Dopamine contributes to the modulation of Bengalese finch song production in different social contexts

Anca Vochin

Integrated Program in Neuroscience

McGill University

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Abstract

Social modulation of vocal signals is central to efficiently transmitting information and shaping interactions between individuals. In social species, like humans, those who lose the ability to communicate vocally can become marginalized and isolated, leading to further detrimental effects on health and well-being. While extensive research has been done to decipher the neural mechanisms underlying vocal communication control, multiple important questions remain unanswered. One of them is how dopamine modulates moment-to-moment vocal motor control. The current data is limited to studies in humans with known dopaminergic disturbances, such as Parkinson's disease, in whom this cannot be reliably ascertained. Like humans, songbirds use vocal signals to communicate, and these vocal signals are regulated by forebrain circuits that are innervated by dopamine. While several studies have shown that the structure and organization of birdsong could be modulated by dopamine, little is known about precisely how dopamine modulates spectral and temporal properties of song. Here we present two pharmacological experiments to reveal the role of dopamine in context-dependent changes to song tempo, syllable sequencing and syllable structure in a songbird, the Bengalese finch. We analysed the effect of peripherally administered D1 and D2 receptor agonists and of centrally administered D1 agonist on song organization. We found that peripheral administration of D1 agonist mimicked social context changes on syllable sequencing, while central administration mimicked social context changes to song tempo. These data lend support to the notion that dopamine contributes to the social modulation of vocal output and hint to potential points of intervention in human speech disorders.

Résumé

La modulation sociale des signaux vocaux est essentielle pour transmettre efficacement des informations et définir les interactions entre les individus. Dans les espèces sociales, comme les humains, ceux qui perdent la capacité de communiquer vocalement peuvent être marginalisés, ce qui entraîne des effets encore plus néfastes sur la santé. Bien que des recherches approfondies ont été réalisées pour déchiffrer les mécanismes neuronaux qui sous-tendent le contrôle de la communication vocale, des questions importantes restent sans réponse. Parmi elles, comment la dopamine module le contrôle moteur vocal à chaque instant. Les données actuelles se limitent à des études sur des humains présentant des troubles dopaminergiques, comme la maladie de Parkinson, chez lesquels ce rôle ne peut être déterminé de manière fiable. Comme les humains, les oiseaux chanteurs utilisent des signaux vocaux pour communiquer, régulés par des circuits du cerveau antérieur innervés par la dopamine. Même si plusieurs études ont montré que la structure et l'organisation du chant des oiseaux pouvaient être modulées par la dopamine, on sait peu sur comment la dopamine modifie les propriétés spectrales et temporelles du chant. Nous présentons ici deux études pharmacologiques pour révéler le rôle de la dopamine dans les changements contextuels du tempo du chant, du séquençage et de la structure des syllabes chez le pinson du Bengale. Nous avons analysé l'effet des agonistes des récepteurs D1 et D2 administrés en périphérie et de l'agoniste D1 administré de façon centrale sur l'organisation du chant. L'administration périphérique de l'agoniste D1 imitait les changements de contexte social sur le séquençage des syllabes, tandis que l'administration centrale imitait les changements de contexte social sur le tempo des chants. Ces données confirment l'idée que la dopamine contribue à la modulation sociale du débit vocal et indiquent des points d'intervention potentiels dans les troubles de la parole chez les humains.

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Contributions of authors

The work presented in this thesis will be submitted for publication as a peer-reviewed manuscript written by me (first author) and Dr. Jon Sakata (second author). Due to the COVID-19 pandemic leading to the research ramp down, my initial research project could not be advanced enough for a thesis. The datasets presented in this thesis were already collected. The peripheral drug administration data were collected by Laura Matheson, while the central infusion data was collected by Gaurav Isola, who performed the surgeries. I processed and analysed the behavioural data (i.e. song), analysed the data and wrote the manuscript. Dr. Jon Sakata designed the experiments and provided guidance and assistance with the data analysis and revised drafts of this thesis.

Chapter 1. Introduction

1.1 Human speech and birdsong

Speech production is intrinsic to successful participation in social interactions. Typically, social interactions involve a delivery or exchange of information, intentions or feelings, tailored to our audience. The efficient transmission of the intended message is not only dependent on word choice but also upon non-verbal properties of speech, such as prosody. Humans modulate these speech parameters depending on social interactions or function. For example, we speak slower when we educate our toddlers, faster when we want to communicate urgency, and softer and with a lower pitch when expressing attraction or affection.

Similar to all motivated behaviours, speech production is hypothesized to be modulated by dopamine (Norel et al., 2020; Simonyan et al., 2013). Support for this hypothesis comes from clinical observations of people with known dopaminergic depletion, as in Parkinson's disease (PD), who display significant impairments in vocal motor modulation. Patients with PD produce speech that is slow and monotone due to a reduction in the variability of the fundamental frequency, and their speech contains inappropriate silences and imprecise articulation (Bowen et al., 2013; Duffy, 2013). Such speech impairments significantly undermine the ability to communicate and lead to social isolation, compounding the impact of the disease (Baylor et al., 2011; Hartelius et al., 2008; Miller et al., 2006).

Levo-dopa, the standard medication used to improve the motor symptoms in PD marginally improves speech, even when it significantly improves the gross motor function (Azevedo et al., 2013; De Letter et al., 2007). Other treatments include combinations of levo-dopa and several dopamine agonists, but side effects of the drugs combination often exceed their

benefits (Azevedo et al., 2013; Pinho et al., 2018). These problems stem from a yet limited understanding of how dopamine modulates the moment-to-moment control of speech and of which speech properties are directly modulated by dopamine. Elucidating dopamine's involvement in modulating vocal motor control could inform better pharmacological treatments for the speech disturbances accompanying PD.

Songbirds offer an excellent model to reveal how dopamine modulates sensorimotor processes like those in human speech. Like humans, songbirds need to learn and practice their vocalizations; consequently, the neural circuits for song learning and production in songbirds are analogous to brain circuits for speech acquisition and control in humans (Brainard and Doupe, 2002; Doupe and Kuhl, 1999). Furthermore, songbirds provide the key advantages of a reliable output of stereotyped motor behavior and an anatomically discrete neural circuit dedicated to song control. This lends great experimental tractability to studying vocal motor control and its neural underpinnings.

1.2 Song learning in songbirds

Akin to human language, birdsong is culturally transmitted and is learned during development through exposure to a tutor's song (Okanoya, 2004, 2015; Sakata and Yazaki-Sugiyama, 2020). The juvenile songbird memorizes the sounds, then develops his song through extensive practice (Catchpole and Slater, 2008; Fee and Scharff, 2010). (Note: in many species, including Bengalese finches, only the males learn and produce complex songs). Adult birds actively maintain their song structure by relying on error correction mechanisms based on auditory feedback (Clayton, 1987; Sakata et al., 2008; Sakata and Yazaki-Sugiyama, 2020; Woolley and Rubel, 2002). Similar to how speech deteriorates following hearing loss, the song

of adult songbirds who can no longer hear their own vocal production deteriorates both spectrally and temporally (Lombardino and Nottebohm, 2000; Woolley and Rubel, 2002).

1.3 Birdsong structure

Male adult Bengalese finches (*Lonchura striata var. domestica*) sing complex songs which remain relatively stable across their adult life (Brainard and Doupe, 2002). Their song mainly contains stereotyped elements, but also displays some variability.

The Bengalese finch song begins with several short, quiet sounds called introductory notes. These are followed by acoustic elements called "syllables", each of which are separated by at least 5 milliseconds of silence (James and Sakata, 2014; Sakata and Brainard, 2008). Syllables have distinct acoustic properties and are arranged in stereotyped sequences called motifs. However, the song also contains more variable sequences, called branch points (Figure 1).

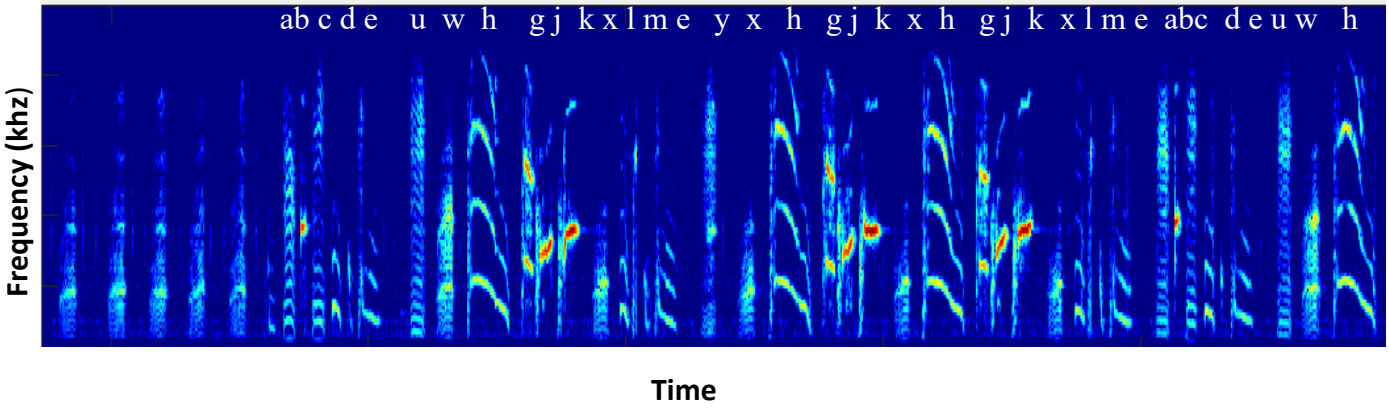

Figure 1. Example spectrogram of the Bengalese finch song. Time is represented on the x-axis, frequency (kHz) on the y-axis and color represents the amplitude of the sound (i.e. blue = quiet, red = loud). Syllables are denoted by letters. Introductory notes appear in the beginning of the song (here, unlabelled). The sequence "abcde" is an example of a stereotyped sequence. The sequence "lme" is an example of a branch point, as it variably transitions to "y" or to "a".

Male Bengalese finches sing in at least two distinct social contexts. They produce song in the context of courtship, directing their song towards a female and this type of song is aptly

named female-directed (FD) song. When the finch sings alone or in the presence of other males, he produces undirected (UD) song, as he does not sing towards an individual in particular. Although hard to detect by our ear, renditions of FD and UD song contain different spectral and temporal characteristics that are salient to other finches (Chen et al., 2016; Woolley and Doupe, 2008). Overall, the UD song is more variable in syllable sequencing and syllable structure. Conversely, the FD song is more stereotyped, with less variable syllable sequencing and structure, is delivered at a faster tempo, and is preceded by more introductory notes (Kao and Brainard, 2006; Matheson and Sakata, 2015; Sakata et al., 2008; Woolley and Doupe, 2008).

