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.

Keywords:

Dopamine, zebra finch, songbird, glutamate, basal ganglia

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 minutes 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 vocalizations [21,22]. In zebra finches, α-syn expression is enriched at synapses in song control nuclei during critical developmental periods associated with vocal learning

68 although its role in synaptic plasticity and adult song behavior is unclear [23]. Here, we
69 **hypothesize** that α-syn contributes to DA signaling in Area X differentially depending on
70 the social context.

71 The gene *GRIN2B* encodes a NMDA 2B glutamate receptor. NMDA 2B receptors
72 initiate synaptic plasticity events in cortical and basal ganglia brain regions. In a
73 previous study, Miller and colleagues [15, Fig. S6] showed that *GRIN2B* is a hub gene
74 in a synaptic plasticity network in Area X activated by two hours of UD singing. As the
75 bird sings, *GRIN2B* mRNA expression increases in Area X and is correlated to a more
76 variable UD song. In adult finches, virally-driven overexpression of *GRIN2B* expression
77 in cortical song nucleus LMAN causes FD song to revert back to its juvenile state of
78 increased variability with more stuttering [24]. However, the effects of social context on
79 GRIN2B protein levels in Area X are unknown including the relationship between
80 GRIN2B mRNA/protein levels in Area X and FD song.

81 Using these molecular markers of synaptic transmission (TH, α-syn) and
82 plasticity (GRIN2B), we first determined whether protein levels were regulated by
83 activity-dependent mechanisms (singing vs. not singing). Given the large range of
84 protein values detected within the singing groups, we **hypothesized** that the amount of
85 song/time spent singing resulted in differences in protein levels for TH, α-syn, and
86 GRIN2B in Area X. Our findings supported our hypotheses indicating a strong
87 relationship between protein levels and social context-dependent singing. TH and
88 GRIN2B protein levels in Area X increased with the amount of UD but not FD singing
89 whereas α-syn protein levels increased with the amount of UD song but fluctuated
90 depending on the time spent singing FD song.

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. 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 hour 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 to elicit changes in protein expression [14]. Any bird that was quiet in the last 30 minutes of the two-hour singing period was excluded from collection given that 30 minutes 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 characterization

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 to α-syn (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: AB_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 a 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 determined on a Bio-Rad RC DC assay as in Miller et al. [14]. 15μg of protein lysate was run at 100V using a 10% polyacrylamide-SDS gel then transferred to 0.2μm PVDF membranes for the Western blotting procedure as in Miller et al. 2008. 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, 1:500, 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: AB_2107445; Proteintech, #10494-1-AP, 1:250, RRID: AB_2263076) [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

205 FD). There were fewer birds in the time spent singing analysis group due to
206 experimenter error (missing song files).
207 *2.5. Statistical analysis*
208 For protein level comparison between the four behavioral groups, the means of
209 each group were compared via the Kruskal-Wallis test (VassarStats, vassarstats.net)
210 because the data did not fit a normal distribution. No data points were deemed outliers
211 in the graphical plots using exclusion criteria based on technical issues (e.g. Western
212 blot running conditions or imaging issues) [26]. Therefore, all data points were
213 determined to be experimentally valid data points and were not excluded from analysis.
214 Regression analyses using the OriginLab graphing program was used to determine
215 if TH, α-syn, and GRIN2B protein levels varied as a function of the number of motifs or
216 time spent singing with R^2 values reported as in previous publications [11,13,14].
217 Scatterplots were made in the OriginLab graphing program, and the optimal fit (either
218 linear or curvilinear) is shown for each data set [26,27]. R^2 values for both linear and
219 curvilinear fit were obtained from Origin and confirmed by IBM SPSS Statistical software
220 package in consultation with our university statistician Mark Borgstrom. Each graph was
221 determined to be linear or curvilinear based on the significant F change value
222 determined by IBM SPSS Statistical software package. If the significant F change value
223 was $p < 0.05$ for either the linear or curvilinear model, the correlating model was used. If
224 neither model had a significant F change value of $p < 0.05$, the linear model was used.
225 Statistics for the linear and curvilinear (quadratic) plots were done using IBM SPSS
226 Statistical software package where x = number of motifs/time spent singing and y =

227 protein levels. The ANOVA F and p-values are reported based on x^2 (x-squared) for the
228 plots.
229
230 **3. RESULTS**
231 *3.1. Antibody validation*
232 The polyclonal antibody against α-syn protein detected bands of similar
233 molecular weights across finch Area X, nidopallium (NP), and mouse basal ganglia
234 (MBG) tissue (Fig. 2A). Incubation of the α-syn antibody with 30 times excess of its
235 peptide (Proteintech, #ag1285) resulted in the absence of protein bands (Fig. 2A),
236 confirming specificity along with a similar molecular weight band detected in MBG. To
237 detect and validate GRIN2B protein in finch Area X, we used commercially available
238 antibodies raised against either the mouse N-terminal peptide sequence (Millipore) or
239 the human C-terminal peptide sequence (Proteintech) (Fig. 2B) and tested them in two
240 different finch brain regions (Area X, NP) and in MBG tissue. Both antibodies detected
241 GRIN2B protein bands at similar molecular weights (~180 kD) in finch and MBG tissue.
242 *3.2. Gene expression of non-singers vs. singers in Area X*
243 In this study, we examined the total protein expression of TH, α-syn, and
244 GRIN2B via Western blots from tissue punches of Area X following four behavioral
245 states: 0 HR NS, 2 HR NS, 2 HR UD, and 2 HR FD (Fig. 2C) in which the 0 HR NS was
246 used as the control condition given the lack of behavioral manipulation [6,14]. There
247 were no significant differences between groups for TH, α-syn, and GRIN2B (Fig. 3, see
248 legend for statistics). Like Miller et al. [14], we pursued the sources of the range in
249 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 seconds singing, but as the bird exceeds around 350 seconds, α-syn levels rise (Fig. 5D). However, the number of FD motifs (Fig. 5C) had no effect on α-syn levels.

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 has also been reported for the transcription factor FoxP2; furthermore, the number of motifs sung during two hours of UD but not FD behavior alters FoxP2 protein expression [14]. In our study, levels of TH, α-syn, and GRIN2B in Area X increased with more UD singing but did not show these same patterns with FD song. With more FD song, TH levels did not change, α-syn levels fluctuated depending on how much the bird sang, and GRIN2B levels trended downwards.

