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Effects of Inactivation of the Periaqueductal Gray on Song Production in Testosterone-Treated Male Canaries (*Serinus canaria*)

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ABSTRACT

Male canaries (*Serinus canaria*) display seasonal changes in the motivation to sing which have been found to be dependent on the action of testosterone (T). During the breeding season when T is high, males sing at a higher rate compared to males with low T. The effect of T on song rate is known to be mediated by the medial preoptic nucleus (POM); however, it is unclear how T-signaling in POM impacts song production. One potential mechanism is via modulation of dopaminergic input into song control nuclei by the periaqueductal gray (PAG). In order to test the role of PAG in T-mediated song production, we treated male canaries with peripheral T implants and implanted a guide cannula targeting the PAG. Through this guide cannula, we transiently inactivated PAG with injections of the $GABA_A$ agonist, muscimol. Each bird received multiple infusions of both muscimol and saline with a 48-hour washout period between treatments. The order of injection type was randomized and counterbalanced between individuals. Muscimol infusion into the PAG, but not nearby regions, increased the

latency to sing post-injection. These results support the hypothesis that PAG is involved in the production of song, potentially mediating the motivation to sing or alternatively interfering with the pre-motor activity of nucleus RA. Other song features were however not affected.

SIGNIFICANCE STATEMENT

Communication is essential for social species relying on coordinated behavior for survival and reproduction. However, the neural mechanisms underlying the motivation to engage in vocal communication are currently unknown. Here, we show that inhibition of the periaqueductal gray (PAG) increases the latency for male canaries to sing, but does not influence features of song quality once they resume singing. These results indicate that the PAG is involved in regulating the motor initiation or the underlying motivation of the complex, learned behavior of singing but not of innate vocalizations, such as calls. Our findings suggest that the PAG is likely involved in transmitting preoptic signals to the song control system, probably via dopaminergic projections from this region to song control nuclei.

INTRODUCTION

For social species, communication between individuals is essential for forming bonds and promoting survival. Without the ability to communicate, the benefits of living in a social group – such as coordinated food gathering, collective defense of self and territory, mating, and caring for young – are unattainable. However, having the ability to communicate verbally is useless if one does not actually do so. Therefore, individuals that live in social groups must be motivated to engage in vocal communication.

Currently, the neural circuits and molecular mechanisms underlying this motivation are largely unknown.

Songbirds are an ideal model for addressing this problem, as they rely on vocal communication to relay biologically significant information, and have a vocal learning process with many parallels to that of humans (Doupe & Kuhl, 1999; Konishi, 2004; Kuhl, 2000, 2003). While song can have a variety of functions, one of its key utilities is in mate attraction (Catchpole & Slater, 2008). Appropriately, in seasonally breeding songbird species such as the canary (*Serinus canaria*), the motivation to sing increases during the breeding season, when attracting a mate is particularly important (Voigt & Leitner, 2008). At this time, males also have increased plasma testosterone (T) (Ball & Balthazart, 2017; Ball, Riters, & Balthazart, 2002; Leitner, Van't Hof, & Gahr, 2003). It is this increase in T that leads to a heightened motivation to sing, in addition to a variety of other effects on song quality (Ball, Castelino, Maney, Appeltants, & Balthazart, 2003).

This effect of T on the motivation to sing appears to occur via activity in the medial preoptic nucleus (POM), since implantation of T specifically in this region in castrated male canaries increases the number of songs per minute and the percentage of time spent singing (Alward, Balthazart, & Ball, 2013). In addition, partially lesioning the POM has the inverse effect, causing male European starlings (*Sturnus vulgaris*) to sing less frequently (Alger, Maasch, & Riters, 2009; Riters & Ball, 1999). However, it is not yet known how T in POM can induce such changes in song behavior.

