The Effects of Nicotine Administration on Spectral and Temporal Features of Crystallized Song in the Adult Male Zebra Finch

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ABSTRACT

Introduction: The process through which the zebra finch (Taeniopygia guttata) acquires and produces crystallized song has long been thought of as highly analogous to the process through which humans acquire and produce speech. Nicotinic acetylcholine receptors are present in song nuclei that play important roles in song production and/or acquisition. We studied the effect of in vivo nicotine on temporal and spectral features of crystallized song.

Methods: We used 16 adult male zebra finches, which were exposed to an established 7-day nicotine or saline treatment. Song behavior was monitored for a 2-month period following the cessation of the nicotine/saline treatment. All animals served as their own control in order to determine whether any song characteristics changed over the observed time period compared to the baseline measurement.

Results: Inter-syllable duration and Wiener entropy were significantly affected by the in vivo administration of nicotine. These observed changes persisted for a 2-month period following the cessation of nicotine exposure. Similar changes were not observed in the age-matching control group.

Conclusions: Nicotine significantly affected tempo and rhythm of the crystallized zebra finch song. We believe that this in vivo nicotine-exposed zebra finch model not only can provide a further understanding of the underlying behavioral mechanisms of the cognitive implications of nicotine dependence but also could help in the development of therapeutics targeting cognitive deficits often observed during nicotine withdrawal.

INTRODUCTION

The zebra finch (Taeniopygia guttata) has long been used as an animal model for memory and cognition due to the natural way through which males acquire and produce song. Song learning in male zebra finches is comparable to human speech development and occurs in two phases, the so-called sensory and the sensorimotor phase (Bohner, 1990; Brenowitz, Margoliash, & Nordeen, 1997; Miller, Hilliard, & White, 2010; Nottebohm, 1972). During the sensorimotor phase, the young finches’ subsong (variable vocalizations) develops into plastic song, which consists of a more structured song. At the end of the sensorimotor phase (around the age of 90–110 days), the song crystallizes and remains relatively stable over the course of the animal’s natural life (Bolhuis & Gahr, 2006). Crystallized song shows little variation in its temporal and spectral properties (Brenowitz et al., 1997; Saar & Mitra, 2008). The temporal properties, such as inter-syllable duration (ISD) and syllable duration (SD), characterize the tempo and rhythm of a song (Glaze & Troyer, 2013) and are suggested to be controlled by the telencephalic nucleus HVC (Hamaguchi & Mooney, 2012). The spectral properties, such as amplitude, Wiener entropy (WE), and pitch, characterize the melody of a song and are comparable to the formants in speech (Ohms, Escudero, Lammers, & ten Cate, 2012). Each male zebra finch produces a unique song, which consists of a series of syllables organized into several motifs (phrases).

Discrete interconnected brain regions, known as song nuclei, control birdsong (Bolhuis & Gahr, 2006; Brenowitz et al., 1997; Marler & Peters, 1982). In the motor pathway (MP) necessary for song production, the HVC projects onto the robust nucleus of the arcopallium (RA), which in turn projects to two separate motor neurons innervating the vocal organ in birds (syrinx) and the respiratory muscles. The anterior forebrain pathway, a basal ganglia–related circuit essential for song learning and plasticity, also originates in the HVC (Wada, 2012). Recent studies have shown that both the HVC and the RA contain nicotinic acetylcholine receptors (nAChRs;
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S. L. T. Cappendijk & M. J. Marks, personal communication, April 4, 2009; Lovell, Clayton, Replogle, & Mello, 2008; Salgado-Commissariat, Rosenfield, & Helekar, 2004; Shea & Margoliash, 2010), which implicate a role of nicotine-related pathways in both song production and song learning and plasticity: Nicotine directly applied to the HVC of anesthetized finches (Shea & Margoliash, 2003) or administered to brain cultures taken from the RA (Salgado-Commissariat et al., 2004) altered the firing rate of neurons. In our previous study (Cappendijk et al., 2010), we showed that in vivo nicotine exposure in adult male zebra finches dose-dependently affected the quantity of produced crystallized song.

