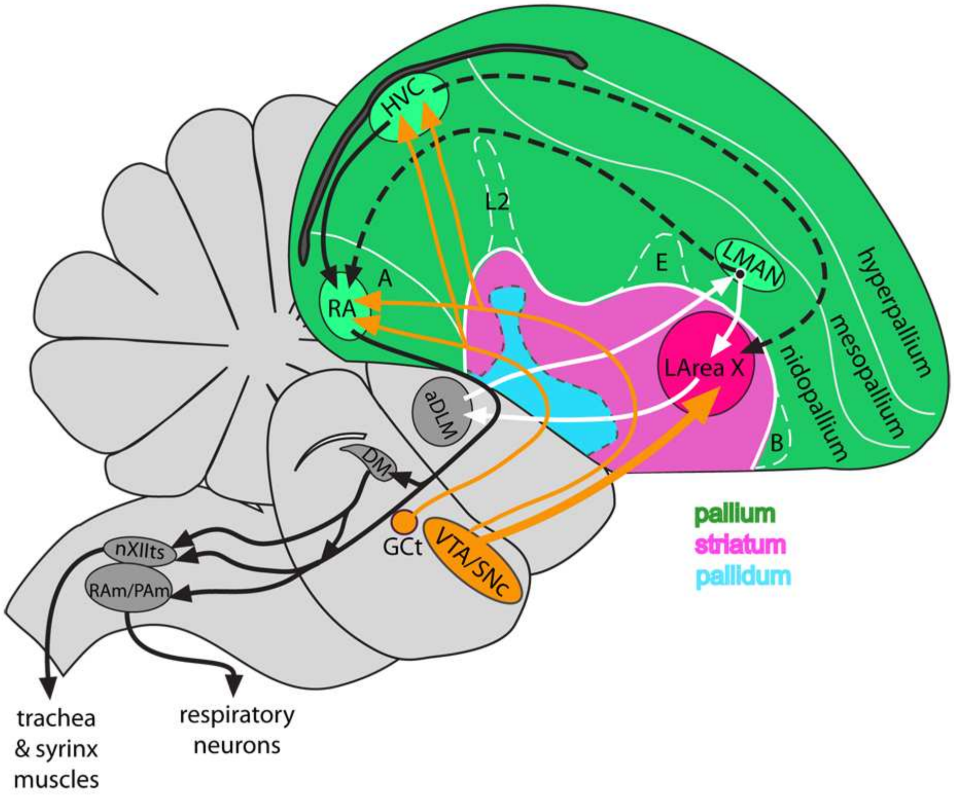

Fig. 1. Diagram of the avian brain highlighting pallial, striatal, and pallidal telencephalic areas and the song system of songbirds. Black arrows: posterior vocal pathway. White arrows: anterior vocal pathway. Dashed arrows: connections between the two pathways. Orange arrows: Dopaminergic (GCt and VTA-SNc) input into the song nuclei. Abbreviations are in the anatomical abbreviations list.
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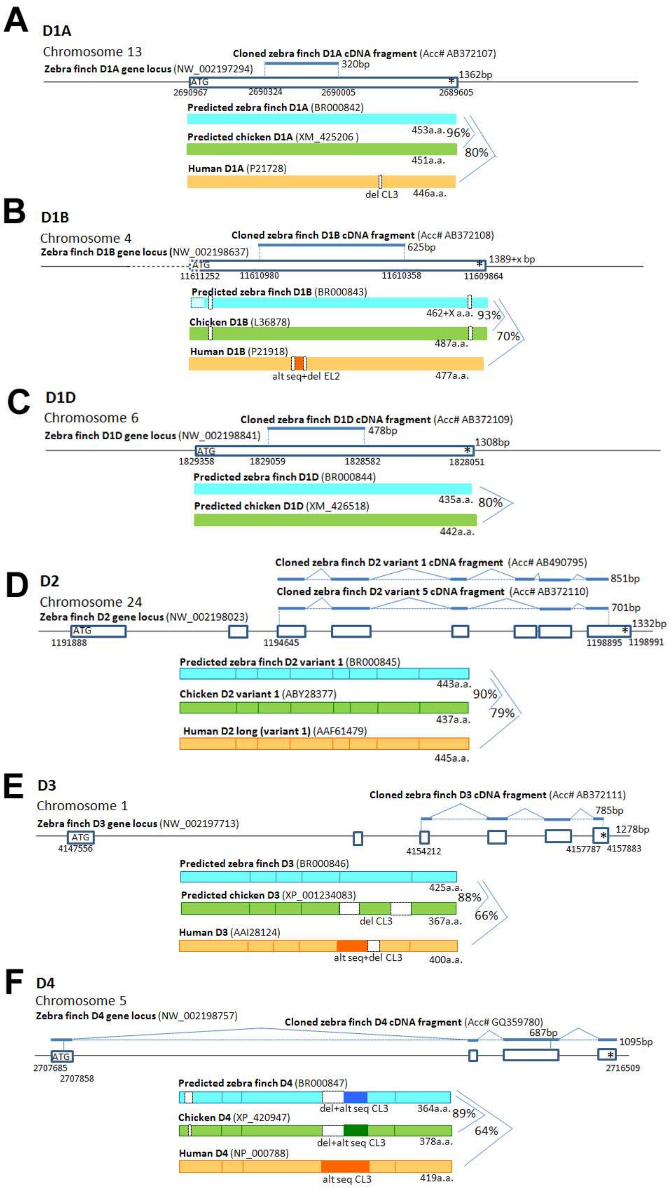

Fig. 2. General structure and sequence comparisons of the six zebra finch dopamine receptors relative the homologous receptors in chicken and human. A-F: For each receptor, shown is the size and position of the cloned zebra finch cDNA fragment (top solid blue lines) aligned to the zebra predicted exon coding (blue open bars) and intron non-coding (black lines) sequence of the zebra finch genome. * is a stop codon. The D1 genes have one coding exon each, D2 has 8, D3 has 6, and D4 has 4. Below the chromosomal sequences are comparisons of the genome-predicted or mRNA-derived protein coding sequences between zebra finch (turquoise), chicken (green), and human (orange bars). The % values indicate percent identities between zebra finch and chicken or zebra finch and human. The lighter blue 5′ end of the zebra finch D1B protein indicates that this region has not been sequenced yet in the genome, but is expected to be present due to the sequence found in the chicken and human genomes. The D4 zebra finch cDNA clone spans the full-length coding region, but the accession number refers to 687bp of cDNA sequence obtained to date; the remaining sequence is inferred from the zebra finch genome. White bars represent deletions (del);