For the male finch, the performance of FD song is crucial for reproductive success, as females respond preferentially to FD song over UD song (Sasaki et al., 2006; Woolley and Doupe, 2008). Hence, integrating external cues (i.e. the presence of a potential mate) to perform a more stereotyped version of song seems to be an essential function of the adult bird's song circuit.

The increased variability of UD song compared to that of FD song is hypothesised to indicate that UD song is a form of vocal motor exploration or "practice" (Woolley, 2016). Through UD song, the bird can explore a limited range of song structural and temporal variations from his repertoire and learn from his mistakes. Contrariwise, when the Bengalese finch sings towards the female, he is engaged in song "performance" (Woolley, 2016), wherein he decreases his vocal exploration and produces a precise, stereotyped song. While these singing behaviours have been thoroughly studied and described, much remains unknown about the neural mechanisms underlying their motor control.

1.4 Neural mechanisms underlying social-context dependent song production

Bengalese finches have an anatomically discrete and specialized neural circuit for song learning and production (i.e. the song circuit), comprising two major pathways: the song motor pathway (SMP) and the anterior forebrain pathway (AFP). The song system has a hierarchical organization, with SMP's premotor nucleus HVC (used as proper name) at the top of the hierarchy, projecting to the downstream motor nucleus RA (robust nucleus of the arcopallium) and to Area X, in the AFP**.** A simplified diagram of these pathways, displaying the main song nuclei, is shown in Figure 2.

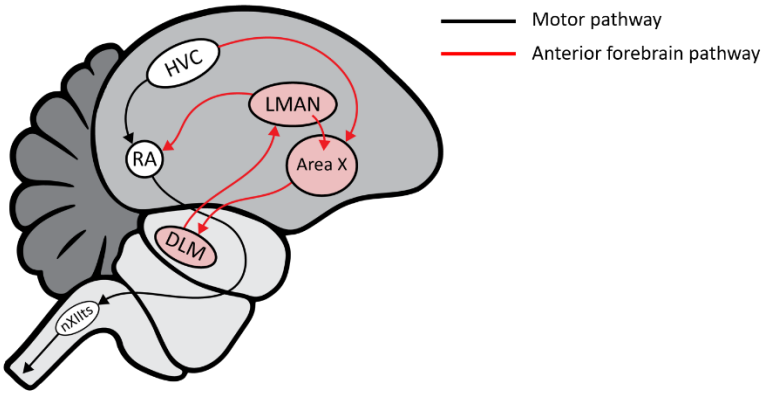

Figure 2. Schematic *diagram of a sagittal section of the brain depicting the song system pathways*

Broadly speaking, the SMP is mainly involved in song production, whereas the AFP underlies song learning in juveniles and song maintenance in adulthood (Ali et al., 2013; Aronov and Fee, 2012; Kao et al., 2015). Lesions within the SMP impair song production, leading to a disrupted song or entirely abolishing it (Simpson and Vicario, 1990; Thompson and Johnson, 2007). In contrast, lesions in the AFP do not degrade crystallized song, but reduce song variability and plasticity (Brainard, 2004; Hampton et al., 2009; Ölveczky et al., 2005). Several lines of evidence point towards a differential role of the SMP and AFP in controlling spectral and

temporal properties of the adult bird's crystallized song (Hampton et al., 2009; Kao et al., 2005; Sakata and Yazaki-Sugiyama, 2020). The global syllable sequence and song tempo is thought to be mainly generated from the SMP (Long and Fee, 2008; Zhao et al., 2019), while the AFP is believed to mainly encode and control the spectral features of song (Ali et al., 2013; Thompson et al., 2011; Xiao et al., 2018).

In top-down models of song production, HVC is central to establishing the precise timing of events in the motor sequence of song, functioning as a "neural clock" (Goldberg and Fee, 2010; Troyer, 2016). The concept is reflected in the sparse activity of the HVC_{RA} projection neurons, time-locked to individual syllables and bursting at a precise time in the song motif (Ali et al., 2013; Hahnloser et al., 2002; Long et al., 2010). In line with the idea that HVC controls song tempo, cooling HVC and thus directly changing neuronal dynamics, slows all temporal aspects of the song, from individual syllables to the motif (Long and Fee, 2008; Zhang et al., 2017). This multi-level decrease in song tempo hints that HVC globally controls all timescales of the song. Adding weight to this hypothesis, pharmacological manipulations in HVC change song tempo. Isola, Vochin and Sakata (2020) have shown that increasing inhibition in HVC by infusing muscimol, a $GABA_A$ agonist, increases the duration of stereotyped song sequence. Additionally, infusions of carbachol, an acetylcholine agonist, shortens sequence durations (Jaffe and Brainard, 2020). Taken together, this rich evidence highlights that HVC is strongly involved in controlling temporal aspects of song.

Additional evidence suggests that HVC activity might encode syllable sequencing through the activity of HVC neurons projecting to Area X (HVC_X neurons) song nucleus in the AFP. These HVC_X neurons burst during variable vocal sequences in Bengalese finches, and will burst selectively during transitions to certain syllables over others (Fujimoto et al., 2011), hence

potentially encoding sequencing. More evidence for the role of HVC in encoding syllable sequencing, comes from Zhang et al. (2017) who not only confirmed the role of HVC in controlling song timing but also used their cooling experiment to study HVC's role in song sequencing. Their evidence suggests that HVC activity might encode syllable sequencing because cooling HVC alters not only song tempo, but also the sequencing of syllables. Specifically, directly affecting HVC's neuronal dynamics in this way increases the transitions of syllable sequences, hence decreasing sequencing stereotypy (Zhang et al., 2017). The positive relationship between the observed decrease in song tempo and sequencing changes suggests that in HVC, neural mechanisms modulating syllable timing could similarly modulate syllable sequencing to affect song stereotypy. Interestingly, additional evidence also highlights the potential existence of neural mechanisms in HVC which independently regulate syllable sequencing and timing. Matheson and Sakata (2015) observed that the number of syllable transitions at branch points (i.e. "nodes" in song where stereotyped sequences have variable transitions) are negatively correlated with the duration of gaps between syllables. These types of changes independently affect song stereotypy, either increasing it or decreasing it, as is the case when the bird transitions from singing UD song to FD song and vice versa.

As previously mentioned, the activity of the song nuclei Area X and LMAN in the AFP, mainly regulate song spectral features. The hypothesis is resonated in extensive studies on pitch learning in adult birds (Ali et al., 2013; Andalman & Fee, 2009; Charlesworth et al., 2011; Warren et al., 2011). Although their song is crystallized, adult birds can learn to shift their pitch in response to white noise playback and to shift it back when the noise is no longer presented. However, birds with inactivated or lesioned LMAN are unable to learn pitch shifting and pitch recovery (Ali et al., 2013; Andalman and Fee, 2009; Charlesworth et al., 2011; Warren et al.,

2011). Similarly, birds with lesions in Area X are unable to learn pitch shifting but are not impaired in pitch recovery if the lesion occurs after pitch learning (Ali et al., 2013). Further evidence towards the role of AFP in encoding syllable structure comes from Hampton et al. (2009), who showed that lesions of LMAN eliminate the social modulation of the variability of syllable structure in adult Bengalese finches. This evidence also speaks towards the role of the AFP in regulating song variability in the adult songbird across social contexts. Indeed, after song crystallization, the activity and acoustic variability in AFP are higher during UD song than FD song (Jarvis et al., 1998; Sossinka and Böhner, 1980), with UD song displaying more variable spike timing (Kao et al., 2008). Substantiating evidence for the modulation in AFP activity across social contexts shows that LMAN lesions reduce the variability in UD song to levels found in FD song (Kao and Brainard, 2006). Moreover, LMAN and Area X not only display significantly reduced neuronal firing during FD song compared to UD song, but also a reduced variability in neuronal activity (Hessler and Doupe, 1999).

While this supports the notion that activity in the AFP underlies social context-dependent increase in adult song variability it is less clear how tempo and syllable sequencing are socially regulated.

1.5 The role of dopamine in the regulation of birdsong

We have seen that song production is controlled by the song system and highly influenced by social factors. However, less is known about how exactly social factors and song circuitry are linked in songbirds.

Song nuclei in the AFP and SMP receive strong dopaminergic inputs from catecholaminergic nuclei in the midbrain (Castelino et al., 2007; Gale et al., 2008; Lewis et al.,

1981). Dopamine is released from the ventral tegmentum (VTA) and substantia nigra pars compacta (SNc) onto neurons in Area X (Gale and Perkel, 2010; Goldberg and Fee, 2010; Saravanan et al., 2019). Additionally, dopaminergic neurons from the VTA/SNc and the periaqueductal grey (PAG) project to the SMP, reaching both HVC and RA; Figure 3).

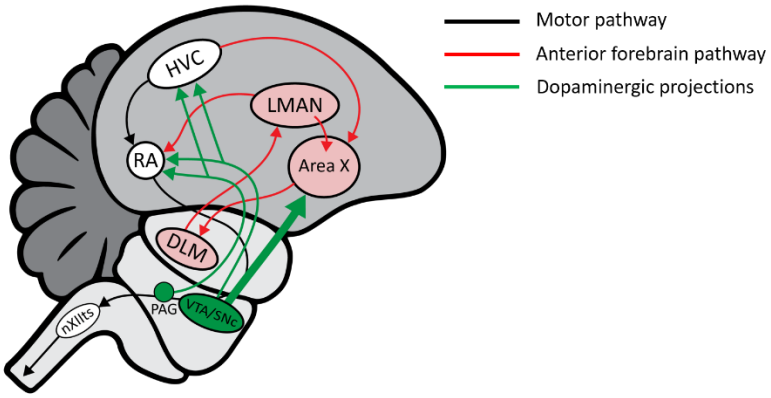

Figure 3. A simplified diagram of the dopaminergic input from the midbrain to the song nuclei.

During development, dopamine modulation underlies the integration of social context to promote song learning (Tanaka et al., 2018). For example, finches learn better from a live tutor, compared to passive tutoring. Correspondingly, dopaminergic neural activity is increased during live tutoring compared to passive tutoring (Chen et al., 2016). The PAG dopaminergic projections are also active during song learning and these projections are strongly activated by a singing tutor. Conversely, blocking dopamine signaling from the PAG to HVC prevents the production of the copied song (Tanaka et al., 2018). This indicates that the PAG-HVC dopaminergic inputs are central to integrating social cues in song learning. In all, the evidence strongly points to a central role of dopamine in incorporating social cues in song learning and production.