Nicotine stimulates brain reward circuits and the activation of dopamine (DA) neurons in mesolimbic DAergic circuits, which have been shown to play a significant role in nicotine reward and nicotine dependence (Balfour 2009; Fowler & Kenny, 2014). Cognitive deficits are considered an important symptom of nicotine dependence and are strong predictors of relapse (Levin, 2006; Paolini & De Biai, 2012). The underlying mechanisms of cognitive deficits observed during nicotine withdrawal are unclear and a better understanding of these deficits will definitely help to improve existing and develop new therapeutics (Ashare, Falcone, & Lerman, 2014). Nicotine dependence treatments, which focus on nAChR subtype-specific receptor agonists and nicotinic cotreatments interacting with the DAergic system, are still being explored with a focus on providing therapeutic treatments for cognitive dysfunction (recent reviews by Levin, 2013; Zurkovsky, Taylor, & Newhouse, 2013). In songbirds, DA receptors are primarily found in the HVC, the RA, and Area X. The RA mainly receives DAergic inputs from the periaqueductal gray and the ventral tegmental area (VTA)–substantia nigra pars compacta and shows expression of D1-like and D2-like receptors (Kubicova, Wada, & Jarvis, 2010; Liao, Wang, Pan, Hou, & Li, 2013). The HVC also projects to Area X, which projects to the VTA/substantia nigra. These latter nuclei provide feedback to the HVC, showing a similar structure as the mammalian cortico-basal ganglia pathway, which is implicated in vocal sequence variability (Hamaguchi & Mooney, 2012). As the song system of the zebra finch contains a similar architecture when compared to mammalian forebrain networks, we believe that the zebra finch model could be used to study cognitive deficits that occur following nicotine dependence.

We hypothesize that in vivo nicotine administration affects the temporal and spectral features of crystallized song, which implies that nicotine-related pathways play a role in song behavior in zebra finches. A better understanding of these pathways could help in the development of therapeutics targeting cognitive deficits often observed during nicotine withdrawal.

METHODS

Animals

All animals used in this study were purchased from Acadiana Aviaries. Upon arrival, the adult male zebra finches (age between 150 and 175 days) were group housed in our aviary for a minimum of 2 weeks. Based on extensive behavioral studies, the selected age of our animals assures that the song pattern is crystallized and therefore not prone to variability as observed due to either development (age younger than 90 days) or to old age (Cooper et al., 2012; Wada, 2012, respectively). Although small variations in syllables have been observed, the crystallized zebra finch song is considered among the least variable of those produced by songbird species (Glaze & Troyer, 2006; Williams, 2004). Animals had free access to food (Vita-Finch) and water throughout the full length of the experiment. They were kept under artificial 14-hr light:10-hr dark cycle with the lights on at 08:00. The ambient temperature was set at 26±2°C (Cappendijk et al., 2010). The experimental protocols used in this study were in line with national and international ethical guidelines. This protocol was approved by the Animal Care and Use Committee of Florida State University (Protocol # 1109).

Experimental Design

Adult male zebra finches (n = 16), weighing between 12 and 15 g, were randomly selected and moved into customized recording cages where the animals were kept in visual, but not auditory isolation (Cappendijk et al., 2010). The experiment was divided into three experimental phases, which were preceded by a 7-day habituation period, allowing the animals to adjust to social isolation. Phase 1: All animals were given injections of physiological saline (0.03 ml/10 g body weight, sc at 7:00 and 19:00) for five consecutive days. This procedure was performed to habituate the animals to handling. Phase 2: Animals were randomly divided into two equal groups, a control and a nicotine-exposed group (n = 8 each). For seven consecutive days, twice a day at 07:00 and 19:00, the control group received injections of saline (0.03 ml/10 g body weight, sc), while the nicotine group was exposed to a nicotine treatment (0.18 mg/kg sc). This dose was selected based upon our prior studies (Cappendijk et al., 2010). Phase 3: Following the cessation of nicotine and saline administration, the animals were monitored for a 2-month observation period to analyze potential long-term effects of nicotine.

