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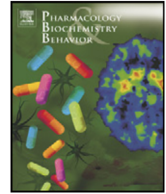

In vivo nicotine exposure in the zebra finch: A promising innovative animal model to use in neurodegenerative disorders related research

S.L.T. Cappendijk [a,*], D.F. Pirvan [a], G.L. Miller [a], M.I. Rodriguez [a], P. Chalise [a], M.S. Halquist [b], J.R. James [b]

[a] College of Medicine, Dept. of Biomedical Sciences, Florida State University, Tallahassee 32306-4300, FL, USA
[b] Virginia Commonwealth University, Dept. of Pharmaceutics, Richmond, VA, USA

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ABSTRACT

Nicotine improves cognitive enhancement and there are indications that neurodegenerative (age-related) cognitive disorders could be treated with nicotine-based drugs. The zebra finch is a well-recognized model to study cognitive functioning; hence this model could be used to study the effects of nicotine in neurodegenerative cognitive disorders. However, nicotine's *in vivo* physiological and behavioral effects have never been studied in the zebra finch. Here we present the first *in vivo* nicotine study in zebra finches. We evaluated the dose–response effects of nicotine on locomotor activity, song production, food intake and body weight. A liquid chromatography tandem mass spectrometry method was developed and validated for quantification of nicotine and cotinine in feces. The subcutaneous nicotine drug regiment (0.054–0.54 mg/kg) induced physiologically significant values of nicotine and cotinine. The mid (0.18 mg/kg) and high (0.54 mg/kg) dose of nicotine promoted the development and expression of a sensitized response of song production and locomotor activity. Food intake and body weight were not affected following nicotine exposure. In conclusion, the zebra finch can be used as an innovative animal model not only in nicotine-related research studying cognitive functioning, but also in studies examining nicotine dependence and addictive mechanisms.

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1. Introduction

Synaptic plasticity has been shown to underlie both early and late changes in cognitive functioning. Alterations in synaptic plasticity could lead to cognitive impairment, which could play a significant role in a number of psychiatric and neurological disorders, such as Alzheimer's Disease (AD), depression, schizophrenia, and attention-deficit/hyperactivity disorder (Rowan et al., 2003; Mattson and Meffert, 2006; Brennaman and Maness, 2008). There are multiple approaches used to study cognitive impairment and enhancement in neurological related disorders. For example, exposure to enriched environmental conditions showed a delay in cognitive dysfunction development associated with AD (Gelfo et al., 2009; Paban et al., 2009). These findings are consistent with the suggestion that lack of cognitive stimulation (education, social interaction and advanced occupational attainment) is a risk factor in the etiology of AD. Enhancement of cognitive stimulation can reduce the risk of age-associated dementia and the onset of AD and other neurodegenerative disorders (Terry et al., 2006, Carletti and Rossi 2008, respectively). However, for patients that are currently suffering from these types of disorders, therapies such as enriched environment might not be an option to stimulate cognitive processes as the process of neurodegeneration might be too much progressed, and other therapies need to be sought for these patients. Lately, there is emerging evidence that nicotine improves cognitive function in humans and nonhuman species and numerous studies are focusing on the development of novel nicotinic treatments for cognitive dysfunction (Levin et al., 2006; Grady et al., 2007; Cincotta et al., 2008; Kadir et al., 2008).

In the present study, we present the first ever successful performed *in vivo* nicotine research with a zebra finch. What significant scientific contribution does this songbird have for research on novel nicotinic treatments for cognitive dysfunctioning? The zebra finch (*Taeniopygia guttata*) is considered as a high order model for cognition. Birdsongs are a naturally learned behavior in the zebra finch. The bird is not born with song, but has to learn how to sing using mechanisms similar to human infants learning to speak (Nottebohm, 2004). Similar functional properties of avian and mammalian thalamic neurons suggest conserved forebrain mechanisms of sensorimotor information processing across vertebrate taxa (Reiner et al., 2004). As with humans, during critical periods of embryonic, juvenile and adult life of songbirds, postnatal neurogenesis occurs (Alvarez-Buylla and Nottebohm, 1988; Scott and Lois, 2007; Nottebohm, 2004).

The ability of many songbirds, including the zebra finch to learn vocal behavior is sexually dimorphic. This behavioral dimorphism is

* Corresponding author. Dept. of Biomedical Sciences, College of Medicine, Florida State University, 1115 West Call Street, Tallahassee, FL 32306-4300, United States. Tel.: +1 850 645 1483; fax: +1 850 644 5781.
E-mail address: susanne.cappendijk@med.fsu.edu (S.L.T. Cappendijk).

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reflected in the songbird brain. A relatively discrete structure of the neural network that controls song learning and production is present in the male brain, whereas in non-singing females these specific song related nuclei and their accompanying axonal pathways are either absent or diminished in size and number (Nottebohm, 2004).

The song system originates in the HVC (formerly known as the high vocal center) and consists of an anterior forebrain pathway (AFP) and a posterior motor pathway. The song nuclei in the posterior motor pathway are involved in song production, and the AFP nuclei are involved in song acquisition (Jarvis et al., 1998).

Neurogenesis continues throughout life in the HVC in the adult songbird neostriatum (Alvarez-Buylla and Nottebohm, 1988). In zebra finches, the initial song motor pattern is acquired by approximately 3 months of age, but new neurons are added to the song motor pathway throughout life.