One potential pathway for transmission of T-modulated activity in POM to the song control system is via the periaqueductal gray (PAG). There are reciprocal projections between PAG and POM (Riters & Alger, 2004). In addition, PAG sends

dopaminergic projections to several nuclei in the song control system – HVC, the robust nucleus of the arcopallium (RA), and Area X (Appeltants, Absil, Balthazart, & Ball, 2000; Appeltants, Ball, & Balthazart, 2002; Castelino, Diekamp, & Ball, 2007; Lewis, Ryan, Arnold, & Butcher, 1981). Neural activity in PAG also appears to be tied to activity in POM, as European starlings with lesions to POM have decreased expression of the immediate early gene ZENK in the PAG (Alger et al., 2009). Furthermore, DOPAC, a metabolite of dopamine, in PAG has been found to correlate with song production (Heimovics, Salvante, Sockman, & Riters, 2011).

In the present study, we examine the role of PAG in the control of singing. We castrated male canaries and implanted them with Silastic implants that continuously release testosterone, ensuring high singing rates. We then implanted guide cannulas to target the PAG for neurochemical manipulation in these males. We conducted six trials per individual, alternating infusion of saline (vehicle) or the $GABA_A$ agonist muscimol into this brain region. We hypothesized that PAG transmits to the song system T-modulated activity from POM and accordingly sends motivational cues to song control nuclei. Therefore, we predicted that during trials in which PAG was transiently inactivated, males would either refrain from singing for some time following infusion or sing less frequently than in control trials. Furthermore, we predicted that this effect would be limited to song, since T-modulated changes in vocal behavior are limited to song, which is learned, and do not affect calls, which are innate.

MATERIALS AND METHODS

All animal procedures were performed in accordance with the University of Maryland, College Park animal care committee's regulations.

Experimental Animals & Pre-experimental Manipulations

Fourteen adult male canaries (*Serinus canaria*) of the American Singer strain were obtained from a local breeder (Maryland Exotic Birds). Upon arrival, birds were housed in grouped aviaries on a short-day photoperiod (8L:16D) to induce photosensitivity (Nicholls & Storey, 1977). All animals were provided with ad libitum food and water. Male birds were castrated under anesthesia (Forane isoflurane, Baxter; Isotex 4 anesthesia machine, Surgivet) through an incision between the last two ribs on each side. Birds were allowed to recover in their home cage for six weeks to allow adequate time for any residual physiological or behavioral effects of endogenous T to clear.

Stereotaxic Implantation

Birds were anesthetized using isoflurane gas and placed in a stereotaxic apparatus modified for use in small birds. Beaks were placed in a custom, 3D-printed beak holder placed at 45° below the horizontal axis of the apparatus. Each bird received a unilateral guide cannula (26 gauge, C315GMNSPC, Plastics One) implant targeting PAG. In order to target PAG without puncturing the ventricle and inducing bleeding, we oriented the stereotaxic arm at a 40° angle to the right from vertical and then used the following stereotaxic coordinates: dorsoventral: – 7.1 mm from the dorsal surface of the brain; anterior-posterior: 0 mm from the most rostral tip of the cerebellum; medial-lateral: 4.5 mm to the right from midline. The cannula was lowered to the target coordinates and dental cement was applied around the implant. The skin was sutured around the implant. At the time of stereotaxic surgery, males were also implanted subcutaneously with a 12mm-long Silastic implant (Dow Corning; internal diameter,

138 0.76mm; external diameter, 1.65 mm) packed with 10mm of T in order to standardize
139 circulating T levels. Following recovery from surgery, males were transferred to
140 individual sound-attenuating chambers (41 cm x 48 cm x 51 cm) set to 14L:10D to
141 simulate breeding photoperiods.

