Social context-dependent singing alters molecular markers of dopaminergic and glutamatergic signaling in finch basal ganglia Area X

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ABSTRACT

Dopamine (DA) is an important neuromodulator of motor control across species. In zebra finches, DA levels vary in song nucleus Area X depending upon social context. DA levels are high and song output is less variable when a male finch sings to a female (female directed, FD) compared to when he is singing by himself (undirected, UD). DA modulates glutamatergic input onto cortico-striatal synapses in Area X via N-methyl-D-aspartate (NMDA) and DA receptor mechanisms, but the relationship to UD vs. FD song output is unclear. Here, we investigate the expression of molecular markers of dopaminergic and glutamatergic synaptic transmission (tyrosine hydroxylase – TH, alpha-synuclein – α-syn) and plasticity (NMDA 2B receptor – GRIN2B) following singing (UD vs. FD) and non-singing states to understand the molecular mechanisms driving differences in song output. We identified relationships between protein levels for these biomarkers in Area X based on singing state and the amount of song, measured as the number of motifs and time spent singing. UD song amount drove increases in TH, α-syn, and NMDA 2B receptor protein levels. By contrast, the amount of FD song did not alter TH and NMDA 2B receptor expression. Levels of α-syn showed differential expression patterns based on UD vs. FD song, consistent with its role in modulating synaptic transmission. We propose a molecular pathway model to explain how social context and amount of song are important drivers of molecular changes required for synaptic transmission and plasticity.

1. Introduction

Dopamine (DA) is an important neuromodulator of motor control, motivation and reward-based behaviors across mammalian and avian species. Insight into the role of DA modulation in neural mechanisms for human vocal motor control has been obtained from studying songbirds [1].

In male zebra finches (Taenopygia guttata) [2] and European starlings (Sturnus vulgaris) [3], levels of DA in the brain vary depending upon the social context in which the bird sings. When the male zebra finch sings to a female, known as female-directed (FD) song, DA levels measured via high-performance liquid chromatography (HPLC) are higher in vocal control region Area X compared to when the male is practicing his song alone, known as undirected (UD) song [2]. Neurons found in Area X, a song-dedicated nucleus in the finch basal ganglia, receive dopaminergic input from the substantia nigra (SN) and ventral tegmental area (VTA) as in mammals (Fig. 1) [4,5]. Pharmacological approaches in adult zebra finches have shown that reduction of pre-synaptic DA input or antagonism of post-synaptic DA receptors in Area X can abolish social context-dependent song differences. Injection of the neurotoxin 6-hydroxydopamine (6-OHDA) into Area X depletes DA nerve terminals and results in UD song resembling the more stereotyped FD song [6]. The application of a D1 receptor antagonist into Area X causes FD song to show more variable pitch changes similar to that of UD song by altering neuronal firing patterns [7,8]. In a related species, Bengalese finch (Lonchura striata domestica), treatment with 6-OHDA in Area X interferes with pitch changes as part of reinforcement-driven vocal plasticity during UD song [9]. The molecular mechanisms through which DA modulates these aspects of UD vs. FD adult song behavior are not well identified.

Based on in vitro slice electrophysiology experiments, activation of D1-like receptors reduced NMDA and non-NMDA receptor-mediated excitatory post-synaptic currents in Area X medium spiny neurons (MSNs) [10]. DA application can also directly increase pallidal (PAL) neuron firing, driving thalamic inhibition and result in a less variable song output associated with FD song [7]. How changes in the amount of...
DA available modify synaptic transmission and plasticity mechanisms at these cortico-striatal synapses in Area X to support UD vs. FD song output requires investigation. One strategy is to identify molecular targets of DA modulation that are differentially expressed based on social context-dependent singing.

Social context has an impact on mRNA and protein expression levels in song nuclei, including Area X. ZENK, an immediate early gene, shows increased mRNA and protein levels in Area X following 30–45 min of UD but not FD song [11,12]. FoxP2, a speech-related gene and transcription factor, shows decreased mRNA expression in Area X following two hours of UD but not FD song, but at the protein level, singing in both contexts drive protein levels down in comparison to non-singers [13,14]. Intriguingly, FoxP2 mRNA levels significantly decrease with higher numbers of UD song motifs over a two-hour period [15, Fig. S5]. By contrast, there is a weak association between FoxP2 mRNA and amount of FD song [13, Fig. 4], but this relationship is absent at the protein level [14, Fig. 5]. Social context and the amount of song are therefore two determinants that can affect FoxP2 expression levels. Both ZENK and FoxP2 are examples of social context-dependent differences in gene expression but the underlying molecular circuitry is not well identified.

