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Neurochemical Characterization of Dopaminoceptive Cells in Song Control Nuclei of Canaries and Their Activation During Song Production: A Multiplex Fluorescent In Situ Hybridization Study

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ABSTRACT

Highly sensitive in situ hybridization procedures (RNAScope) were used to quantify the expression of three dopamine receptors (Drd1, Drd2, and Drd3) in two song control nuclei (HVC and the Area X of the basal ganglia) that are known to receive dopaminergic inputs and in the periaqueductal gray (PAG) of male and female canaries. Both sexes were treated with testosterone to ensure they would sing actively. We also determined the excitatory versus inhibitory phenotype of the cells expressing these receptors as well as their activation following a period of song production. The three receptor types were identified in each brain area, with the exception of Drd3 in Area X. The density of cells expressing each receptor varied as a function of receptor type and brain area. Surprisingly few sex differences were detected; they do not seem to explain the sex differences in testosterone-induced song. Overall, the density of Drd-positive cells was much lower in PAG than in the two song control nuclei. In HVC, the majority of cells expressing the three receptor subtypes were VGlut2-positive, whereas colocalization with Vglut2 occurred in few cells in Area X and in an intermediate proportion of cells in PAG. The number of inhibitory cells expressing dopamine receptors was limited. Most dopaminoceptive cells in Area X did not express either excitatory or inhibitory markers. Finally, cellular activation during singing behavior, as measured by the expression of Egr1, was observed in cells expressing each of the three dopamine receptor subtypes, except Drd3 in the PAG.

1 | Introduction

Song is a complex learned, species-typical vocalization often produced during courtship and reproduction as well as with communication related to other social behaviors (Catchpole and Slater 2008; Rose, Prior, and Ball 2022). Oscine songbirds such as canaries (*Serinus canaria*) exhibit a specialized neural circuit that is involved in the learning, production, and perception of song (Nottebohm 2008; Nottebohm, Stokes, and Leonard 1976). The quality of song is regulated by nuclei in the vocal production

pathway of the song circuit (Fee, Kozhevnikov, and Hahnloser 2004; Fee and Scharff 2010). Androgens and their metabolites act directly in these forebrain nuclei to regulate song quality (Alward et al. 2018). However, the motivation to sing is promoted by androgens acting in the medial preoptic nucleus (POM) (Alward, Balthazart, and Ball 2013; Alward et al. 2016; Ball et al. 2020).

Androgen action in the POM results in changes in activity of the song system via projections to the periaqueductal gray (PAG) that includes the A11 tyrosine hydroxylase cell group, which then sends ascending catecholaminergic inputs to the song control nuclei HVC and Area X. Indeed, in addition to acting directly on the song control nuclei and indirectly in the POM, androgens can also modulate the song system by acting via ascending catecholaminergic inputs from the midbrain and the brainstem that send diffuse dopaminergic projections to song nuclei from a variety of nuclei (Ball, Riters, and Balthazart 2002; Maney, Bernard, and Ball 2001). Therefore, understanding the neurochemical properties of ascending catecholamine inputs is essential to understand how song system activity is modulated via endogenous stimuli such as steroid hormones.

For well over 30 years, it has been hypothesized that the catecholamine system projects to forebrain song control nuclei, initially based on patterns of receptor expression (Ball 1990). In more recent years, catecholamine projections and receptor distribution have been characterized (Appeltants et al. 2000; Appeltants, Ball, and Balthazart 2002; Ben-Tov, Duarte, and Mooney 2023; Kubikova, Wada, and Jarvis 2010; Lewis et al. 1981), and expression of dopamine receptors has also been investigated by single-cell RNA sequencing (Xiao et al. 2021). Functional consequences of dopamine action on song learning, production, and perception have been described (Barr, Wall, and Woolley 2021; Ben-Tov, Duarte, and Mooney 2023; Day et al. 2019; Haakenson, Balthazart, and Ball 2020; Hoffmann et al. 2016; Leblois and Perkel 2012; Leblois, Wendel, and Perkel 2010; Miller et al. 2015; Murugan et al. 2013).

There are five subtypes of dopamine receptors, all of which signal primarily via interaction with GTP-binding proteins (G proteins) (Neve, Seamans, and Trantham-Davidson 2004). The functional consequences of these G protein interactions differ across subtypes (Neves, Ram, and Iyengar 2002). Based on these differences in action, dopamine receptors can be divided into two different classes: those in the D1-like family (*Drd1*, *Drd5*) and those in the D2-like family (*Drd2*, *Drd3*, *Drd4*) (Kebabian and Calne 1979). D1-like receptors couple to the G proteins $G\alpha_s$ and $G\alpha_{olf}$ and activate adenylate cyclase, consequently increasing intracellular concentrations of cyclic adenosine monophosphate. D2-like receptors couple to the G protein $G\alpha_i/\alpha_o$, which has the opposite effect, an inhibition of adenylate cyclase.

In birds, the multiple dopamine receptors have also been split into these two categories (D1- and D2-like), even if the exact nomenclature is somewhat different (Kubikova, Wada, and Jarvis 2010). Three receptor subtypes in the D1-like family have been identified and called D1A, D1B, and D1D (Demchyshyn et al. 1995; Kubikova and Kostal 2010; Kubikova, Wada, and Jarvis 2010; Sun and Reiner 2000), with D1B corresponding to the D5 of mammals (Kubikova, Wada, and Jarvis 2010). Three receptors have also been identified in the D2-like family—the D2, D3, and D4 receptors (Kubikova and Kostal 2010; Kubikova, Wada, and Jarvis 2010; Schnell, You, and El Halawani 1999). In zebra finches (*Taeniopygia guttata*), all these receptors, except D4, are differentially expressed in the song control nuclei as compared to the surrounding tissue. In most cases, there is higher expression in the nucleus, especially in HVC, but there are exceptions (e.g., in LMAN, the lateral magnocellular nucleus of the anterior nidopallium) (Kubikova, Wada, and Jarvis 2010). In that species, receptor expression is also regulated during development and mainly decreases during the earlier phases of song learning (sensory acquisition and sensorimotor phases; Kubikova, Wada, and Jarvis 2010). D1- and D2-like receptors are still present in adulthood, suggesting that they may both contribute to adult song production in addition to a potential role in song acquisition. Accordingly, multiple studies have identified activation of specific dopamine receptors in HVC or in Area X of the basal ganglia in connection to the production of song or to some of the underlying changes in brain function (e.g., Ding and Perkel 2002; Gale and Perkel 2005; Hara et al. 2007; Leblois and Perkel 2012; Leblois, Wendel, and Perkel 2010).

In particular, a recent study indicated that lesioning dopaminergic projections from the PAG in HVC with a local 6-hydroxydopamine injection essentially abolishes female-directed song (Ben-Tov, Duarte, and Mooney 2023). HVC, however, contains three distinct classes of neurons—interneurons, RA projection neurons, and Area X projection neurons—and these neurons are distributed heterogeneously throughout the nucleus (Dutar, Vu, and Perkel 1998; Fortune and Margoliash 1995; Gahr 1990; Margoliash et al. 1994). Interneurons are inhibitory, whereas projection neurons send excitatory, glutamatergic input to RA and Area X (Ding, Perkel, and Farries 2003; Gale and Perkel 2005; Mooney and Konishi 1991; Mooney and Prather 2005; Olveczky, Andalman, and Fee 2005). Thus, depending on which receptors are expressed on which type of neurons, dopamine could influence song production by acting on local inhibitory neurons or via excitatory projections to Area X.

It has been demonstrated that female canaries only rarely sing spontaneously (Ko et al. 2020) but they can be induced to sing at rates somewhat similar to males by treatment with exogenous testosterone (Dos Santos et al. 2022; Hartog et al. 2009; Madison et al. 2015). However, multiple aspects of these testosterone-induced songs do not match male song quality: these songs are shorter, have a smaller bandwidth, include a smaller repertoire of syllables, as well as fewer and shorter trills (Dos Santos et al. 2022; Dos Santos, Ball, et al. 2023; Dos Santos, Logue, et al. 2023). In testosterone-treated birds, the volume of song control nuclei also remains smaller in females than in males (Dos Santos et al. 2022; Madison et al. 2015). These sex differences might therefore be organizational in nature (i.e., result from effects of genetic differences or early steroid action). We asked here whether these differences might be associated and potentially caused by a differential expression of dopamine receptors and/or activation of dopaminoceptive cells.