142 **Microinfusion Procedures**

143 In the week following surgery, birds were handled daily and the dummy injectors
144 were removed and reattached so that the birds would habituate to experimental
145 manipulations. After a male had been observed to sing at least two days in a row, he
146 underwent a mock infusion session to acclimate him to the infusion procedure, in which
147 saline was infused through the internal cannula in the same protocol as later test
148 infusions. Test infusions began two days after the acclimation infusion. Each bird
149 underwent six experimental infusion sessions: three sessions with 0.2 µl 0.9% saline
150 (vehicle) and three sessions with muscimol in 0.9% saline (0.5 µg / 0.2 µl solution, cat #
151 0289, Tocris). Infusions alternated between vehicle and drug, but the starting infusion
152 type was randomized and counterbalanced across individuals. Infusion sessions were
153 separated by at least 48 hours to allow for drug washout. During infusions, the dummy
154 injector was replaced by an internal cannula (33 gauge, C315IMNSPC, Plastics One)
155 connected to a Hamilton microliter syringe via a polyethylene tube. The syringe was
156 loaded into a syringe pump (KDS 220, KD Scientific) programmed to deliver treatment
157 (vehicle or drug) at a rate of 0.1 µl/min for 2 minutes, for a total volume of 0.2 µl.
158 Following infusion, the internal cannula was held in place for an additional minute to
159 allow for diffusion and to avoid reflux of the solution. Birds were then returned to their
160 home chamber. Chambers contained a combination microphone/camera (Mini Spy HD

1000TVL, TPEKKA) connected to a computer running DVRserver (V6.33b; Mammoth Technologies, Austin, TX) designed for real-time video and audio surveillance. Video and audio was recorded for the remainder of the day following infusions.

Behavior Quantification

Videos for each day were downloaded and converted into two filetypes: AVI files for video viewing and WAV files for audio analysis. In order to quantify the latency to sing, we used two independent measures. First, videos were watched from the time a bird was returned to the chamber after drug infusion until the bird was observed singing. The difference in time between infusion and singing was recorded. Audio files were independently inspected in Adobe Audition, and the time between the sound of the bird being returned to the chamber and the start of singing was recorded. These two measures were then compared to ensure that the recorded latency to sing was accurate. In two instances where there were differences between the latency recorded from videos or from audio files, another investigator analyzed the corresponding files to determine which measure was correct and ultimately a consensus was reached on the true time when the first song occurred. For measures of song quality, we used Audition to clip audio files to be one hour in length, starting at the bird's first song post-infusion. These files were analyzed using Avisoft (SASlab Pro, Berlin, Germany). Songs were defined as bouts of vocalizations longer than or equal to 1 s in duration and separated by 500 ms of silence (Alward et al., 2013). One bird was excluded from any analysis of song quality, due to high background noise from the chamber fan. In order to measure the number of calls, experimenters watched video recordings of the hour following return to the chamber following infusions. For each minute of the hour, experimenters

184 counted the number of calls that occurred during that minute and all counts were
185 summed at the end to get the number of calls that occurred during the entire hour. The
186 same procedure was used in order to quantify perch hops, as a measure of general
187 activity following infusion, for 20 minutes of video following return to the chamber. For all
188 behavior quantification procedures, experimenters were blind to treatment and cannula
189 placement.

190 **Verification of Cannula Targets**

191 After birds completed all six test microinfusion sessions, we performed a final
192 microinfusion of 0.2 µl fluorescent-conjugated muscimol (Muscimol BODIPY TMR-X
193 Conjugate, cat # M23400, ThermoFisher) according to the procedure described above.
194 We allowed 30 minutes for muscimol diffusion prior to extracting brains and flash
195 freezing them on dry ice. Brains were stored at -80°C. Brain tissue was sectioned with a
196 cryostat (Microm HM 500 OM) at 50 µm in the coronal plane and directly mounted on
197 slides. We then visualized the spread of fluorescent muscimol and the location of tracts
198 created by the guide cannula to determine if the cannula was accurately targeting PAG
199 for drug infusion.