The HVC consists of a heterogeneous population of inhibitory interneurons and two populations of projection neurons that send axons towards either the robust nucleus of the arcopallium (RA) or the striatal nucleus area X. The RA is located in a brain area that is comparable with layer V of the mammalian prefrontal cortex, a layer in which schizophrenic patients show pathologies of pyramidal neurons (Black et al., 2004). RA neurons display premotor activity during singing that is notable for its precision (Chi and Margoliash, 2001; Leonardo and Fee, 2005; McCasland 1987). Post-hatching neurogenesis has been reported in the RA of nestlings and juvenile zebra finches (Nowicki et al., 1998). New neurons take approximately 28 days to reach RA (Pytte et al., 2007).

Salgado-Commissariat et al. (2004) used adult male zebra finch brain slices and showed that nicotinic cholinergic mechanisms could play a critical role in synaptic plasticity in the zebra finch song system. In particular, they demonstrated a bi-directional synaptic plasticity in the RA, which indicated the involvement of both alpha-4 and alpha-7 nicotinic acetylcholine receptors subtypes. This fore mentioned receptor subtypes are known to be involved in cognitive processes (Picciotto et al, 2001; Leonard and Bertrand, 2001; Levin et al., 2006).

Since the zebra finch has never been used in *in vivo* nicotine research, the focus of this study was to determine a dose–response curve for nicotine in the adult male zebra finch, and to examine the acute effects of nicotine on song production, locomotor activity, body weight and food intake. We chose to quantify song production and not determine the quality of the song production. The reason we chose not to determine the quality of the songs in the current study is that for new neurons to reach the RA takes between 25 and 30 days (Li et al., 2000a; Pytte et al, 2007). Since we examined the acute effects of nicotine in this study, the testing period might not be of sufficient length to show an effect on quality of the song pattern. However, this long-term feature will be evaluated in long-term follow-up studies.

Our *in vivo* treatment of nicotine consisted of multiple injections of nicotine over time. It is well known that repeated injections of nicotine produce progressively larger increases in locomotor activity and stereotypy, an effect referred to as behavioral sensitization (Vezina et al., 2007; Mao and McGehee, 2010). Behavioral sensitization is used as a model to study learning and memory, bipolar disorder, and drug abuse. It has been suggested that the dopamine system underlies behavioral sensitization as well as underlying the mechanism of drug addiction and craving. Recently, it was shown that the dopaminergic system is involved in birdsong learning and maintenance (Kubikova and Kostal, 2010). The results gained from these previous published studies implicate that *in vivo* nicotine administration in the zebra finch could induce behavioral sensitization, which would be demonstrated in changes on locomotor activity and/or song production.

In addition, we developed a liquid chromatography/tandem mass spectrometry (LC/MS/MS) assay for the determination of nicotine and the metabolite cotinine in fecal samples to assess zebra finch exposure and to relate exposure levels to physiologically and behavioral parameters.

This study provides evidence that the zebra finch can be used as an innovative animal model in nicotine-related research studying cognitive functioning and also in studies examining nicotine dependence and addictive mechanisms.

2. Experimental procedures

2.1. Animals and housing

Adult male zebra finches ($n = 54$) with a body weight between 12 and 15 g were purchased from a breeder (Acadiana Aviaries, LA). The animals were group housed (3–4 animals per cage) for a minimum of two weeks in our aviary room. The ambient temperature was nominally set at 26 ± 2 °C and the lighting conditions were set at 14 h light:10 h dark (lights on at 8:00 am). One week before testing, the animals were transferred to our custom-built recording cages (room next door) with identical environmental parameters. Food and water was available *ad libitum* throughout the experiment. The Animal Care and Use Committee at Florida State University approved all procedures.

2.2. Drugs

Nicotine hydrogen tartrate salt was purchased from Sigma Chemical Co. (St. Louis, MO) and was dissolved in physiological saline (0.9% sodium chloride). Nicotine doses were reported as freebase. Since nicotine has never been administered *in vivo* to zebra finches, we gathered data from rodent research (Itzhak and Martin, 1999; Quik and Di Monte, 2001; Shoaib et al., 2002; Collins and Izenwasser, 2004; Sahraei et al., 2004; Biala and Budzynska, 2006; Lutfy et al., 2006) and extrapolated these findings to the metabolism of birds. We performed a crossover study in our laboratory with six adult male zebra finches and found the following doses physiologically relevant: 0.054, 0.18, and 0.54 mg/kg, subcutaneously (referred to as low, mid and high dose of nicotine, respectively).

2.3. Experimental design

A total of 16 individual custom-built recording cages was used in the study. The birds used for this experiment were housed singly, in visual but not auditory isolation. The animals were tested for 6 consecutive hours (8:00–14:00) daily. Song production and spontaneous locomotor activity were automatically recorded. Body weights were registered at 7:00 and 19:00. At 7:00, food was refreshed and weighed (no food restriction). At 14:00 food was reweighed and feces samples were collected on a daily basis. This time point was chosen based on our previous data collection point (Nieves et al., 2008).

One day after the transfer of the animals, the recording of song production and spontaneous activity (perching) started. This period lasted for five days. Following these 5 days of recordings, a stable song production baseline was observed (day-to-day difference less than 10%). All animals were then injected for 5 consecutive days with physiological saline (0.03 ml/10 g body weight, s.c., at 7:00 and 19:00) to reduce stress due to handling (habituation period). After 5 days, the animals were randomly divided into three nicotine (high, mid, and low) and one saline group. Injections were administered 2× daily for 7 days. The injection times were kept at 7:00 and 19:00 in order to mimic the injection times during the habituation period. A total of 32 animals was tested.

2.4. Vocal recording

Digital audio recordings were made using a desktop PC equipped with a multi-channel sound card and Avisoft Recorder (Cappendijk and Johnson, 2005). The Avisoft recording software allowed real-time monitoring and event-triggered recording of birdsong according to

programmed frequency and duration criteria. A song data analysis program was developed in our laboratory to automatically count song production (Miller et al., 2007). Prior to initiation of the experiment, each chamber was calibrated to ensure the accuracy of the sound levels and measurements.