darker color bars represent non-homologous alternate sequences (alt seq). The predicted zebra finch proteins are those that we generated and curated with GENESCAN and the UCSC genome browser. We further curated the predicted ENSEMBLE chicken D3 protein sequence, as it had 300 more 5’ a.a. than all other D3 proteins in the database, which we believe was a computational error. Detailed protein alignments are shown in Fig. S1. CL3 – cytoplasmatic loop 3; EL2 – extracellular loop 2.
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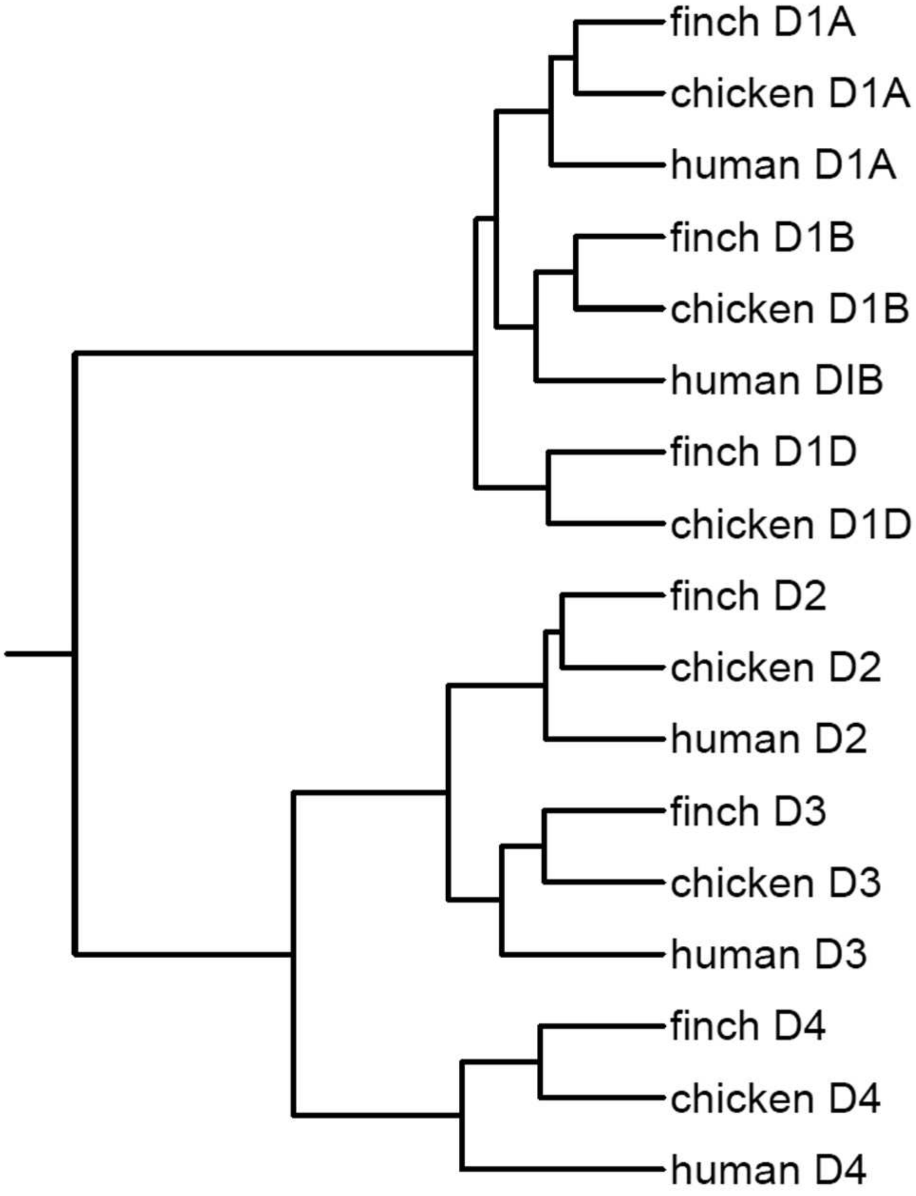

Fig. 3. Phylogenetic analyses of dopamine receptors in the zebra finch, chicken, and human. Shown is a phylogram generated using the full-length protein coding sequences (Fig. S1), dialign alignments (http://bibiserv.techfak.uni-bielefeld.de/dialign/submission.html), and the iTOL tree generating software (http://itol.embl.de/). For D2 and D3, variant 1 sequences were used. Branch lengths represent evolutionary time separating gene relationships (longer branch, more time). The D1 family has shorter branch lengths indicating that they are probably more closely related than the D2 family. All receptor types show closer homologies to each other across species than they do to other receptor types within species.
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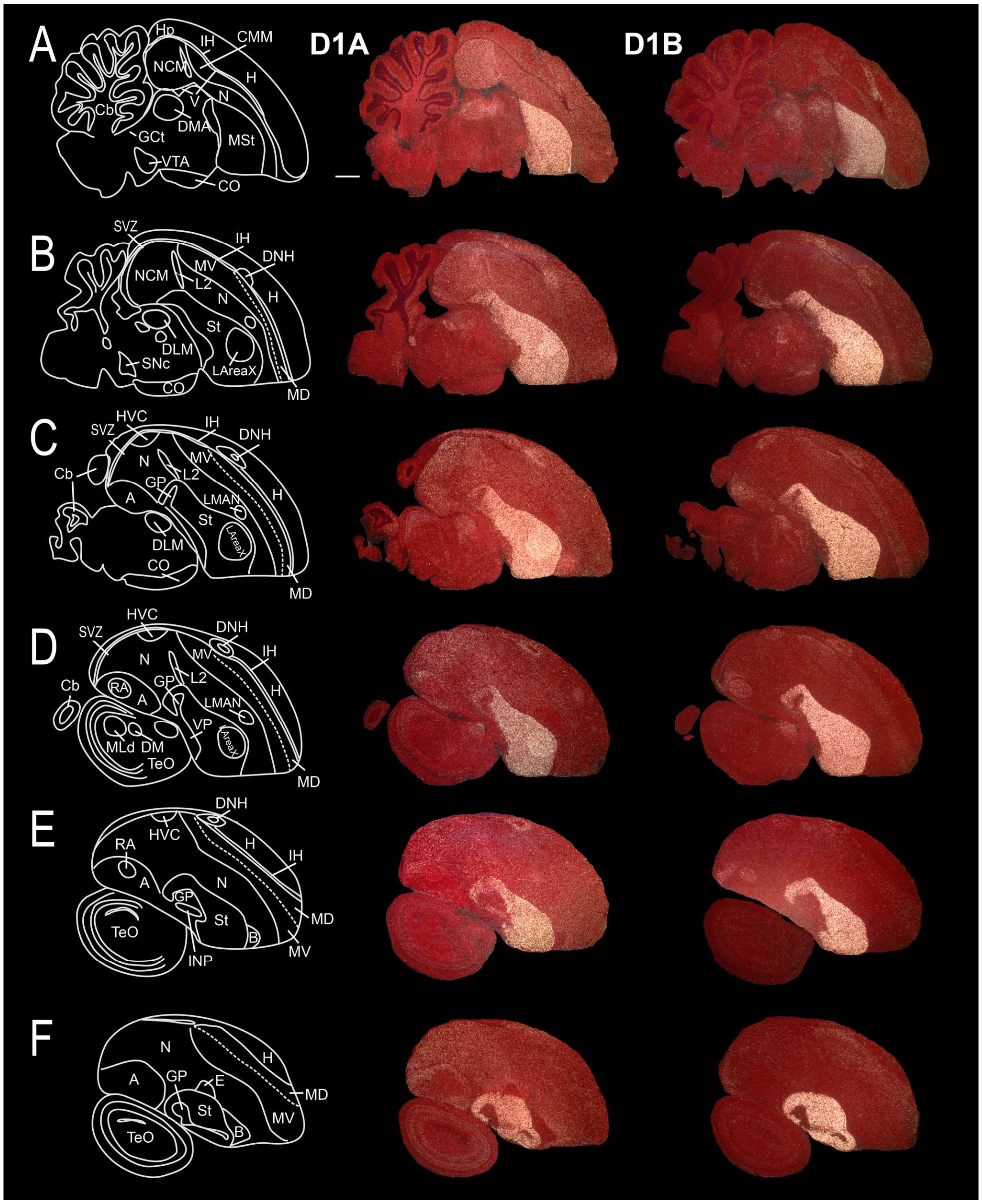

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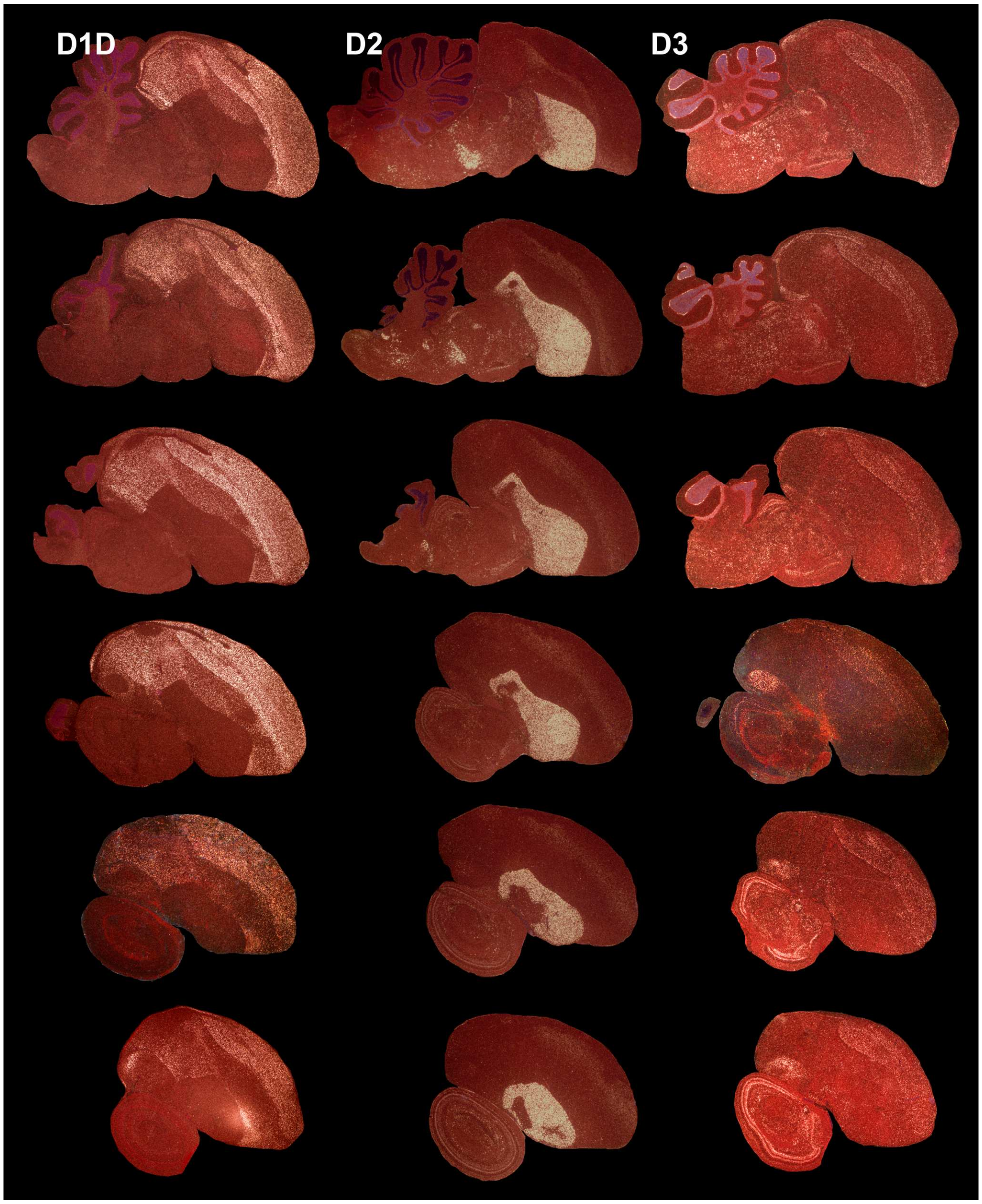