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 Riters [3] looked at FD song for a shorter time period, 30-45 minutes, 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 hours vs. 30 minutes) dedicated to one state only whereas Sasaki et al. [2] alternated

30 minutes 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

319 greater acoustic variability measured as the change in mean Wiener entropy, shown in
320 Fig. 7B. Dopaminergic and glutamatergic input converge on medium spiny neurons
321 (MSNs), the site of cortico-striatal synaptic plasticity. After 30-45 minutes of UD song
322 (black pathway, Fig. 7A), low DA levels exist in Area X [2]. MSNs inhibit pallidal (PAL)
323 neurons so that DLM provides excitatory drive to song nucleus LMAN. LMAN drives
324 song nucleus RA to promote more variability in pitch, etc. in the bird's song, as part of
325 his trial and error learning [33]. Based on findings from our study, we propose the blue
326 molecular pathway (Fig. 7B) that becomes activated with increased UD singing. As the
327 bird continues to sing over the two-hour period (blue pathway), α-syn facilitates
328 increased glutamate release onto MSNs requiring up-regulation of NMDA 2B (GRIN2B)
329 receptors. With increased NMDA 2B receptor levels, MSNs excitability and inhibition
330 over PAL neurons increase to support more UD song rendition-to-rendition variability
331 [15]. However, by two hours from the start of singing, TH levels begin to rise in Area X
332 as a potential mechanism to counter too much acoustic variability, leading to increased
333 DA synthesis.

334 *4.3. Molecular pathway model for FD song*

335 The traditional circuit that supports low acoustic variability in FD song (in
336 comparison to UD song), is represented by the black colored pathway in Fig. 7C. With
337 30-45 minutes of FD song (black pathway), there are high levels of DA from SN/VTA
338 onto Area X, which depress glutamatergic input from LMAN onto Area X MSNs (Fig.
339 7C) [10]. Consequently, PAL neurons are disinhibited and subsequent strong DLM
340 inhibition leads to decreased excitability between LMAN and RA with a less variable,
341 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 (Fig. 7), 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].

ACKNOWLEDGMENTS

JEM and LYS conceived of and designed the experiments for this study. LYS and SJM executed the experiments and the analyses. LYS wrote the manuscript with input from all authors. We thank Dr. Mark Borgstrom and Dr. Elena Plante for statistical consultation and the University of Arizona Animal Care. Declarations of interest: none. This work was supported by the University of Arizona startup funds to J. E. Miller.

REFERENCES

[1] Simonyan, K., Horwitz, B., & Jarvis, E. D. (2012). Dopamine regulation of human speech and bird song: A critical review. *Brain Lang*, 122(3), 142-50.

[2] Sasaki, A., Sotnikova, T. D., Gainetdinov, R. R., & Jarvis, E. D. (2006). Social context-dependent singing-regulated dopamine. *J Neurosci*, 26(35), 9010-9014.

386 [3] Heimovics, S.A., & Riters, L. V. (2008). Evidence that dopamine within motivation
387 and song control regions regulates birdsong context-dependently. *Physiol Behav*, 95,
388 258-266.
389 [4] Gale, S. D., Person, A. L., & Perkel, D. J. (2008). A novel basal ganglia pathway
390 forms a loop linking a vocal learning circuit with its dopaminergic input. *J Comp Neurol*,
391 508(5), 824-839.
392 [5] Gale, S. D., & Perkel, D. J. (2010). A basal ganglia pathway drives selective auditory
393 responses in songbird dopaminergic neurons via disinhibition. *J Neurosci*, 30(3), 1027-
394 1037.
395 [6] Miller, J. E., Hafzalla, G. W., Burkett, Z. D., Fox, C. M., & White, S. A. (2015).
396 Reduced vocal variability in a zebra finch model of dopamine depletion: implications for
397 Parkinson disease. *Physiol Rep*, 3(11), 1-16.
398 [7] Leblois, A., Wendel, B. J., & Perkel, D. J. (2010). Striatal dopamine modulates basal
399 ganglia output and regulates social context-dependent behavioral variability through D1
400 receptors. *J Neurosci*, 30(16), 5730-5743.
401 [8] Leblois, A., & Perkel, D. J. (2012). Striatal dopamine modulates song spectral but not
402 temporal features through D1 receptors. *Eur J Neurosci*, 35(11), 1771-1781.
403 [9] Hoffmann, L. A., Saravanan, V., Wood, A. N., He, L., & Sober, S. J. (2016).
404 Dopaminergic contributions to vocal learning. *J Neurosci*, 36(7), 2176-2189.
405 [10] Ding, L., Perkel, D. J., & Farries, M. A. (2003). Presynaptic depression of
406 glutamatergic synaptic transmission by D1-like dopamine receptor activation in the
407 avian basal ganglia. *J Neurosci,* 23(14), 6086-6085.

[11] Jarvis, E. D., Scharff, C., Grossman, M. R., Ramos, J. A., & Nottebohm, F. (1998). For whom the bird sings: context-dependent gene expression. *Neuron*, 21(4), 775-788.

[12] Mello, C. V., & Ribeiro, S. (1998). ZENK protein regulation by song in the brain of songbirds. *J Comp Neurol*, 393(4), 426-438.

[13] Teramitsu, I., & White, S. A. (2006). FoxP2 regulation during undirected singing in adult songbirds. *J Neurosci*, 26(28), 7390-7394.

[14] Miller, J. E., Spiteri, E., Condro, M. C., Dosumu-Johnson, R. T., Geschwind, D. H., & White, S. A. (2008). Birdsong decreases protein levels of FoxP2, a molecule required for human speech. *J Neurophysiol*, 100(4), 2015-2025.

[15] *Hilliard, A. T., *Miller, J. E., Fraley, E. R., Horvath, S., & White, S. A. (2012). Molecular microcircuitry underlies functional specification in a basal ganglia circuit dedicated to vocal learning. *Neuron*, 73(3), 537-552. *equal contributions

[16] Bottjer, S. W. (1993). The distribution of tyrosine hydroxylase immunoreactivity in the brains of male and female zebra finches. *J Neurobiol*, 24(1), 51-69.

[17] Soha, J. A., Shimizu, T., & Doupe, A. J. (1996). Development of the catecholaminergic innervation of the song system of the male zebra finch. *J Neurobiol*, 29(4), 473-489.

[18] Ciucci, M. R., Ma, S. T., Fox, C., Kane, J. R., Ramig, L. O., & Schallert, T. (2007). Qualitative changes in ultrasonic vocalization in rats after unilateral dopamine depletion or haloperidol: a preliminary study. *Behav Brain Res*, 182(2), 284-289.