2.5. Spontaneous activity

The individual recording cages were equipped to monitor locomotor activity throughout the experiment. A magnetic sensor was installed on each of the two perches (low and high perch). Our laboratory developed a computer program to evaluate automatically the number and length of the events on each perch. In this study we used the number of events as our independent variable.

2.6. Non-invasive fecal sample collection and preparation

Fecal samples were collected from three animals, randomly chosen, from each of the 4 different groups (low, mid, high nicotine and saline group) on a daily basis. The collection took place at the end of the recording period (14:00). This time point was chosen based upon our previous collection point (Nieves et al., 2008). Each fecal sample was collected using a stainless steel spatula that was disinfected between collections to avoid cross-contamination. Fecal samples were placed in plastic vials and frozen at −20 °C until assayed. Our laboratory modified the extraction method of Washburn et al. (2003). Briefly, frozen fecal samples were placed in a lyophilizer (Savant Speedvac) for 24 h. The freeze-dried samples were ground, sifted through a stainless steel mesh (Thickness: 330 µm; Spectrum Labs) into a petri dish, and mixed thoroughly. The samples were weighed, and transferred into labeled tubes. Careful rinsing of the petri dish was performed with 1 ml of 90% methanol in water to minimize any loss of analytes. The samples were then vortexed at high speed in a multitube vortex for 30 min, and centrifuged at 3000 rpm for 20 min. The supernatant was transferred into a microcentrifuge tube and frozen until analysis.

2.7. Liquid chromatography/tandem mass spectrometry (LC/MS/MS) protocol

2.7.1. Standards and reagents

Cotinine, cotinine-d3, and nicotine-d3 were obtained from Cerriliant (Round Rock, TX). Acetonitrile, methanol, tetrahydrafuran, and methyl tertiary butyl ether (MTBE) were purchased from Burdick and Jackson (Muskegon, MI). Nicotine, formic acid, and ammonium formate were purchased from Sigma. Sodium hydroxide was obtained from GFS Chemicals (McKinley, OH).

2.7.2. Instrumentation

The LC/MS/MS analysis was performed on an HPLC system consisting of liquid chromatograph, Shimadzu, System Controller, SCL-10A Vp, Pumps, LC 10 AD Vp, Solvent Degasser, DGU14A (Columbia, MD). The autosampler consisted of an HTC Pal, Leap Technologies (Carrboro, NC). The mass spectrometer was a Waters Quattro API Micro, Waters Corp. with a data acquisition, Masslynx version 4.1 installed on IBM think center computer, Waters Corporation (Milford, MA), which was operated in the electrospray ionization (ESI) positive multiple reaction monitoring (MRM) mode. Quanlynx was used for the quantification of the results. Chromatographic separation was achieved using a Polaris Si-A column (50 mm × 3.0 mm; 5 um, Agilent Technologies, Palo Alto, CA). Chromatographic separation was employed by using hydrophilic interaction liquid chromatography (HILIC), (Alpert, 1990). HILIC was performed with a gradient initially of 100% 1:1 acetonitrile:methanol with 0.05% formic acid slowly changing to 90% over 3 min and 10% 10 mM ammonium formate with 0.05% formic acid.

The following optimized conditions were used: collision energy at 20 eV, with capillary voltage at 3.0 kV, cone voltage at 20 V, source temperature at 150 °C, and desolvation temperature at 400 °C. The desolvation gas flow was set at 300 l/h. The collision gas was argon.

2.7.3. Calibration standards and quality control samples

Calibration standards and quality control samples were prepared in 90% methanol and stored at −20 °C. The calibration curve utilized a linear regression, with weighting $1/x^2$, and range for the assay was 2–75 ng/ml for nicotine and 1–3000 ng/ml for cotinine. Cotinine and its deuterated internal standard were eluted at approximately 1.65 min and nicotine and its internal standard was eluted at approximately 3.0 min. Peak area ratios for the nicotine and cotinine peak versus each of the compounds' deuterated internal standards' were employed for calibration of the system and unknown sample concentrations were extrapolated from the calibration curve. All samples were analyzed in singlicate. Post-column infusion studies were performed to evaluate matrix effects and adjust chromatography as needed (Matuszewski et al., 2003).

2.7.4. Sample preparation

A volume of 50 ul supernatant was isolated using a single liquid extraction, evaporated to dryness, and diluted with mobile phase for analysis. Deuterated internal standard, base, and 90:10 methyl-t-butyl ether:tetrahydrafuran were added to each sample and nicotine was extracted. The organic layer was poured onto reconstitution solution and evaporated to dryness under a nitrogen stream. The samples were then reconstituted with 1% formic acid in acetonitrile and a volume of 10 ul was injected into the LC/MS/MS.

2.7.5. Validation

The method was validated for precision and accuracy. The interrun accuracy was determined by replicate analysis of quality control samples and at low quality control samples that were extracted from the sample batch. Accuracy was defined as the percent difference from nominal (%DFN) and assay precision was presented by percent relative standard deviation (%RSD).

2.7.6. Validation of the subcutaneous nicotine administration, a 36 h study

In order to validate our choice of drug administration in the zebra finch, we examined the presence of nicotine and cotinine in feces samples collected every 4 h for 36 h. The first collection of feces started 13 h before the first nicotine administration. Sixteen adult male zebra finches ($n = 4$ per treatment group) were used. This experiment was performed under the same conditions as described under the Experimental design section. The parameters (song production, locomotor activity, and food intake) were not evaluated, due to the frequent entry of personnel (every fourth hour) into the recording room to collect feces samples.