We propose that molecular mechanisms of synaptic transmission and plasticity mediate social context-dependent song output associated with differences in DA levels in Area X (e.g. low in UD; high in FD). As a starting point, we have identified three potentially important molecular markers in Area X. We selected two related pre-synaptic molecular markers (tyrosine hydroxylase – TH, alpha-synuclein – α-syn) and one post-synaptic marker (NMDA 2B receptor – GRIN2B) based on prior work implicating them in synaptic transmission and plasticity mechanisms [15].

TH is an enzyme required for DA biosynthesis. TH is found in terminals of dopaminergic neurons that project from the SN/VTA to Area X [16,17]. TH is commonly used as a marker to detect changes in DA signal in finch Area X and rat basal ganglia that are correlated to altered vocalizations including parkinsonian-like output [3,6,18,19]. As a biomarker of DA synthesis, we hypothesize that total TH protein levels in Area X will mimic the low vs. high levels of DA present during UD vs. FD singing [2] but that TH will also be influenced by how much the bird sings.

In mammalian models, the pre-synaptic protein α-syn normally regulates TH enzymatic activity in dopaminergic terminals, synaptic vesicle function and DA release into the synaptic cleft, and helps traffic DA active transporter to the membrane surface [20]. In rodents, virally-driven overexpression of α-syn leads to reductions in DA release due to dopaminergic terminal loss in the basal ganglia as well as changes in social context-dependent singing. One strategy is to identify molecular targets of DA modulation that are differentially expressed based on social context-dependent singing.

2. Materials and methods

2.1. Animal model and experimental design

All animal use was approved by the Institutional Animal Care and Use Committee at the University of Arizona. Non-breeding adult male
zebra finches (n = 42) between 120 and 300 days post-hatch were used in this study. Male finches were moved to individual sound attenuation chambers and acclimated for three days under a 13.5:10.5 h light:dark cycle during which UD song was continuously recorded using Song Analysis Pro [25]. After acclimation, birds were collected for one of four behavioral groups starting at lights-on in the morning (7:30 am) and following the time points established by Miller et al. [14]. Two groups of non-singers were collected at lights-on (0 HR NS) or two hours after lights-on (2 HR NS). For the 2 HR NS condition, an experimenter observed the bird and if the bird attempted to sing, which happened infrequently, they tapped on the cage to prevent the bird from singing. Our prior publication showed that neither cage tapping nor the presence of the investigator significantly alters serum corticosterone levels between NS, undirected (UD) or female-directed (FD) behavioral groups [14]. Birds collected as 2 HR NS occasionally completed motifs on an average of 4.2 ± 2.3 motifs within the two-hour period. The two singing groups sang either two hours of undirected (2 HR UD) or female directed (2 HR FD) song. We excluded birds that sang less than 90 motifs in a two-hour period because previous work showed this was the minimum number of motifs needed to elicit changes in protein expression [14]. Any bird that was quiet in the last 30 min of the two-hour singing period was excluded from collection given that 30 min of quiet after singing can reduce ZENK expression back to baseline levels [11]. FD song was elicited by the presentation of female zebra finches over the two-hour period [14]. For 2 HR FD birds, live video streaming and investigator observations were used to ensure that the bird sang only FD and not UD song. At the completion of the non-singing or singing assignments, finches were euthanized using an overdose with isoflurane in order to collect the brain tissue.

2.2. Song analysis

The two hours of singing prior to collection was used for song analysis. Motifs were identified as repeated sequences of syllables separated by silent periods. The numbers of motifs were manually counted throughout the two-hour span. Time spent singing was calculated by averaging the motif length of 20 randomly selected motifs within the two-hour period for each bird and then multiplying by the total number of motifs sung in the two hours. This random sampling approach has been successfully employed in previous publications as being representative of the total time spent singing [14,15]. To confirm the validity of this random sampling approach in the current study, we compared data obtained using this method to a ‘summed method’ in the same bird where the length of every motif was measured and added together over the two-hour recording period to obtain time spent singing. No statistically significant difference was detected between the two methods when comparing n = 3 birds per UD and FD groups (paired t-test, p = 0.215).