To address this question, we quantified in canaries cells expressing three different dopamine receptors (*Drd1* that belongs to the D1-like group, *Drd2*, and *Drd3* that are part of the D2-like group) in HVC and Area X with the implementation of a very sensitive fluorescent in situ hybridization (ISH) (RNAScope) technique.

We examined cells expressing these dopamine receptors for co-expression of a marker of excitatory projection neurons, vesicular glutamate transporter 2 (*VGlut2*), and a marker for GABAergic inhibitory interneurons, glutamate decarboxylase 2 (*Gad2*). The D1-like receptors have been functionally implicated in song/auditory modulation (Barr, Wall, and Woolley 2021; Leblois and Perkel 2012), and evidence from mammals suggests that D1A is functionally more important than the D1B/D5 (Beaulieu and Gainetdinov 2011), so we focused on the D1A (encoded by the *Drd1* gene) and not the D1B/D5. The D1D and the D4 receptor subtypes have a low expression level in the HVC of zebra finches (Kubikova, Wada, and Jarvis 2010), so we did not measure them here. We also determined to what extent cells that express these three dopamine receptor subtypes are active during singing, as measured by expression of the immediate early gene *Egr1*. Specific attention was paid to the possible existence of sex differences affecting all these variables, as substantial sex differences are apparent in relation to multiple aspects of singing in testosterone-treated canaries (Dos Santos et al. 2022; Dos Santos, Ball, et al. 2023; Dos Santos, Logue, et al. 2023; Madison et al. 2015). Results from HVC were then compared to similar data collected in Area X and in the PAG, which is the origin of some of the ascending catecholaminergic inputs to song control nuclei.

2 | Materials and Methods

2.1 | Experimental Animals and Pre-Experimental Manipulations

Male and female canaries (HVC: $n = 12$, six males and six females; Area X: $n = 6$, three males and three females) of the American Singer strain were obtained from a local breeder (Maryland Exotic Birds). All birds were implanted subcutaneously with a 12 mm-long Silastic implant (Dow Corning; internal diameter, 0.76 mm; external diameter, 1.65 mm) packed with 10 mm of testosterone (T) in order to standardize circulating T levels and produce high rates of singing (Alward, Balthazart, and Ball 2013; Madison et al. 2015). The goal of the present study was indeed to search for neurochemical correlates of the sex differences in song quality produced by males and females when they are treated with exogenous testosterone (see Section 1).

Three weeks after T implantation, when birds were observed to be singing regularly, they were placed in sound-attenuated chambers. These chambers contained a combination microphone/camera (Mini Spy HD 1000TVL, TPEKKA) connected to a computer-running DVR server (V6.33b; Mammoth Technologies, Austin, TX) designed for real-time video and audio surveillance while simultaneously recording for the duration of time birds were present. Birds were placed in the chambers overnight for acclimation to the new environment and were observed from the time lights turned on the next morning until they sang. Thirty minutes following initiation of song, birds were deeply anesthetized with isoflurane and perfused transcardially with 4% paraformaldehyde (PFA). Brains were extracted and postfixed in 4% PFA overnight. This duration of singing was selected because previous work indicated that this reliably induces expression of the immediate early gene Egr1 (also known as Zenk in birds) in song control nuclei (Jarvis and Nottebohm 1997). Brains were then transferred to a 30% sucrose solution overnight and then flash frozen on dry ice and stored at − 80°C until used. Brains were cut at 45 μm in the coronal plane using a cryostat at − 20°C (Microm HM 500 OM). All animal procedures were performed in accordance with the University of Maryland, College Park animal care and use committee's regulations.

2.2 | ISH by RNAScope

Two sections from each bird containing HVC or Area X were mounted on slides and processed using the RNAScope Multiplex Fluorescent Reagent Kit V2 (ACD, #323100). Custom RNA probes were synthesized by Advanced Cell Diagnostics for use in the canary (see Table 1).

Slides were incubated for 30 min at 60°C, dehydrated in a series of increasing concentrations of ethanol (50%, 70%, and 100%) for 5 min per step, and allowed to air dry. They were then incubated with hydrogen peroxide for 10 min. Heat-mediated retrieval was performed by a 5-min incubation with target retrieval reagent (ACD) at 95°C and incubation in Protease III solution (ACD) for 30 min at 40°C. Slides were then washed with wash buffer (ACD) at room temperature, and probes were added to allow for hybridization for 2 h at 40°C. After two more 2-min washes, slides were stored in 5× saline-sodium citrate solution (Fisher, #BP1325-1) overnight. The next day, slides were washed and then amplified with AMP1 (ACD) for 30 min, AMP2 (ACD) for 30 min, and AMP3 (ACD) for 15 min at 40°C, with 2 × 2 min washes in between each step. Next, the Channel 1 probe (D1R, D2R, or D3R) was developed. Slides were incubated at 40°C with HRP-C1 (ACD) for 15 min, the corresponding Opal dye (Akoya Biosciences) for 30 min, and HRP blocker (ACD) for 15 min, with 2 × 2-min washes between each of these steps. This same procedure was followed for Channels 2–4 (HRP-C2 through HRP-C4). Sections were then counterstained for DAPI by incubating for 5 min in 1:2000 Hoechst 33342 (ThermoFisher, #H3570) at room temperature, washed with wash buffer, and coverslipped using ProLong Gold Antifade Mountant (ThermoFisher, #P36934). The slides were then imaged using a Nikon W1 spinning disk at 20× magnification, capturing images with five-color channels.

2.3 | Data Quantification

The best section from each bird (excluded due to the absence of staining or other artifacts) was then used to quantify the six different mRNAs of interest and assess their colocalization. Two types of measures of mRNA expression were collected. The first set of analyses quantified the percentage of area covered by each of the three dopamine receptor transcripts in 200 × 200 μm^2 positioned in the center of HVC. In a second set of analyses, the number of cells expressing the different markers under investigation (Drd1-3, VGlut2, Gad2, and Egr1) and their colocalization within HVC were quantified. Image Z-stacks were opened as five-color hyperstacks in Fiji, an image processing package distribution of ImageJ that includes additional plugins (RRID:SCR_002285). ImageJ's auto brightness/contrast algorithm was applied to each channel individually. Researchers then identified cells visually, utilizing DAPI to identify nuclear boundaries of each cell nucleus and examining each color channel to determine which markers were present within this boundary. The "Cell Counter" plugin

TABLE 1 | Custom probes for in situ hybridization in the canary (*Serinus canaria*) and the corresponding dye used for visualization.

Probe target	NCBI number	Catalogue #	Channel	Opal dye
Drd1	XM_018924404.2	1070791-C1	C1	520 (green)
Drd2	XM_030233706.1	1070801-C1	C1	520 (green)
Drd3	XM_030234687.1	1071641-C1	C1	520 (green)
Egr1	XM_018915688.2	1070811-C2	C2	690 (pink)
VGlut2	XM_009100660.2	1070821-C3	C3	570 (orange)
Gad2	XM_018909302.2	1070831-C4	C4	620 (red)

was then used to identify the combination of markers visible in each cell (Drd only, Drd and Egr1, Drd and Egr1 and Vglut2, etc.).

2.4 | Statistical Analyses

All results were analyzed by two-way ANOVA with the sex of the birds and the receptor type as factor. Significant effects of receptor type were further investigated by Tukey's post hoc tests. Effects were considered significant for $p < 0.05$. Results in the three brain areas were also compared by *t*-tests with Benjamini–Hochberg correction. All data in this text are represented by their mean and standard error of the mean but all bar graphs also include individual data points.

3 | Results

The RNAScope procedure successfully labeled each of the three separate dopamine receptors together with VGlut2, Gad2, and Egr1 (Figure 1). At higher magnification, most of the RNA label was distributed in cells in a discrete (punctate) manner and rarely filled the entire cell body with the possible exception of the Egr1 RNA that appeared to be densely expressed in the entire cell.