2.8. Statistical analysis

The effect of nicotine on baseline locomotor activity, food intake, body weight, song production and nicotine/cotinine fecal values was analyzed with a two way nested analysis of variance (ANOVA). The factors used in the ANOVA were treatment (nicotine or saline) and the various dose levels (control, low, mid, and high). All data were analyzed as a function of the dose. There was no effect of birds observed within the doses, therefore, this was not further addressed. The data collected in the 36 h study were not statistically evaluated, but were presented as nicotine/cotinine being present or absent in the fecal samples.

The effect on body weight for nicotine-treated animals was also analyzed with a two way nested ANOVA for within-subjects repeated measures. Statistically significant interactions were further analyzed

with Tukey's post hoc comparisons, with a correction for multiple comparisons performed if applicable. The Tukey's post hoc tests were conducted on the main effects from the ANOVA. The criterion for significance was set at a five-percentage level ($p \leq 0.05$).

Our analysis program was developed to analyze parameters using bins as small as 1 min. This type of representation of data however did not contribute to the clearness of the results and is not presented. We opted to analyze the song production and locomotor activity data for the total period of 6 h (8:00–14:00) and separately for the first hour of the light period (8:00–9:00) and for the second hour (9:00–10:00). These time points were chosen based on the relatively short half-life of nicotine and the zebra finch's high metabolism. In addition, as repetitive nicotine administration is known to induce behavioral sensitization among species (Mao and McGehee, 2010), we analyzed the song production and the locomotor activity data recorded on day 1 versus day 7 of the nicotine treatment for the total recording period of 6 h, and for the first and second hours, using a one-tail paired *t*-test.

3. Results

3.1. Behavior

The first exposure of nicotine in adult male zebra finches dose-dependently induced a flat posture, air gasps, and head twitch responses. In addition, a few animals demonstrated obsessive counter clock-wise turns. These signs occurred within 4 min following the nicotine administration and faded dose-dependently within 20–30 min.

3.2. Song production (Fig. 1)

There was a significant effect of dose on total song production over the length of the nicotine exposure ($F(3,38) = 5.54$, $p = 0.0029$). Post-hoc analysis showed that the control group (saline) and the low and the high nicotine group showed no differences in the song production, but the mid dose of nicotine induced a significant effect on the song production. Since no pharmacokinetic or pharmacodynamic data are available for nicotine metabolism in the zebra finch, we also analyzed the data after the first and second hour of testing. The song production in the first hour of the light period (8:00–9:00) was significantly different for treatment levels ($F(3,38) = 7.85$, $p = 0.0003$). Post-hoc analysis showed that song production was significantly different for the dose pairs control-mid dose and the control-high dose of nicotine. Consistent results were obtained for song production records, registered in the second hour (9:00–10:00) of testing. The second hour song production showed that the treatment was significantly different ($F(3,38) = 4.5$, $p = 0.0084$), due to significance between the mid and low dose of nicotine treatment.

3.3. Locomotor activity (Fig. 2)

Analysis of the total locomotion activity showed no difference in the dose-dependent treatment ($F(3, 38) = 0.68$, $p = 0.57$), while the analysis of the first hour of testing (8:00–9:00) showed a significant dose-dependent treatment effect ($F(3,38) = 4.35$, $p = 0.0098$). Post-hoc analysis showed that the high nicotine dose induced a significant decrease in locomotor activity. The analysis for the second hour of testing was not significantly different when saline exposed controls were compared to nicotine-exposed animals.

3.4. Behavioral sensitization (Table 1)

Comparing the first (D1) and the last (D7) day of the nicotine treatment showed that during the first hour of testing (08:00–09:00) a significant increase of song production and locomotor activity occurred in the high nicotine-exposed group, while in the mid nicotine-exposed group a tendency for significance was observed in the locomotor activity ($p = 0.07$). During the second hour of testing (09:00–10:00), a significant increase in song production was observed in the high nicotine-exposed group, while in the mid nicotine-exposed group the locomotor activity was significantly increased. Song production registered for the total recording time of 6 h, showed a significant increase in song production in the mid nicotine-exposed group, while there was a tendency to significance was registered in the high nicotine-exposed group ($p = 0.06$). No significant effects were observed in the group of animals exposed to the low dose of nicotine, although the average values measured for the locomotor activity during the first and the

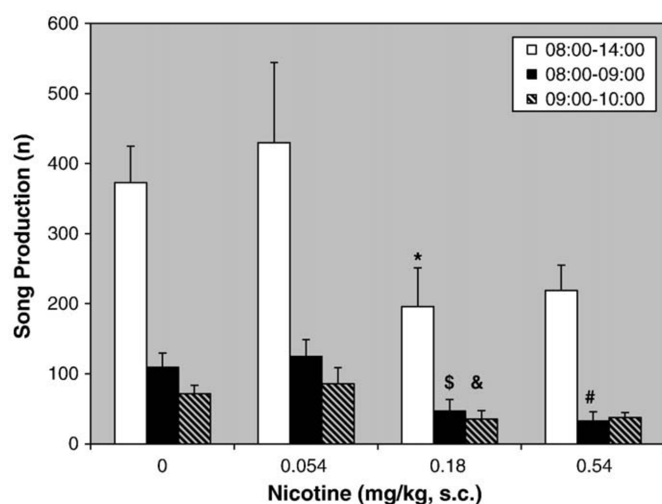

Fig. 1. The number of vocalizations of the zebra finches, presented for the total daily recording period (8:00–14:00, white bars), the first hour of the recording period (8:00–9:00, black bars) and the second hour (9:00–10:00, striped bars). $N = 8$ each group. The bars represent mean ± S.D. On the *x*-axis, the doses of nicotine and saline are presented. Post-hoc analysis of the total recording time showed that the song production in 0.18 mg/kg nicotine group differed significantly from the saline group (*). Post-hoc analysis of the song production registered in the first hour (8:00–9:00) showed a significant difference for the control-mid dose ($) and the control-high dose of nicotine (#). Consistent results were obtained for the analysis of the data gained in the second hour of the recording period (9:00–10:00). A significant difference was observed, due to a difference between the mid and low dose of nicotine treatment (&).