Song Recordings

Vocal recordings were made using two desktop PCs and Avisoft Recorder (Cappendijk et al., 2010). The desktop PCs were each equipped with a 2TB external hard drive, which allowed for the storage, mobility, and analysis of the song data. Recording took place on a daily basis (08:00–14:00) and started on the first day of Phase 1 and ended on Day 7 of Phase 3. Due to the high volume of data collected and storage space needed for the full length of our experiment, recordings for Phase 3 continued after the seventh day into a 3 times per week recording schedule until the end of the experiment. Each recording period was also from 08:00 to 14:00.

Analysis of Song Data

Song data were run through an analysis program custom-built for the Cappendijk Lab (Miller, Cappendijk, & van Engelen, 2007). This analysis allowed for quantification of sound data and the removal of noise files (recordings triggered by cage sounds that were not vocally produced by the test animal). Sound data were then edited using Adobe Audition to remove possible microphone feedback (F. Johnson, personal communication, April 4, 2009). Following these steps, song data were analyzed manually using the explore and score feature of Sound Analysis Pro (SAP) Version 2A.04 (Tchernichovski, Lints, Deregnaucourt, Cimenser, & Mitra, 2004). A spectrogram of
an adult male zebra finch song is shown in Figure 1. In general, in nature, the highest activity in singing is registered at dawn, which is attributed to better feeding conditions, vacant territories, and fertility of the females. Studies in a laboratory environment have shown that singing behavior and song tempo are affected by the time of the day (Glaze & Troyer, 2006). The first hour of the light cycle (8:00–9:00) was selected to analyze temporal and spectral features of the undirected song behavior, based on previous results from our lab (Cappendijk et al., 2010). A recent study by Aronov and Fee (2012) demonstrated that zebra finch brain temperature started rising 2 hr after the lights came on, which affected directed song variability. A similar variation in the undirected song could occur under our experimental conditions. However, since we analyzed undirected song data recorded during the first hour of the light cycle, major variability in song patterns should not be attributed to the time of recording, but to treatment effects.

Spectral and Temporal Features

We focused on two temporal features of song: SD, measuring the length of each produced syllable, and ISD, quantifying the length of the gap in-between two syllables, which are produced in the same motif. Therefore, SD and ISD determine not only the overall length of song being produced but also the overall rhythm and tempo. SD and ISD are used as standard parameters by multiple research groups as they provide information about the syllables being produced, how they are organized in a song motif, and how long motifs and song bouts last (Glaze & Troyer, 2013; Long & Fee, 2008; Vu, Schmidt, & Mazurek, 1998). Both temporal features were measured in milliseconds (ms). The spectral features selected for this study were amplitude, WE, and pitch. Amplitude is indicative of the intensity of the sound being produced and is measured in decibels (dB). Pitch (measured in Hertz or Hz) is indicative of the frequencies being produced within each syllable (Tchernichovski, Nottebohm, Ho, Pesaran, & Mitra, 2000). WE reflects a measurement of chaos in a produced sound. In essence, WE measures how much energy is spread out at a given time in the spectrum of a sound. A high value (WE = 0) is registered when the energy is equally distributed across frequencies (as seen in white noise) and a low value when the energy is concentrated in a small number of narrow frequency bands (as seen in more tonal sounds, such as a plucked harp string); as such, WE acts as an audio fingerprint of a produced syllable and helps track changes in energy distribution over multiple song events. All characteristics of song were recorded using SAP and visualized using Excel and Graphpad Prism.