3.1 | HVC

3.1.1 | Comparative Expression of Dopamine Receptor Subtypes in HVC

Quantification of the percentage area of a 200 × 200 μm square that had fluorescent label for each of the three dopamine receptors in both males and females indicated that Drd1 was the most widely expressed receptor, followed by Drd3 and then Drd2 (Figure 2A).

A two-way ANOVA of these data with dopamine receptor subtypes and sex as factors identified a significant effect of receptor subtype ($F_{(2,27)} = 17.17$, $p < 0.001$) but no sex difference ($F_{(1,27)} = 0.91$, $p = 0.347$) and no interaction between the two factors ($F_{(2,27)} = 1.91$, $p = 0.168$). Post hoc Tukey HSD tests showed that all three subtypes significantly differ from the others (Drd1–Drd2, $p < 0.001$; Drd1–Drd3, $p = 0.008$; Drd2–Drd3, $p = 0.037$).

Dopamine receptor expression was also quantified in HVC by counting the number of cells that expressed the mRNA and dividing these numbers by the area considered (Figure 2B). The distribution of the three different types of these receptors was very similar in the two sexes. Analysis of the density of each subtype by two-way ANOVA (receptor type and sex as factors) identified no sex difference and no interaction between sex and receptor type, although there was a main effect of receptor type (see Table 2 for the detail of all statistical results). Post hoc tests comparing the different conditions indicated a difference in density between Drd1 and Drd2 ($p = 0.013$) but other comparisons were not significant (Drd1–Drd3: $p = 0.080$, Drd2–Drd3: $p = 0.699$).

These two measures of receptor density thus agree reasonably well. The small differences between the two measures of receptor expression are likely due to the spatial distribution of these

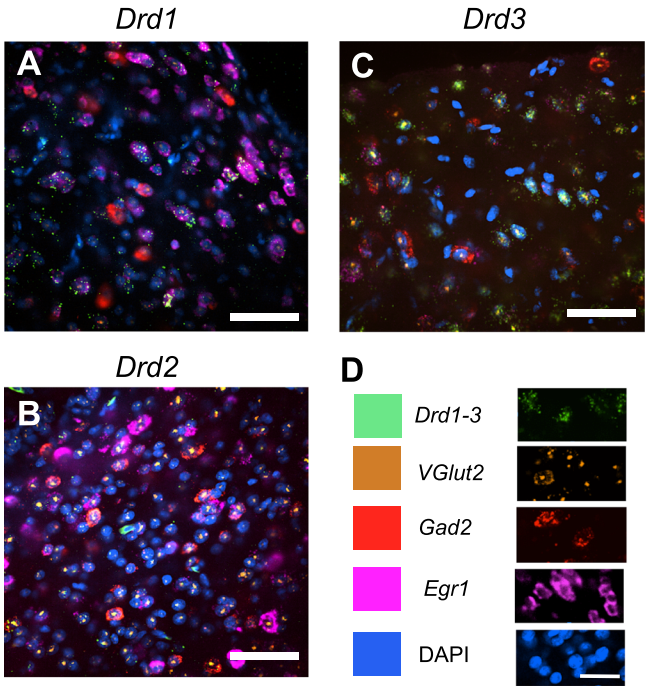

FIGURE 1 | Photomicrographs illustrating the colocalization of the three dopamine receptors with the markers of excitatory (VGlut2) or inhibitory (Gad2) neurotransmission and with the immediate early gene Egr1 in a male HVC. DAPI was used to mark cell nuclei in all sections. Panels A–C, respectively, illustrate the colocalizations with Drd1, Drd2, and Drd3, and Panel D summarizes the color codes used in the photomicrographs. Magnification bar = 50 μm in the three large panels and 25 μm in the small panels illustrating the cellular localization of the labels.

TABLE 2 | Statistical results.

		HVC		Area X		PAG	
		$F_{(df)}$	p	$F_{(df)}$	p	$F_{(df)}$	p
Drd/mm^2	Sex	$F_{(1,27)} = 1.24$	0.275	$F_{(1,8)} = 0.39$	0.564	$F_{(1,13)} = 0.02$	0.889
	Receptor	$F_{(2,27)} = 5.04$	**0.014**	$F_{(1,8)} = 0.09$	0.769	$F_{(2,13)} = 5.54$	**0.018**
	Interaction	$F_{(2,27)} = 2.64$	0.09	$F_{(1,8)} = 0.13$	0.726	$F_{(2,13)} = 0.24$	0.790
% Vglut2	Sex	$F_{(1,27)} = 0.058$	0.812	$F_{(1,8)} = 0.67$	0.437	$F_{(1,13)} = 10.11$	**0.007**
	Receptor	$F_{(2,27)} = 12.61$	**< 0.001**	$F_{(1,8)} = 0.32$	0.568	$F_{(2,13)} = 10.67$	**0.002**
	Interaction	$F_{(2,27)} = 0.45$	0.640	$F_{(1,8)} = 0.21$	0.660	$F_{(2,13)} = 1.97$	0.178
% Gad2	Sex	$F_{(1,27)} = 0.039$	0.845	$F_{(1,8)} = 1.84$	0.212	$F_{(1,13)} = 0.05$	0.818
	Receptor	$F_{(2,27)} = 16.96$	**< 0.001**	$F_{(1,8)} = 0.001$	0.991	$F_{(2,13)} = 4.24$	**0.038**
	Interaction	$F_{(2,27)} = 0.17$	0.844	$F_{(1,8)} = 0.61$	0.458	$F_{(2,13)} = 0.68$	0.522
% Egr1	Sex	$F_{(1,27)} = 0.32$	0.579	$F_{(1,8)} = 0.79$	0.399	$F_{(1,13)} = 0.11$	0.748
	Receptor	$F_{(2,27)} = 1.71$	0.218	$F_{(1,8)} = 11.79$	**0.009**	$F_{(2,13)} = 11.19$	**0.002**
	Interaction	$F_{(2,27)} = 0.34$	0.713	$F_{(1,8)} = 0.25$	0.633	$F_{(2,13)} = 0.67$	0.528

Note: Summary of two-way ANOVA results for each region and probe, with sex and receptor subtype as factors. Bold values are statistically significant $p < 0.05$.
Abbreviation: PAG, periaqueductal gray.

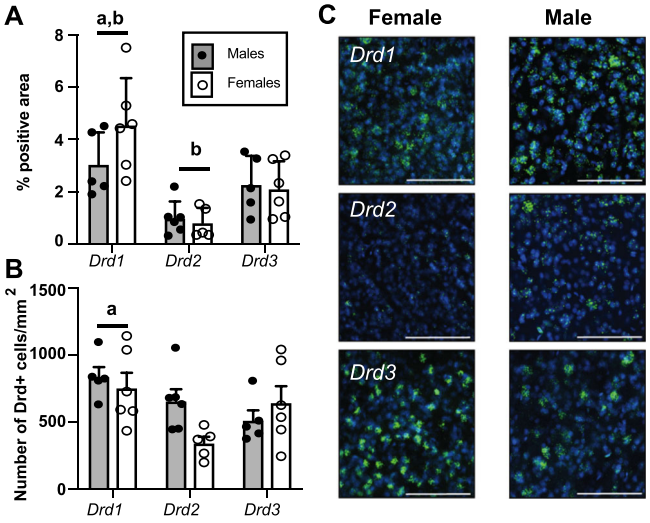

FIGURE 2 | Relative expression of dopamine receptor types in the HVC of males and females. (A) Percentage of cells within a 200 × 200 μm^2 in HVC that had fluorescent label for the three dopamine receptor subtypes. (B) Density of cells (numbers/mm^2) that expressed the mRNA of each dopamine receptor subtype. All bar plots represent means + *SEM* of all data points which are also indicated separately. *a*, *b* = $p < 0.05$ versus Drd2 or Drd3, respectively (collectively for both sexes). (C) Example photomicrographs of receptor densities in males and females. DAPI-labeled nuclei are blue, and dopamine receptor expression is labeled in green. White scale bars indicate 100 μm.

receptors. The significantly lower density of Drd2 compared to Drd3 observed in the percentage areas covered but not in the cell numbers is possibly explained by the fact that Drd3 expression was highly clustered within cells, such that cells that had Drd3 fluorescence typically had a high level of expression (Figure 2C). In contrast, Drd2 was more diffuse, and cells with some expression often had only a few detectable transcripts. Expression of Drd1 was variable, with some cells exhibiting high expression and some cells lower expression.