Fig. 4. Expression profiles of dopamine receptor types in sagittal series from adult male zebra finch brain. A-F: Rows showing medial to lateral series across two pages of this report with the respective drawings on the left. Columns are labeled on the top for each receptor (D4 pattern is shown separately in Fig. 6). The images were taken under darkfield microscopy. White silver grains, dopamine receptor mRNA expression; red, cresyl violet stain. Rostral is right, dorsal is up. Scale bar, 1 mm. The sequences of the cDNA probes used are in Genbank (Accession #s AB372107, AB372108, AB372109, AB3490795, AB327111, for D1A, D1B, D1D, D2 transcript variant 1, and D3 respectively; Table 1).
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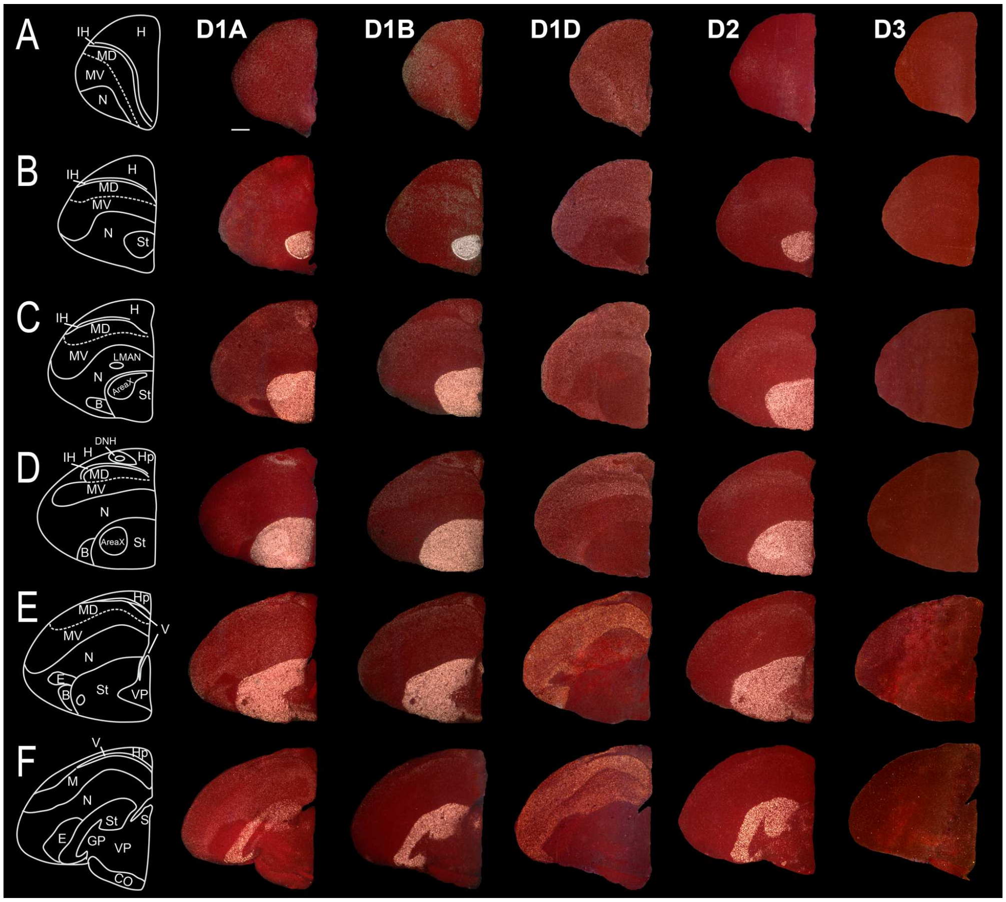

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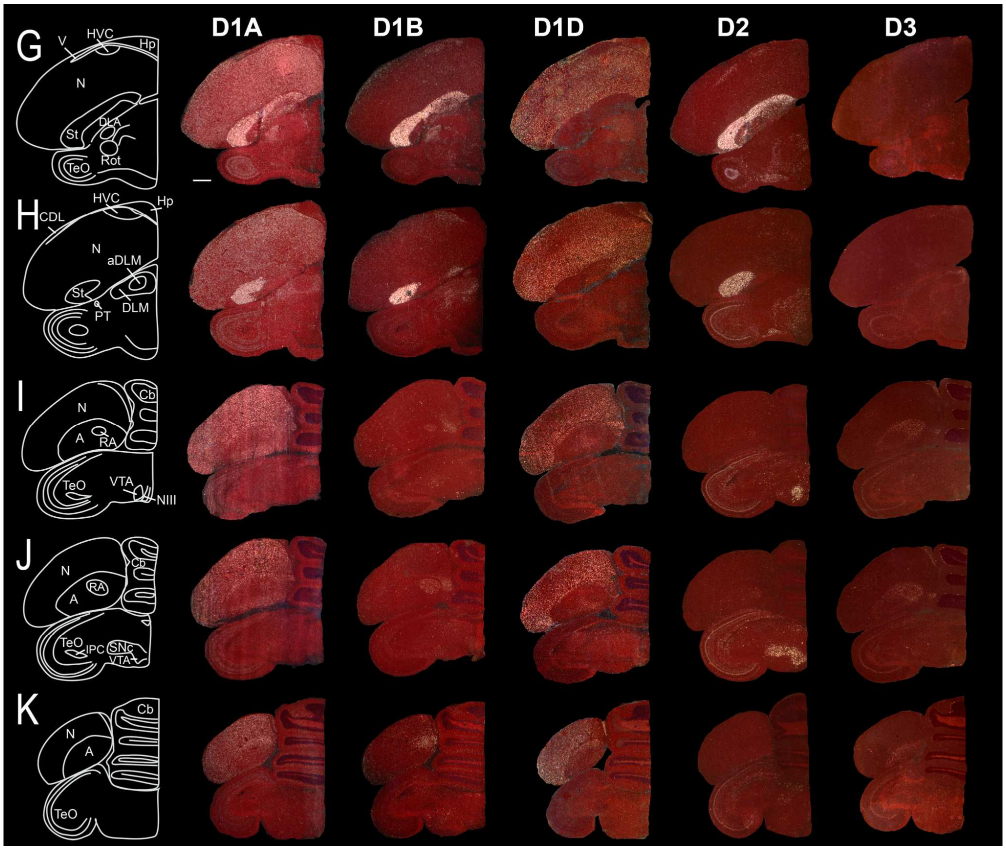


Fig. 5. Expression profiles of the five dopamine receptor types in frontal series of sections of one brain hemisphere from adult male zebra finch brain. A-K: Rows showing rostral to caudal series with the respective drawings on the left. Columns are labeled on the top for each receptor. The images were taken under darkfield microscopy. White silver grains, dopamine receptor mRNA expression; red, cresyl violet stain. Dorsal is up, medial is right. Scale bar, 1 mm.
172x145mm (300 x 300 DPI)