[19] Grant, L. M., Barnett, D. G., Doll, E. J., Leverson, G., & Ciucci, M. (2015). Relationships among rat ultrasonic vocalizations, behavioral measures of striatal dopamine loss, and striatal tyrosine hydroxylase immunoreactivity at acute and chronic

431 time points following unilateral 6-hydroxydopamine-induced dopamine depletion. *Behav*
432 *Brain Res*, 291, 361-371.
433 [20] Venda, L. L., Cragg, S. J., Buchman, V. L., & Wade-Martins, R. (2010). α-Synuclein
434 and dopamine at the crossroads of Parkinson's disease. *Trends Neurosci*, 33(12), 559-
435 568.
436 [21] Decressac, M., Mattsson, B., & Björklund, A. (2012). Comparison of the behavioural
437 and histological characteristics of the 6-OHDA and α-synuclein rat models of
438 Parkinson's disease. *Exp Neurol*, 325(1), 306-315.
439 [22] Gombash, S. E., Manfredsson, F. P., Kemp, C. J., Kuhn, N. C., Fleming, S. M.,
440 Egan, A. E., Grant, L. M., Ciucci, M. R., MacKeigan, J. P., & Sortwell, C. E. (2013).
441 Morphological and behavioral impact of AAV2/5-mediated overexpression of human
442 wildtype alpha-synuclein in the rat nigrostriatal system. *PloS One*, 8(11), 1-15.
443 [23] George, J. M., Jin, H., Woods, W. S., & Clayton, D. F. (1995). Characterization of a
444 novel protein regulated during the critical period for song learning in the zebra finch.
445 *Neuron*, 15(2), 361-372.
446 [24] Chakraborty, M., Chen, L. F., Fridel, E. E., Klein, M. E., Senft, R. A., Sarkar, A., &
447 Jarvis, E. D. (2017). Overexpression of human NR2B receptor subunit in LMAN causes
448 stuttering and song sequence changes in adult zebra finches. *Sci Rep*, 7(1), 1-18.
449 [25] Tchernichovski, O., Nottebohm, F., Ho, C. E., Pesaran, B., & Mitra, P. P. (2000). A
450 procedural for an automated measurement of song similarity. *Anim Behav*, 59(6), 1167-
451 1176.
452 [26] Scheff, S. W. (2016). *Fundamental statistical principles for the Neurobiologist a*
453 *survival guide.* Cambridge, MA: Academic Press.

454 [27] Myers, J. L., & Well, A. D. (2003). Testing for curvilinearity in regression. In D.
455 Riegert (Eds.) *Research design and statistical analysis (*2nd ed., pp.598-601). Mahwah,
456 NJ: Lawrence Erlbaum Associates
457 [28] Yavich, L., Jäkälä, P., & Tanila, H. (2006) Abnormal compartmentalization of
458 norepinephrine in mouse dentate gyrus in alpha-synuclein knockout and A40P
459 transgenic mice. *J Neurochem*, 99(3), 724-732
460 [29] Gureviciene, I., Gurevicius, K., & Tanila, H. (2007). Role of alpha-synuclein in
461 synaptic glutamate release. *Neurobiol Dis*, 28(1), 83-89.
462 [30] Watson, J. B., Hatami, A., David, H., Masliah, E., Roberts, K., Evans, C. E., &
463 Levine, M. S. (2009). Alterations in corticostriatal synaptic plasticity in mice
464 overexpressing human alpha-synuclein. *Neurosci*, 159(2), 501-513.
465 [31] Woolley, S. C., & Kao, M. H. (2015). Variability in action: contributions of a songbird
466 cortical-basal ganglia circuit to vocal motor learning and control. *Neurosci*, 296, 39-47.
467 [32] Kao, M. H., Doupe, A. J., & Brainard, M. S. (2005). Contributions of an avian basal
468 ganglia-forebrain circuit to real-time modulation of song. *Nature*, 433(7026), 638-643.
469 [33] Sober, S. J., Wohlgemuth, M. J., & Brainard, M. S. (2008). Central contributions to
470 acoustic variation in birdsong. *J Neurosci*, 28(41), 10370-10379.
471 [34] Xiao, L., Chattree, G., Oscos, F. G., Cao, M., Wanat, M. J., & Roberts, T. F. (2018).
472 A basal ganglia circuit sufficient to guide birdsong learning. *Neuron,* 98(1), 208-221.
473

474 **TABLE LEGEND**

475 **Table 1. Primary and secondary antibody information.** All primary and secondary
476 antibodies used in the study and relevant information are listed in the table.

FIGURE LEGENDS

Figure 1. Schematic of the song circuitry. Individual brain regions are denoted by circles/ovals. The anterior forebrain pathway is comprised of a cortico-basal-thalamic-cortico loop (LMAN, Area X, DLM). Area X receives dopaminergic input from SN/VTA and glutamatergic inputs from cortical nuclei HVC and LMAN. DLM – dorsal lateral nucleus of thalamus; HVC – higher vocal center; LMAN – lateral magnocellular nucleus of anterior nidopallium; RA – robust nucleus of arcopallium; SN – substantia nigra; VP – ventral pallidum; VTA – ventral tegmental area. [4]

Figure 2. Validation blots for α-syn and GRIN2B antibodies and a representative western blot with four different behavioral states.

A: Western blot with α-syn protein detected at ~16 kD in Area X, nidopallium (NP), and mouse basal ganglia (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. **B**: Western blot with GRIN2B protein detected at ~180 kD in Area X, NP, and MBG. 15 µg of protein loaded per lane. ‡Antibody 1 for GRIN2B (Millipore, #06-600) and §antibody 2 for GRIN2B (Proteintech, #21920-1-AP) both detect protein bands at similar molecular weights, confirming specificity of the antibody to GRIN2B. **C**: Representative blot of 15 µg of tissue lysate per lane from bilateral tissue biopsies of Area X. Two birds were run per behavioral group. Blot shows protein levels of TH (~55 kD, Miller et al. 2015), GRIN2B (~180 kD), GAPDH (~35 kD), and α-syn (~16 kD); finch molecular weights obtained from: www.ncbi.nlm.nih.gov/protein. Protein signals in each lane were normalized to

GAPDH, the loading control. Dotted lines denote where the blots were cut prior to antibody incubation to avoid cross-reactivity between antibodies, weak signals, and to minimize antibody usage.

Figure 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).

Figure 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 sang 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.

Figure 5. Changes in Area X α-syn protein levels with UD and FD song.

α-syn protein levels of UD singers and FD singers plotted against the number of motifs sang 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.

Figure 6. Differential expression of Area X GRIN2B protein levels in response to UD and FD song.

GRIN2B protein levels of UD singers and FD singers plotted against the number of motifs sang within the two-hour period (A, C) or time spent singing (B, D). GRIN2B protein levels were dependent upon the number of motifs sung during UD (n = 8, ANOVA, F = 13.18, p = 0.011) but not FD (n = 8, ANOVA, F = 1.13, p = 0.329). In addition, protein levels were dependent upon the time spent singing UD song (n = 8, ANOVA, F = 12.22, p = 0.013) but not FD song (n = 7, ANOVA, F = 5.21, p = 0.071). Although the time spent singing FD song did not have a significant effect on GRIN2B levels, a downward trend was evident. Each point represents an individual bird. Gray

546 lines denote linear regression curves drawn as best fits to the data based on the
547 significant F change value.
548 **Figure 7. Molecular pathway models for UD and FD song.**
549 **A**: Black lines represent the circuit activity after 30-45 minutes of UD song. **B**: Blue lines
550 denote circuit activity at the end of two hours of UD song in relation to how much the
551 bird sang. A bird that sang more over the two hours of UD song had increased
552 expression of TH, α-syn, and GRIN2B (up arrows) potentially leading to more
553 acoustically variable song. The increased levels of TH at two hours may be a self-
554 regulatory check to prevent too much variability in song beyond this time point. **C**: Black
555 lines represent the circuit activity after 30-45 minutes of FD song. **D**: Blue lines denote
556 circuit activity at the end of two hours of FD song in relation to how much the bird sang.
557 A bird that sang more over the two hours of FD song had the same levels of TH
558 denoted as ~, fluctuating levels of α-syn (up and down arrow), and reduced levels of
559 GRIN2B (down arrow). The rendition-to-rendition variability of FD song compared to UD
560 song after two hours is unknown (denoted as ?). DLM – dorsal lateral nucleus of
561 thalamus; LMAN – lateral magnocellular nucleus of anterior nidopallium; MSN –
562 medium spiny neurons; PAL – pallidal neurons; RA – robust nucleus of arcopallium; SN
563 – substantia nigra; VTA – ventral tegmental area.