Several studies have also implicated dopamine as a key modulator of vocal motor control. Song stereotypy has been shown to be influenced by the activity of the VTA dopaminergic neurons projections. In adult birds, dopamine neurons in the VTA are more active during FD song production (Matheson and Sakata, 2015) and dopamine released from the VTA into Area X is higher during FD song production (Sasaki et al., 2006). Additionally, experimentally manipulating dopamine levels in the AFP leads to changes in song stereotypy. Specifically, infusion of dopamine in Area X reduces the neuronal firing variability and increases the stereotypy of the vocal production, whereas dopamine antagonists infused in Area X decreases song stereotypy (Leblois et al., 2010).

As in mammals, dopamine acts through D1- and D2-family receptors in songbirds, both of which are highly expressed throughout the song system (Kubikova et al., 2010). Similar to their modulatory activity in mammals, in songbirds D1- and D2- family receptors have opposite effects on the activity of the medium spiny neurons (MSN): D1 receptor activation enhances their activity, while D2 receptor activation reduces it (Kubikova et al., 2010; Simonyan et al., 2012). Since the MSN are the most numerous neurons in the striatum of songbirds (Ali et al., 2013; Mooney and Prather, 2005; Saravanan et al., 2019), dopamine is poised to be a powerfully modulating signal for vocal motor control in the striatal song nuclei. This is because in songbirds, HVC expresses both D1 and D2 dopamine receptors, with higher levels of D2 receptors than Area X (Kubikova et al., 2010), indicating that dopamine could differentially modulate neural activity in the song nuclei of the SMP and AFP and affect the stereotypy of the song in distinct ways. Indeed, it has been shown that adult birds lacking D1 receptors in Area X are unable to modulate their song stereotypy or to switch from UD to FD song (Murugan et al., 2013). Additionally, the neurons in Area X containing D1 receptors increase the immediate early

genes (IEG) expression when male zebra finches sing UD song but decrease it in neurons containing D2 receptors (Kubikova et al., 2010). Compellingly, electrophysiological recordings have shown that the HVC_{RA} projection neurons receive a constant stream of mainly depolarizing inputs from afferent and local excitatory sources (Hamaguchi and Mooney, 2012; Mooney, 2000). Given the sparse, burst firing of HVC projection neurons and the known role of D2 receptors in generally inhibiting neural activity, the role of dopamine in HVC shapes itself as a modulator of the incoming depolarizing activity, molding it into an efficient, precise output.

In all, studies of context-dependent singing have shown that striatal dopamine is critical to the modulation of cortico-striatal activity, leading to changes in vocal motor control. However, it is not yet clear how dopamine acting in the SMP modulates the various song parameters known to change between FD song and UD song.

Overview of study rationale

Matheson and Sakata (2015) demonstrated that systemic injections of amphetamines mimicked the effect of social context, leading to the production of UD songs with FD song characteristics. The UD songs produced following amphetamine administration were preceded by more introductory syllables and consisted of more stereotyped syllable sequencing and syllable structure. Because amphetamines elevate catecholamine concentrations in the brain, this study strongly indicates that catecholamines, including dopamine have a modulatory effect on vocal motor control. However, amphetamines increase not only dopamine levels in the brain but also norepinephrine and other neuromodulators; therefore, it remains unclear whether the observed song changes are mediated by dopamine. In addition, because amphetamines were administered systemically, the study did not allow for precise localization of the effect in the brain.

Here, I present two experiments conducted to further our understanding of the role of dopamine in vocal motor control. Based on the findings from Matheson and Sakata (2015) and the putative role of dopamine in birdsong motor control, we hypothesise that 1) dopamine increase in song nuclei changes vocal motor control towards the production of songs resembling FD songs and that 2) dopamine increase in the pre-motor nucleus HVC is involved in modulating song tempo and song sequencing. Our first aim was to analyse how peripheral administration of D1 and D2 receptor agonists affects the spectral and temporal properties of Bengalese finch song. We investigated the effects of either D1 or D2 receptor activation on song motor control because although they have been shown to have opposite effects on MSN activity (Kubikova et al., 2010), it is not yet known how this affects song properties. Specifically, it is not yet clear to what extent activation or inhibition of MSN in cortical areas can increase or decrease the stereotypy of different song features. Second, we aimed to localize dopamine's effect on song tempo and sequencing by centrally infusing a D1 receptor agonist into the pre-motor nucleus HVC. Ultimately, the knowledge derived from these experiments could lead to better treatments for speech disorders such as those accompanying Parkinson's disease. Since catecholamine deficiencies can underlie these speech difficulties, investigating the locus of dopaminergic action in motor control of sequenced movements like birdsong could be a stepping-stone towards the development of better treatments.

Chapter 2. Effects of peripheral injections of a D1 agonist (SKF81297) and D2 agonist (quinpirole) on song production

2.1 Introduction

The results obtained by Matheson and Sakata (2015) support the hypothesis that catecholamines, including dopamine contribute to social context-dependent vocal control modulation. The notion that dopamine is involved in the transition from undirected (UD) to female-directed (FD) song has been previously explored by Sasaki et al. (2006), showing that more dopamine is released in Area X during the production of FD song than UD song. These studies point to an important role of catecholamines such as dopamine in the modulation of vocal control in songbirds. However, the results of the studies do not clarify dopamine's role in modulating the various spectral and temporal changes observed during FD song.

In order to assess how elevated levels of dopamine affect vocal motor behaviour, we injected birds with a D1 receptor agonist, SKF81297 or with a D2 receptor agonist, quinpirole. We investigated their effects on song features known to be modulated by social context. Specifically, we assessed the effect of D1 and D2 receptor agonists on the variability of syllable structure, number of introductory notes, song tempo and sequence variability in Bengalese finches. We predicted that these manipulations would cause UD song to more closely resemble FD song.

2.2 Methods

Animals

Adult male Bengalese finches (n=18; ages: 5 - 20 months old) that were obtained from an outside breeder were used for this study. Birds were housed in same-sex group cages, on a 14:10 light:dark cycle and provided food and water *ad libitum*. All procedures were approved by the McGill University Animal Care and Use Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Testing conditions

Prior to the experiment, the birds were housed individually in sound-attenuating boxes, under the same light, water, and food conditions as in the group cages. Their vocalizations were recorded using an omnidirectional microphone positioned above their cage and a computerized, sound-activated recording system (Sound Analysis Pro v.1.04). To obtain a baseline song, the birds were recorded for 48-hrs pre-surgery.

Twelve birds received an injection of 30 µl of a low dose of SKF (1.0 mg/kg), a high dose of SKF (5.0 mg/kg) and vehicle (phosphate-buffered saline (PBS)). Both SKF doses were based on studies in other species showing significant effect on behaviour at doses in this range (Larkin et al., 2016; Pezze et al., 2016; Zahrt et al.,1997). The order of the injections was pseudorandomized and balanced based on a Latin square design. All injections were administered in the inguinal fold and at approximately the same time of the day to control for circadian variation in some song features (Kojima and Doupe, 2009). The injections for a given bird were separated by median = 10 ± 3.5 days.

In a separate pharmacological manipulation, 12 birds received 30 µl of a low dose of the D2 agonist quinpirole (0.1 mg/kg), a high dose of quinpirole (1.0 mg/kg) and vehicle. Six birds from this experimental condition were also part of the SKF injection group, with 3-12 months gap between the two manipulations. The doses of quinpirole were selected based on previous publications indicating a range of doses sufficient to induce behavioural changes in rodents (Lénárd et al., 2017; Messias et al., 2016; Péczely et al., 2016). The method of delivery and injection schedules were the same as those for SKF81297 injections.

Song analysis

Delimitation and labelling of song syllables were identical to those of previously published studies (James and Sakata 2014, 2015; Sakata et al. 2008; Sakata and Brainard 2006; Warren et al. 2012; Isola et al., 2020; James et al., 2020). Following amplitude-based syllable segmentation, syllables were manually labeled based on visual inspection of spectrograms using custom-written MATLAB software. Individuals labelling song were unaware of the experimental condition.

Similar to the approach used by Matheson and Sakata (2015) following peripheral injections of amphetamine, we aimed to label and analyse the first 30 songs produced following the injections of D1- and D2-receptor agonists. However, some birds did not produce 30 songs for the remainder of the day (3 out of 36 instances for SKF81297 and 6 instances out of 36 for quinpirole). In these cases, we labelled and analysed as many songs as the birds produced.

Following amphetamine administration, Matheson and Sakata (2015), found that syllable sequencing was significantly affected by the experimental treatment. To assess whether peripheral D1- and D2-receptor agonist administration also modulate sequencing, we analysed syllable sequencing at variable sequences of syllables called “branch points” (Okanoya and

Yamaguchi ,1997; Sakata and Brainard, 2006, 2008; Warren et al., 2012; Wohlgemuth et al., 2010). Typically, a branch point can have between two to five types of sequence transitions, and we examined the effect of SKF81297 and of quinpirole on variability changes in sequence transitions. The variability at a branch point is quantified by calculating the transition entropy. Branch points were independently identified and analysed in the songs following injections; this allowed us to identify sequences that could be variable under one condition (e.g., following saline) but stereotyped in another (e.g., following SKF). To compute transition entropy, we first computed the probability of different syllable transitions immediately following the branch point sequence. For each branch point, sequence variability was quantified as the transition entropy:

$$\text{transition entropy} = \sum -p_i \cdot \log2(p_i)$$

where the sum is over all possible transitions, and p_i is the probability of the i^{th} transition across all songs (Gentner and Hulse 2000; Gil and Slater 2000; James and Sakata 2014, 2015; Sakata et al. 2008; Sakata and Brainard 2006). Branch points with transitions that are more variable (i.e., closer to uniform probability) have higher transition entropy scores. Sequences in which the most prevalent (i.e., dominant) transition occurred 95% of the time (or more) under both drug and PBS conditions were not considered branch points. Instances in which song ended following the branch point were not included in the calculation of entropy, and only branch points that occurred $\geq$15 times were analysed to help ensure the reliability of estimates (Isola et al. 2020; James and Sakata 2014, 2015; James et al.,2020; Matheson et al. 2016; Matheson and Sakata 2015; Toccalino et al. 2016).