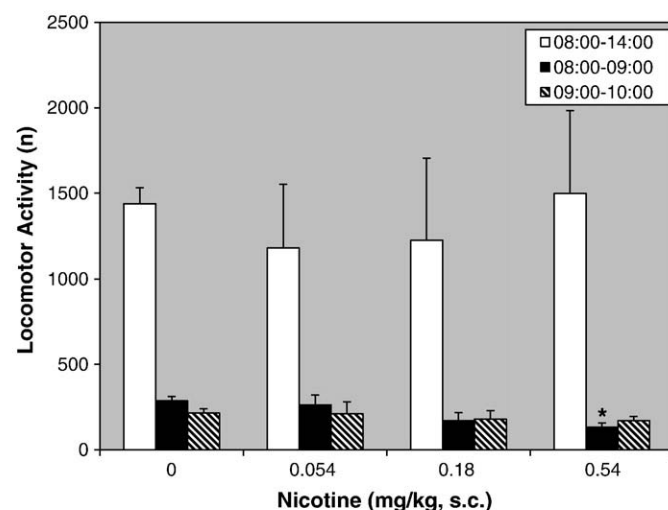

Fig. 2. Total locomotor activity (8:00–14:00, white bars), locomotor activity produced in the first (8:00–9:00, black bars) and the second hour (9:00–10:00, striped bars) of the recording period. $N = 8$ each group. The bars represent mean ± S.D. Analysis of the total locomotion activity showed no difference in the dose-dependent treatment, while the analysis of the first hour of testing showed a significant dose-dependent treatment effect. Post-hoc analysis showed that the high nicotine dose induced a significant decrease in locomotor activity (*).

Table 1
The number of vocalizations (VOC, part A) and locomotor activity (LOC, part B) presented for the total daily recording period (8:00–14:00), the first (8:00–9:00) and the second (9:00–10:00) hour, measured on the first (D1) and on the last day (D7) of the saline/nicotine treatment. Doses of nicotine (0.054, 0.18, 0.54 mg/kg, s.c.) are referred to as low, mid and high nic, resp. The values represented are mean ± SEM. $N = 8$ each group.

Treatment	A. VOC			B. LOC		
	8:00–14:00	8:00–9:00	9:00–10:00	8:00–14:00	8:00–9:00	9:00–10:00
Saline						
D1	353 ± 45	113 ± 5	73 ± 12	1462 ± 209	216 ± 28	239 ± 63
D7	407 ± 61	112 ± 17	81 ± 12	1310 ± 160	281 ± 37	211 ± 29
p-value	0.19	0.41	0.35	0.36	0.18	0.48
Low nic						
D1	342 ± 98	115 ± 29	72 ± 22	1555 ± 544	212 ± 65	167 ± 58
D7	398 ± 144	105 ± 28	70 ± 24	1188 ± 189	279 ± 47	268 ± 54
p-value	0.19	0.20	0.29	0.13	0.25	0.22
Mid nic						
D1	191 ± 68	50 ± 24	38 ± 17	1142 ± 404	123 ± 59	109 ± 52
D7	269 ± 78	54 ± 18	64 ± 32	790 ± 189	125 ± 34	164 ± 50
p-value	0.02	0.42	0.17	0.42	0.07	0.03
High nic						
D1	164 ± 39	22 ± 12	30 ± 9	1367 ± 618	104 ± 38	127 ± 41
D7	231 ± 37	46 ± 19	54 ± 11	1361 ± 454	166 ± 40	201 ± 43
p-value	0.06	0.01	0.05	0.48	0.005	0.14

second hour of testing were slightly elevated. In the saline treated group no significant effects were observed between mean values of vocalization and locomotor activity from day 1 versus day 7.

3.5. Food intake

No significant effects on food intake were found when comparing the nicotine dose-dependent treatment with the control ($F(3,38) = 1.1$, $p = 0.36$). Saline animals had an average food intake of 3.5 ± 0.1 g (mean ± SD), while nicotine-exposed animals ate an average of 3.2 ± 0.3, 3.6 ± 0.3, and 3.4 ± 0.1 g (low, mid, high dose, respectively). However, when comparing saline with all nicotine-exposed animals a significant effect was observed ($F(1, 38) = 4.66$, $p = 0.037$). Throughout the experiment, no spillage of food was registered on the floor of the cages.

3.6. Body weight

We compared the body weights measured in the morning (7:00) during the 5 days of saline injections (habituation period) versus the 7 days of nicotine injections. The first number corresponds with the average body weight (g) registered during the habituation period, while the second number corresponds with the average body weight registered during the nicotine/saline treatment: saline 15.0 ± 0.8, 14.9 ± 0.9; low 13.5 ± 0.4, 13.2 ± 0.4; mid 14.6 ± 0.6, 14.5 ± 0.5; high 14.8 ± 0.8, 14.7 ± 0.6. No interaction effect between body weight and nicotine treatment was observed (within-subjects).

Table 2
Interrun ($n = 6$) Precision and Accuracy of Nicotine and Cotinine. The accurancy is defined as percent difference from nominal (%DFN), precision by percent relative standard deviation (%RSD).