200 **Data Analysis**

201 We analyzed latency, song measures, and call data using repeated-measures
202 ANOVA, with cannula placement and treatment (muscimol, saline) as factors. In
203 addition, we performed estimation based on confidence intervals using the Data
204 Analysis using Bootstrap-Coupled Estimation (*dabestr*) package, written for use in the R
205 programming language (Ho, Tumkaya, Aryal, Choi, & Claridge-Chang, 2019). For song
206 measures, which encompassed singing behavior in the hour after the bird began

singing, we combined data from multiple songs to create a single value for each acoustic feature, averaged across all vocalizations in the hour of audio quantified for each individual and trial. Total number of songs was calculated by adding the number of vocalizations that met our criteria for song. Time spent singing was calculated by adding the duration of each of these songs. For measures that concerned each individual song (song duration, interval between songs, number of song elements, energy, peak to peak amplitude, root mean square (RMS) amplitude, entropy, fundamental frequency, bandwidth, peak amplitude, and peak frequency) we averaged the values of each measure across the songs sung within the one hour song file for a given trial. These measures were chosen in order to quantify a range of song features including stereotypy, volume, and frequency range.

RESULTS

Due to variation in accuracy of stereotaxic implantation of guide cannulas, subjects were categorized in three groups based on the location: PAG, intercollicular nucleus (ICo), and misses (*Figure 1*). Five males were categorized in the ICo group, five in the miss group, and four in the PAG group. The fluorescent muscimol spread an average of 533 μm in the ventral-medial direction away from the end of the guide cannula tract and an average of 225 μm in the perpendicular direction. In individuals where the guide cannulas targeted PAG, this spread was sufficient to cover at least some portion of the contralateral side, in addition to affecting the hemisphere containing the guide cannula. When guide cannulas targeted regions that are located more laterally (ICo and misses), this spread only affected the hemisphere ipsilateral to the guide cannula. To determine if muscimol diffused into the aqueduct and was

transported to other brain regions via the ventricular system, we assessed fluorescence around the aqueduct and across brain regions in the telencephalon and diencephalon. We did not observe fluorescence above standard autofluorescence in any brain regions outside the mesencephalon near cannula tracts.

Song Latency

There was a significant difference in latency to begin singing between muscimol and saline trials ($F(1,11) = 5.775$, $p = 0.035$, $\eta 2 = 0.046$) and a significant interaction between cannula placement and treatment ($F(2,11) = 19.222$, $p < 0.001$, $\eta 2 = 0.303$) (*Figure 2*). There was no significant main effect of cannula placement alone on latency to sing ($F(2,11) = 1.873$, $p = 0.200$, $\eta 2 = 0.143$). The effect of treatment was driven by differences in the group with guide cannulas targeting PAG. In birds with cannulas targeting PAG, there was a large increase in time to sing post-infusion for muscimol trials compared to saline trials (mean muscimol latency minus saline latency (ΔL) = 125.75 min, SE = 13.50), while differences in latency in birds with cannula targeting ICo (ΔL = 20.10 min, SE = 14.34) and in birds with cannula targets classified as misses (ΔL = -41.50 min, SE = 20.43) were smaller. We found a similar pattern of results by performing estimation statistics with the *dabestr* R package. We created 5000 bootstrapped sample distributions representing the difference in latency to sing for muscimol trials minus saline trials, such that a positive estimation would indicate a larger latency to sing following muscimol infusions compared to saline infusions. The PAG group had a 95% confidence interval that indicated a larger latency to sing following muscimol trials compared to saline trials (126 min, 95% CI [71.8; 184]). The 95% confidence interval for the ICo group (28 min, 95% CI [-3.21;113]) included 0,

indicating that there is likely no difference in latency to sing between muscimol and saline trials. The Miss group's 95% confidence interval (-38.8 min, 95% CI [-99.5; -9.51]) was close to zero, but did not include zero – indicating a small increase in latency to sing for saline trials.

Song Measures

In the hour after birds began singing, we did not find any significant differences between treatments (saline or muscimol) or between cannula placements for the amount of time spent singing (total number of songs, total amount of time spent singing) or measures of song quality (song duration, interval between songs, energy, number of elements, peak to peak frequency, fundamental frequency, entropy, bandwidth, or amplitude). (*Figure 3, see Table 1 for results of statistical analyses*). We did observe a significant interaction between treatment and placement for RMS amplitude ($F(2,10) = 4.828$, $p = 0.0341$). However, the lack of significant main effects of cannula placement ($F(2,10) = 0.485$, $p = 0.629$) or treatment ($F(1,10) = 0.312$, $p = 0.589$) and the small effect size ($\eta 2 = 0.012$) suggests that this may be a spurious effect. Furthermore, the 95% confidence interval of bootstrapped differences between muscimol and saline trials included zero in all groups, supporting the idea that this is not a real effect (PAG: -2.43 RMS power, 95% CI [-5.19; 0.123]; ICo: 0.528 RMS power, 95% CI [-0.002; 1.52]; Miss: 0.796 RMS power, 95% CI [-0.942; 2.31]).