Figure 1

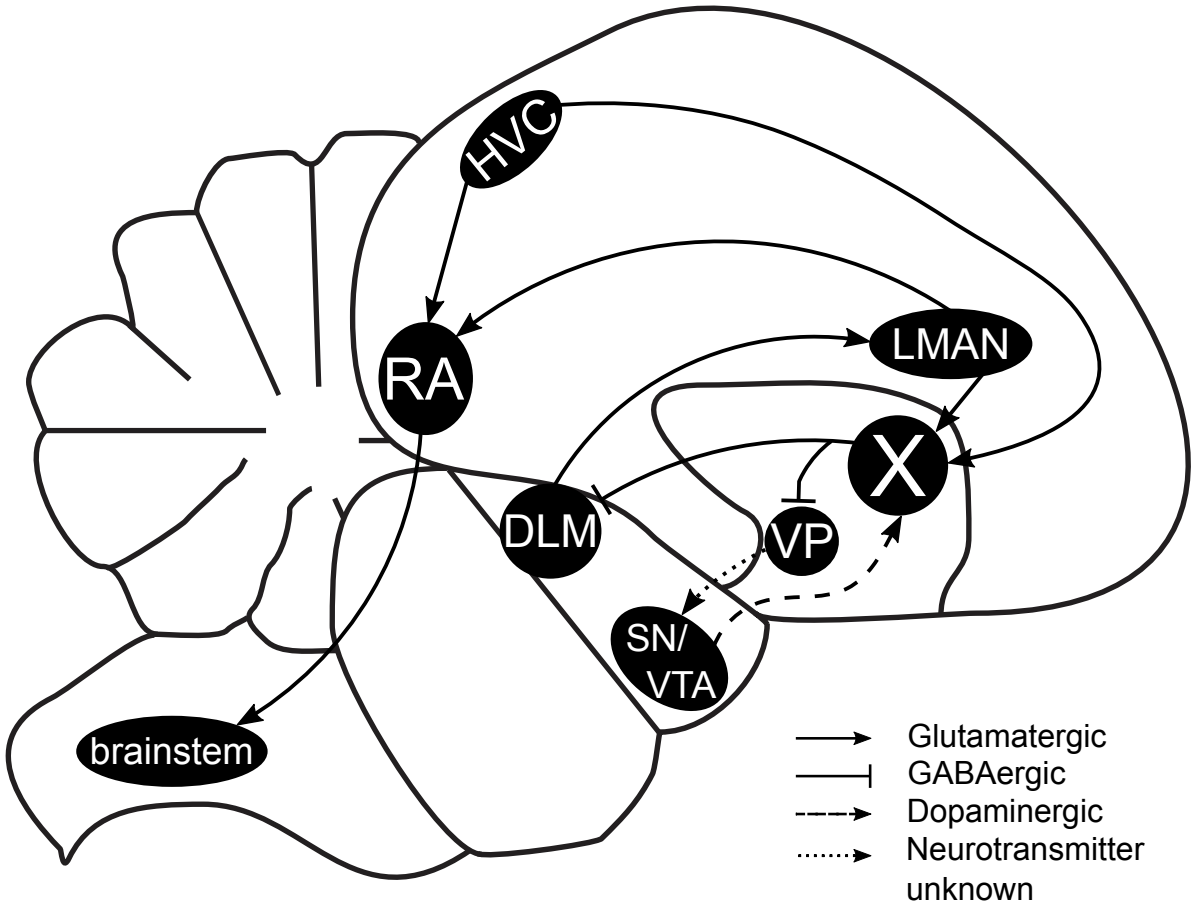

HVC
RA
LMAN
X
DLM
VP
SN/
VTA
brainstem
Glutamatergic
GABAergic
Dopaminergic
Neurotransmitter
unknown