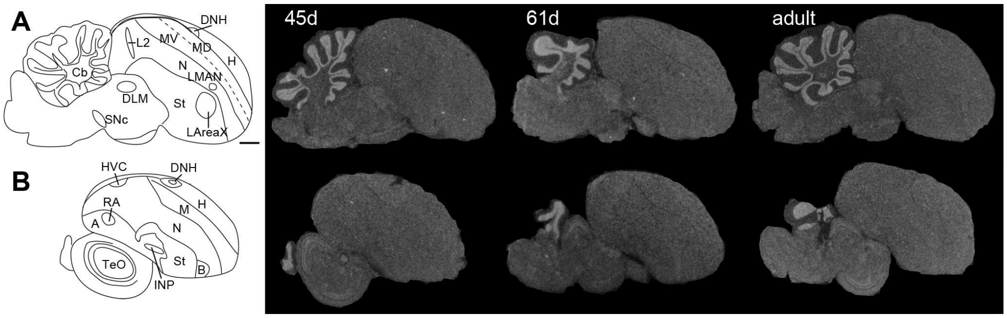


Fig. 6. Expression profile of the D4 dopamine receptor in the sagittal plane from male zebra finch brains during development and in adulthood. Images were taken from film autoradiograms and inverted. Only several sections are shown as there was not much differential expression of D4 receptor in the telencephalon. White, dopamine receptor mRNA expression. Dorsal is up, rostral is right. Scale bar, 1 mm. Genbank accession number of probe sequence is GQ359780 (Table 1).
172x53mm (300 x 300 DPI)

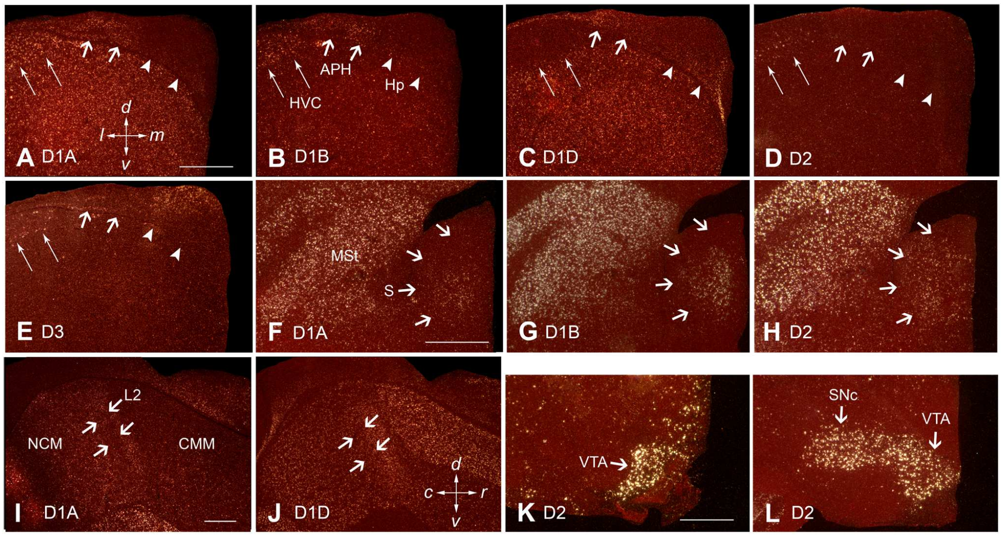


Fig. 7. Higher power images of differential dopamine receptor subtype expression in specific zebra finch brain regions. A-E: the hippocampal formation; F-H: the septum; I-J: Field L2, NCM and CMM; K-L: the midbrain dopaminergic cell groups VTA and SNc. Arrowheads in A-E point to the ventricle. All sections are coronal, except for I and J which are sagittal. White silver grains, dopamine receptor mRNA expression; red, cresyl violet stain. Scale bars, 0.5 mm.
172x91mm (300 x 300 DPI)

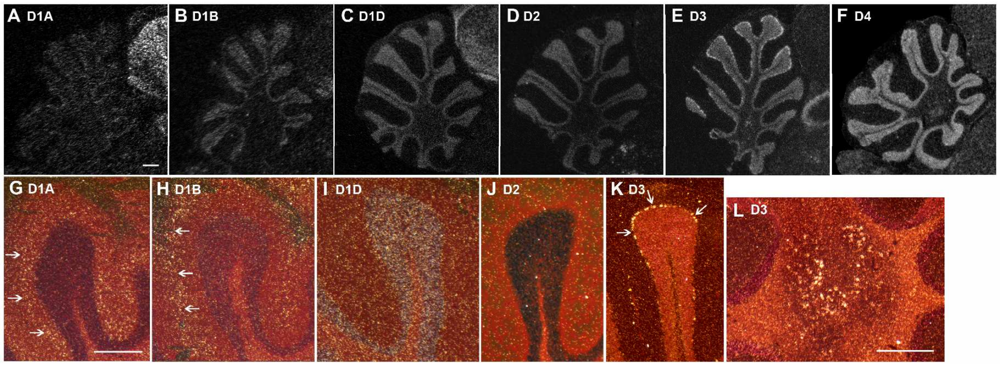


Fig. 8. Higher power images of differential dopamine receptor subtype expression in the cerebellum. The top row shows film autoradiogram images that were inverted, where expression can be seen in the granular layer for all receptors; the Nissl staining of the dense granular layer in the in-situ hybridizations masks the label. The bottom row shows Nissl stained images in darkfield, where the differential expression (white silver grains) of several receptors (D1A, D1B, and D3) can be seen in the inner and outer halves of the molecular layer and in Purkinje cells, respectively (arrows). Scale bars, 0.25 mm.
172x62mm (535 x 535 DPI)