2.3. Antibody characterisation

Tyrosine Hydroxylase (TH)-This antibody for TH (Millipore, #AB152, RRID: AB_390204, Table 1) was used to detect dopaminergic positive terminals in Area X and has been previously validated by Miller et al. [6], detecting a protein band ~55 kD in finches.

Alpha-synuclein (α-syn)-This antibody (Proteintech, #10842-1-AP, RRID: AB_2192672, Table 1) was chosen due to 86% homology to zebra finch α-syn (~16 kD, NCBI accession number NP_001041718). To validate this antibody, a preadsorption control was performed with the original antigen via Western blot (Fig. 2A).

Glutamate ionotropic receptor NMDA subunit 2B (GRIN2B, NR2B)-This antibody was chosen due to 95% homology to zebra finch GRIN2B (~180 kD, NCBI accession number XP_002195885). Because the peptide was not available to do a preadsorption control, we validated a commercially available GRIN2B primary antibody made against the N-terminus of the mouse peptide (Millipore, #06-600, RRID: AB_310193, Table 1) and compared it to another commercially available antibody made against the C-terminus of human GRIN2B (Proteintech, #21920-1-AP, RRID: AB_11232223, Table 1) in Western blots to detect protein bands at similar molecular weights (Fig. 2B). Mouse basal ganglia (MBG) and finch nidopallium (NP, non-basal ganglia region) were used as positive controls alongside Area X. Both antibodies detected protein bands at the predicted molecular weight. For all subsequent blots, Millipore’s GRIN2B antibody was used to detect protein levels.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-The antibody for GAPDH (Millipore, #MAB374, RRID: RB_2107445, Table 1) was used to normalize for equal protein loading across the lanes and was previously validated by Miller et al. [14] as detecting a protein band of ~35 kD in finch Area X. Over the course of the study, we detected a weakening of the antibody signal despite testing different lots. Therefore, we also used an antibody to GAPDH from Proteintech (Proteintech, #10494-1-AP, RRID: AB2263076, Table 1) that also detects a protein band at the same molecular weight in Area X and yielded the same results.

2.4. Brain sample preparation, western blotting, and quantification

Directly following overdose with isoflurane, brains were dissected out and flash frozen with liquid nitrogen. Brains were cryosectioned until Area X was visualized and bilateral tissue biopsies of Area X were obtained by punching 1 mm deep using a syringe attached to an intramedic luer-stub 20-gauge adapter (catalog #427564, BD Medical Technology). Post-hoc Nissl staining of 40 μm coronal brain sections was performed to validate accuracy of Area X targeting [14]. Tissue biopsies obtained from Area X were homogenized and isolated for total protein using modified RIPA lysis buffer, and concentrations were determined on a Bio-Rad DC RC assay as in Miller et al. [14]. 15 μg of protein lysate was run at 100 V using a 10% polyacrylamide-SDS gel then transferred to 0.2μm PVDF membranes for the Western blotting procedure as in Miller et al. [14]. Primary antibodies were incubated on separate portions of the blot to prevent cross-reactivity (refer to Fig. 2C). Primary antibodies incubated overnight at 4 °C were TH (Millipore, #AB152, 1:5000, RRID: AB_390204), α-syn (Proteintech, #10842-1-AP, 1:100-1:250, RRID: AB_2192672), GRIN2B (Millipore, #06-600, 1:100-1:500, RRID: AB_310193), and GAPDH (Millipore, #MAB374, 1:5000, RRID: AB2192672), Proteintech, #10494-1-AP, 1:250, RRID: AB2263076) [14,15]. After TBST washes, the blots were incubated at room temperature for two hours in secondary HRP-conjugated rabbit antibody (GE Healthcare-Amersham, #NA934, RRID: AB_772206, Table 1) at concentrations 1:2000 (TH, GRIN2B, GAPDH) and 1:1000 (α-syn), and secondary HRP-conjugated mouse antibody (GE Healthcare-Amersham, #NA931, RRID: AB_772210, Table 1) at concentration 1:1000 (GAPDH). Blots were developed using chemiluminescence and imaged using a Bio-Rad system. Quantifications were performed on Quantity One (Bio-Rad) by an experimenter blind to the behavioral states. A rectangular band was drawn surrounding the signal of interest (raw value) and the same-sized band was placed just above or below the band in the same lane to obtain the “background”. The corrected value was calculated by subtracting the background from the raw value. Corrected values were obtained for TH, α-syn, GRIN2B, and GAPDH. Corrected values for TH, α-syn, and GRIN2B were divided by the corrected GAPDH value in the corresponding lane to get normalized values to control for equal protein loading. GAPDH was used as the normalization control because it is not behaviorally regulated and there was no difference in expression of GAPDH between behavioral groups (Kruskal-Wallis, p = 0.34), replicating previous findings [14,15]. The protein signals of interest/GAPDH values were then divided by the average of the two lanes of the control condition, 0 HR NS, within the
same blot for normalization (set = 1). The 0 HR NS birds are not experimentally manipulated and provide a good control without any singing. This normalization step enables inter-blot comparisons for a total of 5 separate blots. Each western blot consisted of two different birds per behavioral state. For protein levels from Western blots, there were 10 birds per behavioral group for TH and α-syn and eight birds per behavioral group for GRIN2B (Fig. 3). For song analysis, the total numbers of birds are as follows: TH and α-syn (n=10 for number of UD/FD motifs, n=9 for time spent singing UD, n=8 for time spent singing FD); GRIN2B (n=8 for number of UD/FD motifs and time spent singing UD, n=7 for time spent singing FD). There were fewer birds in the time spent singing analysis group due to experimenter error (missing song files).