Calls

We also quantified the number of calls birds made in the hour immediately following infusion and found no significant differences between treatments ($F(1,11) = 4.783$, $p = 0.054$), cannula placements ($F(2,11) = 0.468$, $p = 0.639$), or an interaction

between the two ($F(2,11) = 2.549$, $p = 0.127$) (*Figure 4*). Likewise, 95% confidence intervals of the difference between muscimol and saline trials included zero for all three cannula placements (PAG: -53.9 calls, 95% CI [-140; 0.75], ICo: -24.3 calls, 95% CI [-92.3;53], Miss: 4.31 calls, 95% CI [-18.2; 44.2]).

General Activity

We did not find a difference in the number of perch hops following infusion between cannula placements ($F(2,11) = 0.546$, $p = 0.594$), between saline and muscimol trials ($F(1, 11) = 0.228$, $p = 0.643$), or an interaction between placement and treatment for any cannula placement. ($F(2, 11) = 0.942$, $p = 0.419$). Likewise, the 95% confidence intervals of difference between muscimol and saline trials included zero for all conditions (PAG: -19.3 perch hops, 95 CI [-68.9; 0.1], ICo: -5.6 perch hops, 95 CI [-27.1; 3.4], Miss: 11.3 perch hops, 95 CI [-8.27; 37.2]).

DISCUSSION

This study found that transiently inactivating the PAG increases the latency to sing in castrated male canaries treated with exogenous T. Since muscimol infusion into this region increased the latency to sing, while having no effect on other aspects of song production, PAG seems to be specifically involved in the initiation of singing behavior. This could be due to PAG conveying cues from the POM to the song control system. The interpretation of these results depends critically on the assumed duration of muscimol infused into the PAG. Based on past pharmacological studies, it is likely that the dose of 0.5 µg muscimol we infused in the PAG activated $GABA_A$ receptors for a duration of one or two hours. Indeed a previous study found that an intraperitoneal injection of muscimol produced an analgesia in the hot plate test that was fully effective

for 40 min and was already decreasing at 60 min, with an extrapolated return to baseline before 2 hours post injection (Sawynok & Labella, 1982). Using an approach more similar to the present work, muscimol infused into the preoptic area decreased the lordosis quotient of female rats at 10 and, to a lower extent, 30 min post infusion, but the behavior returned to baseline at 60 min. A similar time course was observed for the stimulation of lordosis by muscimol injected into the medial hypothalamus (McCarthy, Malik, & Feder, 1990). These durations of action depend on the doses that are injected but studies combining doses and duration are rare and, to our knowledge, do not exist for brain injections. Based on these data, it seems likely that our injections of a low dose of muscimol modulated $GABA_A$ activity for a duration of about one-two hours which explains why singing activity resumed 125 min later but the quality of the songs produced at that time was not affected. If $GABA_A$ activity was still increased when singing activity recovered, this would mean that PAG does not control song quality, since song was normal at that time. However, we do not believe that this interpretation is very likely, because it would then be difficult to understand why singing activity recovered.

In mammalian species, electrical stimulation of the PAG has been shown to elicit innate, species-specific calls (Jürgens, 1994). However, little is known concerning the function of PAG in regulating learned vocalizations, such as song. Projections from the PAG to HVC have been found to be important during zebra finch (*Taeniopygia guttata*) development and song learning – allowing juveniles to detect the presence of a tutor and encode the tutor song (Tanaka, Sun, Li, & Mooney, 2018). Our results indicate that

the medial PAG has additional influence on adult song – presumably by transmitting signals from POM to the song system.