Introductory notes are short, low-amplitude vocalizations that are repeated before the onset of song. The number of times introductory notes are repeated before song onset is increased in FD song than in UD song (Hampton et al., 2009; Matheson et al., 2016; Sakata et al., 2008). To

calculate the number of introductory notes per song bout, we counted backward from the first introductory note preceding the first motif of a song bout until there was >500 ms of silence.

It has been shown that song tempo changes across social context and that FD songs have a faster tempo (Kao and Brainard, 2006; Sakata et al., 2008). We computed song tempo by calculating the duration of stereotyped sequences produced in the bird's song. The duration of these sequences was defined as the interval from the onset of the first syllable to the onset of the last syllable in the sequence. Only sequences produced n>10 times under both control and experimental conditions were included in the analysis.

To determine whether peripheral injections of D1- and D2-receptor agonists affected the acoustic structure of song, we computed the fundamental frequency (FF) of syllables. We focused on syllables with flat, harmonic structure because the median and variability of FF of such syllables are modulated by neural activity in focal circuits (Ali et al. 2013; Hampton et al. 2009; James and Sakata 2014, 2015; Kao and Brainard 2006; Kojima et al. 2018; Matheson et al. 2016; Sakata et al. 2008). We defined the FF as the distance, in Hz, between the zero-offset peak and the highest peak in the autocorrelation function. To compute the FF, we calculated the autocorrelation of a segment of the sound waveform. To improve the resolution and accuracy of frequency estimates, we performed a parabolic interpolation of the peak of the autocorrelation function. The syllables were screened to ensure that only examples devoid of sound artifacts were analysed, and only syllables that were produced >10 times in both control and experimental conditions were included in the analysis.

Statistical analysis

Our experimental design was a within-subject design with song performance on a day of drug infusion compared to the song performance by the same bird on the PBS (control) day

(Andalman and Fee 2009; Hamaguchi and Mooney 2012; Leblois and Perkel 2012; Stepanek and Doupe 2010; Tanaka et al. 2016). We computed and analysed the median of temporal and spectral song features since medians are less affected by extreme values than other measures of central tendencies. Similarly, we computed and analysed the interquartile region (IQR) normalized by the median ("normIQR") as our measure of variability because the IQR is less affected by extreme values than other estimates of variability like standard deviation (James and Sakata, 2015).

We used mixed effects models to investigate the effect of SKF81297 and of quinpirole on song motor control. This model was chosen to account for the fact that each bird served as their own control (Andalman and Fee, 2009; Charlesworth et al., 2012; Hamaguchi and Mooney, 2012; Sasaki et al., 2006; Stepanak and Doupe, 2010; Tanaka et al., 2016; Warren et al., 2011). For all analyses, the fixed effect was dose. When features for multiple syllables (FF) or for multiple sequences (sequence durations or branch points) were measured across control and experimental conditions within the same bird, syllable ID nested within bird ID or sequence ID nested within bird ID was the random variable. Otherwise, bird ID was the only random variable. This is a powerful approach because acoustic features are heterogeneous across syllable types (e.g., some syllables have high FFs, some have low FFs) or sequences (e.g., some sequences are longer than others), hence including syllable or sequence ID as a factor removes these sources of variation.

All statistics were computed using JMP Pro 13.0 (SAS, Cary, NC), with $\alpha=0.05$ for all analyses. Planned post-hoc contrasts were computed for all cases where $p < 0.05$.

Despite that we analysed raw data, we plotted the change in median and normIQR values of song features to visualize the effect of dopamine agonists (proportion change for median or

difference for normIQR) from the control (saline) day. Python's Seaborn data visualization module was used to create swarmplots and boxplots for visualization.

2.3 Results

Effects of peripheral D1 agonist (SKF81297) on the variability of syllable sequencing

Figure 4a provides an example of how transition entropy values change between experimental and control days for one Bengalese finch. Under control conditions, the branch point sequence "gbcdef" can transition to "x", "a" or "k". The transition to "x" has the highest probability of occurrence, and this probability increased at both doses of the D1 agonist, thus increasing song stereotypy. The calculated transition entropy, encompassing the transition probabilities was lower for both SKF81297 doses compared to vehicle, in a dose-dependent manner for this bird. This pattern of changes to syllable sequencing was consistent across birds; consequently, we observed that peripheral injections of SKF81297 significantly affected the branch points transition entropy ($F_{2,106}$=5.5, p=0.0052; Figure 4b). Branch points had significantly lower transition entropies following administration of both 1 and 5 mg/kg dose of SKF relative to their transition entropies following vehicle injections (Tukey's HSD, $p<0.02$ for both).

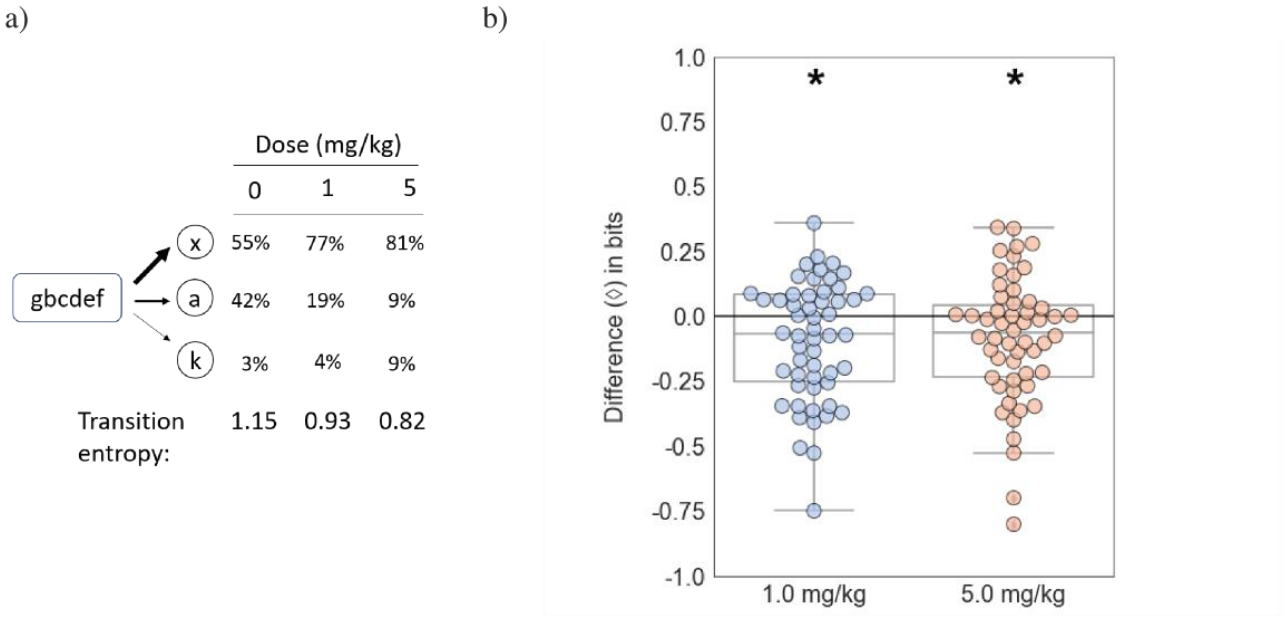

Figure 4. Change in syllable sequencing after peripheral administration of 1 mg/kg and 5 mg/kg SKF, from baseline. a) Example of a branch point's transition entropy value for vehicle and experimental days. Transition entropy decreased at 1 mg/kg and 5mg/kg SKF, indicating a reduction in the variability of syllable sequencing. b) Plot of the difference in transition entropy from baseline at 1 and 5 mg/kg dose of SKF. "" denotes $p<0.05$*

Additionally, there was a trend for SKF81297 to increase the number of introductory notes preceding song ($F_{2,22}=3.2$, $p=0.0607$; Figure 5). Both the 1 mg/kg and 5 mg/kg dose of SKF81297 increased the median number of introductory notes by ~10% ($p = 0.0865$ for the low dose and $p = 0.1062$ for the high dose).

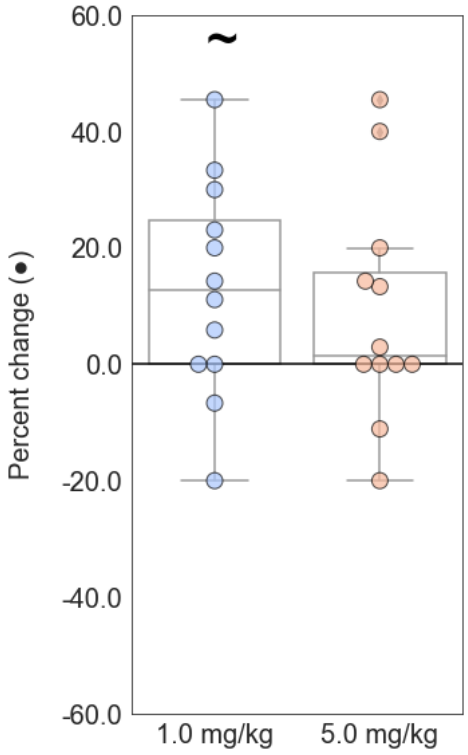

Figure 5. Change in the number of introductory notes preceding song after peripheral administration of 1 mg/kg and 5 mg/kg SKF81297. The percent change was calculated by subtracting the median number of introductory notes under SKF conditions from the median number of introductory notes under PBS conditions, divided by the median under PBS conditions and multiplied by 100. "~" denotes p <0.10

Effect of peripheral D1 agonist (SKF81297) administration on song tempo

We analysed song tempo by computing the median duration of stereotyped sequences within the Bengalese finch song, and overall, peripheral SKF81297 injections did not significantly affect song tempo ($F_{2,22}$=1.3, p=0.2940; Figure 6).

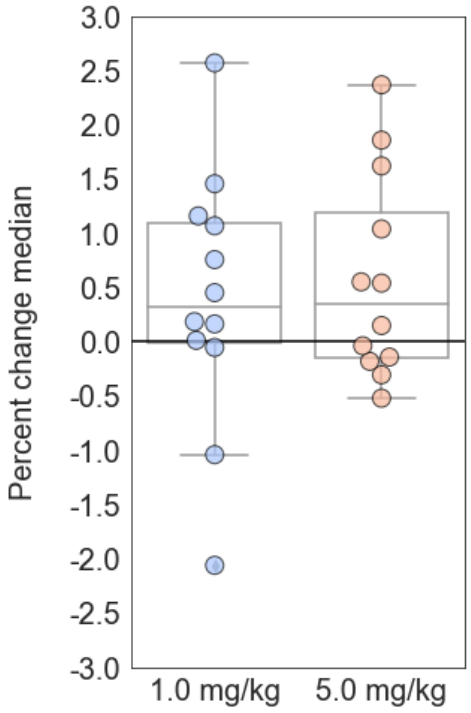

Figure 6. Plot of the percent change in the median duration of stereotyped sequences. Each data point represents a sequence produced by a bird. For all birds, several stereotyped sequences were analysed and are all shown in the plot. The change was calculated by subtracting the average of the string duration measures under SKF conditions from the average of the string duration measures under PBS conditions, divided by the average under PBS/PBS+DMSO conditions and multiplied by 100.