2.5. Statistical analysis

For protein level comparison between the four behavioral groups, the means of each group were compared via the Kruskal-Wallis test (VassarStats, vassarstats.net) because the data did not fit a normal distribution. No datapoint were deemed outliers in the graphical plots using exclusion criteria based on technical issues (e.g. Western blot running conditions or imaging issues) [26]. Therefore, all data points were determined to be experimentally valid data points and were not excluded from analysis.

Regression analyses using the OriginLab graphing program was used to determine if TH, α-syn, and GRIN2B protein levels varied as a function of the behavioral state.

### Table 1

Primary and secondary antibodies used in the study and relevant information are listed in the table.

<table>
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<tr>
<th>Antibody</th>
<th>Reference</th>
<th>RRID</th>
<th>Antigen</th>
<th>Concentrations</th>
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<td><strong>Primary antibody</strong></td>
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<tr>
<td>Anti-tyrosine hydroxylase antibody (rabbit polyclonal)</td>
<td>AB152 (Millipore)</td>
<td>AB_390204</td>
<td>Denatured tyrosine hydroxylase from rat pheochromocytoma (denatured by sodium dodecyl sulfate)</td>
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<tr>
<td>Anti-NR2B (GRIN2B) antibody (rabbit polyclonal)</td>
<td>06-600 (Millipore)</td>
<td>AB_310193</td>
<td>a.a. 1437-1456 of mature mouse NR2B or 1463-1482 of NR2B precursor; signal is a.a. residues 1-26 [KFNGSSNGHVYEKLSSIEDV]. This sequence is identical to a.a. 1437-1456 of mature rat NR2B and a.a. 1465-1484 of human NR3, containing a N-terminal lysine</td>
<td>1:100-1:500</td>
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<td>GRIN2B (NR2B) antibody (rabbit polyclonal)</td>
<td>21920-1-AP (Proteintech)</td>
<td>AB_11232223</td>
<td>human GRIN2B-GST fusion protein YLDQFRKTKENGPHVHVLDTJYKERSDD-FRRD5VSGGPPCTNRSHEIKHTGDHKVG-YSGVPAWPKENLTIVNWEVD8SGNGFCRSC-CP65LHJN5TVVYTGQNSGRQAICREACK-KAGNLYDIESEDSNQELDQPAAPVAVTNS-ASTTGYQSPNTPSKAQNKRNRLRPPQHY-DTFPDLQKEAAALAPR1SVLDKDGRFMHDG-SPVNHFBMSAGESTFAANNKSVPTAGH-HHHNPQG5GYMLSKSYBDFYOTQPSF-TPGDQDIPLLHSKSSYPRPOTVAGASKAR-PDFRALTVNPV5ALHVGAPVARQKDCIC-IGNQSNCPVPNKNPRAFNSSNGHVYE-KLSSIEDV (1133-1484 aa encoded by BC113620)</td>
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<tr>
<td>Alpha-synuclein antibody (rabbit polyclonal)</td>
<td>10842-1-AP (Proteintech)</td>
<td>AB_2192672</td>
<td>human SNCA-GST fusion protein MDVFMGLGSLKAKEGVEVAAEGTKQGV-AEGKRDKEVGYVGSRTKEVGVATYA-ETKEmQGTGAVTTGATVAGKTVTTEG-AGSIAATGKVKEQDLGKNERGAPCITGL-EDMPVPDPNEAYMEPSEGQYDYEPEA (1-140 aa encoded by BC013293)</td>
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<td>GAPDH antibody (rabbit polyclonal)</td>
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<td>AB_2263076</td>
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<td>Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody, clone 6C5 (mouse, monoclonal)</td>
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<td>AB_772210</td>
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function of the number of motifs or time spent singing with R² values reported as in previous publications [11,13,14]. Scatterplots were made in the OriginLab graphing program, and the optimal fit (either linear or curvilinear) is shown for each dataset [26,27]. R² values for both linear and curvilinear fit were obtained from Origin and confirmed by IBM SPSS Statistical software package in consultation with our university statistician Mark Borgstrom. Each graph was determined to be linear or curvilinear based on the significant F change value determined by IBM SPSS Statistical software package. If the significant F change value was p < 0.05 for either the linear or curvilinear model, the correlating model was used. If neither model had a significant F change value of p < 0.05, the linear model was used. Statistics for the linear and curvilinear (quadratic) plots were done using IBM SPSS Statistical software package where x = number of motifs/time spent singing and y = protein levels. The ANOVA F and p-values are reported based on x² (x-squared) for the plots.