Analyte	Concentration (ng/ml)	Estimated Mean ± S.E.	Precision (%RSD)	Accuracy (%DFN)
Nicotine	4.0	4.6 ± 0.6	12.5	14.6
	20.0	19.2 ± 1.6	8.6	−4.1
	60.0	56.2 ± 1.9	3.4	−6.4
Cotinine	3.0	3.0 ± 0.3	8.8	−0.6
	500.0	519.1 ± 24.7	4.8	3.8
	1500.0	1513.4 ± 70.3	4.6	0.9

3.7. Bioassay nicotine/cotinine (Tables 2 and 3)

The interrun assay precision and accuracy values were less than 15%, and therefore met the established acceptance criteria given by the US Department of Health and Human Services, Food and Drug Administration (2001).

There was no significant dose-dependent nicotine effect on the nicotine ($F(3,10) = 2.0$, $p = 0.17$) and cotinine ($F(3,10) = 1.8$, $p = 0.2$) levels measured in the feces. However, if the levels of nicotine and cotinine in the control group were compared with nicotine-exposed animals, it showed that both nicotine ($F(1,10)$, $F = 5.4$, $p = 0.04$) and cotinine ($F(1,10) = 5.18$, $p = 0.045$) were significantly different. The mean values for nicotine-exposed animals for nicotine and cotinine were 0.468 and 0.128 ng/mg, respectively. The mean values used for the control animals for statistical purposes were the limit of quantitation (LOQ) values.

3.8. Validation of the subcutaneous nicotine administration: nicotine and cotinine in fecal samples collected over 36 h (Figs. 3 and 4)

One hour before the last saline administration (habituation period) feces samples were collected (6:00). Following this first feces collection, samples were collected every 4 h, totaling 36 h. Thirteen hours later the animals, which were randomly divided in three nicotine groups and one saline group, received the first nicotine/saline administration (19:00). Samples collected at 22:00 did not show any presence of nicotine and cotinine. In the samples collected at 2:00, the presence of nicotine was confirmed in two animals treated with the mid dose of nicotine, while in the high nicotine group cotinine was measured in one animal. Four

Table 3
Nicotine (ng/mg) and Cotinine (ng/mg) measured in non-invasive collected fecal samples. Three animals per group were chosen at random for analysis. Nicotine was given subcutaneously, 2x/day, with a 12 hour interval, for 7 days. Samples were collected on a daily basis throughout the experiment. BLQ = below limit of quantitation.

Groups	Nicotine (ng/mg)	Cotinine (ng/mg)
Saline ($n = 3$)	BLQ	BLQ
Low nicotine ($n = 3$, 0.054 mg/kg, s.c.)	0.164 ± 0.17	0.052 ± 0.084
Mid nicotine ($n = 3$, 0.18 mg/kg, s.c.)	0.76 ± 0.93	0.338 ± 0.28
High nicotine ($n = 3$, 0.54 mg/kg, s.c.)	0.259 ± 0.39	0.125 ± 0.12

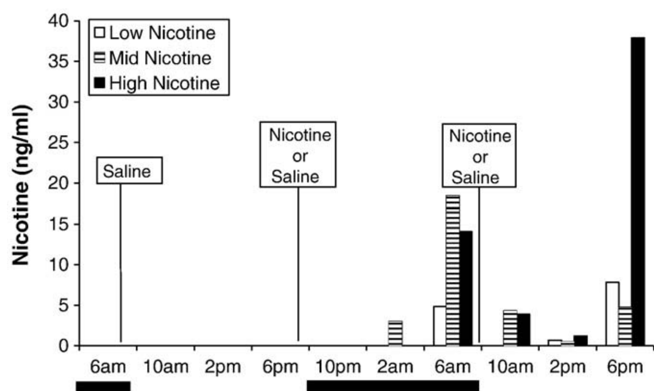

Fig. 3. Nicotine (ng/ml) measured in feces samples, collected from low (0.054 mg/kg, s.c., white bars), mid (0.18 mg/kg, s.c., striped bars) and high nicotine (0.54 mg/kg, s.c., black bars) exposed animals. Note that 11 h following the first nicotine exposure both nicotine and cotinine are present in all nicotine-treated groups, and that the expression levels fluctuate over time, but never reach zero. Nicotine and cotinine expression values in the feces collected from control animals could not be measured, and this group is therefore not presented in these figures. The black bar beneath the *x*-axis represents the dark period (lights off 22:00–lights on 8:00).

hours later (6:00), the presence of nicotine and cotinine was confirmed in the three groups of nicotine animals, but not in the control group (saline). In the following 12 h, nicotine and cotinine were present in all nicotine-treated groups but not in the saline groups. These data demonstrate that the drug regimen used in our study is able to produce a fluctuating level of nicotine and cotinine in the zebra finch feces, starting 11 h following the first nicotine exposure.

4. Discussion

Our study confirms that the zebra finch can be used for *in vivo* nicotine-related research. Our strongest finding is that the subcutaneous nicotine drug regiment induced values of nicotine and cotinine that are comparable with values measured in human and rodent models of nicotine exposure. Based upon this finding we conclude that the use of non-invasive collected fecal samples is justified in this animal model. We can also conclude that the zebra finch metabolizes nicotine. In respect to our parameters, we found that song production, which might be considered as a measurement for synaptic plasticity, was acutely affected by mainly the mid dose and to some extent the high dose of nicotine compared to the control group and the low nicotine group. Spontaneous locomotor activity, which was registered in the same recording period as song production was only significantly affected by the high dose of nicotine following the first hour of administration, when analyzing the combined data from the total treatment. Another important finding is the demonstration of behavioral sensitization of song production and locomotor activity in zebra finches exposed to the mid and the high nicotine dose. The low dose of nicotine did not induce a significant increase in the song production and the locomotor activity. However, values for locomotor activity were slightly higher in the first and second hour of recording. Behavioral sensitization is known to occur following the repetitive administration of nicotine across species (Vezina et al., 2007; Mao and McGehee, 2010; DiFranza and Wellman, 2007; Sax and Strakowski, 2001). Our findings support our statement that the zebra finch can be used to examine the *in vivo* effects of nicotine.