Statistical Analysis

For group measurements, the averages of song characteristics for independent syllables were taken for each experimental phase and normalized to baseline (Phase 1) using the following equation:

\[
\left(\frac{|P_x_{Ave} - P_{1_{Ave}}| + 1}{P_{1_{Ave}}} \right) * 100\%,
\]

where \(P_x_{Ave}\) stands for the average from the phase being examined, \(P_{1_{Ave}}\) stands for the average taken from Phase 1 (average baseline measurements). This calculation allows for a baseline of 100% with all changes in song characteristics resulting in an increased percentage. Then, the normalized syllable characteristics for all animals were averaged together for each experimental phase to allow for overall characteristic comparison across the phases. The absolute value bars in the numerator and divisor prevent the negative values of entropy from altering the design of the equation. The temporal and spectral features of this experiment were analyzed across phases by analysis of variance and Dunn’s multiple comparisons test, where \(p < .05\) was considered statistically significant.

RESULTS

The effects of nicotine and saline on the temporal features, ISD and SD, are shown in Figure 2. The values for each individual syllable were normalized and transformed into a percentage of their baseline measurement as described in the Methods section. These percentages were then averaged together to allow for a comparison of control and nicotine-exposed groups over the three experimental phases. The cessation of nicotine induced a significant change in ISD (\(p = .007\)). This change was not observed in the age-matching control group (\(p = .4701\)). This finding indicates a long-term change to ISD, which seems to be initiated by the nicotine administration. Figure 2 also shows that neither the control nor the nicotine administration induced a significant change in SD during any of the experimental phases. Further examination of the individual birds and syllables for each experimental group indicates that the change in ISD was not caused by an overall decrease or increase in duration, but rather a combination of increases and decreases (data not shown). This indicates that the change in ISD is actually an increase in overall variability, rather than an overall tendency toward faster or slower song.

The effects of nicotine and saline on the spectral features, amplitude, pitch, and WE, are shown in Figure 3. Neither saline
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Figure 2. The effects of in vivo nicotine and saline exposure on the temporal features of adult male zebra finch song: inter-syllable duration (ISD) and syllable duration (SD). Temporal features of zebra finch song for each syllable were converted to a percentage of their baseline measurements. These percentages were compared over three phases of the experiment. Before (Phase 1, baseline set at 100%), during (Phase 2), and after (Phase 3) drug administration for both the saline and the nicotine-exposed group (n = 8, each group). Values are mean ± SE. Nicotine significantly altered ISD (p = .007, vertical striped bar). There was no group effect observed for SD. * represents groups, which are significantly changed (p < .05) compared to baseline measurements.

Figure 3. The effects of in vivo nicotine and saline exposure on the spectral features of adult male zebra finch song: amplitude, pitch, and Wiener entropy (WE). Spectral features of zebra finch song for each syllable were converted to a percentage of their baseline measurements. These percentages were compared over three phases of the experiment. Before (Phase 1, baseline set at 100%), during (Phase 2), and after (Phase 3) drug administration for both the saline and the nicotine-exposed group (n = 8, each group). Values are mean ± SE. The amplitude was not affected by nicotine and saline over the course of the experiment. Nicotine and saline significantly altered pitch during Phase 3 (p = .0146, black bar, p = .0292, vertical striped bar, respectively). Nicotine exposure significantly changed WE (p = .007, gray bar) in Phase 3, while WE remained unaffected in the age-matching control group. * represents groups, which are significantly changed (p < .05) compared to baseline measurements.
nor nicotine treatment induced a statistically significant change in amplitude. A statistically significant difference in pitch during the third experimental phase for both the control ($p = .0146$) and the nicotine-exposed group ($p = .0292$) was measured. While a significant change does take place after the cessation of injections, it cannot be attributed to the nicotine administration as it also takes place in the control group. Nicotine cessation induced a significant change in WE ($p = .007$) during the third experimental phase, which is absent in the control group ($p = .1767$), and thus can be attributed to the nicotine exposure.