3.1.2 | Dopaminoceptive Neurons in HVC Are Mostly Excitatory

The majority of cells expressing any of the three dopamine receptor subtypes were excitatory, as measured by VGlut2 expression (Figure 3). More than 80% of the dopaminoceptive cells expressed VGlut2. This percentage of colocalization was, however, slightly lower in Drd2+ cells. This was statistically confirmed by the two-way ANOVA that detected a significant effect of the receptor type but no sex difference (and no sex-by-receptor type interaction). Post hoc tests indicated that these colocalization values were significantly lower for Drd2+ cells compared to both Drd1 and Drd3 (Drd1–Drd2: $p < 0.001$; Drd1–Drd3: 0.999; Drd2–Drd3: $p < 0.001$).

The colocalization with Gad2 (inhibitory neurons) was much more restricted (maximum mean of 20% of cells). This colocalization also differed slightly by receptor type, with Drd2+ cells having now a significantly higher percentage of colocalization than the two other types (Drd1–Drd2: $p < 0.001$; Drd1–Drd3: $p = 0.770$; Drd2–Drd3: $p < 0.001$). There was similarly no sex difference and no sex-by-receptor type interaction.

Very few Drd+ cells simultaneously expressed VGlut2 and Gad2 (less than 5% on average and these numbers were similar for the three subtypes and both sexes). There was also no interaction between the two factors (all $p < 0.122$).

3.1.3 | Immediate Early Gene Expression in HVC Drd± Cells

HVC cells expressing mRNA for each of the three dopamine receptor subtypes had similar proportions of co-expression with

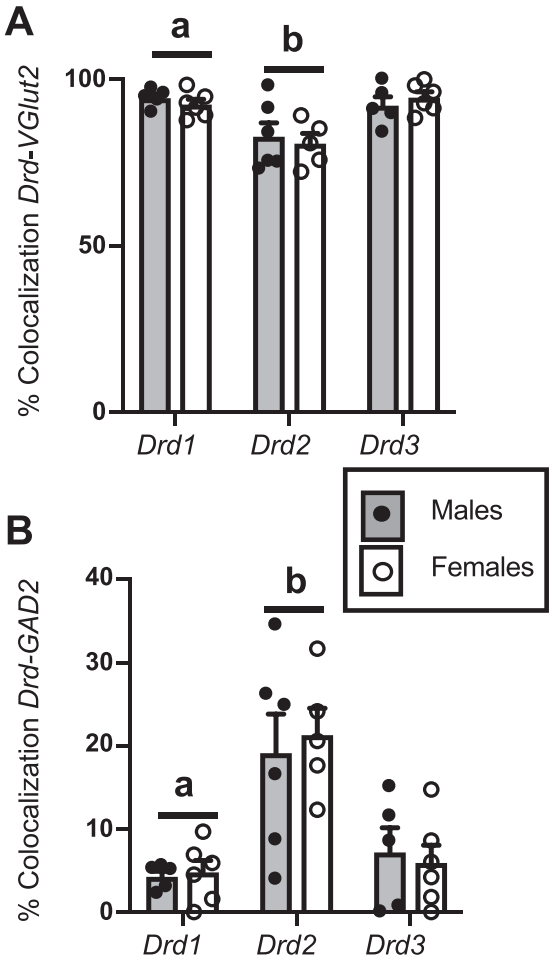

FIGURE 3 | The majority of HVC cells that express dopamine receptors are excitatory. (A) Percentage of cells that expressed fluorescent label for one of the dopamine receptor subtypes colocalized with VGlut2, a marker for excitatory neurons, or (B) with Gad2, the marker of inhibitory neurons. All bar plots represent means + *SEM* of all data points that are also indicated separately. *a*, $b = p < 0.05$ versus Drd2 or Drd3, respectively (collectively for both sexes).

the immediate early gene Egr1, and this activation did not seem to differ in males and females. A two-way ANOVA with dopamine receptor subtype and sex as factors indicated that there was no significant effect of receptor subtype, sex, or interaction between the two on the percentage of dopaminoceptive cells expressing Egr1 (Figure 4).

3.2 | Area X

3.2.1 | Relative Distribution of Dopamine Receptors in Area X

Both Drd1 and Drd2 mRNA were also densely expressed in Area X. As previously demonstrated by Kubikova, Wada, and Jarvis (2010), Drd3 expression in Area X was very low. In most sections, we were unable to distinguish Drd3 mRNA expression from background signal, precluding quantification. The density of dopaminoceptive cells (Drd1 and Drd2 subtypes) was similar in the two sexes (Figure 5A).

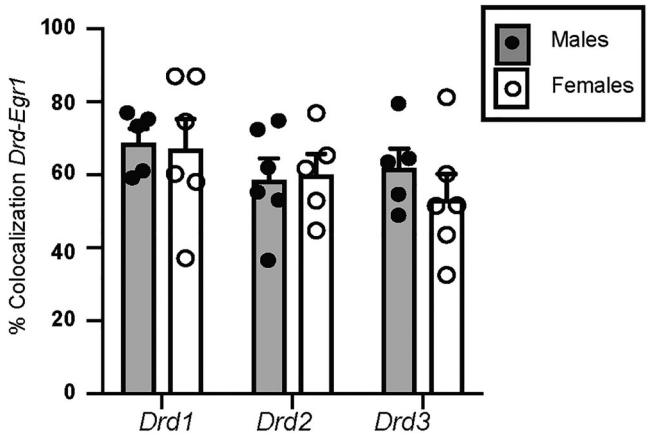

FIGURE 4 | Similar proportions of cells expressing dopamine receptor subtypes are active during song in HVC. Number of cells that had fluorescent label for one of the dopamine receptor subtypes colocalized with the immediate early gene Egr1. All bar plots represent means + *SEM* of all data points that are also indicated separately.

The two-way ANOVA of the density of cells expressing each receptor indicated no significant difference between receptor types or sexes and no interaction between these two factors.

3.2.2 | Colocalization of Area X Drd± Cells With Excitatory or Inhibitory Markers

The majority of Area X Drd1 or Drd2 cells did not express Vglut2 or Gad2 (Figure 5B,C; see Figure 6 for representative photomicrographs illustrating this very low colocalization). Less than 3% of cells expressed both markers together. The vast majority (80% or more) thus did not express any of these markers. Two-way ANOVAs analyzing these percentages of colocalization of Drd1 and Drd2 with Vglut2 identified no significant difference related to receptor type, sex, or their interaction (Figure 5B). A similar conclusion was reached for the colocalization with Gad2 (no effect of receptor type, sex, or their interaction; Figure 5C).

3.2.3 | Immediate Early Gene Expression in Area X Drd Cells

The proportion of Drd1+ cells in Area X that co-expressed the immediate early gene Egr1 was higher compared to Drd2+ cells (Figure 5D). A two-way ANOVA with dopamine receptor subtype and sex as factors indeed confirmed that there was a significant effect of receptor subtype on the percentage of cells expressing Egr1, but there was no sex difference and no interaction between sex and receptor type.