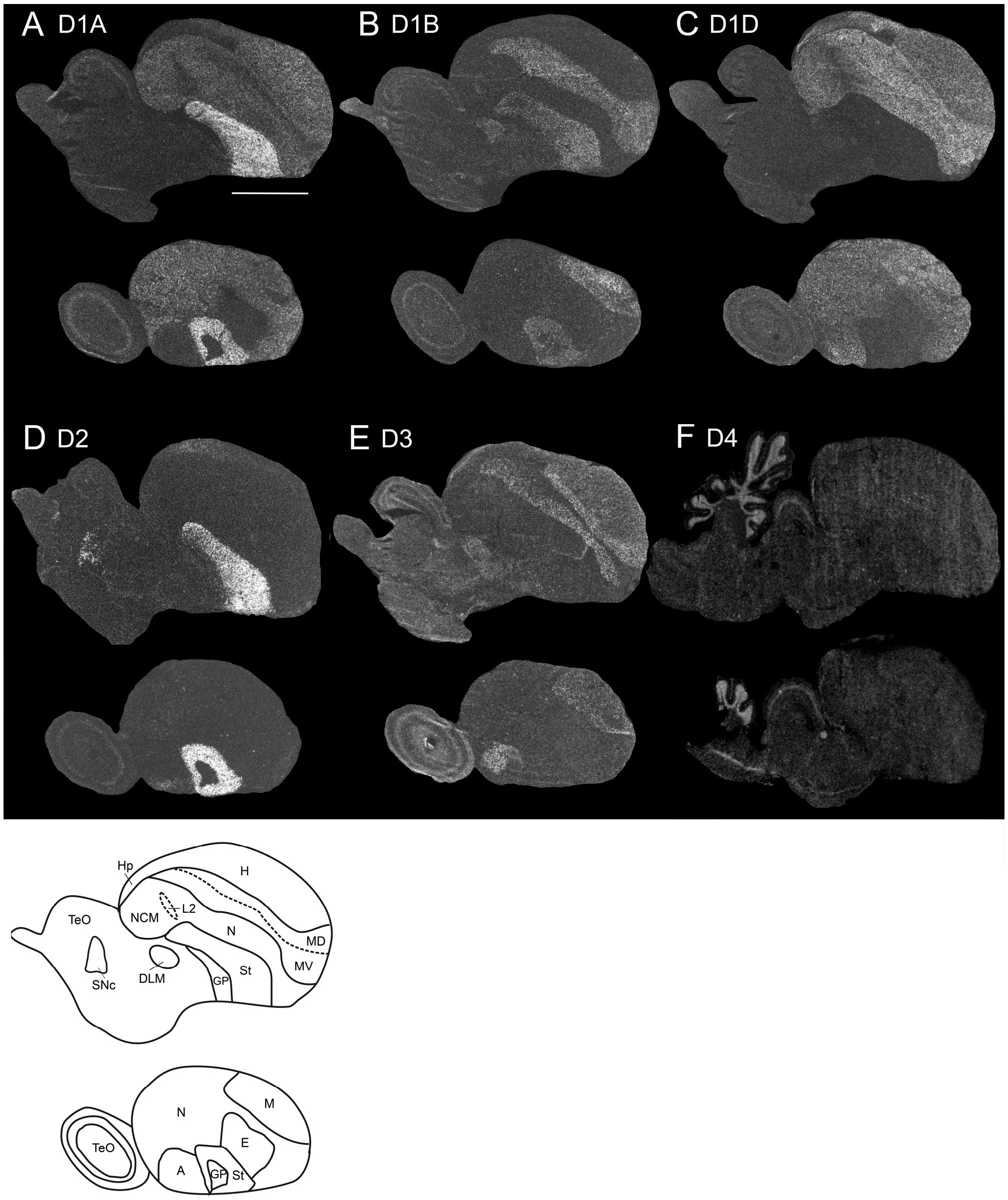


Fig. 9. Expression profiles of dopamine receptor subtypes in adult chicken brain. Images were taken from film autoradiograms and inverted; white, mRNA signal. Two sagittal sections are shown per receptor; the distance from the midline is ~1.5 (top) and 3.5 mm (bottom). A-C: Upper rows show the D1 receptor family (D1A, D1B, and D1D). D-F: Lower rows show the D2 receptor family (D2, D3, and D4). The hybridizations were done with the zebra finch S35-UTP labeled cRNA riboprobes using the same high stringency conditions as for the zebra finch in-situ hybridizations. Scale bar, 0.5 cm.
173x207mm (600 x 600 DPI)

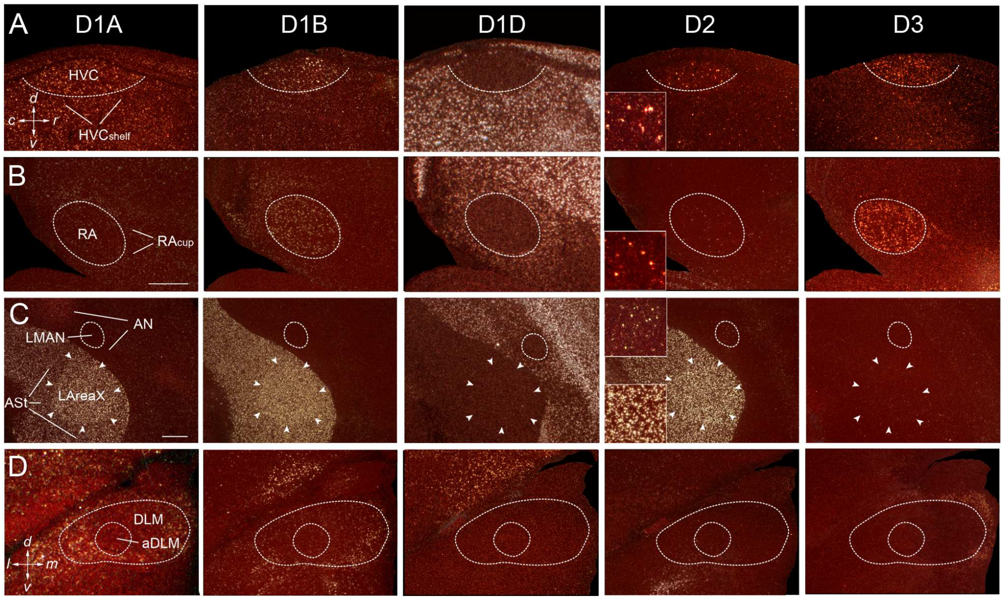


Fig. 10. Higher power images of differential expression of different dopamine receptor subtypes in the song nuclei HVC, RA, LMAN, LAreaX, and aDLM of an adult male zebra finch. Insets for the D2 receptor show higher power images of isolated labeled cells in pallial song nuclei (HVC, RA, LMAN); inset for LArea X is shown for comparison. All sections are sagittal except for the last row showing aDLM, which is coronal. White silver grains, dopamine receptor mRNA expression; red, cresyl violet stain. Scale bar, 0.5 mm (insets magnified 2.7 more times).
172x103mm (300 x 300 DPI)

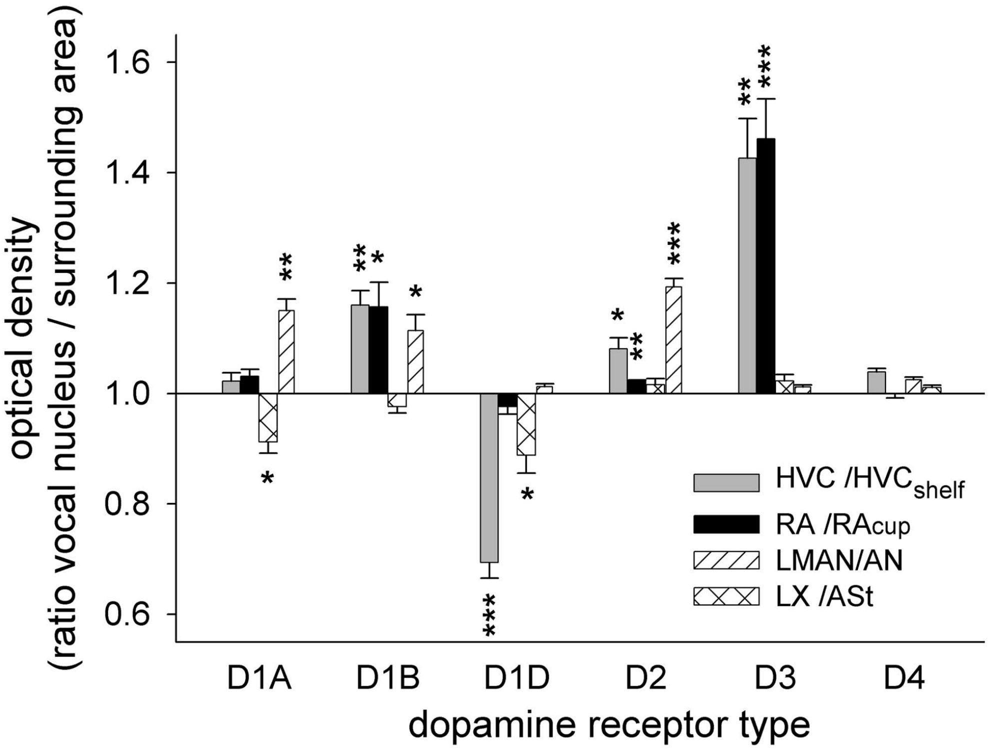


Fig. 11. Quantification of the expression levels of the dopamine receptors in the song nuclei HVC, RA, LMAN, and LAreaX relative to their surrounding brain regions of HVCshelf, RAcup, AN, and ASt in adult male zebra finches. Each bar represents mean ± SEM measured from film autoradiograms. Statistical analysis was done by ANOVA followed by Fisher's PLSD post hoc test. Asterisks above or below bars represent p-values for the post-hoc test: * p<0.05, ** p<0.01, and *** p<0.001.
83x63mm (600 x 600 DPI)