Figure 4 shows the spectrograms of a representative control (S-3) and a representative nicotine-exposed (N-3) animal. Comparing Phase 1 with Phase 3 in the nicotine-exposed animal shows that the ISD changed, which was shown in Figure 2 ($p = .007$), indicating a change in the song tempo and rhythm. A similar change was not observed in the age-matching control group.

**DISCUSSION**

The findings in this study support our hypothesis that in vivo nicotine administration affects the qualitative properties of crystallized song. In particular, nicotine induced a statistically significant alteration of the temporal feature ISD and the spectral feature WE. These changes manifested themselves during Phase 3, which corresponds to the cessation of the nicotine treatment, and were not seen in age-matching controls. In both cases, the changes that were observed were indicative of a general increase in variability, rather than an overall increase or decrease in observed values. We also found that pitch was significantly altered in both saline and nicotine-exposed groups; as such, this change cannot be attributed to the nicotine treatment.

Glaze and Troyer (2013) have shown that the temporal features of song become less variable over the course of the first year of a zebra finch’s life. Since we used adult zebra finches, which were aged between 150 and 175 days at the beginning of the experiment, it can be assumed that little variation would be present in the temporal features of their crystallized song. This assumption was confirmed in our control group over a time period of approximately 3 months (Phase 1 vs. Phase 3) and can be seen in temporal and spectral features of crystallized song (Figures 2 and 3). In contrast, when adult male zebra finches are exposed to nicotine, the overall characteristics of the crystallized song become more variable (Figures 2–4). Cooper et al. (2012) demonstrated a correlation between variability of the song and age of a Bengalese finch (more than 5 years old). These authors speculated that the observed variability, which expressed itself as a slowing of the song due to a lengthening of the ISD, reflected an age-related loss of DAergic function in the basal ganglia. Since we observed a change in ISD, we

**Figure 4.** Spectrograms of a representative control (S-3) and a representative nicotine-exposed (N-3) animal over the length of the behavioral experiment. The spectrograms of a control (S-3, left) and a nicotine-exposed (N-3, right) animal are shown during each phase of the experiment. The song of S-3 consists of four syllables represented by the boxes: A–D; the song of the N-3 consists of five syllables: a repeating introductory syllable A (first two boxes), B–D, and E, which also repeats. No difference can be observed in inter-syllable duration or syllable duration for S-3 when comparing all phases. N-3 shows an increase in the gap between the two A syllables, and between syllables A and B, while the durations of all syllables remain constant. Time is plotted across the x-axis in ms. Each tick mark represents a time span of 50 ms. Frequency is plotted across the y-axis.
could argue that the DAergic function in the basal ganglia (Area X) was changed following the withdrawal of nicotine, which is in line with the general consensus that mesolimbic DAergic circuits are playing a significant role in nicotine reward and nicotine dependence (Balfour, 2009; Fowler & Kenny, 2014). Additional support for the involvement of D1 receptors in Area X in nicotine dependence processes derives from the fact that these receptors were shown to play a role in social context–related song variability (Bosíková, Koštál, Cvíková, Bílčík, & Niederová-Kubíková, 2012; Leblois & Perkel, 2012). To further examine this possibility, analysis of D1 receptors during nicotine dependence processes could provide insight to the mechanisms involved in the observed changes of song variability.

Long and Fee (2008) showed that altering the temperature of only the HVC induced changes in temporal features of song, wherein colder temperatures led to longer ISDs and warmer temperatures led to shorter ISDs. As our results show an overall change in the ISDs, we hypothesize that the HVC is affected directly by the nicotine treatment. This hypothesis is supported by a study performed by Shea and Margoliash (2003), showing that nicotine directly applied to the HVC of anesthetized finches altered the firing rate of cholinergic neurons. Exposing the zebra finches to nicotine could have altered the DA release/functioning in the basal ganglia (Area X) through the nAChRs in the HVC (Lovell et al., 2008). Area X projects to the VTA/substantia nigra. The VTA/substantia nigra provide feedback to the HVC, showing a similar structure to the mammalian cortico-basal ganglia pathway, which is implicated in vocal sequence variability (Hamaguchi & Mooney, 2012).