3.3 | The PAG

Some of the sections stained by RNAScope for HVC also contained the medial PAG, a region historically referred to in avian species as the midbrain central gray, containing the A11 tyrosine hydroxylase cell group. This represents only a portion of the PAG: previous work comparing immunohistochemical markers

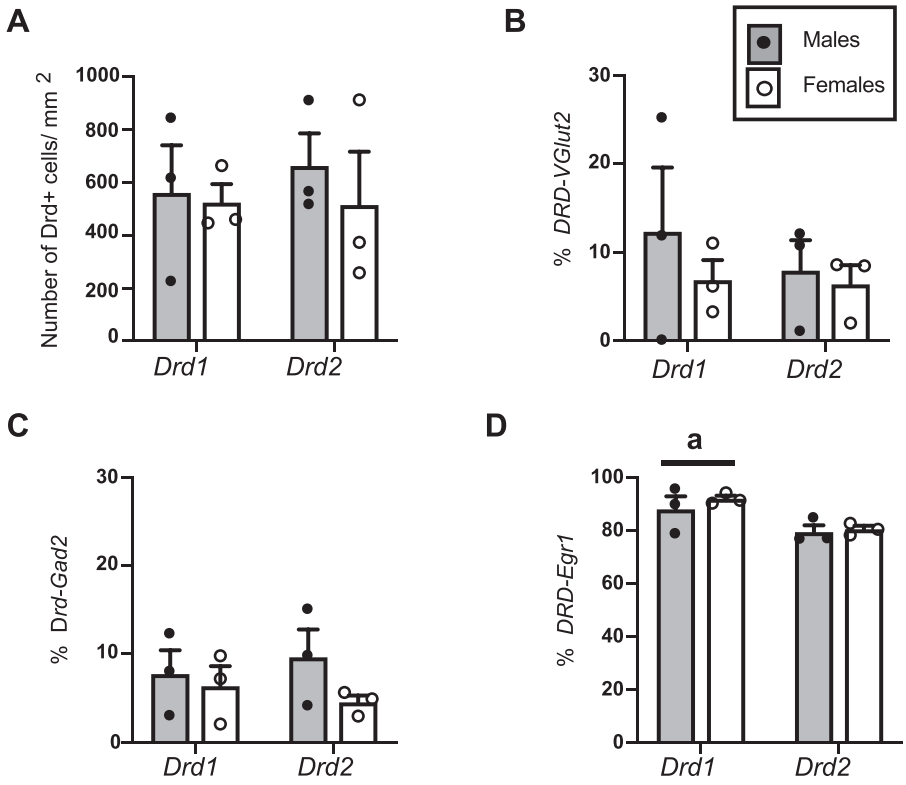

FIGURE 5 | D1 and D2 dopaminoceptive cells in Area X and their colocalization with the neurotransmitter markers VGlut2 and Gad2 and with Egr1. (A) Density of cells that expressed the mRNA of each of the dopamine receptor subtypes. (B and C) Percentage of Drd1+ or Drd2+ cells that expressed fluorescent label for Vglut2, a marker for excitatory neurons (B) or for Gad2, a marker of inhibitory neurons (C). (D) Percentage of Drd1+ and Drd2+ cells that expressed Egr1. All bar plots represent means + *SEM* of all data points that are also indicated separately. $a = p < 0.05$ versus Drd2 (collectively for both sexes).

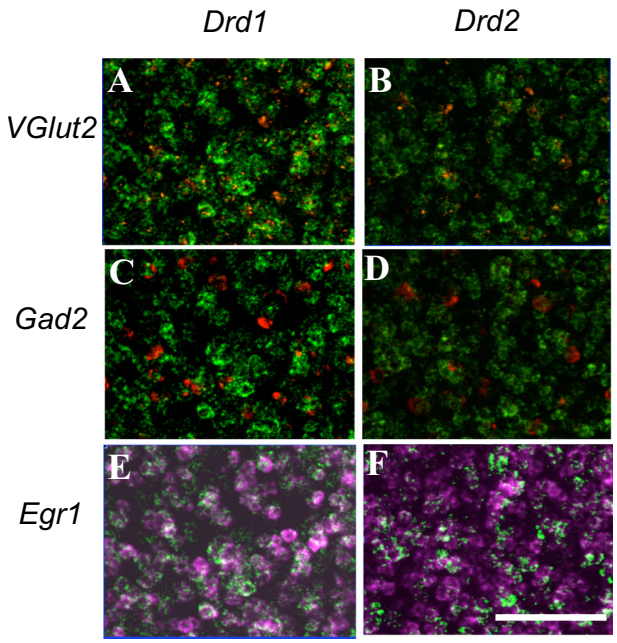

FIGURE 6 | Colocalization of Drd1 and Drd2 with VGlut 2, GAD2, and Egr1 in Area X. All photomicrographs come from male sections. The six panels illustrate the colocalization of Drd1 (A, C, E) and Drd2 (B, D, F) in green with respectively VGlut2 (A, B) in orange, GAD2 (C, D) in red, and Egr1 (E, F) in pink. Magnification bar = 100 μm.

and Fos expression between mice and finches, suggested that the avian central gray and the DM/ICo are organized like the mammalian PAG but unfolded open (Kingsbury et al. 2011). The medial central gray, the region examined in this study (see Figure 7A), exhibits similarities to the mammalian ventral PAG, whereas the lateral central gray extending through DM/ICo resembles the mammalian dorsal PAG. Work from several labs has supported the hypothesis that one function of the avian medial PAG, formerly referred to as the midbrain central gray, is to facilitate song production (Asogwa et al. 2023; Ben-Tov, Duarte, and Mooney 2023).

The same quantifications were therefore performed in the medial part of this nucleus, as defined by Kingsbury et al. (2011) that is known to send dopaminergic projections to the song control nuclei (see Section 1). Data reported below suggest a few interesting aspects, although they must be considered with caution given their limited number (two to four data points per condition).

Representative photomicrographs of the different markers are presented in Figure 8. The density of dopaminoceptive cells in medial PAG was lower than that seen in HVC and Area X. The medial PAG contained similar numbers of cells expressing each dopamine receptor type in both sexes, and the ANOVA of these data accordingly did not identify any difference related to the sex of the birds or the interaction of sex with receptor