The nature of the information transferred from POM to PAG would however require additional investigations. In canaries, we have collected extensive evidence indicating that testosterone action in the POM is necessary for singing behavior to occur but does not modify song quality (Alger et al., 2009; Alger & Riters, 2006; Alward et al., 2013; Alward, Bournonville, et al., 2016; Alward, Madison, Parker, Balthazart, & Ball, 2016; Riters & Ball, 1999; Vandries et al., 2019). We have therefore hypothesized that the POM modulates the motivation to sing, but it could also be postulated that these effects reflect a modulation of the motor aspects of singing. It is challenging to distinguish between these two hypotheses. The terms motor control are usually used for mechanisms that are relatively close to the effector muscles, including for example the motor magnocellular neurons directly projecting to the spinal chord or possibly neurons in the PAG. The preoptic area is rather considered as an integration area that modulates higher order processes, including motivation. It is often assumed in the literature on male sex behavior in rodents that an increased latency to show sexual behavior after introduction of a female reflects a decrease in motivation (Melis & Argiolas, 1995) and we think that the delay in singing initiation reflects here a similar mechanism. The interpretation of the present experiments is thus probably not that inactivating PAG directly blocks a motor transmission to the muscles of the syrinx (such a direct projection is not known to exist) but rather that this inactivation interrupts transmission of a “motivational” signal from POM to HVC and/or RA. It is however true that RA is myotopically organized based on the muscles of the syrinx (Vicario, 1991) so

344 an indirect modulation of the motor control of the syrinx via the PAG projection to RA
345 could potentially contribute to the effects we have observed in this study.
346 The observation that the general activity of the birds (i.e. perch hopping) and the
347 rate of calling behavior were not affected by muscimol during the period when song was
348 inhibited supports the specificity of the effect on song and argues against an
349 interpretation that would be based on a general inhibition of activity or on a non-specific
350 stress response. However, given the difficulties of ascribing a strictly motivational role to
351 the observed effect of transiently inactivating PAG, additional data would be required to
352 completely dissociate this region's effects on motivation as compared to the motor
353 control of the syrinx.
354 Notably, it appears that the medial PAG, and not ICo, regulates these
355 motivational signals. Birds with cannulas targeting this region took an average of 125
356 minutes longer to begin singing after muscimol infusions, presumably refraining from
357 singing until the muscimol wore off, while birds with cannulas targeting other regions did
358 not display a large difference in song latency between muscimol and saline trials.
359 Immunohistochemical analysis has indicated that this region, referred to as the
360 mesencephalic central gray in some older publications (e.g. Stokes, Leonard, &
361 Nottebohm, 1974), is organized like a folded open ventral mammalian PAG, while the
362 laterally adjacent ICo appears to be homologous to the dorsal mammalian PAG
363 (Kingsbury, Kelly, Schrock, & Goodson, 2011). In addition, the medial PAG contains the
364 A11 group of catecholaminergic neurons, sending projections to the song control
365 system (Appeltants et al., 2000, 2002). We hypothesize that the motivational cues from
366 the POM are transmitted to the song system via these projections. Future studies

should investigate these catecholaminergic inputs into song control nuclei and their influence on song behavior.

These results indicate that PAG is involved in the initiation of singing behavior and may regulate the motivation to produce song, but cannulas misplaced in the ICo served as a valuable control as ICo has been implicated in production of innate calls (Nieder & Mooney, 2020; Popa & Popa, 1933). Due to this literature, we expected a decrease in calling behavior following muscimol infusion into this region. However, we did not observe such a difference, likely due the placement of cannula. For cannulas targeting PAG, the medial position of the nucleus allowed muscimol to spread and to affect the bilateral extent of the structure. In contrast, when the cannula was located in ICo, a nucleus located more laterally, muscimol was not able to spread to the contralateral side in order to induce bilateral inactivation. Bilateral lesions of ICo decrease ring dove (*Streptopelia risoria*) nest coos, but only unilateral activation of this area with steroid hormones are required to induce an increase in nest-cooing behavior (Cohen & Cheng, 1981, 1982). Therefore, it is possible that using two cannulas to bilaterally inactivate this region would reduce calling behavior, a hypothesis that could be tested in future experiments. In addition, the cannulas classified as being in ICo in this study targeted the medial, rather than lateral, portion of ICo. Since many of the electrical stimulation studies implicating ICo as a regulator of calling behavior targeted the lateral region of the nucleus close to the dorsomedial nucleus of ICo (DM), it is more likely that this lateral portion is responsible for innate vocalizations (Seller, 1980). Therefore, targeting muscimol treatment in this lateral portion of ICo may potentially result in an inhibition of calling behavior.