Further study is needed to confirm and understand the link between the DAergic system and the song system of the zebra finch and how this is related with the cognitive/speech processes affected by nicotine dependence. Kubicova et al. (2010) showed that cells in Area X expressed both D1A and D2 receptors. During undirected singing, the majority of the D1A-only-containing neurons expressed immediate early genes, such as egr1 (avian zenk). Egr1 has an inverse correlation with FOXP2 (Thompson et al., 2013), which was identified as the first gene functionally linked to human speech/vocalization. A decline in the density of positive labeled FOXP2 cells in Area X correlates with increased song stereotypy. Murugan, Harward, Scharff, and Mooney (2013) showed that FOXP2 knockdown interferes with the DA modulation of the cortico- striatal pathway (mainly through D1 receptors), which implies that D1 receptors could play a role in the expression of song variability. FOXP2 can adapt cortico-basal ganglia circuits, resulting in speech and language impairment and a decreased central DA level (Murugan et al., 2013) due to disrupting the reinforcement learning mechanisms. A similar finding was observed after repeated nicotine exposure in rats (Quick, Olausson, Addy, & Taylor, 2014). FOXP2 is claimed to be one of the genes involved in human speech processes affected by nicotine dependence (Eicher et al., 2013). The protein sequence of zebra finch FoxP2 is 98% identical with mouse and human FoxP2 (Haesler et al., 2004).

WE was significantly changed following the nicotine treatment. Pytte et al. (2012) observed that an increase in WE was correlated with a decrease in new neurons in the HVC after deafening adult zebra finches. These authors proposed that the newly formed neurons enhanced the function of the vocal MP and as such could contribute to song stability. In rats, it has been shown that nicotine can deactivate transcription factors that regulate neurogenesis and other plasticity-related processes necessary for learning and memory (Wei et al., 2012). A recent study in adult mice showed that targeting the α7 nAChR inhibited proliferation and stimulated neuronal differentiation of neural stem/progenitor cells in the subventricular zone (Narla et al., 2013). An in vitro study by Shea and Margoliash (2010) identified that α7 nAChRs were present in the HVC, and it has been shown that nAChRs and in particular the α7 nAChR play a role in synaptic plasticity and sensory gating (Callahan, Hutchings, Kille, Chapman, & Terry, 2013; Fucile, 2004). In addition, studies have shown that diminished sensory gating in the frontal brain region are indications of a deficient inhibitory cortical function in nicotine-dependent smokers (Brinkmeyer et al., 2011). However, the underlying mechanisms are unclear and in that respect the use of zebra finches exposed to nicotine might help understand this phenomenon. To further examine the potential role of α7 nAChRs in the spectral and particularly the temporal features of song behavior, future studies will focus on the anterior frontal pathway with its output nucleus LMAN (lateral magnocellular nucleus of the anterior nidopallium). The reason for focusing on the LMAN is the fact that inactivation of the LMAN can reverse shifts in syllable pitch, induced by distorted auditory feedback (Warren et al., 2010). Both the control and nicotine-exposed groups demonstrated a significant change in pitch in Phase 3 of the experiment (Figure 3). Further studies are needed to clarify this finding.

In conclusion, we have shown that in vivo nicotine administration significantly affected the temporal and spectral features, ISD and WE, respectively, in the adult male zebra finch. We speculate that nicotine affects the cholinergic signaling in the HVC (both anterior forebrain and posterior MP) and/or DAergic signaling in Area X (basal ganglia circuit), introducing a long-term song variability.

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DECLARATION OF INTERESTS
None declared.

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