Effect of peripheral D1 agonist (SKF81297) administration on syllable structure

Finally, we analysed the effect of the D1 agonist on the variability of fundamental frequency (FF) of syllables with flat, harmonic structure. We found that peripherally injecting SKF81297 did not significantly affect the variability of syllable structure ($F_{2,84}$=1.5, p=0.2228) nor the median of the FF ($F_{2,84}$=1.8, p=0.1687).

Overall, these results suggest that peripheral administration of D1 receptor agonist, leading to increased dopaminergic transmission in song nuclei mimics some of the social effects on birdsong stereotypy. Specifically, it modulates the stereotypy of syllable sequencing and the

number of introductory notes preceding song. However, no modulatory effect is apparent of the stereotypy of song tempo or syllable structure through this specific dopaminergic manipulation.

Effects of D2 agonist quinpirole on song performance

The peripheral administration of quinpirole did not significantly impact any of the analysed song features, raising the possibility that song stereotypy is modulated by dopamine mainly through the D1 receptor. Specifically, there was no significant effect on sequence variability at branch points ($F_{2,101} = 0.2$, $p = 0.8821$), the repetition of introductory notes preceding song ($F_{2,21} = 1.4$, $p=0.2628$), song tempo ($F_{2,21} = 0.3$,$p = 0.7753$), median FF ($F_{2,65} = 2.6$, $p = 0.0590$) or the variability of FF ($F_{2,65} = 1.8$, $p = 0.1746$).

Chapter 3. Effects of central infusion of D1 agonist (SKF81297) in HVC

3.1 Introduction

In songbirds, dopamine plays a primary role in courtship song directed towards females. Song produced in a social context appears to be highly rewarding, as dopamine levels are elevated in the striatum of birds during directed singing (Sasaki et al., 2006). Specifically, dopamine levels in Area X, a song nucleus in the anterior forebrain pathway (AFP) are higher during female-directed (FD) song than undirected (UD) song (Sasaki et al., 2006). Moreover, infusions of dopamine antagonists in Area X increases variability during FD song (Leblois et al., 2010), hinting that dopamine may function as a regulator of AFP activity. We also know that the AFP encodes spectral song properties, as demonstrated from studies on pitch shifting in adult songbirds (Charlesworth et al.,2011; Warren et al.,2011). This hints to a potential effect of dopamine in the AFP to modulate the variability of spectral song properties between FD and UD song. However, it is not yet clear where dopamine exerts its effects on vocal motor control to also affect the temporal properties of FD song compared to UD song.

In Chapter 2, we peripherally administered a D1 receptor agonist to test the hypothesis that dopamine increase mimics social-context effect on song production. Although we found similar changes to syllable sequencing as those observed in FD song, the results did not speak to a localization of the effect of dopamine to a specific area in the song system. Here we manipulated dopamine levels in the pre-motor nucleus HVC to further assess dopamine's role in modulating vocal motor control of spectral and temporal song features between FD and UD song.

3.2 Methods

Animals

We assessed the effects of D1 agonist infusions into HVC in male adult Bengalese finches (n=11; ages: 1.9-4.1 yrs old) that were born and raised in our breeding colony at McGill University, where they were housed in same-sex group cages, on a 14:10 light:dark cycle and provided food and water *ad libitum*. For the experiment, the birds were housed individually, in sound-attenuating boxes, under the same circadian and feeding conditions as in the colony. The birds were recorded using an omnidirectional microphone positioned above their cage and a computerized, sound-activated recording system (Sound Analysis Pro v.1.04). To obtain a baseline song, the birds were recorded for 48-hrs pre-surgery. All procedures were approved by the McGill University Animal Care and Use Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Cannulae implantation in HVC

Adult Bengalese finches were pre-anesthetized with ketamine (0.02 mg/g; IM) and midazolam (0.0015 mg/g; IM). Gaseous isoflurane (0.2–4.0% in O2) was administered to induce and maintain anesthesia. Birds were placed in a stereotaxic device for surgery. After lidocaine was injected under the scalp, the scalp was dissected along the midline, and a craniotomy was made over HVC (0.2 mm rostral, 1.9-2.1 mm lateral from the caudal edge of the bifurcation of the midsagittal sinus). Guide cannulae with dummy probes (Harvard Apparatus, Hollison, MA, CMA 7) were lowered until they just penetrated the brain and were then fixed to the bird's skull with epoxy and dental cement. After 5-7 days of recovery, microdialysis probes (Harvard Apparatus, Hollison, MA, CMA 7) were inserted into the guide cannula. CMA 7 microdialysis

probes consist of a reservoir that is connected to an inlet and outlet tube. Inlet and outlet tubes were cut to 4-6 cm in length and outfitted with a cap to allow for fluid to fill the reservoir to allow for passive drug diffusion while the birds were being recorded unrestrained (Andalman and Fee 2009; Hamaguchi and Mooney 2012; Tanaka et al. 2016; Isola et al., 2020).

Insertion of probes into the brain can cause transient changes to song production as the neural tissue experiences some compression. Until the song recovered to resemble baseline levels, both probes were filled daily with 40 μL of 0.025M phosphate-buffered saline (PBS; pH=7.4) using a syringe pump (Harvard Apparatus, Pump 11 Elite) to prevent clogging.

Central infusion of D1-receptor agonist (SKF81297) in HVC

To assess the contribution of dopamine in HVC to vocal control, birds were infused with different doses of SKF (0.2 ,0.4 ,0.5 ,1.0 and 3.0 mM) dissolved either in sterile PBS or in sterile PBS + DMSO ("vehicle") and the dosing was established in a pseudo-random manner. These concentrations have been used to manipulate neural activity in various regions of the songbird and rodent brain (Kelley and Delfs, 1991; Larkin et al., 2016; Leblois et al., 2010; Pezze et al., 2016; Zahrt et al., 1997). The SKF infusion days were interleaved with days of vehicle infusions. Each pair of the vehicle and corresponding SKF dose was considered a "block" (see below for analysis). Birds were infused at similar times of day after the lights came on to control for circadian variation in some song features, including song tempo (Kojima and Doupe, 2009). As studies suggested that the infused drug effects become variable after four hours (Andalman and Fee, 2009; Naie and Hahnloser, 2011), only audio recordings during the four hours between the infusion and wash-out ("experimental period") were analysed.

Tissue collection and histology

At the end of the experiment, experimental birds were deeply anaesthetised with isoflurane vapor. They were then transcardially perfused with 25 ml of heparinized saline (100 IU/mL) followed by 150 mL of 3.7% formalin or 4% paraformaldehyde (pH=7.4). Brains were removed, post-fixed for at least 4 hrs at 4°C in 3.7% formalin or 4% paraformaldehyde and then transferred to a 30% sucrose solution at 4°C for cryoprotection. Sagittal sections were cut at 40 µm using a freezing microtome (Leica Biosystems, Wetzlar, Germany) and stored in 0.025M PBS with sodium azide at 4°C until processing. Brain sections were mounted and stained with cresyl violet to visualize and confirm the location of probes and cannulae.

Song analysis

The song features analysed were the same as those described in chapter 2 *"Song analysis"* section (i.e. variability of syllable sequencing, introductory notes, song tempo and variability of syllable structure), using the same analysis method. However, as previously described, here we analysed all songs produced in a 4-hour "experimental period" after SKF infusion in HVC, rather than the first 30 songs. Additionally, we further expanded our analysis of song tempo by computing the effect of SKF on gaps and syllables durations. As in our previous analysis on the effects of the peripherally administered SKF, we only analysed branch points that occurred $\geq$15 times and stereotyped sequences produced n>10 times under both control and experimental conditions.

Statistical analyses

Our experimental design was a within-subject design with song performance on a day of drug infusion compared against song performance by the same bird on the adjacent (generally preceding) control day (Andalman and Fee, 2009; Hamaguchi and Mooney, 2012; Isola et al., 2020; Leblois and Perkel, 2012; Stepanek and Doupe, 2010; Tanaka et al., 2016). Just as for the data analysis from the peripheral injection of SKF (see Chapter 2 *"Statistical analyses"*), we used mixed-effects models to analyse the effect of SKF on song. The effect of SKF was not different when PBS or PBS+DMSO was used as the vehicle, so this was removed from the model to streamline the analysis. The pairs of control PBS and corresponding SKF dose were defined as a "block" and used as a random factor in each statistical model. We used syllable ID or sequence ID nested within bird ID as the random variable to analyse FF, sequence durations and branch points. All statistics were computed using JMP Pro 13.0 (SAS, Cary, NC), with $\alpha=0.05$ for all analyses. Planned post-hoc contrasts were computed for all cases where $p < 0.05$.

To visualize the effect of SKF, we plotted the change in the median and normIQR of song features under SKF conditions (proportion change for median or difference for normIQR) from the control (saline) day. Python's Seaborn data visualization module was used to create swarmplots and boxplots for visualization.

3.3 Results

Effects of D1 agonist (SKF81297) infusion in HVC on the variability of syllable sequencing

Mixed model effects indicated that SKF does not have a significant effect on branch point (BP) entropy ($F_{5,64} = 0.2$, $p = 0.9749$; Figure 7).