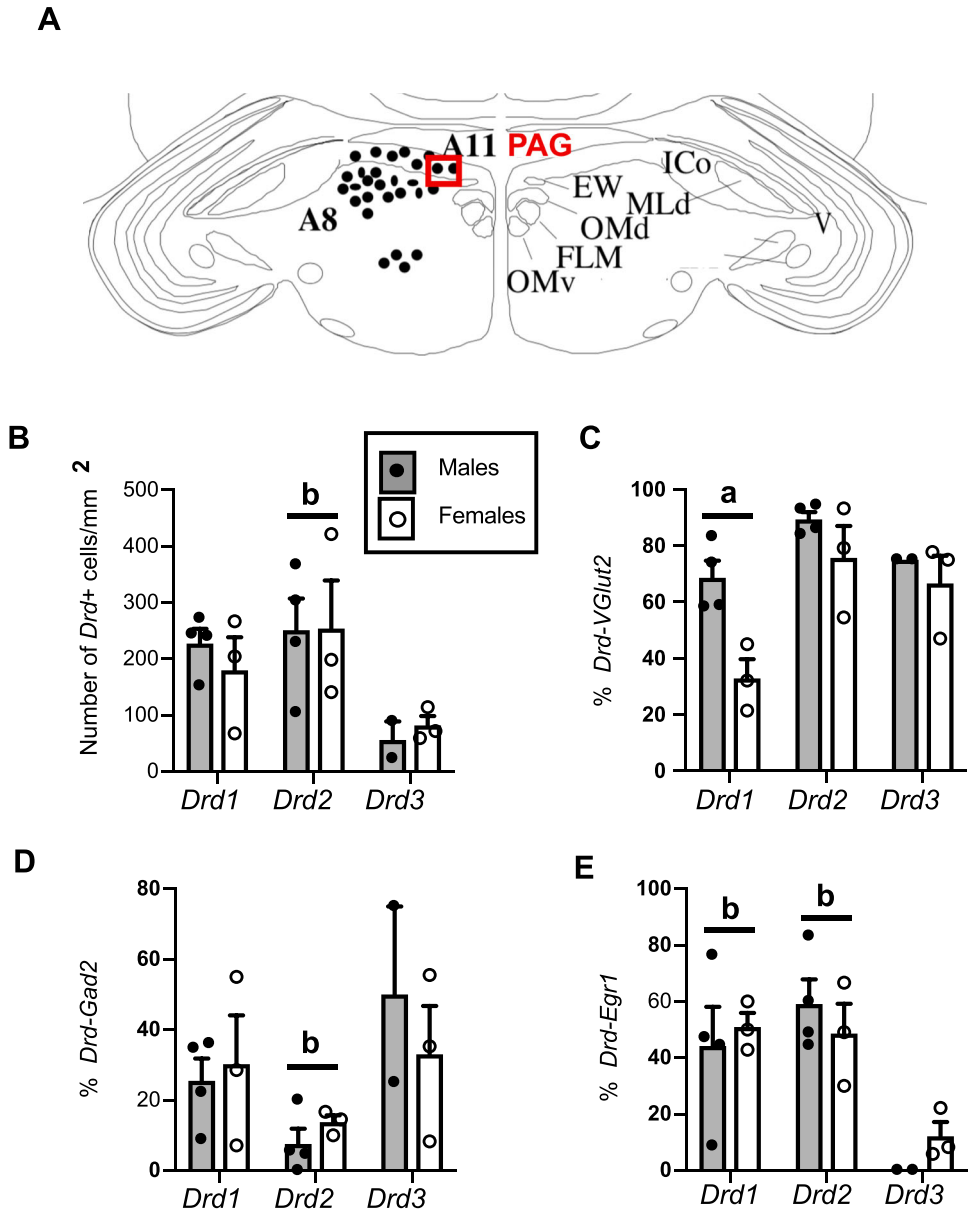

FIGURE 7 | Relative density of dopaminoceptive cells in the medial PAG and their colocalization with the neurotransmitter markers VGlut2 and Gad2 and Egr1. (A) Schematic drawing of the ventral part of the brain at the level of the PAG illustrating the distribution of tyrosine hydroxylase positive cells (black dots) and localization of the area where quantification of dopamine receptors was performed (red square). (B) Number of cells that expressed the mRNA of each of the dopamine receptors. (B–D) Percentage of Drd+ cells that expressed fluorescent label for Vglut2, a marker for excitatory neurons (C), or for Gad2, a marker of inhibitory neurons (D). (E) Percentage of Drd+ cells that expressed Egr1. All bar plots represent means + *SEM* of all data points that are also indicated separately. *a*, *b* = $p < 0.05$ versus Drd2 or Drd3, respectively (collectively for both sexes). A8–A11, dopaminergic cell groups (A11 corresponds to PAG); EW: nucleus of Edinger–Westphal; FLM: fasciculus longitudinalis medialis; ICO: nucleus intercollicularis; MLD: nucleus mesencephalicus medialis, pars dorsalis; OMd-v: oculomotor nucleus dorsal–ventral; PAG: periaqueductal gray; V: ventricle.

type (Figure 7B). However, there was a difference related to the receptor type. *Drd3* receptor density was significantly lower than that observed for *Drd2* ($p = 0.015$ by Tukey post hoc), and a similar pattern, although not statistically significant, was observed for the difference between *Drd3* and *Drd1* ($p = 0.076$).

The colocalization of these dopaminoceptive cells with *Vglut2* appeared to be influenced by receptor and sex (Figure 7C). Overall, more dopaminoceptive cells colocalized with *VGlut2* in males as compared to females, and there was also an overall significant difference between receptor types. Tukey post hoc tests indicated a significant difference in the amount of *Drd1* versus *Drd2* ($p = 0.002$) and a trend for a difference in the amount of *Drd1* versus *Drd3* ($p = 0.052$) but no difference in the amount of *Drd2*–*Drd3* ($p = 0.309$). There was, however, no significant interaction between these two factors.

Degree of colocalization with *Gad2* was specific to receptor type, but similar in males and females with no interaction between these two factors (Figure 7D). Post hoc tests identified

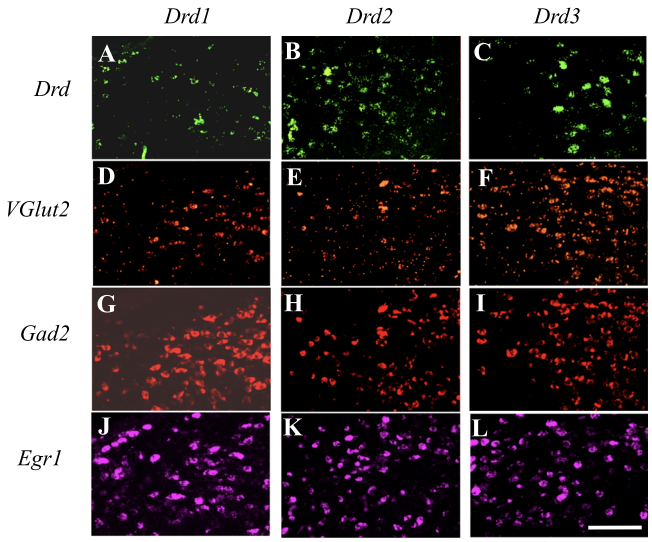

FIGURE 8 | Photomicrographs illustrating the densities of Drd1, Drd2, VGlut 2, GAD2, and Egr1 in the medial part of PAG. All photomicrographs come from female sections. The 12 panels illustrate the localization of *Drd1* (A, D, G, J), *Drd2* (B, E, H, K), and *Drd3* (C, F, I, L) in green with, respectively, *VGlut2* (D–F) in orange, *GAD2* (G–I) in red, and *Egr1* (J–L) in pink. Magnification bar = 100 μm.

a significant difference in the colocalization of *Drd2* versus *Drd3* ($p = 0.033$) but not between the two other pairs of receptor types (*Drd1–Drd2*: $p = 0.221$, *Drd1–Drd3*: $p = 0.436$).

The dopamine receptor-positive cells were also differentially activated during singing with a substantial activation being observed in *Drd1* and *Drd2*-positive cells but not in the *Drd3* cells (Figure 7E). There was thus a significant difference related to the receptor type but no sex difference and no interaction. As is apparent upon inspection of the figure, the post hoc Tukey tests indicated significant differences in the amount of activation of *Drd1*+ cells versus *Drd3*+ cells ($p = 0.005$) and *Drd2* and *Drd3* ($p = 0.002$) but not between *Drd1* and *Drd2*.

3.4 | Comparisons of Brain Areas

Given that no significant sex difference and no significant interactions between sex and receptor type were detected in all previously reported analyses, with the exception of a sex difference in colocalization of *Drd* with *VGlut2* in the medial PAG (see Table 2), it became possible in a final analysis to compare all results between the three brain areas after pooling the data from males and females, summarized in Figure 9. Because *Drd3* was not detected in Area X, an overall analysis of these results could not be performed by two-way ANOVA; instead, multiple *t*-tests were performed for each receptor type to compare the three brain areas (HVC vs. Area X, HVC vs. PAG, and Area X vs. PAG) and a Benjamini–Hochberg correction applied to the resulting probabilities.

As is obvious from the figure and confirmed statistically, the density of the three dopamine receptor subtypes was lower in the medial PAG compared to the two other areas. The colocalization of these receptors with *VGlut2* was much lower in Area X than in the two other brain regions, whereas the colocalization with *Gad2* was in general more frequent in the PAG (with the exception of *Drd2* vs. HVC or Area X). Finally, cells in medial PAG exhibited a much lower pattern of gene activation than the other two areas, as revealed by *Egr1* colocalization. Note that the significant sex difference affecting the colocalization of *Drd* with *VGlut2* in the PAG (males > females) does not impact the present conclusions because the magnitude of the sex difference is much smaller than the differences between brain nuclei.

4 | Discussion

In this study, we quantified the mRNA expression of three dopamine receptors (Drd1, Drd2, and Drd3) in three brain nuclei in canaries: HVC, Area X, and medial PAG. We determined the excitatory or inhibitory nature of the cells expressing these receptors as well as their activation following a period of song production. Major differences between brain nuclei and between receptor types were identified but, somewhat surprisingly, very few sex differences were detected. The three receptor types were present in a substantial number of cells in all three nuclei, with the exception of Drd3, which was not detected in Area X. These data confirm the overall distribution of dopaminergic receptors that had been previously observed in the HVC and Area X of zebra finches (Kubikova, Wada, and Jarvis 2010). Overall, the density of dopaminoceptive cells was much lower in PAG than in the two song control nuclei. In HVC, the majority of cells were VGlut2-positive for the three dopamine receptor subtypes, but this was not the case in Area X, whereas PAG was intermediate. In contrast, the number of inhibitory cells expressing dopamine receptors was much more limited. Most dopaminoceptive cells in Area X did not express either marker, and therefore, their neurochemical status thus remains uncertain. Finally, it is interesting to note that cellular activation during singing behavior, as measured by the expression of Egr1, included cells expressing each of the three dopamine receptors across all three brain nuclei, with the exception of cells expressing Drd3 in the PAG. All these observations have important functional consequences that deserve more comment.