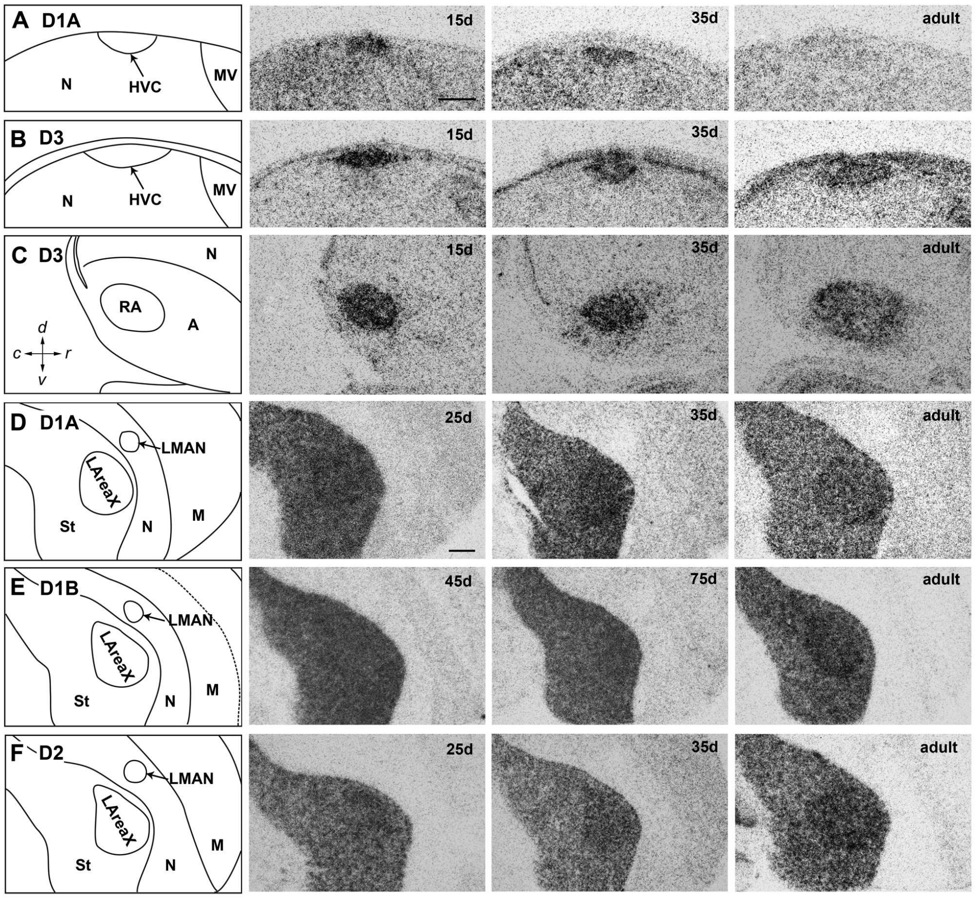


Fig. 12. Images of differential dopamine receptor subtype expression in the song nuclei during development. A: D1A receptors in HVC; B: D3 in HVC; C: D3 in RA; D-F: D1A, D1B and D2 in LArea X and LMAN. The age of each bird in days is labeled in the top right corner of each image. All sections are sagittal. The images were taken from film autoradiograms. Black, mRNA signal. Scale bars, 0.5 mm.
172x156mm (300 x 300 DPI)

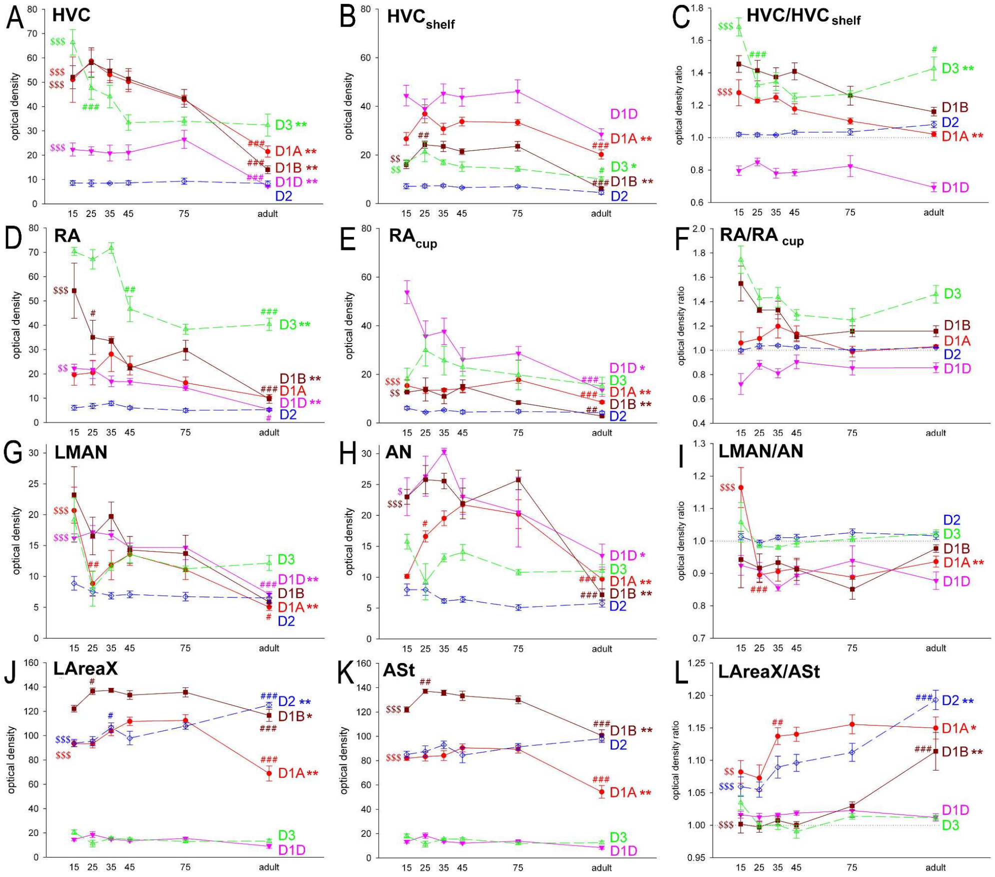


Fig. 13. Quantification of dopamine receptor subtype expression in the song nuclei (A, D, G, J), surrounding areas (B, E, H, K), and their ratios (C, F, I, L) during development. Each point represents mean ± SEM. * $p<0.05$ and ** $p<0.01$, significant changes in densities during the whole of development assessed by ANOVA with Bonferroni correction. Fisher's PLSD post hoc tests revealed significant differences between the individual time points, but for the sake of clarity, in the figure are shown only the differences between 15 day old birds and adults as $$ $p<0.01$ and $$$ $p<0.001$, and differences between two adjacent time points (marked above the later one) as # $p<0.05$, ## $p<0.01$, and ### $p<0.001$.
173x152mm (600 x 600 DPI)

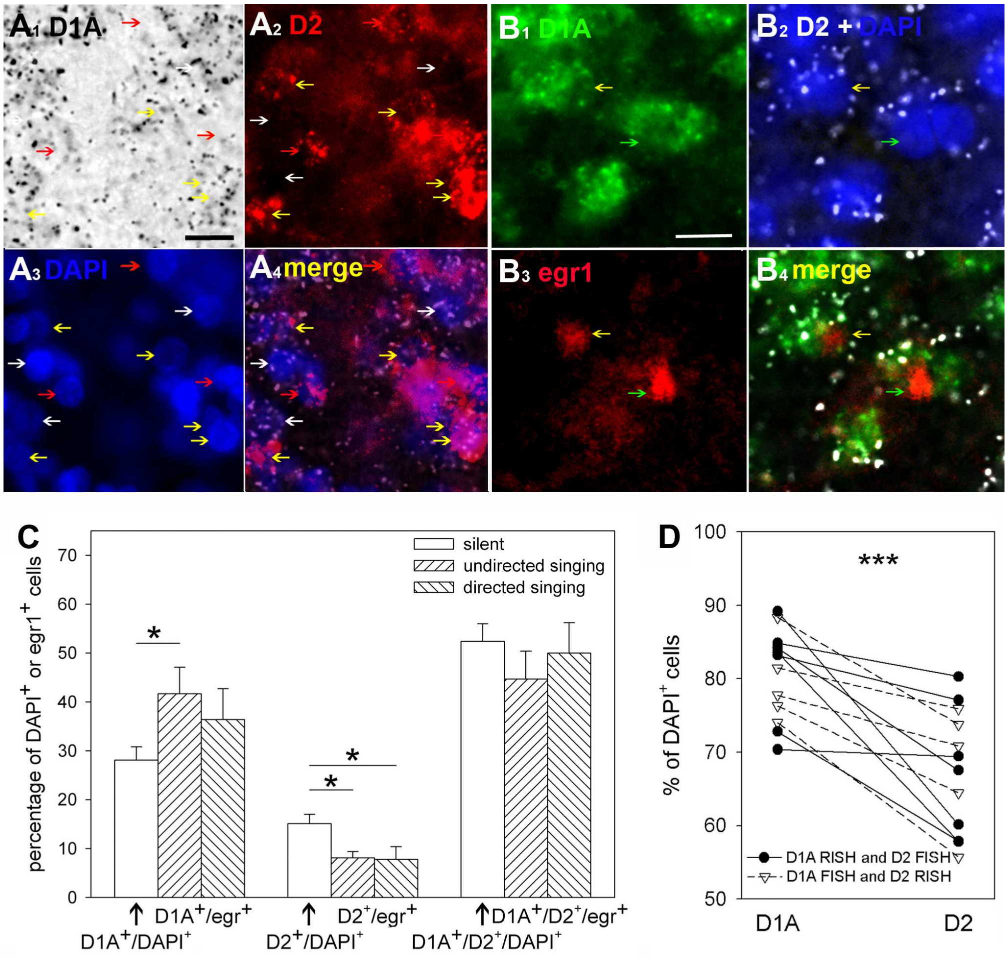
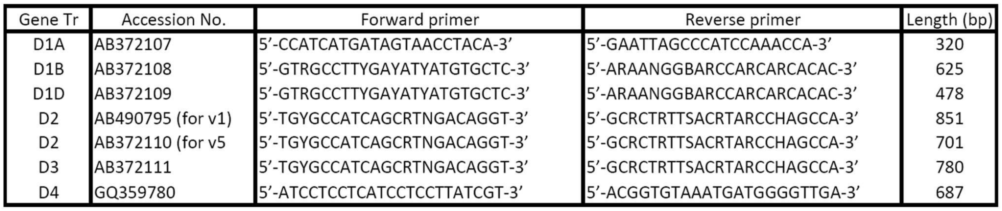