3. Results

3.1. Antibody validation

The polyclonal antibody against α-syn protein detected bands of similar molecular weights across finch Area X, nidopallium (NP), and mouse basal ganglia (MBG) (Fig. 2A). Incubation of the α-syn antibody with 30 times excess of its peptide showed that the bands disappeared, confirming specificity of the antibody to α-syn. A Western blot with GRIN2B protein detected at ~180 kDa in Area X, NP, and MBG. 15 μg of protein loaded per lane. *Preadsorption control: incubation of the α-syn antibody with 30 times excess of its peptide showed that the bands disappeared, confirming specificity of the antibody to α-syn.

3.2. Gene expression of non-singers vs. singers in Area X

In this study, we examined the total protein expression of TH, α-syn, and GRIN2B via Western blots from tissue punches of Area X following four behavioral states: 0 HR NS, 2 HR NS, 2 HR UD, and 2 HR FD (Fig. 2C) in which the 0 HR NS was used as the control condition given the lack of behavioral manipulation [6,14]. There were no significant differences between groups for TH, α-syn, and GRIN2B (Fig. 3, see legend for statistics). Like Miller et al. [14], we pursued the sources of the range in protein values observed within groups for the singers.

3.3. Rise in TH levels in Area X coincides with UD song amount but has no relationship to FD song

The number of motifs and time spent singing were plotted against TH protein levels in Area X (Fig. 4). With UD song, TH levels significantly increased with the number of motifs (Fig. 4A) and trended in the same direction with time spent singing (Fig. 4B). However, neither the number of motifs (Fig. 4C) nor time spent singing FD song (Fig. 4D) had an effect on TH levels.

3.4. Rise in α-syn levels in Area X coincides with number of UD motifs; α-syn levels follow a U-shaped curve in response to time spent singing FD song

With UD song, α-syn levels in Area X significantly increased with the number of motifs (Fig. 5A) with an exponentially stronger increase with a higher number of motifs sung. Time spent UD singing had no significant effect on α-syn levels (Fig. 5B). The α-syn levels plotted against time spent singing FD song follow a U-shaped curve where protein levels decrease in birds that spend <350 s singing, but as the bird exceeds around 350 s, α-syn levels rise (Fig. 5D). However, the number of FD motifs (Fig. 5C) had no effect on α-syn levels.
Fig. 3. Behavioral regulation of Area X TH, α-syn, and GRIN2B protein levels.

A: Bar graph of the mean TH protein levels by behavioral condition with standard error bars (n = 10 birds per group; 0 HR NS: 1 ± 0.04, 2 HR NS: 1.65 ± 0.49, 2 HR UD: 1.19 ± 0.27, 2 HR FD: 1.5 ± 0.40). Each point represents an individual bird from five different blots (n = 10/group). There was no statistically significant difference between the four groups (Kruskal-Wallis test, p = 0.76).