4.1 | The Lack of Sex Differences Related to Dopamine Receptors

It was interesting to note that with one exception (*VGlut2* colocalization in HVC), no sex difference was detected in a species that is known to exhibit substantial sex differences in singing behavior (Dos Santos et al. 2022; Dos Santos, Ball, et al. 2023; Dos Santos, Logue, et al. 2023; Madison et al. 2015). Indeed, in canaries, males sing abundantly during the reproductive season, and more broadly during the year in some breeds, whereas females sing only very rarely (Ko et al. 2020). It is of course possible that our failure to detect sex differences was caused by the limited power of the present studies (six males and six females compared for HVC, three and three for Area X). A careful inspection of the individual data points in all bar graphs suggests, however, that there is no trend toward such differences. Male and female data are completely interspersed, and this is confirmed by the lack of statistical significance for the main effect of sex in all two-way ANOVAs conducted (all $p > 0.2$; see Table 2).

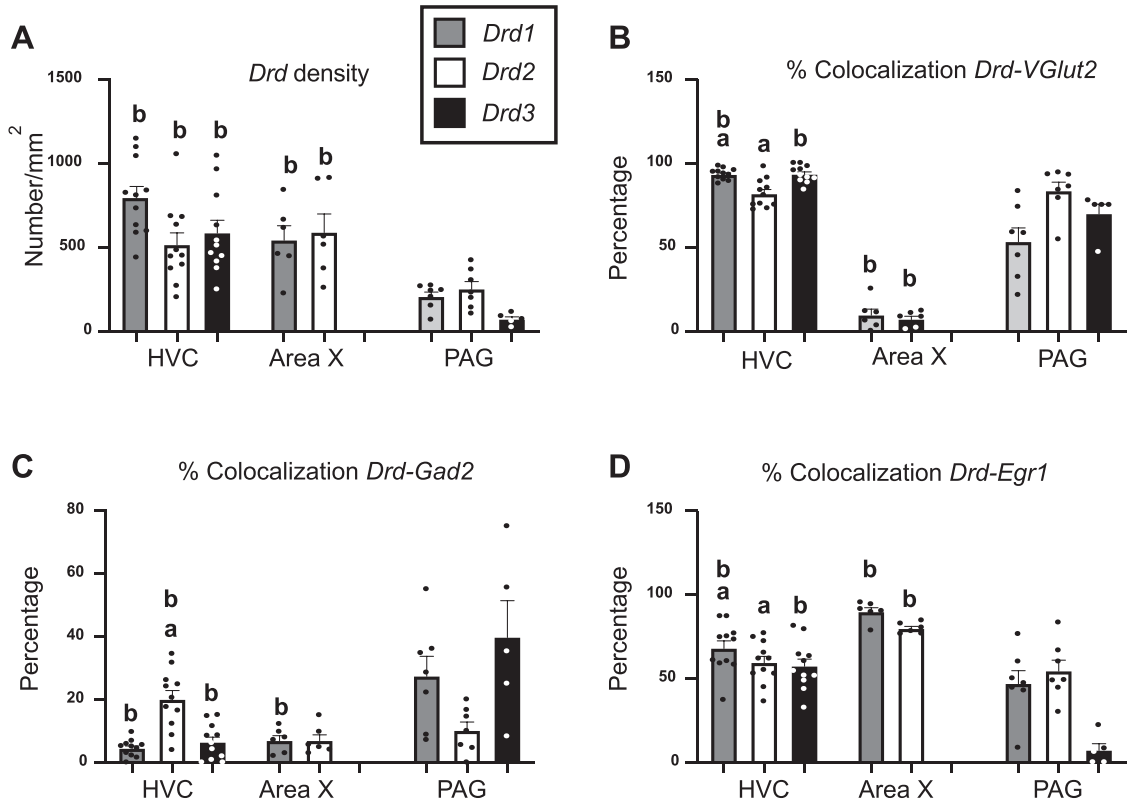

FIGURE 9 | Comparison of the density of dopamine receptors (A) and colocalization with VGlut2 (B), Gad2 (C), or Egr1 (D) across the three investigated brain regions. $a = p < 0.05$ versus Area X; $b = p < 0.05$ versus PAG by Benjamini–Hochberg corrected multiple *t*-tests. PAG, periaqueductal gray.

A possible explanation for the lack of sex differences relates to the fact that all females in the present study were treated with testosterone and were thus singing at high rates. For the largest sample of birds in which HVC was analyzed (n = 6 males + 6 females), analysis of the recordings made before brain collections indicated that the number of songs produced by the two sexes did not differ (males: 80 ± 15, females: 52 ± 16; $t_{10} = 1.26$, $p = 0.235$) nor did the total duration of singing in seconds (males: 344 ± 61, females: 288 ± 61; $t_{10} = 0.544$, $p = 0.599$). In line with this, there are a number of published studies indicating that treatment with testosterone induces substantial singing activity in female canaries (Dos Santos et al. 2022; Hartog et al. 2009; Madison et al. 2015; Nottebohm 1980). However, even after treatment with testosterone, singing activity is not the same in males and females. The sex differences that persist in these conditions vary as a function of the study considered and the breed of canaries analyzed, but they involve the singing rate, the song power, the size of the song repertoire, or the capacity to produce rapid repetitions of short song element (=trills), among other differences (Dos Santos et al. 2022; Dos Santos, Ball, et al. 2023; Dos Santos, Logue, et al. 2023; Madison et al. 2015). It is also interesting to note that under similar endocrine conditions, the volume of song control nuclei (HVC, RA, and Area X) remains larger in males than in females (Dos Santos et al. 2022; Madison et al. 2015). This is also true for the mass of the syrinx and the size of its muscle fibers (Dos Santos et al. 2022; Dos Santos, Logue, et al. 2023). Moreover, the genes expressed in HVC by birds in similar endocrine conditions also remain different (Ko et al. 2021). Although dopamine action in HVC and Area X is clearly implicated in the control of singing activity and appears to do so in males as well as in females, after treatment with exogenous T, the present data therefore suggest that dopamine action in these key forebrain song nuclei relates to total song production but not to the sex differences in song quality and the potentially associated morphological differences.

4.2 | Excitatory and Inhibitory Characteristics of DR-Positive Cells in HVC

The majority of cells (> 93% for Drd1 and Drd3, 81% for Drd2) expressing any of the three DA receptor subtypes in HVC were positive for VGlut2. This is in good agreement with the results of a recent single-cell RNA sequencing study demonstrating that many cells in the zebra finch HVC are glutamatergic (Colquitt et al. 2021). Neurons in HVC send glutamatergic projections to both RA and medium spiny neurons in Area X (Ding, Perkel, and Farries 2003; Mooney and Konishi 1991; Olveczky, Andalman, and Fee 2005; Perkel 2004). We conclude that HVC projection neurons, either to RA or Area X, are the primary target of DA modulation in this region, but this awaits further confirmation.

Although the majority of cells expressing all DA receptor types were positive for the excitatory marker VGlut2, there were a greater proportion of Drd2 cells that expressed the inhibitory marker Gad2 compared to other receptor subtypes. These Gad2-positive, Drd2-expressing cells might constitute a population of inhibitory interneurons. The larger population of Drd2-positive

cells may also include a combination of excitatory projection neurons and projection neurons that co-express both receptor types. D1 and D2 receptors are co-expressed on the same cells in a variety of systems, including in the songbird Area X (Aizman et al. 2000; Ding and Perkel 2002; Kubikova, Wada, and Jarvis 2010; Lee et al. 2004). Additional studies are needed to examine this possibility and determine if these Drd2+/Gad2+ cells are interneurons. In HVC, subclasses of interneurons can be identified by the calcium-binding protein they express: parvalbumin (PV), calbindin, or calretinin (Wild et al. 2005). If these cells are part of the PV+ interneuron subtype, they may serve as another key modulator in this system, as this specific subclass of interneurons inhibits neurons that project to Area X (Mooney and Prather 2005).