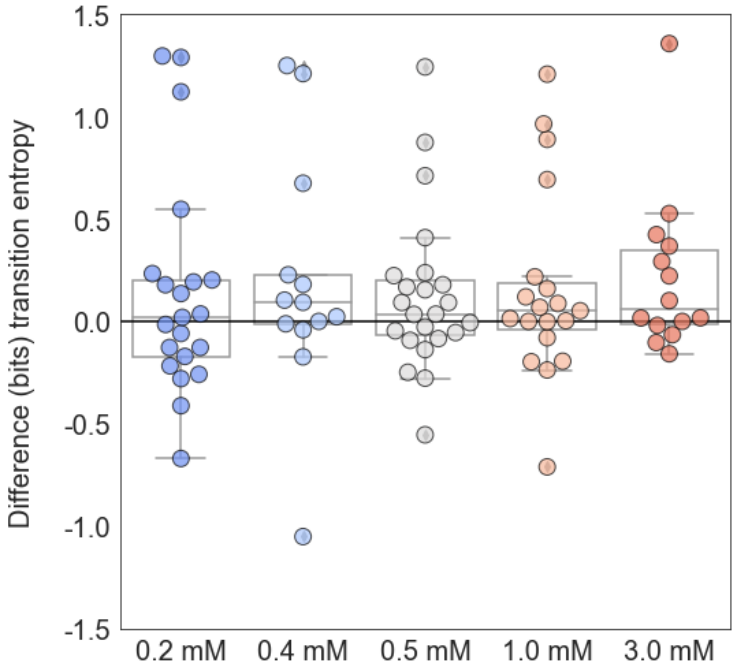

Figure 7. Plot of the change from baseline of the transition entropy at branch point syllables, across SKF doses, for each bird. Multiple branch points were analysed per bird and are all shown in the plot. The change was calculated by subtracting transition entropy under SKF conditions from transition entropy measured under PBS conditions, divided by the average under PBS/PBS+DMSO conditions and multiplied by 100

Another song feature analysed was the number of introductory notes preceding song bouts, which are more numerous in FD song than in UD song. We found that the median number of introductory notes ($F_{5,53} = 0.8$, $p = 0.5244$; Figure 8) was not affected by intracranial infusions of the D1 agonist.

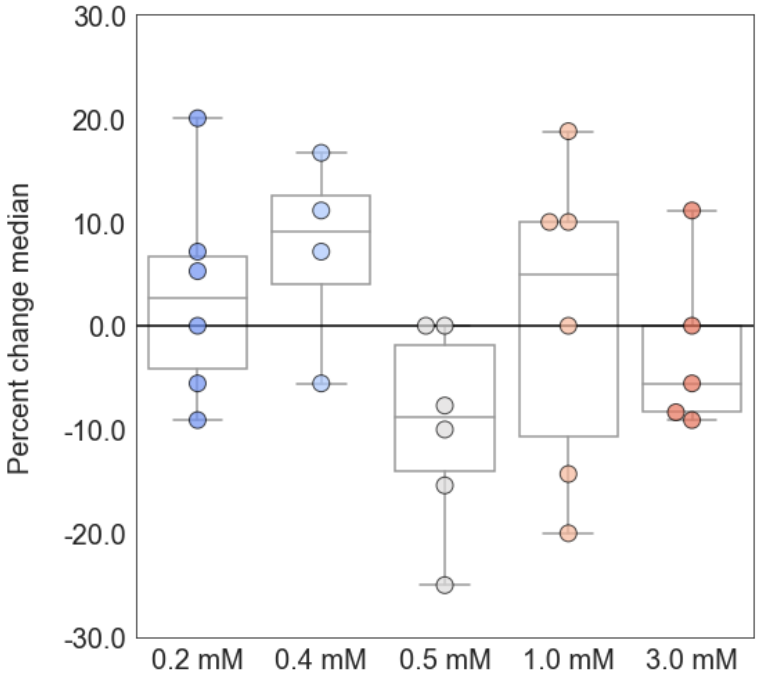

Figure 8. Plot of the percent change in the average number of introductory syllables sung by each bird after SKF infusion in HVC across SKF doses. The change was calculated by subtracting the average number of introductory notes under SKF conditions from the average number of introductory notes under PBS/PBS+DMSO conditions, divided by the average under PBS/PBS+DMSO conditions and multiplied by 100.

Effect of D1 agonist (SKF81297) infusion in HVC on song tempo

Figure 9a provides an example of the changes to song tempo observed after the central infusion of the D1 agonist SKF81297 in HVC. A stereotyped sequence sang by one bird under both control and experimental conditions had a shorter duration after SKF infusion. This pattern was observed across all birds and SKF significantly affected the average duration of stereotyped sequences of syllables ($F_{5,140} = 4.6$, $p = 0.0006$; Figure 9b). Our findings parallel the well-documented FD song characteristic of high tempo delivery, with short stereotyped sequence durations (Jarvis et al., 1998; Kao and Brainard, 2006; Sakata et al., 2008). Further investigation using planned contrasts revealed that sequence duration was significantly decreased by the 0.5

mM (Tukey's HSD; p = 0.0004) and 1.0 mM doses (Tukey's HSD; p=0.0023). To verify that the increase in song tempo was not influenced by variation of stereotyped sequence durations on the control days for 0.5 mM and 1.0 mM, we compared sequence duration on all control days. However, we found no significant difference in the stereotyped sequence durations between the control infusion days ($F_{1,18}$ = 1.7, p = 0.2090), indicating that the effect found on song tempo was not due to variations of control days measurements.

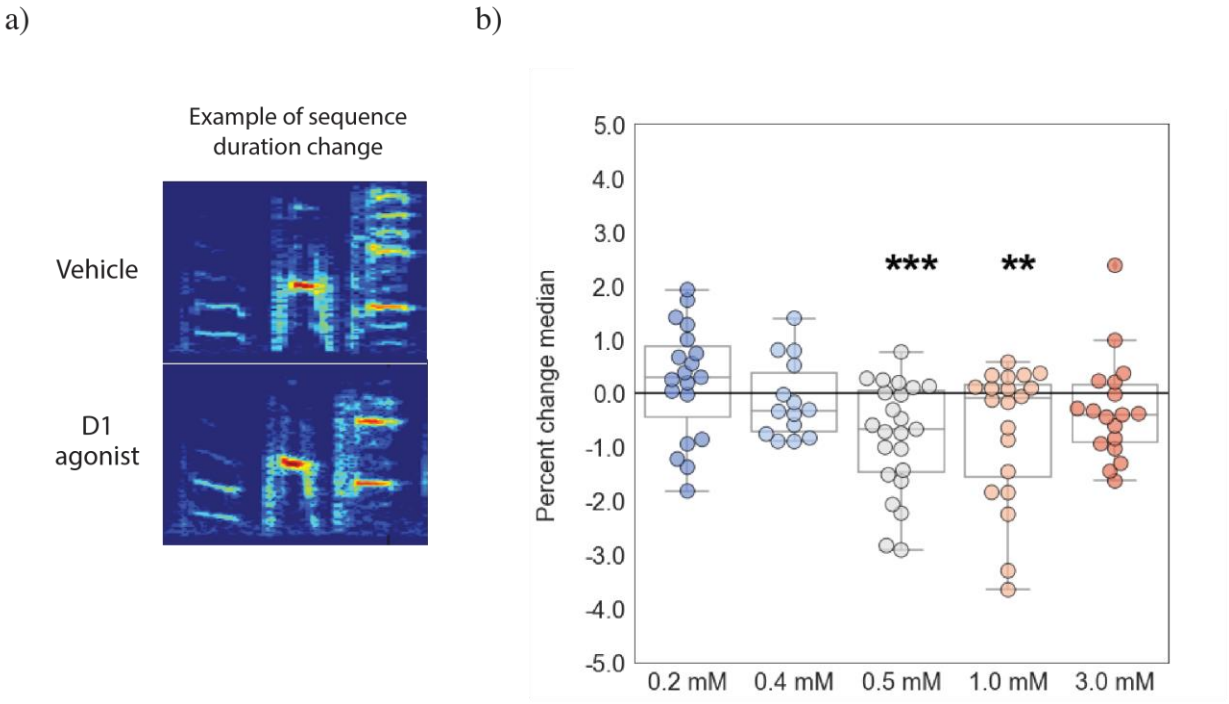

Figure 9. Tempo change from baseline after central infusion of D1 agonist in HVC. a) Example of a stereotyped sequence sung under experimental and control days. The sequence duration is shorter after SKF infusion than after PBS. B) Plot of the percent change of the median duration of stereotyped sequences sung by each bird across SKF doses. Each data point represents a sequence produced by a bird. For all birds, several stereotyped sequences were analysed and are all shown in the plot. The change was calculated by subtracting the average of the string duration measures under SKF conditions from the average of the string duration measures under PBS/PBS+DMSO conditions, divided by the average under PBS/PBS+DMSO conditions, multiplied by 100.

We further investigated whether the decrease in sequence durations was driven by a decrease in syllable durations and/or gaps duration within stereotyped sequences. We found that syllable durations significantly decreased ($F_{2,49} = 5.9$, $p = 0.0052$) at the 0.5 mM (Tukey's HSD; $p = 0.0053$) and 1.0 mM doses (Tukey's HSD; $p = 0.0083$; Figure 10), but gap durations were not significantly affected ($F_{2,49} = 2.2$, $p = 0.1238$; Figure 10).

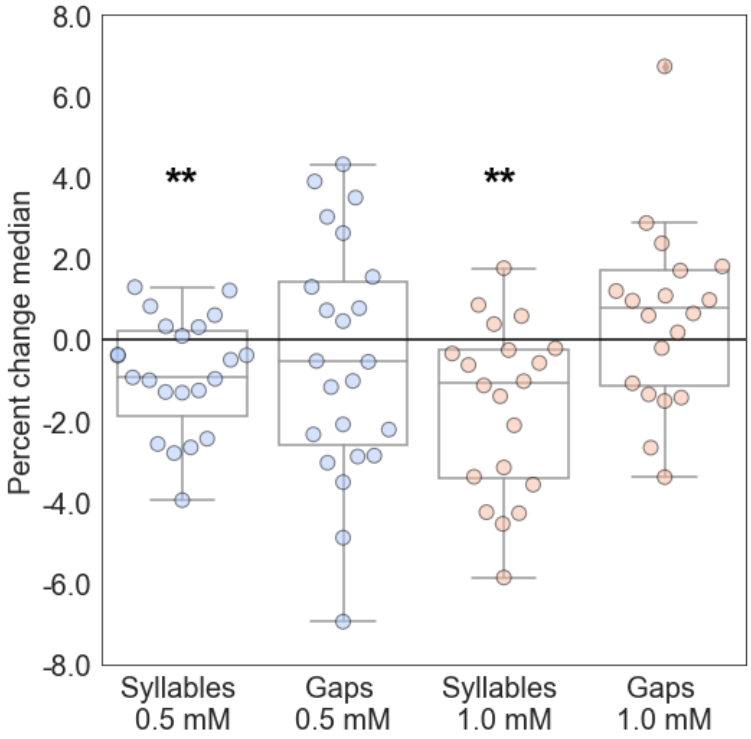

Figure 10. Plot of percent change in the median duration of the syllables and gaps within stereotyped sequences, at 0.5 mM and 1.0 mM SKF. The change was calculated by subtracting the average of the string duration measures under SKF conditions from the average of the string duration measures under PBS conditions, divided by the average under PBS conditions.