B: Mean α-syn protein levels by behavioral condition with standard error bars (n = 10 birds per group; 0 HR NS: 1 ± 0.06, 2 HR NS: 2.36 ± 0.76, 2 HR UD: 1.82 ± 0.65, 2 HR FD: 1.88 ± 0.53). Each point represents an individual bird from five different blots (n = 10/group). There was no statistically significant difference between the four groups (Kruskal-Wallis test, p = 0.24).

C: Mean GRIN2B protein levels by behavioral condition with standard error bars (n = 8 birds per group; 0 HR NS: 1 ± 0.03, 2 HR NS: 2.1 ± 0.92, 2 HR UD: 1.58 ± 0.29, 2 HR FD: 2.68 ± 1.31). Each point represents an individual bird from four different blots (n = 8). There was no statistically significant difference between the four groups (Kruskal-Wallis test, p = 0.45).

Fig. 4. Changes in Area X TH protein levels with UD and FD song.

TH protein levels of UD singers and FD singers plotted against the number of motifs sung within the two-hour period (A, C) or time spent singing (B, D). TH protein levels were dependent upon the number of motifs sung during UD (n = 10, ANOVA, F = 18, p = 0.002) but not FD (n = 10, ANOVA, F = 0.11, p = 0.746). Both time spent singing UD (n = 9, ANOVA, F = 3.68, p = 0.097) and FD (n = 8, ANOVA, F = 0.02, p = 0.887) did not have a significant effect on TH levels. Each point represents an individual bird. Gray lines denote curvilinear regression curve for A and linear regression curves for B, C, and D drawn as best fits to the data based on the significant F change value.
3.5. GRIN2B levels in Area X increase with the amount of UD song but not FD song

With UD song, GRIN2B levels significantly increased with the number of motifs (Fig. 6A) and time spent singing (Fig. 6B). Compared to UD song, GRIN2B levels with FD song showed a trend for decreased protein expression with increased time spent singing (Fig. 6D), but these trends were not statistically significant. The number of FD motifs (Fig. 6C) had no effect on GRIN2B levels.

4. Discussion

Here, we characterized the effects of social context and how much the bird sang on the expression of molecular markers of synaptic transmission and plasticity in zebra finch Area X. Our Western blot analyses did not show differences in the mean protein levels of TH, α-syn, and GRIN2B between social context but revealed strong differences dependent on the social context when comparing the relationship between protein levels and how much the bird sang (amount of song and time spent singing). The lack of overall mean differences in protein levels between UD vs. FD singers plotted against the number of motifs sung within the two-hour period (A, C) or time spent singing (B, D). α-syn protein levels were dependent upon the number of motifs sung during UD (n = 10, ANOVA, F = 16.81, p = 0.002) but not FD (n = 10, ANOVA, F = 0.038, p = 0.85). α-syn levels were dependent upon time spent singing FD (n = 8, ANOVA, F = 6.29, p = 0.043) but not UD (n = 9, ANOVA, F = 1.41, p = 0.274). Each point represents an individual bird. Gray lines denote curvilinear regression curves for A and D and linear regression curves for B and C drawn as best fits to the data based on the significant F change value.

4.1. Social context and amount of song

TH protein levels in Area X increased with more UD song but remained unchanged with increased FD song. Heimovics and Ritters [3] looked at FD song for a shorter time period, 30–45 min, and also did not find changes in TH levels using immunocytochemistry with how much time the non-breeding male birds sang FD [3]. In our current study, birds that sang more UD song motifs had higher TH levels. Sasaki et al. [2] found there was no relationship between DA levels and number of UD or FD song motifs. However, our data had a broader range of singers over a longer time course (2 h vs. 30 min) dedicated to one state only whereas Sasaki et al. [2] alternated 30 min of UD with FD song. Furthermore, we took a different methodological approach to Sasaki et al. [2] by measuring changes in TH, the enzyme for DA biosynthesis, using Western blotting following the song collection period vs. in vivo microdialysis.

As a pre-synaptic protein involved in vesicle-mediated release of neurotransmitter, fluctuating levels of α-syn modulate DA, glutamate, and norepinephrine release [28–30]. Given that singing amount in both social contexts drove α-syn levels, fluctuating levels of α-syn may play a modulatory role in synaptic transmission and plasticity as the bird sings.

Previously, GRIN2B mRNA levels were shown to increase with UD song and with the amount of song; however, the authors did not look at the relationship with FD song [15]. Our results show that GRIN2B protein levels increase with more UD song but tends to decrease with FD song suggesting that synaptic plasticity mechanisms may be differentially activated based on social context.