We decided on giving nicotine subcutaneously to the animals. The reason for choosing this type of administration was that giving an intraperitoneal injection of nicotine goes through first pass metabolism, while subcutaneous injections bypass this first pass metabolism. The current study is primarily concerned with nicotine's effects in the CNS and thus bypassing first pass metabolism is an important criterion. The first exposure of nicotine in adult male zebra finches induced a flat posture, air gasps, and head twitch responses, which were dose-dependent. The occurrence of these signs could point toward the involvement of the central serotonergic system (Yasuda et al., 2002). Previously, we cloned a 5-HT1B receptor in the zebra finch (Cappendijk and Thompson, 2005); this receptor plays a role in the modulation of acetylcholine release (Buhot et al., 2003) and could interact with the effect of nicotine. In addition, in some animals obsessive contralateral moves were observed. This could be attributed to a dysbalanced central dopaminergic system (Ikeda et al., 2007). Like the mammalian striatum, area X, which is the striatal component in the anterior forebrain pathway in songbirds (Gale and Perkel, 2009), receives a dense dopaminergic innervation from the midbrain. It is known that this song nucleus is involved in motor control.

The goal of this study was to demonstrate that the subcutaneous nicotine treatment regimen is able to induce CNS effects, which was confirmed. To examine the role of the serotonergic and/or dopaminergic system in respect to nicotine dependence and addictive mechanisms (tolerance, sensitization, and reward) in the zebra finch does not fall within the scope of this study. Our observed behavioral sensitization however, confirms the possibility of using the zebra finch in studies focusing on drug dependence and addictive mechanisms. Currently, there are no reliable behavioral test matrices available for examining the processes nicotine dependence and addictive mechanisms (reward, sensitization, withdrawal, and tolerance) in the zebra finches. Our laboratory plans to develop some of the behavioral test matrices and ultimately incorporate these types of studies in future work.

There is a wide spectrum of plasma levels observed in human nicotine users and in rodent models. Factors such as dose, route of administration, length of treatment, gender and/or age of subjects play a role in the way that nicotine is processed. Plasma levels in humans peak at around 15–40 ng/ml following cigarette smoking (Russell et al., 1986), a value that was confirmed in a more recently performed study by Moriya and Hashimoto (2003). These authors showed that in smokers, nicotine and cotinine levels were 4–62 and 50–217 ng/ml in blood, respectively. Birds exposed to second hand smoke (smoking households) showed significantly higher plasma cotinine levels (4.3–37.8 ng/ml) than control birds from nonsmoking households (0–3.6 ng/ml). These high levels of cotinine are similar to those reported in humans with clear evidence of clinical alteration and resultant disease from environmental tobacco smoke (Cray et al., 2005). Shoaib and Stolerman (1999) showed that in rats self-administering nicotine (0.06 mg/kg per injection) the nicotine levels ranged from 40 to 120 ng/ml.

Due to the small blood volume of the zebra finch and the accompanying induced stress, we considered the daily withdrawal of blood

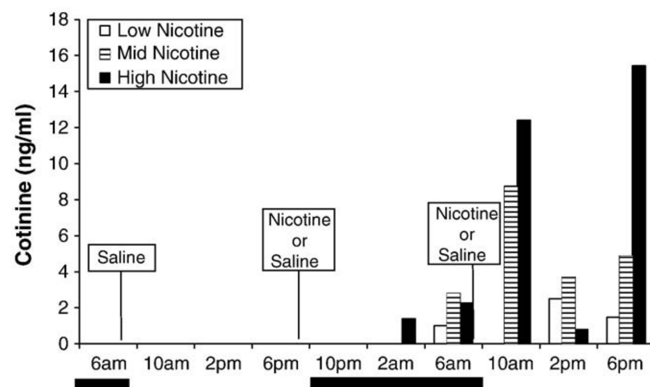

Fig. 4. Cotinine (ng/ml) measured in feces samples, collected from low (0.054 mg/kg, s.c., white bars), mid (0.18 mg/kg, s.c., striped bars) and high nicotine (0.54 mg/kg, s.c., black bars) exposed animals. Note that 11 h following the first nicotine exposure both nicotine and cotinine are present in all nicotine-treated groups, and that the expression levels fluctuate over time, but never reach zero. Nicotine and cotinine expression values in the feces collected from control animals could not be measured, and this group is therefore not presented in these figures. The black bar beneath the *x*-axis represents the dark period (lights off 22:00–lights on 8:00).

samples for measuring nicotine and cotinine levels not feasible, and thus opted for a non-invasive fecal sample collection. This method of measuring was validated in an earlier published paper by our laboratory, in which we measured corticosterone levels in the feces (Nieves et al., 2008).

The validation interrun assay showed that the non-invasive sample collection is a validated method to measure nicotine and cotinine in fecal samples of the zebra finches. In respect to the values of nicotine and cotinine measured in the bioassay, the most striking effect is the apparent reversal of concentrations between the middle and high group (inverted U-shaped dose–effect relationship, Baldi and Bucherelli, 2005). At this moment, the underlying mechanism of action is unclear, but there are many possible explanations; at high doses the nicotine becomes bound to some type of protein in organ or other tissue. At high doses some enzymatic cascade may be disrupted interfering with the normal metabolism of nicotine and or cotinine (Rubinstein et al., 2007). Further analysis of these dose effects will have to be studied in order to determine if these results are due to some type of protein binding and/or tissue disposition.