Effects of D1 agonist (SKF81297) infusion in HVC on syllable structure

Similar to the results of the peripherally administered D1 agonist, intracranial infusion of SKF81297 in HVC did not significantly affect the variability of the FF of flat harmonic syllables ($F_{5,232} = 1.6$, $p = 0.1617$) nor their median ($F_{5,232} = 1.3$, $p = 0.2734$).

Overall, our results suggest that dopamine signalling through the D1 receptor in the pre-motor nucleus HVC has an important role in modulating the stereotypy of song tempo, by reducing the duration of syllables in stereotyped sequences. However, it does not seem to primarily modulate the stereotypy of the other FD song characteristics (i.e. syllable sequencing, syllable structure) or the number of introductory notes.

Comparing the effect of systemic vs. intracranial SKF injections

A number of findings in this chapter contrast with those reported in Chapter 2, wherein the effect of peripheral injections of SKF was analysed. For example, peripheral administration of the D1 agonist SKF81297 significantly decreased transition entropy at branch points and tended to increase the production of introductory notes, but central administration of this same agonist into HVC did not. Conversely, song tempo was significantly increased after central infusions of the D1 agonist into HVC, but not after its peripheral administration. One potential source for the non-overlapping effects could be due to differences in the time window in which songs were labelled and analysed. In particular, in Chapter 2 we analysed the first 30 songs after the injection (to be consistent with previous analyses of systemic drug administration: Matheson and Sakata, 2015), and most songs were produced within 1 hr after injection. In contrast, data in this chapter came from songs analysed within 4 hrs after infusion (to be consistent with previous analyses of pharmacological manipulations in the song system: e.g., Isola et al., 2020). To assess

the degree to which differences in the timing of the songs analysed (relative to administration) accounts for differences between experiments, we further investigated the effect of SKF in the first hour after infusion in HVC. Similar to our previous analyses, to assess the effect of SKF on these song parameters we used mixed-model effects with SKF dose as the fixed effect.

Consistent with our previous analysis of the entire 4-hour experimental period, infusions of SKF into HVC did not significantly affect transition entropy in the first hour after infusion ($F_{6,111}$ = 0.9, p = 0.5047) or the median number of introductory notes produced in the first hour after the infusion ($F_{5,36}$ = 0.5, p = 0.7715).

Also consistent with the song analysis over the 4-hour period, we found that song tempo is significantly increased in the first hour after the SKF infusion in HVC ($F_{2,47}$ = 7.7, p = 0.0013; Figure 11). Specifically, both the 0.5 mM (Tukey's HSD ; p = 0.0251) and the 1.0 mM dose of SKF (Tukey's HSD; p = 0.0054) decrease the median duration of stereotyped sequences.

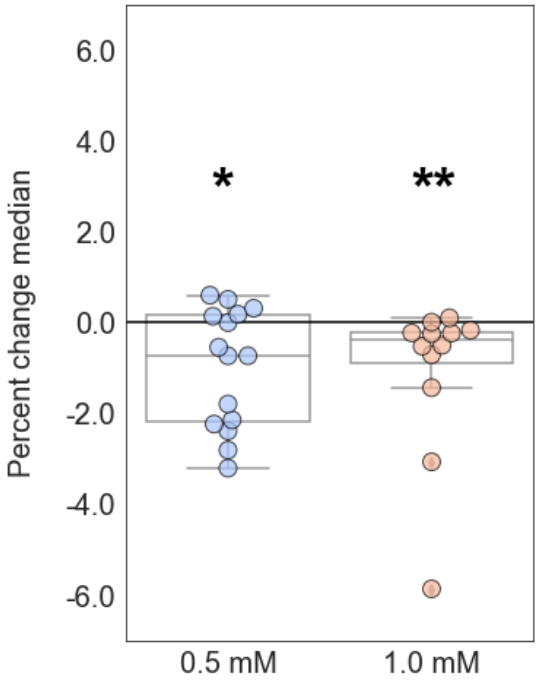

Figure 11. Plot of the change from baseline in the median duration of stereotyped sequences, across 1-hour blocks. Each data point represents a sequence produced by a bird. For all birds, several stereotyped sequences were analysed and are all shown in the plot. The change was calculated by subtracting the average of the string duration measures under SKF conditions from the average of the string duration measures under PBS/PBS+DMSO conditions, divided by the average under PBS/PBS+DMSO conditions, multiplied by 100.

Chapter 4. Discussion

Several lines of evidence strongly indicate that dopamine underlies the changes in vocal motor control for the production of song in different social contexts (i.e. UD and FD song) (Leblois et al., 2010; Matheson and Sakata, 2015; Sasaki et al., 2006). However, it is not yet clear which of the varying song features between the two types of song are directly modulated dopamine. We analysed the effect of SKF81297, a D1 receptor agonist, on four song features well-documented to change between UD and FD song (Kao et al., 2005; Sakata et al., 2008): the variability of syllable sequencing, the number of introductory notes, song tempo and the variability of syllable structure. We found that peripheral and central administration of the D1 agonist SKF81297 differentially mimicked aspects of social-context effect on song organization. The peripheral injection of the D1 receptor agonist SKF81297 significantly decreased the variability of syllable sequencing and tended to increase the number of introductory notes. Conversely, central infusion in the pre-motor nucleus HVC significantly increased song tempo but did not affect syllable sequencing.

Similar to human speech, birdsong organizes fundamental acoustic elements into sequences. In Bengalese finches, these sequences retain some variability of the transitions between syllables into adulthood. It has been shown that syllable transitions occur according to probabilistic neural firing sequences in the sensory-motor system (Bouchard and Brainard, 2013). Compellingly, pharmacological manipulations which can directly change the neuronal dynamics in HVC in some way have been shown to affect the probability of sequence transitions. For example, increasing the inhibition in HVC increases the variability of syllable sequencing at branch points (Isola, Vochin and Sakata, 2020), whereas increasing the amount of acetylcholine in HVC decreases this variability (Jaffe and Brainard, 2020). These results suggest that HVC

could be directly involved in encoding the adult birdsong sequence variability. However, we found that directly manipulating dopamine levels in HVC did not affect the variability of syllable sequencing yet increasing dopamine levels throughout the brain significantly decreased syllable sequencing at branch points. Added to the existing corpus of knowledge (Hamaguchi and Mooney, 2012; Tanaka et al., 2018), our results indicate that there could be different loci of control for sequencing variability in the brain of Bengalese finches, each mainly modulated by different neurotransmitters. Such divergence in the control of a song feature could be justified in terms of evolutionary fitness. As song production with specific acoustic parameters carries a high reproductive fitness value, it is feasible that specific song features are redundantly encoded to prevent immediate loss in case of neural damage. However, given the rapid changes to motor control in birdsong, such redundancy would be more likely to be encoded in the same population of neurons, potentially located in HVC.

Hence, another possibility explaining our results is that HVC is the locus of control for the variability of syllable sequencing, but dopamine acting only locally in HVC is not the immediate modulator for this song property. HVC contains a large population of inhibitory GABA interneurons (Armstrong and Abarbanel, 2016; Mooney and Prather, 2005) and the previously mentioned manipulation of GABA and acetylcholine modulation in HVC could act on these inhibitory interneurons to directly affect neuronal dynamics in HVC. However, in songbirds, dopamine mainly acts on the medium spiny neurons (Kubikova et al., 2010; Simonyan et al., 2012) and could act as a gating signal in HVC, preparing neurons to respond in a specific way. Yet, it might require synergistic action of dopamine in another song nucleus to modulate synaptic input in HVC. This synergistic activity could be required to shift synaptic weights and change the firing probability of specific HVC neurons, causing a change in

sequencing variability. Indeed, dopamine has been shown to act as a gating signal in sensorimotor systems for simple behaviours like prepulse inhibition (PPI), across species, from larval zebra fish (Burgess and Granato, 2007) to rodents (Ralph-Williams et al., 2003; Swerdlow et al., 2001) and humans (Castellanos et al., 1996; Laruelle et al., 2003). In mice, sensorimotor gating of the PPI is disrupted by apomorphine or quinpirole, a D2 receptor agonist and is reversed by D2 receptor antagonists (Ralph-Williams et al., 2003). Conceivably, dopamine acts on "gatekeeper" neurons to regulate the flow of sensory information and modulate moment-to-moment motor decisions. Interestingly, some studies found that peripherally administered dopamine agonists disrupted PPI significantly more than when centrally infused (Swerdlow et al., 2016; Swerdlow et al., 1990; Swerdlow et al., 1992). In songbirds, dopamine has been shown to modulate the excitability of the projection neurons in RA (Liao et al., 2013) and to modulate their transmission at excitatory synapses (Wang et al., 2020), indicating that it can act as a gating signal for the incoming and outgoing neural activity.

Additionally, dopamine has been shown to require synergistic action on either both types of dopamine receptors (Robertson, 1992) or between different populations of dopamine neurons located in different brain areas (Heymann et al., 2020) to induce behavioural changes. In songbirds, dopaminergic population of neurons located in the SMP and the AFP could show such synergy, with each population modulating the flow of information in a specific way. Tian and Brainard (2017) inactivated the AFP and revealed a hierarchical organization of AFP and SMP. They showed that the SMP encodes a moment-to-moment representation of a syllable, while the AFP modulates this representation through top-down biasing signals. Potentially, dopamine acting in the AFP could be a major contributor to the biasing signal. Additionally, computational models have shown that dopamine acting in the cortex sharpens the tuning of neurons (Holca-

Lamarre et al., 2017) potentially offering support to dopamine's role as a "readying" signal. A potentially synergistic action of dopamine or dopamine acting as a gating signal in HVC aligns with our results. Such activity could explain the significant change in the variability of syllable sequencing after peripheral administration of SKF, which affects all song nuclei, but not after local infusion in HVC.

Of course, we must also consider that our pharmacological manipulation in HVC did not fully simulate the effect of endogenous dopamine, which could act both on D1 and D2 receptors in HVC. After peripherally administering quinpirole, a D2 receptor agonist, we found no significant effect on any song feature analysed, indicating that dopamine could exert its effect in HVC mainly through the D1 receptor. Given that HVC expresses significantly higher levels of D2 than of D1 (Kubikova et al., 2010), quinpirole's lack of effect is quite intriguing. However, we also did not find an effect on syllable sequencing after infusing D1 receptor agonist in HVC. Hence, we cannot discount that in HVC, dopamine's synergistic action on both types of receptors could be necessary to shift synaptic weights, change the firing probability of neurons encoding specific syllables and significantly change the variability of syllable sequencing.