The extensive co-expression of VGlut2 with Drd+ cells supports the hypothesis that most DA-receptive cells are projection neurons; however, additional questions remain open concerning the neurochemical features of these neurons. One other potential marker of these neurons would be indicators of acetylcholine action. HVC and Area X receive cholinergic inputs, as these two nuclei express muscarinic acetylcholine receptors (Ball et al. 1990; Ryan and Arnold 1981), and nicotinic cholinergic receptors are expressed in HVC as well (Watson et al. 1988). Single-cell RNA sequencing has additionally demonstrated an even greater diversity of both glutamatergic and GABAergic cells in HVC that actually co-express a wide variety of multiple markers (Colquitt et al. 2021). Future research should determine whether additional neurotransmitters and markers are produced by dopaminoceptive cells and what their downstream targets are.

4.3 | Characteristics of Area X Dopaminoceptive Cells

To make a direct comparison between dopaminoceptive cells in HVC and Area X, we also labeled Drd-positive cells for VGlut2, but the overwhelming majority of Drd-positive cells in this region did not express VGlut2, unlike in HVC. Previous findings are mixed regarding the relative expression of VGlut2 mRNA in Area X of songbirds. Images in the zebra finch brain atlas suggest a population of Area X neurons express VGlut2 mRNA ("Zebra Finch Expression Brain Atlas [ZEBrA]," Oregon Health and Science University; www.zebrafinchatlas.org). In contrast, some studies indicate that there is little VGlut2 mRNA expression in this region (Karim, Saito, and Atoji 2014). Electrophysiological parameters identified a population of spontaneously active VGlut2-positive cells (Budzillo et al. 2017). These cells are modulated by D1 receptor agonists and provide excitatory input that is tightly coupled with inhibitory input and contributes to variability in pallidal neuron firing (Budzillo et al. 2017). The population of Drd1+/VGlut2+ cells we observed in Area X may therefore correspond to this population of cells.

The majority of Drd+ cells in Area X also did not express Gad2. Although the lack of VGlut2 co-expression was somewhat expected as described above, the small proportion of Drd+ cells expressing Gad2 came as a surprise because Gad2 is densely expressed in this brain region (Luo and Perkel 1999b; Pinaud and Mello 2007). In contrast to the excitatory glutamatergic projections that HVC sends to other nuclei in the song control system, one of the primary projections of Area X is an inhibitory GABAergic projection to the dorsolateral nucleus of the anterior thalamus (DLM) (Luo and Perkel 1999a, 1999b). These results suggest that, in contrast to what is observed in HVC, DA action in Area X likely affects network activity within the nucleus rather than directly modulating projections to downstream nuclei.

Subpopulations of interneurons in Area X, including GABAergic PV+ interneurons, interneurons expressing PV and the neurotensin-related hexapeptide LANT6, and cholinergic interneurons, have been identified (Rochefort et al. 2007). Additional research analyzing the expression of these markers would provide insight into which specific subpopulations are modulated by DA and, consequently, elucidate how these cells modulate Area X activity. Much of the previous research on dopamine receptor distribution and action in Area X has focused on Drd1 receptors, and more work is needed to determine which cell populations also express Drd2. There is a significant proportion (> 50%) of Area X cells that simultaneously express both D1 and D2 receptors, but Area X also contains some cells that express only one of these two receptors (Kubikova, Wada, and Jarvis 2010).

4.4 | Immediate Early Gene Expression in Dopaminoceptive Cells

We previously demonstrated that a proportion of dopaminergic (tyrosine hydroxylase-positive) neurons from the PAG project to HVC, RA, and Area X, suggesting dopaminergic modulation of singing behavior in canaries (Appeltants et al. 2000; Appeltants, Ball, and Balthazart 2002; Lewis et al. 1981). Inactivation of the PAG by injection of the $GABA_A$ agonist, muscimol, increases the latency to sing in male canaries (Haakenson, Balthazart, and Ball 2020). Accordingly, in zebra finches, dopaminergic inputs to HVC originating in PAG play a key role in the control of female-directed singing (Ben-Tov, Duarte, and Mooney 2023). It was therefore of interest to observe here that a majority of cells expressing dopamine receptors in HVC and in Area X also expressed *Egr1* and were therefore presumably active during song. Unexpectedly, in HVC, there was no significant difference in the percentage of cells expressing the different DA receptor subtypes (*Drd1*, *Drd2*, and *Drd3*) that co-expressed *Egr1*. This is surprising, as activation of cells expressing D1 receptors in mammalian striatum results in increased *Egr1* expression, whereas the activation of cells expressing D2 receptor leads to a reduction in *Egr1* (Gerfen 2000, Gerfen, Keefe, and Gauda 1995). There are also differences in binding affinity of these receptor subtypes for dopamine, suggesting differences in activity between these populations (Richfield, Young, and Penney 1989). One explanation of this similarity in the activation of cells expressing these different receptor subtypes is that the receptor subtypes may be co-expressed in the same cells.

In contrast, in Area X, Egr1 expression was significantly more frequent in Drd1- than Drd2-positive cells. This finding is in line with previous research indicating that in Area X, Egr1 is more frequently expressed in cells expressing D1 than in cells expressing D2 receptors during undirected song (Kubikova, Wada, and Jarvis 2010). We also found that the percentage of DA-receptive cells active during song was higher in Area X than in

HVC. This may be partially explained by an overall difference in neuronal activity between these two regions, as song-driven Egr1 induction is greater in Area X than in HVC (Jarvis and Nottebohm 1997). However, differences in DA innervation of these regions may also contribute to this effect. Both HVC and Area X receive dopaminergic input from PAG, but this is the primary source of DA in HVC, whereas Area X also receives substantial DA input from VTA (Appeltants et al. 2000; Lewis et al. 1981; Soha, Shimizu, and Doupe 1996). Differences in DA input and consequent modulation of neuronal excitability may also result in differential Egr1 induction.

Finally, the expression of *Egr1* in the medial PAG is consistent with the role of the projections of this brain area to song control nuclei and control of singing behavior. This cellular activation was globally lower than in HVC and Area X and interestingly did not seem to involve cells expressing *Drd3*. This receptor has been implicated in the rate of neuronal recovery in Area X following a lesion, but its participation in the direct control of song remains to be established (Lukacova et al. 2016).

One potential limitation of these data is that *Egr1* expression was here considered to relate to song activation due to its anatomical localization and to the fact that birds had been singing actively during the period preceding brain collection. Multiple studies have shown that immediate early gene expression in HVC for example is motor driven based on song production (Jarvis and Nottebohm 1997; Kimpo and Doupe 1997). It is clear, however, that other factors might have contributed to *Egr1* expression. Additional controls such as measures of *Egr1* expression in relation to movement or to various sensory inputs should be performed to firmly confirm the relationship with singing. This being said, these additional controls would not invalidate the conclusion that activation of dopaminoceptive cells was not different in males and females.

In conclusion, the present study identifies important new evidence about the neurochemical phenotype and organization of dopaminoceptive cells in key areas related to vocal behavior in the canary brain. This study provides a critical neurochemical characterization that could now guide pharmacological investigations of the role of dopamine receptors subtypes in the activation of singing behavior.

Author Contributions

Chelsea M. Haakenson: conceptualization, investigation, data curation, formal analysis, visualization, writing–original draft preparation, funding acquisition. **Jacques Balthazart**: formal analysis, visualization, writing–original draft preparation. **Jonathan W. VanRyzin**: methodology, investigation, writing–review and editing. **Ashley E. Marquardt**: methodology, writing–review and editing. **Sydney E. Ashton**: methodology, writing–review and editing. **Margaret M. McCarthy**: resources, supervision, funding acquisition, writing–review and editing. **Gregory F. Ball**: conceptualization, supervision, writing–original draft preparation, funding acquisition.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Peer Review

The peer review history for this article is available at https://publons.com/publon/10.1002/cne.25675.

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