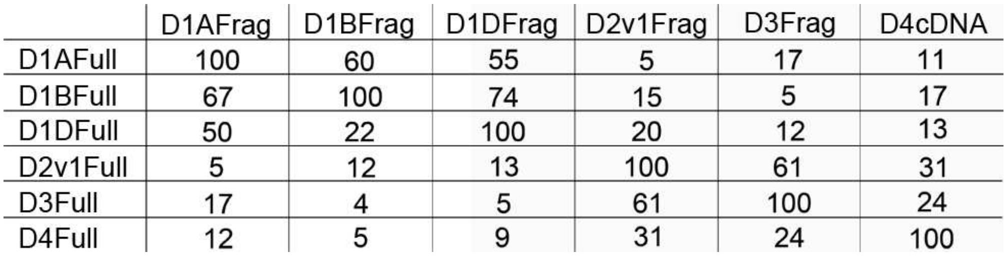
Fig. 14. Images of D1A receptor, D2 receptor, and singing-driven egr1 co-localization in LArea X of the striatum. A1-A4: Co-localization of double labeled D1A and D2 cells. A1, D1A receptor mRNA labeled with silver grains (black) in brightfield view using radioactive in situ hybridization (RISH); A2, D2 receptor mRNA labeled red using fluorescent situ hybridization (FISH); A3, cell nuclei labeled blue with DAPI; A4, merged image of D1A, D2, and DAPI; the D1A receptor signal is inverted and now silver grains are white. White arrows, D1A+/DAPI+ cells; red arrows, D2+/DAPI+ cells; yellow arrows, D1A+/D2+/DAPI+ cells. B1-B4: Co-localization of double and triple labeled neurons with D1A, D2, and undirected singing-driven egr1 expression. B1, D1A receptor mRNA in the cytoplasm labeled green using fluorescent in situ hybridization; B2, D2 receptor mRNA in the cytoplasm labeled with silver grains (white) above cell nuclei labeled blue by DAPI using radioactive in situ hybridization (image taken in darkfield view); B3, egr1 protein labeled red in the nucleus using immunocytochemistry. B4, merged image showing overlap of the D1A, D2, and/or egr1. Green arrow, D1A+/egr1+ neuron; yellow arrow, D1A+/D2+/egr1+ neuron. Scale bars, 10 μm. C: The proportion of cells that express only D1A, only D2, or D1A and D2 receptors. Open bars show the proportion of cells for each receptor type relative to all cells (DAPI+, n=1226 across 6 birds); hatched bars show the proportions relative to all egr1 labeled neurons after singing (after undirected singing, n=747 egr1+ cells total or 124.5+29.5 average egr1+ cells per bird, n=6 birds; or after directed singing, n=198 egr1+ cells total or 49.5+13.2 average of egr1+ cells per bird, n=4 birds). *p<0.05, ANOVA followed by Fisher's PLSD post hoc test. D: Percentages of D1A and D2 expressing cells comparing radioactive (RISH) and fluorescent (FISH) in situ hybridizations that were alternated for the D1A and D2 receptors. No differences in RISH vs. FISH results were found

(p=0.86 for D1A; p<0.30 for D2 t-test, n=6 animals, averaged over several sections). Regardless of probe combination, there is a higher percentage of D1A relative to D2 expressing cells (*** p<0.001, paired t-test between the percentages of D1A and D2 DAPI-labeled cells, single or double labeled, within LArea X of each animal).
172x164mm (300 x 300 DPI)



Gene Tr	Accession No.	Forward primer	Reverse primer	Length (bp)
D1A	AB372107	5’-CCATCATGATAGTAACCTACA-3’	5’-GAATTAGCCCATCCAAACCA-3’	320
D1B	AB372108	5’-GTRGCCTTYGAYATYATGTGCTC-3’	5’-ARAANGGBARCCARCARCACAC-3’	625
D1D	AB372109	5’-GTRGCCTTYGAYATYATGTGCTC-3’	5’-ARAANGGBARCCARCARCACAC-3’	478
D2	AB490795 (for v1)	5’-TGYGCCATCAGCRTNGACAGGT-3’	5’-GCRCTRTTSACRTARCCHAGCCA-3’	851
D2	AB372110 (for v5	5’-TGYGCCATCAGCRTNGACAGGT-3’	5’-GCRCTRTTSACRTARCCHAGCCA-3’	701
D3	AB372111	5’-TGYGCCATCAGCRTNGACAGGT-3’	5’-GCRCTRTTSACRTARCCHAGCCA-3’	780
D4	GQ359780	5’-ATCCTCCTCATCCTCCTTATCGT-3’	5’-ACGGTGTAAATGATGGGGTTGA-3’	687

Table 1. Accession Numbers and Primers. Listed are the cloned dopamine receptor cDNA gene transcripts (Gene Tr), their accession numbers (No), the forward and reverse primers used to clone them, and the clone lengths. The primers for D1B and D1D as well for D2 v1, D2 v5, and D3 receptors were identical.
172x35mm (300 x 300 DPI)



	D1AFrag	D1BFrag	D1DFrag	D2v1Frag	D3Frag	D4cDNA
D1AFull	100	60	55	5	17	11
D1BFull	67	100	74	15	5	17
D1DFull	50	22	100	20	12	13
D2v1Full	5	12	13	100	61	31
D3Full	17	4	5	61	100	24
D4Full	12	5	9	31	24	100

Table 2. Sequence percent identities of cDNA fragments (D1A, D1B, D1D, D2, D3) and full-length cDNA (D4) for the dopamine receptors used for in-situ hybridization when compared with the predicted full-length zebra finch genomic sequence of each dopamine receptor.
82x20mm (300 x 300 DPI)

Fig. S1. Comparative protein sequence analyses of zebra finch dopamine receptor subtypes relative to chickens and humans. A-F: Receptor alignments for D1A, D1B, D1D, D2, D3, and D4, respectively. The protein coding sequences were aligned with clustalW http://www.ebi.ac.uk/Tools/clustalw2/ and then manually edited to correct errors in alignments. Text color coding: Red, small+hydrophobic, including aromatic (-Y) amino acids; Blue, acidic a.a.; Magenta, basic a.a.; Green, hydroxyl+amine+basic (-Q) a.a.. Receptor domains were predicted with TMHMM software (http://www.cbs.dtu.dk/services/TMHMM/) based on the zebra finch sequences. The exact predictions can slightly differ for different species, based on sequence differences. TM, transmembrane domain (boxed regions); CL, cytoplasmic loop; EL, extracellular loop. Outside and inside domains are the amino- and carboxy-terminal regions, respectively, that are positioned outside and inside of the cell, respectively. Note that the TM domains are mainly hydrophobic. For D1B (B), the first ~23 a.a. of the zebra finch protein has not yet been sequenced from the zebra finch genome or cloned as a cDNA, and thus the sequence shown is partial. For D1D (C), the

chicken sequence shown is the one annotated by some sources as D1C. For D2 (D), variant 1 for birds and the variant long of humans are aligned. For D3 (E), the prediction algorithms generated a longer protein at the amino terminal end in chicken than that supported by avian EST evidence and homologies to zebra finch and other vertebrate species (our analysis). Thus, we truncated the chicken sequence at the start site for zebra finch. The closest human D3 variant (variant 1) to the zebra finch protein was aligned. For D4, the zebra finch sequence between the arrows was determined from the cDNA clone of this study (part of which has not yet been sequenced yet in the genome), whereas the remaining sequence was determined from the genome. Accession numbers of the clones used are shown in Fig. 2.
172x307mm (194 x 194 DPI)