Figure 2

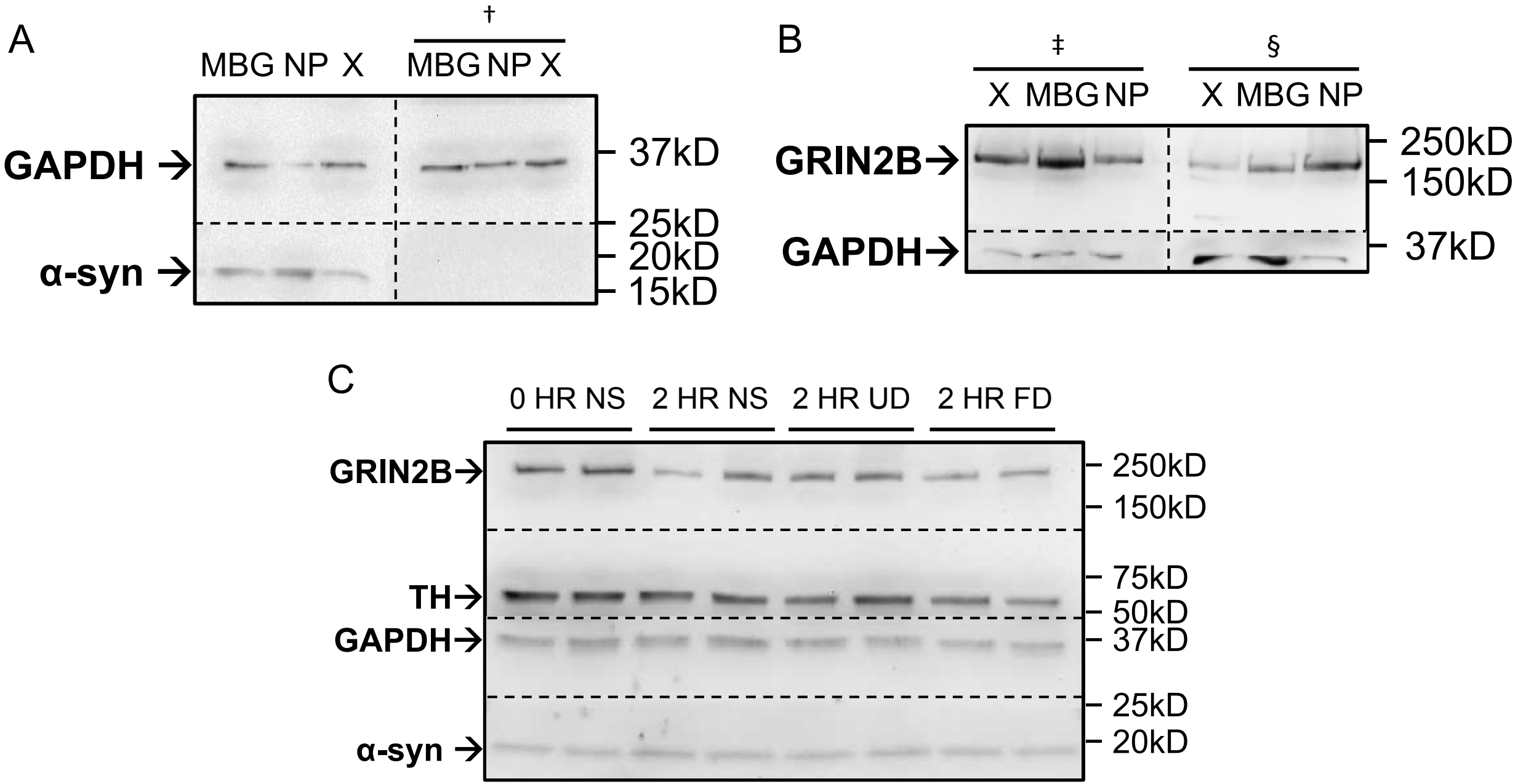

A
†
MBG NP X
MBG NP X
GAPDH →
α-syn →
37kD
25kD
20kD
15kD
B
‡
§
X MBG NP
X MBG NP
GRIN2B→
GAPDH→
250kD
150kD
37kD
C
0 HR NS
2 HR NS
2 HR UD
2 HR FD
GRIN2B→
TH→
GAPDH→
α-syn →
250kD
150kD
75kD
50kD
37kD
25kD
20kD

Figure 3

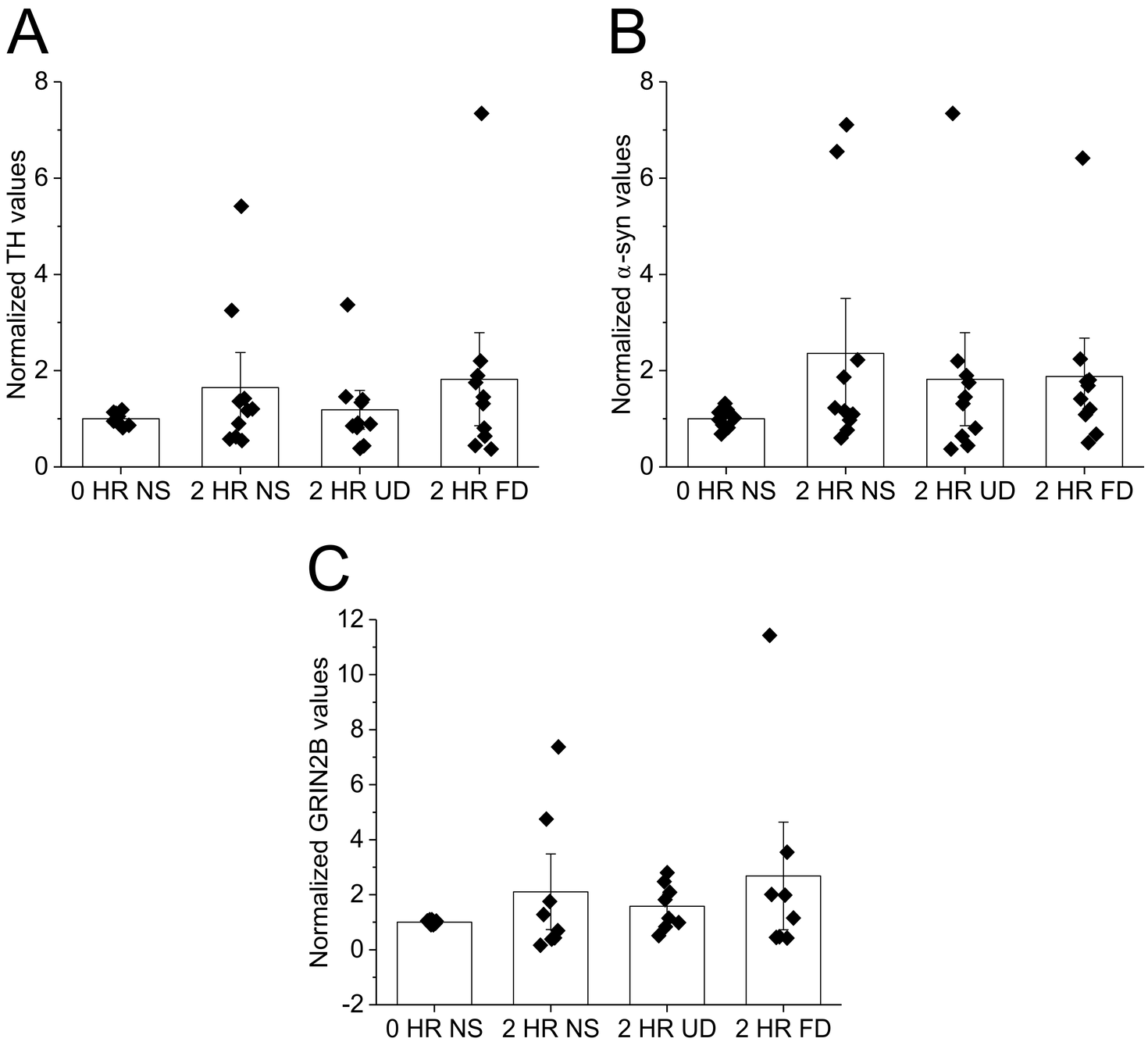

A
Normalized TH values
0
2
4
6
8
0 HR NS
2 HR NS
2 HR UD
2 HR FD
B
Normalized α-syn values
0
2
4
6
8
0 HR NS
2 HR NS
2 HR UD
2 HR FD
C
Normalized GRIN2B values
-2
0
2
4
6
8
10
12
0 HR NS
2 HR NS
2 HR UD
2 HR FD