Alongside syllable sequencing, another song feature known to change in FD song renditions compared to UD song is the increased number of introductory notes preceding song. We found no significant effect of the peripheral or central administration of SKF81297 on the number of introductory notes. However, peripheral injection of SKF tended to increase the number of repeated syllables introducing song. This finding somewhat parallels that from Matheson and Sakata (2015), where peripheral administration of amphetamines significantly increased the number of introductory notes. Since amphetamines increase norepinephrine as well as endogenous dopamine, it is feasible that dopamine acting through the D1-receptor alone does

not fully modulate the motor control of this song parameter. The repetition of syllables, such as introductory notes, is a form of repetitive motor behaviour. In mice and rats, different repetitive behaviours such as compulsory grooming, circling or gnawing have been shown to result from different patterns of activation of the D1 and D2 receptors. Microinjection of a D1 receptor agonist into the striatum increases circling behaviour in mice (Ishiguro et al., 2007), D2 receptor agonists induce repetitive sniffing and licking in rats (Canales and Graybiel, 2000; Delfs and Kelley, 1990), while apo-morphine, a non-selective dopamine agonist induces compulsory gnawing (Ernst and Smelik, 1966). This divergent action of dopamine onto the D1 and D2-class receptors to induce specific types of repetitive behaviours could also occur in Bengalese finches. The increased repetition of introductory notes could occur only when both types of dopaminergic receptors are activated, as was noted by Matheson and Sakata (2015) after injection of amphetamines. Additionally, similar to the potential control of syllable sequencing, a synergistic role of dopamine as a "readying" signal and as a "biasing" signal could be required to increase the number of repeated notes. Direct infusion of the D1 agonist SKF81297 alone into HVC did not have any effect on the number of introductory notes nor did direct infusion of only quinpirole, a D2 agonist, lending some support to the above hypotheses.

We found song tempo to be significantly increased after SKF infusion in HVC, but not after its peripheral administration. Our results of the SKF infusion in HVC align with multiple other lines of evidence which principally implicate HVC as the main generator of the birdsong tempo. This is because direct manipulation of the neural dynamics in HVC results in changes to song tempo. Increasing inhibition in HVC (Isola et al., 2020) and cooling HVC (Zhang et al., 2017) decrease song tempo, while tempo is accelerated following acetylcholine infusion in HVC (Jaffe and Brainard, 2020) . Additionally, electrical stimulation of HVC and of the HVC-RA

projection neurons distorts the temporal sequence of the ongoing syllable, and of the subsequent motif, shortening them (Vu et al., 1994). Such neuronal dynamics are hypothesised to be driven by the activity of the HVC projection neurons, which form a synfire chain in which the neurons active only at a certain time directly activate another population of neurons, which will be active only at the following time (Long et al., 2010; Mooney and Prather, 2005)**.** Long et al. (2010) have offered support to this model by showing that the membrane potential of HVC projection neurons only changes right before the beginning of a burst.

In the synfire chain model, the population of neurons activating each other through the song motif become linked by strong synaptic connections (Long et al., 2010). Any modulatory change which equally increases the excitability of these linked neurons will increase action potential propagation, which translates behaviourally to an increase in song tempo (Glaze and Troyer, 2007). However, if the linked population of neurons receive different modulatory influences, leading to different changes in excitability, action potentials will have unequal propagation speed along the synfire chain (Glaze and Troyer, 2007). Such activity would not necessarily result in globally increased song tempo. This model of neuronal dynamics in HVC might explain our results, as infusion of SKF in HVC could simultaneously change the state of neurons in specific populations, while SKF injection could unequally change the excitability along the synfire chain.

The neuronal dynamics in HVC could have also been affected through a change in temperature in HVC following the D1 agonist infusion. Aronov et al. (2012) have discovered that during FD song, the temperature of HVC increases, which does not happen during singing comparable numbers of UD song. The increase in temperature is strongly correlated with an increase in song tempo (Aronov and Fee, 2012). A potential mechanism through which

dopamine increases HVC's temperature could be through increase in blood flow to the area. Seminal studies have established that dopaminergic activity is directly linked to blood flow (Iadecola, 1998; Iadecola, 2004; Krimer et al., 1998). These studies have shown that in mammals, dopaminergic neurons are in very close contact with capillaries and arterioles and that their activity directly affects vasoconstriction, increasing blood pressure and delivering a higher volume of blood to a specific area. Hence, a potential increase in the blood pressure delivered to HVC could be enough to increase its temperature.

Interestingly, in our study only two (0.5 and 1.0 mM) of the five different doses of SKF (0.2, 0.4, 0.5, 1.0 and 3.0 mM) infused in HVC significantly increased song tempo, creating a U-shaped dose-response curve. Pharmacological and toxicological studies indicate that this curve is typical for substances with a homeostatic range of action, such as hormones, vitamins and neurotransmitters like dopamine (Calabrese and Baldwin, 1997; Calabrese and Baldwin, 2001; Davis and Svendsgaard, 1990). The biphasic dose-response relationship reflects both a direct beneficial effect of the substance at certain doses and an overcompensation to disruption in homeostasis at following doses (Calabrese and Baldwin, 2001). Dopamine levels have been shown to cause a biphasic dose- response, where both too much and too little dopamine impairs performance. For example, older monkeys display improved spatial working memory at low doses of SKF81297, but no improvement or impaired memory at high doses (Cai and Arnsten, 1997). Similarly, central infusion of low doses of SKF81297 causes rats to perform well on a maze memory test administered 12 hours after the training phase, a time when performance is usually poor. However, high doses of SKF81297 impair performance on the same memory task (Floresco and Phillips, 2001). Evidence suggests that exogenous dopamine is additive with endogenous tonic dopamine levels, increasing baseline dopamine (Cools and D'Esposito, 2011;

Kroener et al., 2009). High baseline dopamine levels are known to affect the excitability of a network (Kroener et al., 2009). Such an effect would translate to impaired or lack of desired behavioural output. Conversely, too low doses of exogenous dopamine do not contribute enough to baseline dopamine and have been shown to have no effect on existing network up-states, unless baseline dopamine was depleted (Kroener et al., 2009). A similar mechanism could account for the lack of effect of both low doses and high doses of the D1 receptor agonist SKF81297 on song tempo in Bengalese finches.

Alternatively, we must consider the effect of dopamine agonists on synaptic plasticity mechanisms. Brief stimulation of the D1 receptors have been shown to increase insertion of AMPA receptors into the neuronal membrane, increasing long-term potentiation (LTP) (Gao et al., 2006; Sun et al., 2005; Wolf et al., 2004), leading to a stronger behavioural response to the same dose of drug. However, prolonged activation of D1 receptors decreases their expression on the neuronal membrane (Sun et al., 2008; Wolf et al., 2004) through receptor internalization mechanisms, reducing the response for the same dose of substance. As our birds received several doses of the D1 agonist, potential synaptic changes could have led to a lack of effect at later doses. Additionally, the observed effect at the 0.5 mM and 1.0 mM of SKF could have been potentiated by earlier doses. But such an effect would have extended to following days (Wolf et al., 2004), affecting our control infusions, adjacent to the experimental day, an effect we did not observe. Therefore, any potential synaptic changes did not translate in a behavioural effect on song production that could have confounded the results.

Another consistent difference between the FD song and UD song of Bengalese finches is the change in the variability of the fundamental frequency of flat harmonic syllables. However, neither the peripheral nor the central administration of SKF81297 had an effect on this song

feature. Additionally, the peripheral administration of the D2 agonist quinpirole had no effect on this song parameter. However, Matheson and Sakata (2015) showed that pharmacological manipulation of catecholamines through peripheral injection of amphetamines mimicked social-context effect on song, significantly decreasing the variability of syllable structure. This suggests that either D1-receptor modulation alone or only D2-receptor modulation in the song system could be insufficient to change the vocal motor control of this song feature or the effect could be unrelated to dopamine.

We found that the effects of the D1 receptor agonist SKF81297 on vocal motor production mimic some of the social-context effects on song production. Interestingly, the effects on song following peripheral administration of SKF did not overlap with those of the central infusion of SKF in the premotor nucleus HVC. This persisted even after controlling for differences in the length of the experimental window for song collection. Peripherally administered D1 agonist significantly reduced the variability of syllable sequencing and tended to increase the number of introductory notes, while centrally infused D1 agonist in HVC only significantly increased song tempo. These results indicate that vocal motor control of song tempo can be modulated by dopamine acting locally in HVC through the D1 receptors. Our results also suggest that syllable sequencing is modulated through the D1 receptor, but possibly not through local action of dopamine in HVC alone. These findings could offer support to studies investigating speech disturbances occurring in people with dopaminergic disorders, like Parkinson's disease.

People suffering with Parkinson's disease display significant speech disturbances, which often occur prior to the gross motor symptoms (Azevedo et al., 2013). The currently used pharmacological treatments are not an efficient therapy for the speech problems (Azevedo et al.,

2013; De Letter et al., 2007) as our understanding of dopamine's involvement in moment-to-moment speech is still limited. A very common speech problem in people with Parkinson's is slowed speech tempo (Duffy, 2013; Tjaden, 2008) and monotony of speech due to a significant reduction in the variability of the fundamental frequency of speech (Bowen et al., 2013; Duffy, 2013).Speech tempo is improved by levo-dopa treatments in earlier-stage patients (Norel et al., 2020), but this treatment has mixed results on the variability of the fundamental frequency, mostly offering marginal improvement (Goberman et al., 2005; Skodda et al., 2011). These findings draw a very interesting parallel to our results regarding the effect of dopamine on song tempo and its lack of effect on the variability of syllable frequency. Taken together with the finding that catecholamines affect the variability of fundamental frequency in birds (Matheson and Sakata, 2015) our results hint at likely new pharmacological strategies. Potentially using mixed-drug therapy differentially targeting dopamine receptors as well as other catecholamines like norepinephrine could help improve multiple speech parameters affected in Parkinson's disease.

To further our results and enrich the existing corpus of knowledge regarding the modulation vocal motor control, future research should consider simultaneous use of D1 and D2 receptor agonists to investigate the possibility of synergistic action of dopamine in HVC modulating this song parameter. Locally modulating dopamine in other song nuclei using dopamine agonist and antagonists would further refine our knowledge of dopamine's role in vocal motor control.

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