Now that PAG has been identified as a region essential for the initiation of song, and potentially controlling the motivation to produce learned vocalizations, future research is required to identify the underlying cellular and molecular mechanisms. Systematically exploring how this region interacts with the song control system and the POM will advance our understanding of the biological underpinnings of social communication.

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FIGURE LEGENDS

Figure 1. Experimental Procedures. (a) Timeline of experimental procedures (left) and image of cannula trajectory (right) created with BioRender. **(b)** Coronal sections

528 demonstrating final placement of cannula targets after surgery – 4 in PAG, 5 in ICo, and
529 5 misses. **(c)** Example images of muscimol spread for an individual where cannula
530 placement was on the lateral edge of PAG. Left is a Nissl brightfield photomicrograph
531 showing the location of PAG and right is the corresponding fluorescent
532 photomicrograph.

533 **Figure 2. Change in latency to begin singing post-infusion.** Differences in latency
534 for each individual across trial type. Each line connects the average latency to sing
535 following muscimol infusion to the average latency following saline infusion for an
536 individual bird. Black lines indicate summary statistics (mean and standard error of the
537 mean) while colored lines indicate the average latency for each individual bird.
538 Individual points indicate the latency for each single trial. The three sections of the
539 graph represent cannula placement (ICo, miss, or PAG). In order to determine if an
540 ANOVA was appropriate to determine differences between treatments, we tested the
541 assumption of homoscedasticity (Fig 2-1a). To further assess the differences between
542 groups and treatments, we used bootstrapped estimation statistics of 95% confidence
543 intervals (Fig 2-1b).

544 **Figure 3. Example song quality measurements. (a)** The amount of time in minutes
545 spent singing in the one hour after singing began. There were no significant differences
546 between treatments or cannula placement. The three sections of the graph represent
547 cannula placement (ICo, miss, or PAG). **(b)** The average duration of songs in seconds.
548 There were no significant differences between treatments or cannula placement. **(c)**
549 Average change in RMS Amplitude between treatments (muscimol minus saline). There
550 was a significant interaction between treatment and cannula placement, but no main

effect. Each line connects the average data following muscimol infusion to the average data following saline infusion. Black lines indicate summary statistics (mean and standard error of the mean) while colored lines indicate the average data for each individual bird.

Figure 4. There were no significant differences in call rate in the hour following infusion. Difference between muscimol and saline trials (average number of calls following muscimol infusion minus average number of calls following saline infusion) for individual birds. Colors represent cannula placement. Each line connects the average call rate following muscimol infusion to the average call rate following saline infusion. Black lines indicate summary statistics (mean and standard error of the mean) while colored lines indicate the average data for each individual bird.