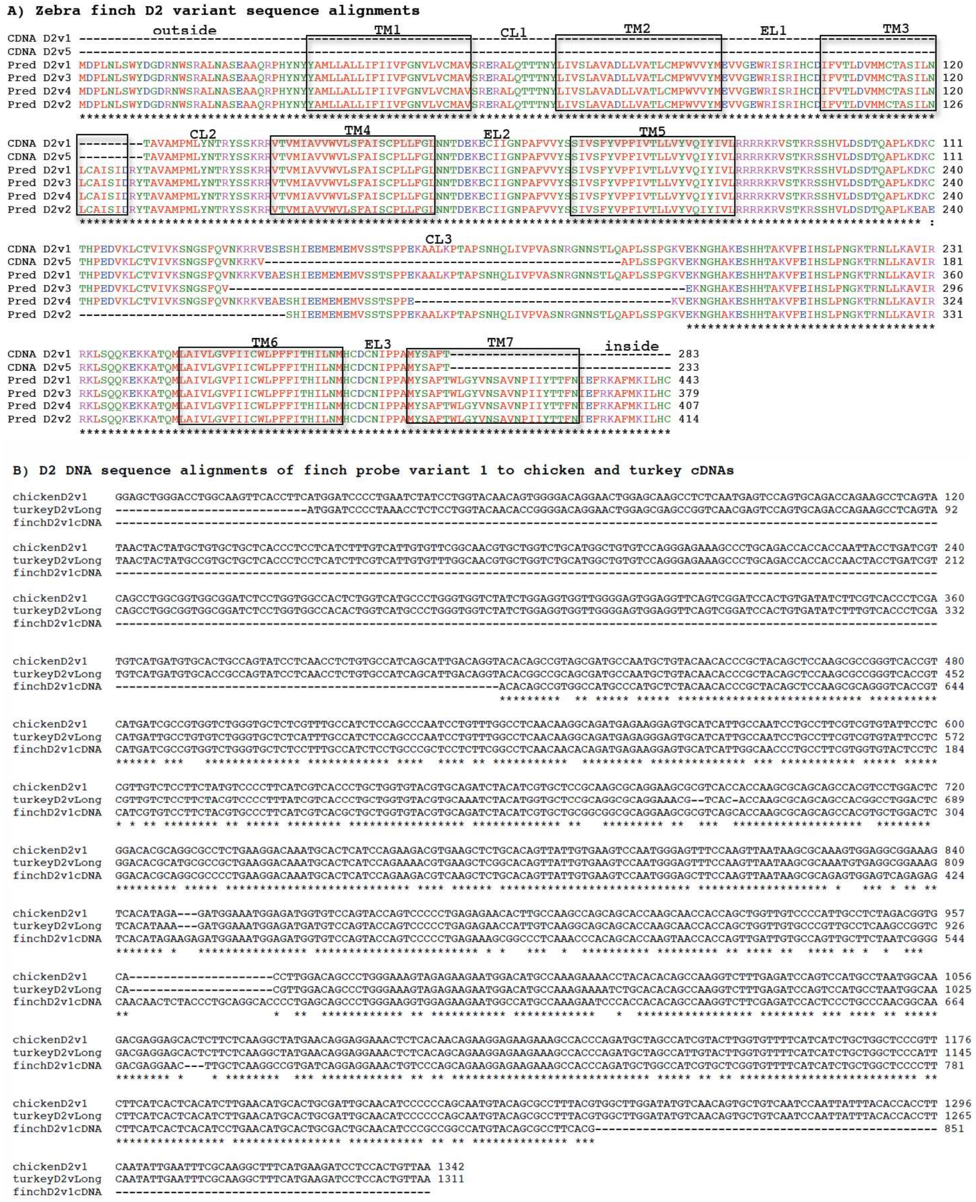


Fig. S2. Zebra finch D2 receptor variant alignments. A: Protein sequence alignments of cDNA supported and genomic predicted protein sequences of D2 splice variants. The cDNA inferred protein variants 1 and 5 (D2v1 and D2v5) were cloned in this study. The variants 3-4 (D2v1-D2v4) were predicted by ENSEMBLE and are in NCBI Genbank. Color-coding and labels follow the format described in the legend of Fig. S1. Note the splice variations in the 3rd cytoplasmic loop (CL3). B: Alignments of the zebra finch D2 variant 1 used for in situ hybridizations in this study with chicken D2 variant 1 and the turkey D2 long variant used by Schnell et al (1999).
173x214mm (213 x 213 DPI)

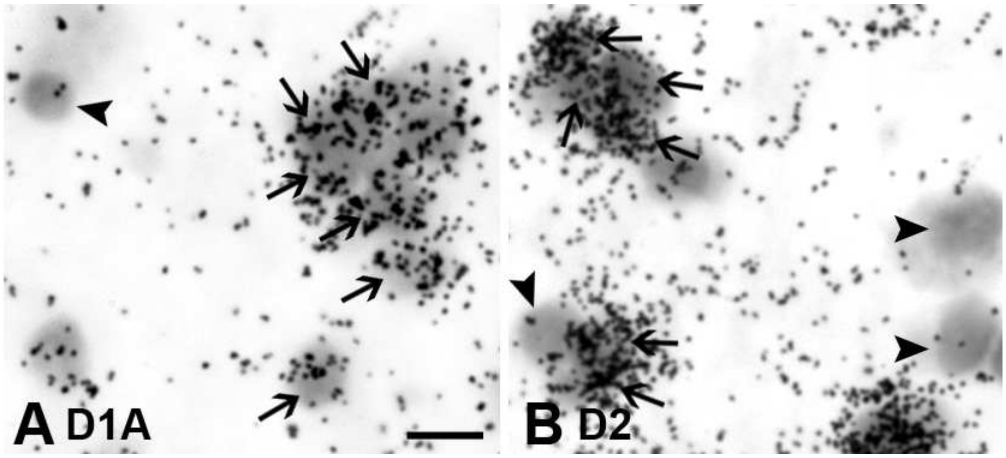
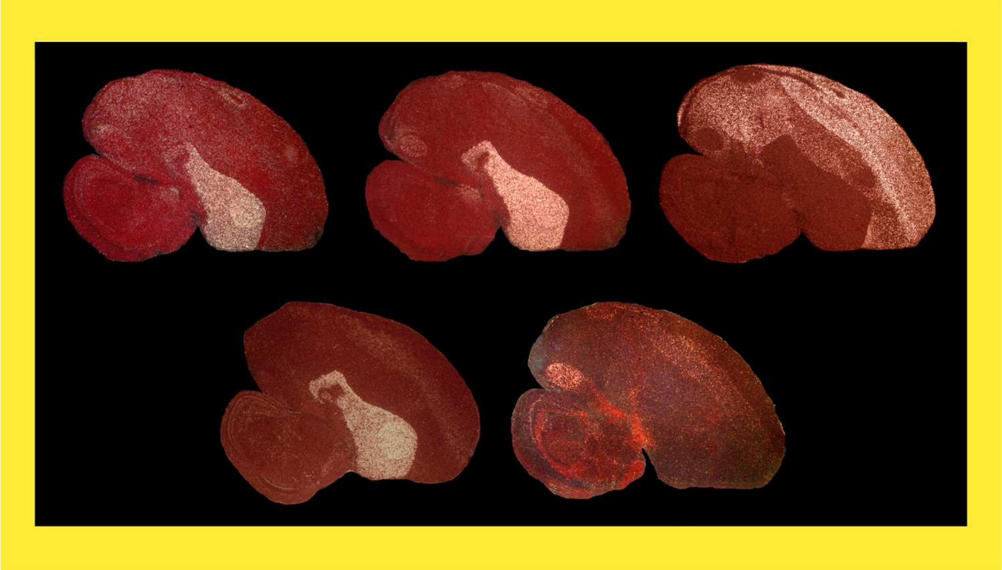


Fig. S3. Images from single label radioactive in-situ hybridization showing A: D1A and B: D2 receptor mRNA (silver grains in emulsion; black dots) above Nissl labeled cells (grey) in Area X of the striatum in zebra finch. Arrows, labeled cells; arrow heads, non-labeled cells. Scale bar, 10 microm.
70x31mm (300 x 300 DPI)



Cover photo. Expression pattern of D1A, D1B, D1D (first raw), D2, and D3 (second raw) dopamine receptors in song nuclei and brain of zebra finch. White is in-situ hybridization mRNA signal, red is background Nissl staining.
127x72mm (300 x 300 DPI)