Figure 4

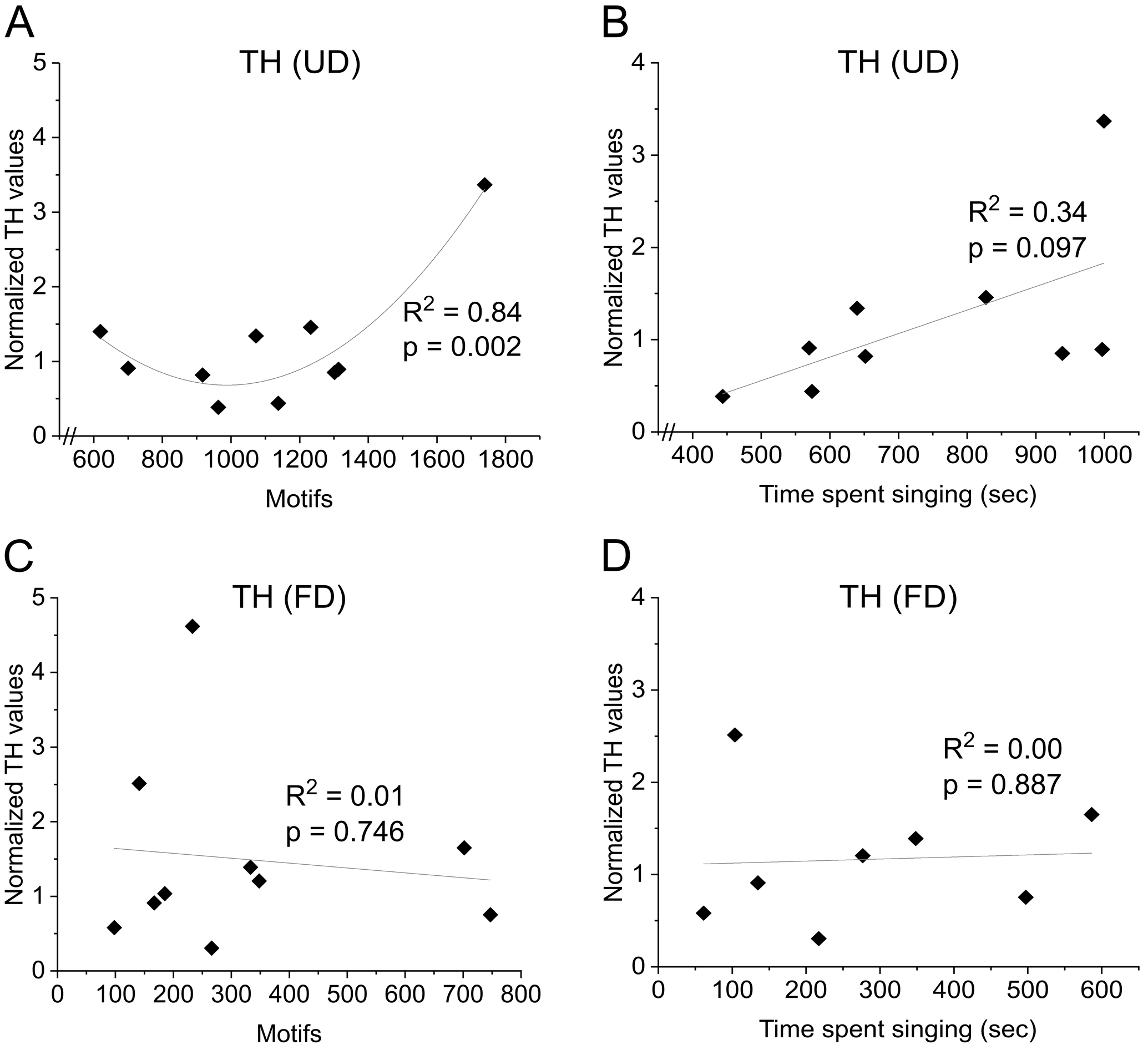

A
TH (UD)
Normalized TH values
Motifs
R^2 = 0.84
p = 0.002
B
TH (UD)
Normalized TH values
Time spent singing (sec)
R^2 = 0.34
p = 0.097
C
TH (FD)
Normalized TH values
Motifs
R^2 = 0.01
p = 0.746
D
TH (FD)
Normalized TH values
Time spent singing (sec)
R^2 = 0.00
p = 0.887