4.2. Molecular pathway model for UD song

Based on our findings in the current study, genetic and electrophysiological evidence from published work [2,7,10–12], we propose a molecular pathway model for circuit-level changes that occur over a two-hour period to drive differences in UD vs. FD song output. A model
for the UD song circuit is depicted in Fig. 7A–B highlighting the roles of Area X, DLM, and LMAN in driving more acoustic variability during UD song [31]. Acoustic variability has been defined as greater pitch changes from rendition to rendition of UD song compared to FD song [31,32]. Hilliard, Miller et al. [15] previously showed birds that sang the most number of motifs of UD song in a two-hour period had greater acoustic variability measured as the change in mean Wiener entropy. Dopaminergic and glutamatergic input converge on medium spiny neurons (MSNs), the site of cortico-striatal synaptic plasticity. After 30–45 min of UD song (black pathway, Fig. 7A), low DA levels exist in Area X [2]. MSNs inhibit pallidal (PAL) neurons so that DLM provides excitatory drive to song nucleus LMAN. LMAN drives song nucleus RA to promote more variability in pitch, etc. in the bird’s song, as part of his trial and error learning [33]. Based on findings from our study, we propose the blue molecular pathway (Fig. 7B) that becomes activated with increased UD singing. As the bird continues to sing over the two-hour period (blue pathway, Fig. 7B), α-syn facilitates increased glutamate release onto MSNs requiring up-regulation of NMDA 2B (GRIN2B) receptors. With increased NMDA 2B receptor levels, MSNs excitability and inhibition over PAL neurons increase to support more UD song rendition-to-rendition variability [15]. However, by two hours from the start of singing, TH levels begin to rise in Area X as a potential mechanism to counter too much acoustic variability, leading to increased DA synthesis.

4.3. Molecular pathway model for FD song

The traditional circuit that supports low acoustic variability in FD song (in comparison to UD song), is represented by the black colored pathway in Fig. 7C. With 30–45 min of FD song (black pathway), there are high levels of DA from SN/VTA onto Area X, which depress glutamatergic input from LMAN onto Area X MSNs (Fig. 7C) [10]. Consequently, PAL neurons are disinhibited and subsequent strong DLM inhibition leads to decreased excitability between LMAN and RA with a less variable, more stereotyped song output. Based on our findings, we propose a new molecular pathway in blue that becomes activated (Fig. 7D). As the bird sings to the female (blue pathway, Fig. 7D), high levels of DA with FD song and fluctuating α-syn levels mediate decreases in GRIN2B protein expression in MSNs, decreasing their excitability and supporting PAL neuron inhibition of DLM. In this manner, low acoustic variability from one song rendition to the next would be preserved but has not been determined. TH levels during FD song do not change in response to the number of motifs or time spent singing because of endogenously high DA levels.

4.4. Future directions

Our study identified differential protein expression patterns for three molecular markers in Area X based on the amount of singing in UD vs. FD song states. We found differences in total protein levels suggestive of modifications in dopaminergic and glutamatergic synaptic transmission in Area X by social context. To verify that MSNs are the cellular target of glutamatergic-dependent changes in synaptic plasticity in response to social context and song amount, it will be necessary to measure anatomical changes at cortico-MSN synapses. We would predict that two hours of UD song will lead to increased numbers of dendritic spines and glutamatergic synapses that persist to maintain the flexibility of the song circuitry needed for vocal motor exploration. By contrast, FD song would be associated with decreased activation of synaptic plasticity mechanisms to promote stability in the song circuitry and therefore, maintain a more stereotyped song output. A direct test of the role of GRIN2B in differential activation of synaptic plasticity mechanisms in UD vs. FD song would involve using a viral vector to overexpress/knockdown its expression [24]. Based on our model
virally-driven increases in GRIN2B expression should make FD song more variable, abolishing social context-dependent differences. The role of DA modulation of glutamatergic pathways in Area X during UD vs. FD song is not known but TH clearly plays a role. Depletion of pre-synaptic TH levels by the neurotoxin 6-OHDA results in a less variable UD song output [6]. Therefore, whether the reduced variability is driven by changes at the Area X glutamatergic synapses requires investigation. Alternatively, optogenetic activation/inhibition of the dopaminergic input of VTA neurons onto Area X can be used as a tool to drive changes in the glutamatergic plasticity circuit and evaluate disruption of UD vs. FD song output [34].

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