Song Measure	Factor	df	F	p	Estimation Statistics *difference (muscimol - saline)*		
Number of Songs	Cannula Placement	2	0.328	0.728	ICo	8.5	95 CI [–6.67; 22.3]
	Treatment	1	0.005	0.944	Miss	–23.4	95 CI [–9.83; 140]
	Interaction	2	1.855	0.206	PAG	25.2	95 CI [–9.83; 140]
Time Spent Singing	Cannula Placement	2	0.574	0.581	ICo	24.6	95 CI [–58.9; 89.3]
	Treatment	1	0.130	0.726	Miss	-117	95 CI [–229; -20.2]
	Interaction	2	1.359	0.301	PAG	230	95 CI [–56.5; 1240]
Avg. Song Duration	Cannula Placement	2	0.662	0.537	ICo	–0.88	95 CI [–3.34; 1.35]
	Treatment	1	0.164	0.694	Miss	0.089	95 CI [–1.06; 1.55]
	Interaction	2	0.626	0.554	PAG	0.256	95 CI [–1.56; 1.77]
Avg. Inter-song Interval	Cannula Placement	2	0.757	0.494	ICo	–13.6	95 CI [–153; 43.4]
	Treatment	1	0.0	0.985	Miss	9.61	95 CI [–147; 166]
	Interaction	2	0.2	0.822	PAG	39	95 CI [–180; 203]
Avg. Song Elements	Cannula Placement	2	0.747	0.499	ICo	1	95 CI [–1.78; 6.86]
	Treatment	1	0.407	0.538	Miss	–0.661	95 CI [–14; 14]
	Interaction	2	0.354	0.710	PAG	1.3	95 CI [–1.89; 6.2]
Avg. Energy	Cannula Placement	2	0.607	0.564	ICo	0.0002	95 CI [0.00004; 0.0006]
	Treatment	1	0.001	0.989	Miss	–0.000008	95 CI [–0.0009; 0.0001]
	Interaction	2	2.541	0.128	PAG	–0.0003	95 CI [–0.0009; 0.0001]
Avg. Peak to Peak Amplitude	Cannula Placement	2	1.12	0.372	ICo	0.014	95 CI [0.001; 0.038]
	Treatment	1	0.753	0.411	Miss	0.003	95 CI [–0.014; 0.026]
	Interaction	2	2.616	0.134	PAG	–0.037	95 CI [–0.083; 0.008]

Avg. RMS	Cannula Placement	2	0.485	0.629	ICo	0.528	95 CI [–0.002; 1.52]
	Treatment	1	0.312	0.589	Miss	0.796	95 CI [–0.942; 2.31]
	Interaction	2	4.828	0.034*	PAG	–2.43	95 CI [–5.19; 0.123]
Avg. Entropy	Cannula Placement	2	0.007	0.993	ICo	0.0009	95 CI [–0.010; 0.008]
	Treatment	1	1.683	0.224	Miss	–0.0009	95 CI [–0.007; 0.002]
	Interaction	2	0.171	0.845	PAG	0.0004	95 CI [–0.014; 0.012]
Max. Fundamental Frequency	Cannula Placement	2	0.385	0.690	ICo	–52.4	95 CI [-363; 225]
	Treatment	1	1.274	0.285	Miss	–80	95 CI [–221; 85.2]
	Interaction	2	0.014	0.986	PAG	–44.3	95 CI [-613; 614]
Max. Bandwidth	Cannula Placement	2	0.343	0.718	ICo	110	95 CI [-129; 495]
	Treatment	1	0.084	0.778	Miss	–43.4	95 CI [–286; 37]
	Interaction	2	0.747	0.498	PAG	–79.4	95 CI [–348; 271]
Max. Peak Amplitude	Cannula Placement	2	0.641	0.547	ICo	0.332	95 CI [–1.94; 2.43]
	Treatment	1	0.512	0.491	Miss	0.15	95 CI [–3.64; 2.84]
	Interaction	2	2.214	0.160	PAG	–2.88	95 CI [–6.2; –0.045]
Max. Peak Frequency	Cannula Placement	2	1.033	0.391	ICo	7.83	95 CI [-217; 149]
	Treatment	1	1.184	0.302	Miss	–182	95 CI [–749; 45.9]
	Interaction	2	0.236	0.794	PAG	–148	95 CI [–730; 492]

Table 1. Results of two-way repeated measures ANOVA of song quality measures.

Figure 2-1. Plots supporting statistical analysis of latency data. **(a)** Plot showing homoscedasticity assumption for ANOVA . Distribution of residuals are plotted against treatment and brain region targeted. **(b)** Plot of confidence intervals of differences between muscimol trials and saline trials bootstrapped 5000 times.