Figure 5

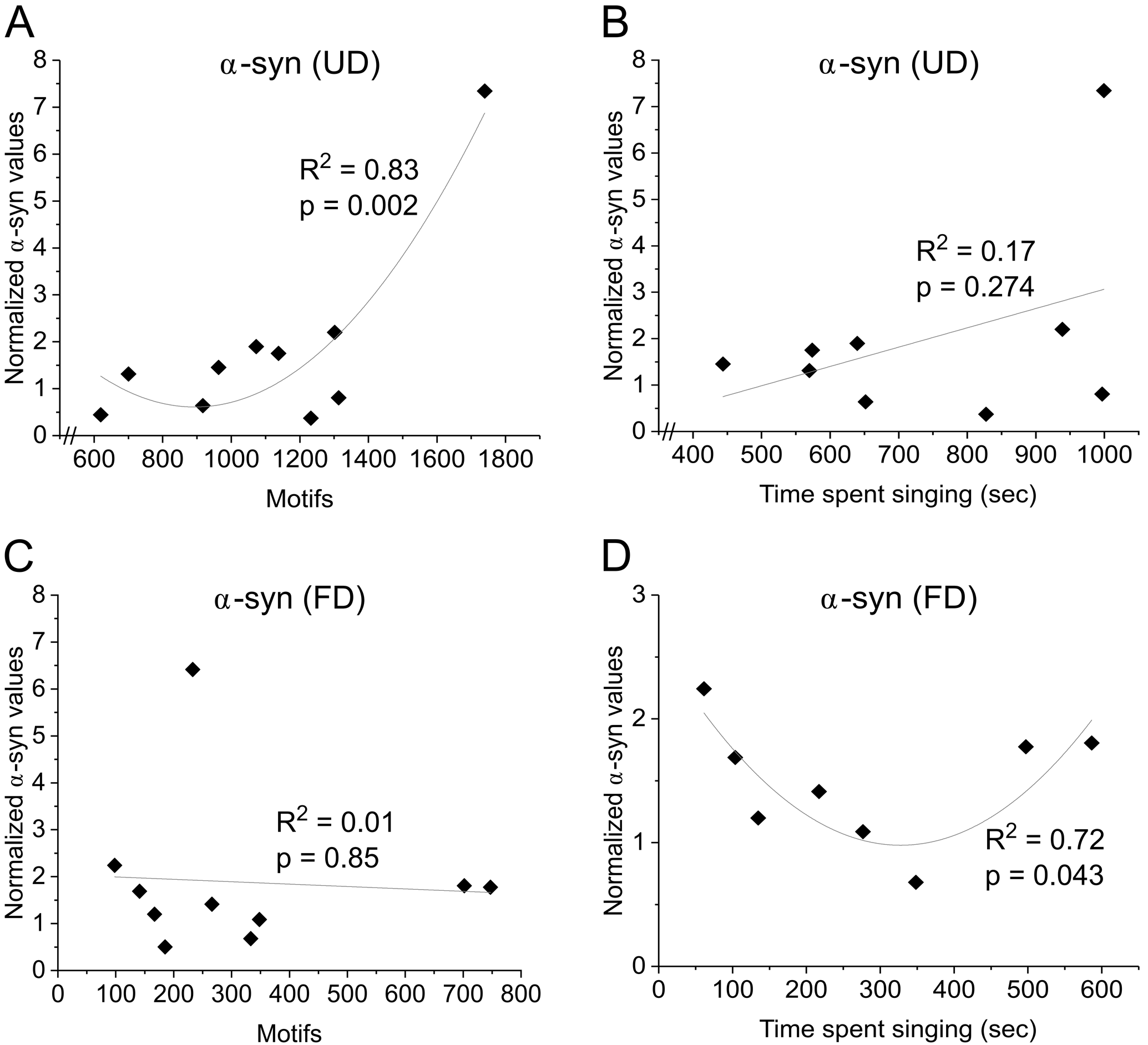
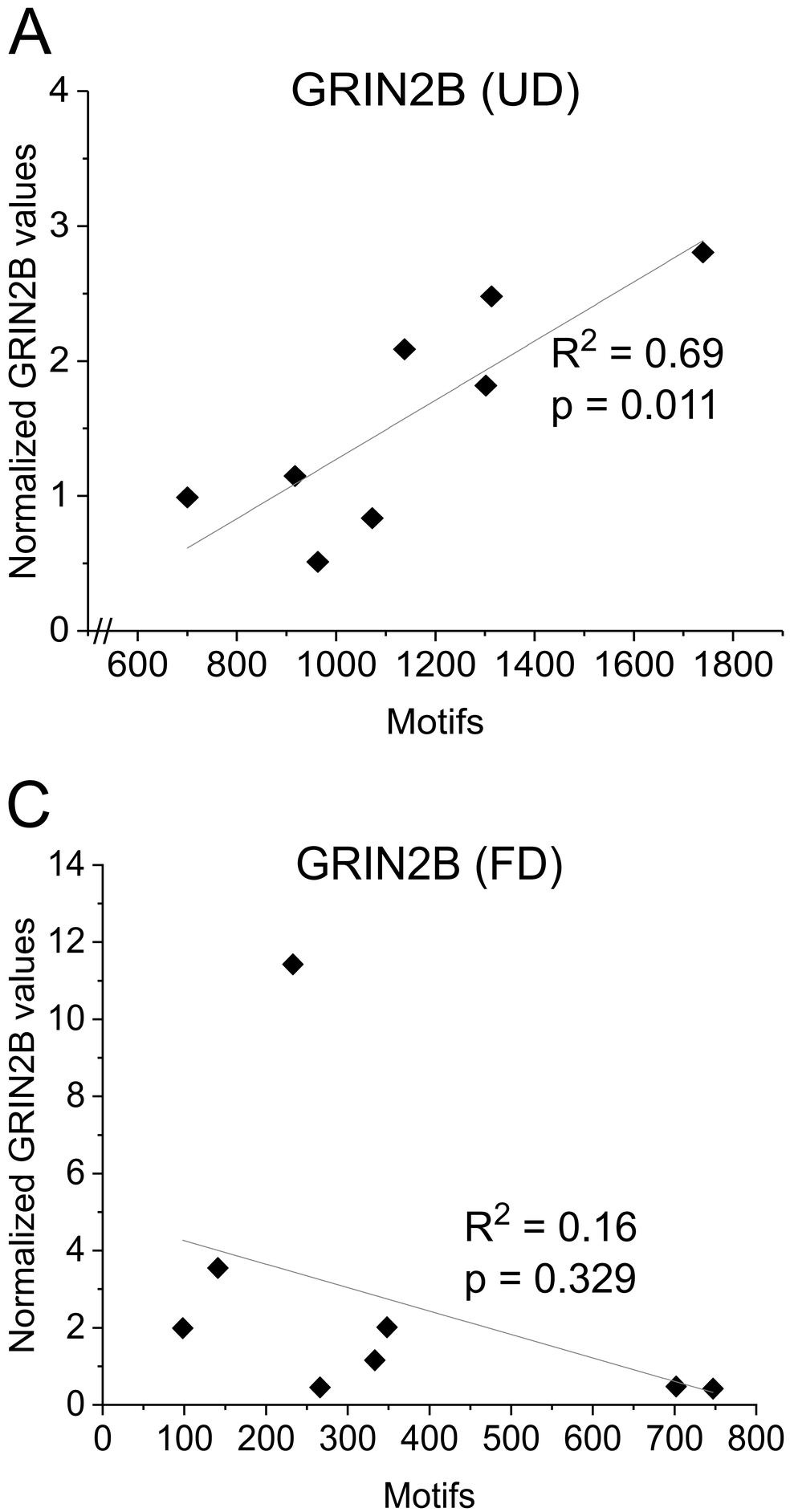
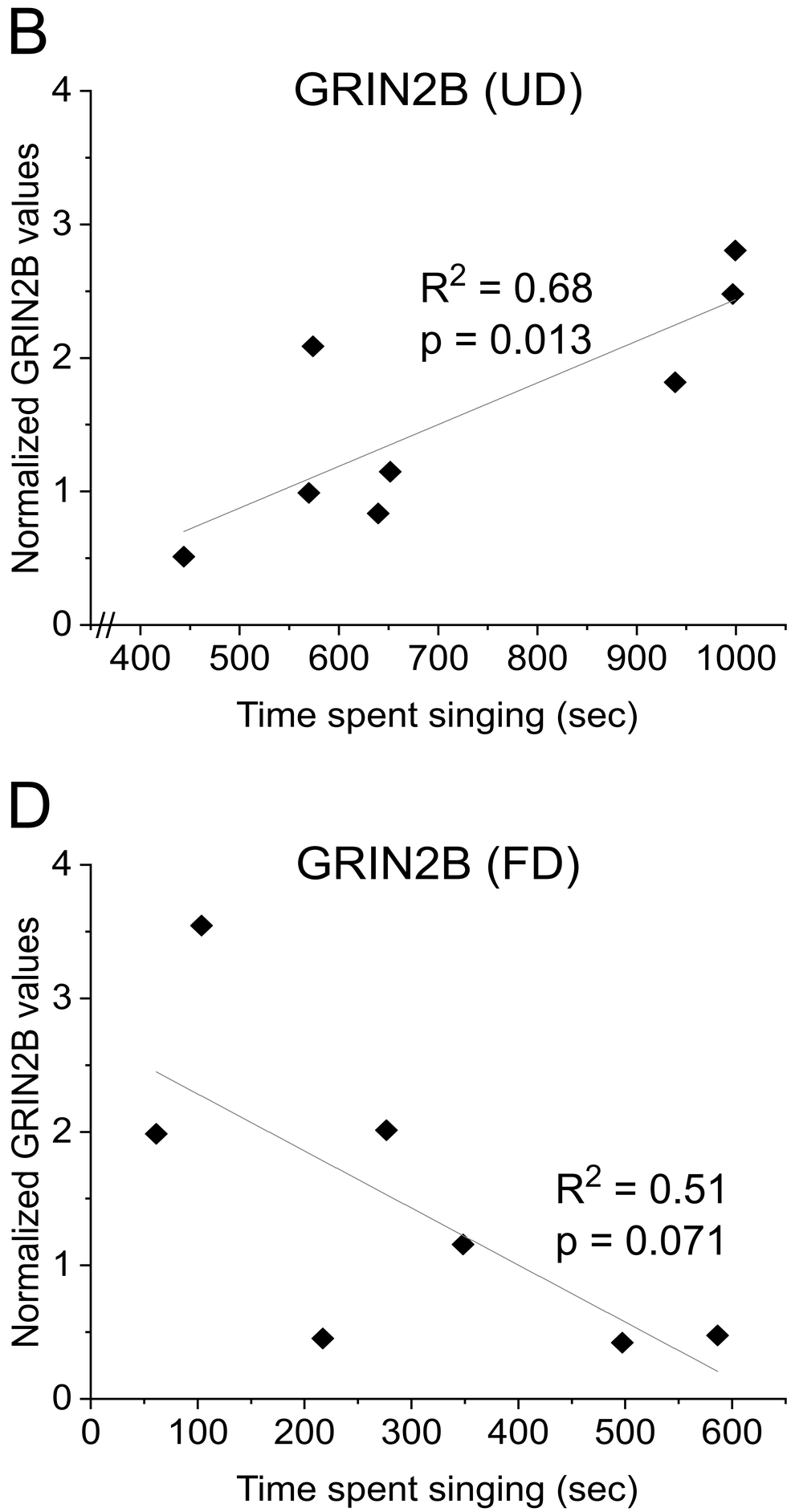

A
α-syn (UD)
R2 = 0.83
p = 0.002
Normalized α-syn values
Motifs
B
α-syn (UD)
R2 = 0.17
p = 0.274
Normalized α-syn values
Time spent singing (sec)
C
α-syn (FD)
R2 = 0.01
p = 0.85
Normalized α-syn values
Motifs
D
α-syn (FD)
R2 = 0.72
p = 0.043
Normalized α-syn values
Time spent singing (sec)

Figure 6

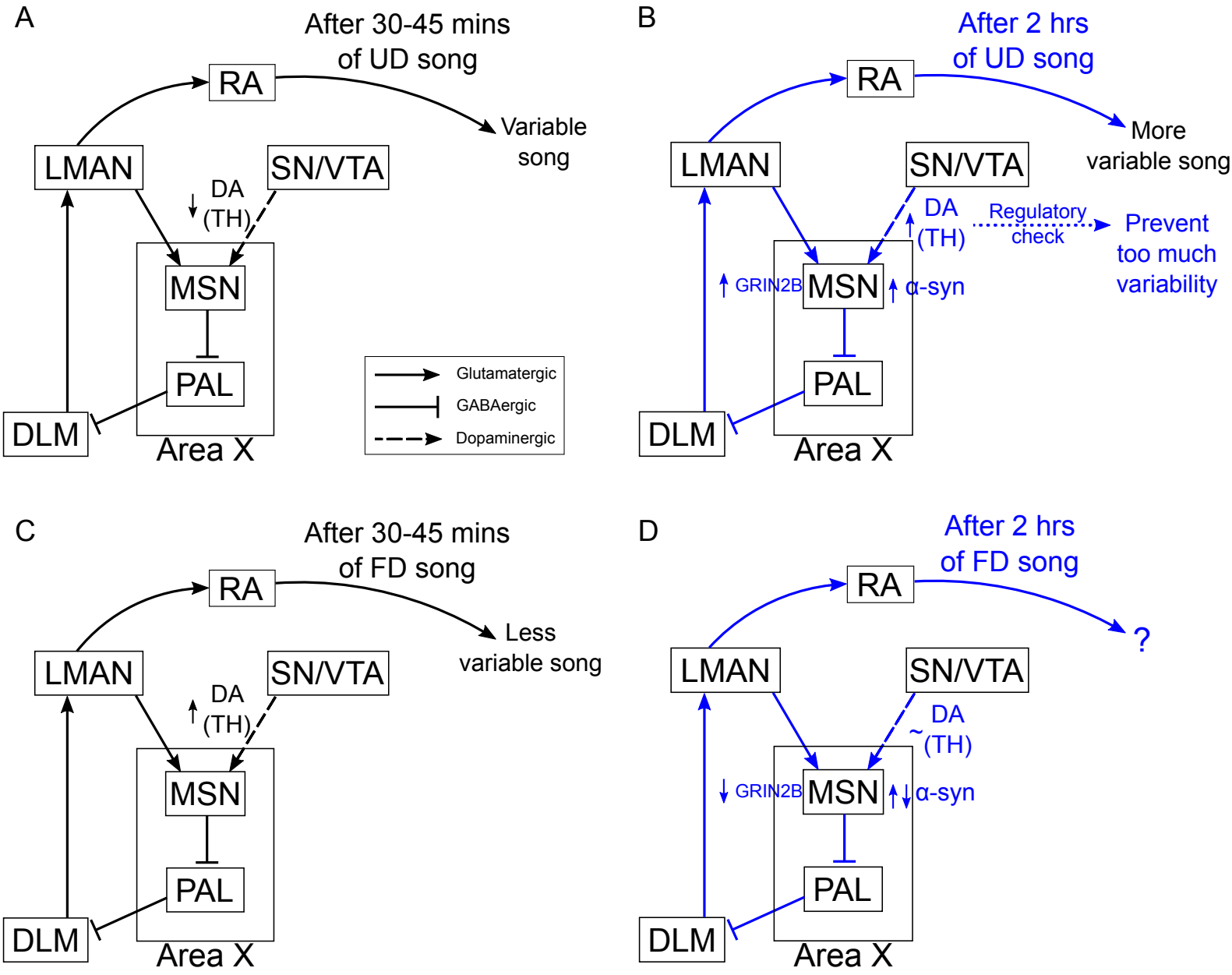

A
GRIN2B (UD)
Normalized GRIN2B values
R^2 = 0.69
p = 0.011
Motifs


B
GRIN2B (UD)
Normalized GRIN2B values
R^2 = 0.68
p = 0.013
Time spent singing (sec)

C
GRIN2B (FD)
Normalized GRIN2B values
R^2 = 0.16
p = 0.329
Motifs

D
GRIN2B (FD)
Normalized GRIN2B values
R^2 = 0.51
p = 0.071
Time spent singing (sec)

Figure 7


A
After 30-45 mins
of UD song
RA
Variable
song
LMAN
SN/VTA
DA
(TH)
MSN
PAL
DLM
Area X
Glutamatergic
GABAergic
Dopaminergic
B
After 2 hrs
of UD song
RA
More
variable song
LMAN
SN/VTA
DA
(TH)
Regulatory
check
Prevent
too much
variability
GRIN2B
MSN
α-syn
PAL
DLM
Area X
C
After 30-45 mins
of FD song
RA
Less
variable song
LMAN
SN/VTA
DA
(TH)
MSN
PAL
DLM
Area X
D
After 2 hrs
of FD song
RA
?
LMAN
SN/VTA
DA
~
(TH)
GRIN2B
MSN
α-syn
PAL
DLM
Area X