The zebra finch is one of the few animal models that exhibit vocal development as a naturally learned process. Bird song and human speech originate both in the telencephalon, which is the center for the highest central nervous functions: e.g., sensory perception; evaluation of sensations; initiation and regulation of muscle movements; language; thought, learning and memory. Our results showed that exposure of adult male zebra finches to a low, mid or a high dose of nicotine (s.c., twice per day for 7 days) dose-dependently affected the song production. Two main neural pathways are involved in song production and song learning. The “motor pathway” controls the vocal motor program through the hierarchical organization of several premotor nuclei. A key nucleus in the motor pathway is the robust nucleus of the arcopallium (RA), which projects to brainstem nuclei controlling the vocal and respiratory muscles. During singing, RA neurons in adult birds generate a highly stereotyped sequence of bursts, which appear to be driven by precisely timed inputs from a higher premotor vocal area, the HVC (Hahnloser et al., 2002). In addition, the RA also receives input from the anterior forebrain pathway, a circuit homologous to the basal ganglia thalamo-cortical loops, which may be involved in controlling motor behavior and stereotypy in mammals (Canales and Graybiel, 2000; Gale and Perkel, 2009). A cholinergic innervation is present in the song system (Ryan and Arnold, 1981). One *in vitro* study showed nicotine-mediated plasticity in the RA and suggested the presence of alpha-4 and alpha-7 nicotinic acetylcholine receptor subtypes (nAChR, Salgado-Commissariat et al., 2004). At this point, the nature of the interaction of nicotine and/or cotinine with the song system is unknown, but our *in vivo* data suggest that nicotine exposure affects the number of vocalizations. We observed a U-shaped dose–effect relationship with the quantity of the song. One of the possible explanations could lie in the finding that Salgado-Commissariat et al. (2004) demonstrated a bi-directional synaptic plasticity in the RA, which was attributed to a possible activation of both alpha-4 and alpha-7 nAChRs. These receptor subtypes are known to be involved in cognitive processes (Picciotto et al, 2001; Leonard and Bertrand, 2001; Levin et al., 2006). Nicotine has a different dissociation constant for the alpha-4 and alpha-7 nAChRs, with the higher affinity for alpha-4 (Tribollet et al., 2001; Damaj et al., 2007). Therefore, we can argue that stimulation of these two receptor subtypes with different concentrations of nicotine could affect the metabolic rate, sensitivity presenting itself as a stimulatory (low) and as an inhibitory (mid more pronounced compared to high) stimulus. nAChRs affinity studies with zebra finch tissue will be performed as follow-up studies.

The results from the 36 h study in which we evaluated the presence of nicotine and cotinine in fecal samples collected every 4 h showed that a continuous, yet fluctuating, level of nicotine and cotinine could be measured in the feces (Figs. 3 and 4, respectively). The first presence of nicotine and cotinine was confirmed 7 h following the first mid and high dose of nicotine exposure, respectively. The low nicotine-exposed animals showed the first presence of nicotine and cotinine 11 h following the first administration. This finding confirms that nicotine is processed in the zebra finch and that the main metabolite cotinine is measurable. Follow-up studies to examine the involvement of the heme-thiolate proteins (CYP450 family) in the metabolism of nicotine and cotinine in the zebra finch are planned.

In rodent models, a variety of locomotor activity models is available to examine the effects of nicotine on cognitive functioning, such as spatial memory tasks (review Carrasco et al., 2006). To study spatial memory task, zebra finches can be tested in a so-called dry Water Maze model (Watanabe and Bischof, 2004). We opted not to test the animals in this model, but rather make use of the natural perch hopping behavior of the zebra finch. Adding the dry maze would have complicated our design of the study, but it will certainly be included for the follow-up studies.

The body weight of the animals did not change during the treatment with nicotine, nor was an overall significant effect on food intake observed. This is an important observation, as it is known that acute nicotine administration can induce appetite suppression (review Chen et al., 2007). Why was a weight loss not observed in the nicotine-exposed zebra finches? Several internal and external factors alone or combinations thereof could attribute to this observation. For example, the drug regiment and/or the administered nicotine dose and route of administration (Kramer et al., 2007; McNair and Bryson, 1983; Levin et al., 1987) could affect the body weight. In the study performed by Kramer et al. (2007), rats received four times a day for 6–7 days in total, intraperitoneal injections of 35 mg/kg nicotine. The observed weight loss was blocked after the application of the nicotinic antagonist mecamylamine into the fourth ventricle. These authors proposed that the perifornical hypothalamus lies on the basis of the weight loss effect of nicotine. It has been shown that catecholaminergic neurons adjacent to this fourth ventricle contain nicotinic receptors that directly innervate the perifornical hypothalamus. It is currently unknown whether there is a structure present in the zebra finch that is identical/comparable with the perifonical hypothalamus in rodents. In addition, the effect of nicotine on the release of catecholamines in the zebra finch might be different than in rodents (Wellman, 1992). However, no data are available on the relationship between catecholamines release in zebra finches and food intake. Factors such as neuropeptide Y and orexin released by the hypothalamus are involved in food intake in rodents (Li et al., 2000b; Kane et al., 2000) but have not examined in the zebra finch in respect to food intake. At this point, we also need to mention that food intake patterns for zebra finches are most likely not similar to the food intake pattern of that observed in rats or other species. A difference in metabolism among different species could play an important role. As we mentioned earlier in this discussion, we are planning to perform pharmacokinetic tests in the zebra finch in order to get an understanding of the metabolism of nicotine and the formation of cotinine under *in vivo* and *in vitro* conditions. These types of studies will lay the foundation and hopefully provide insight about the underlying mechanism of action of nicotine on body weight in zebra finches.

In summary, this study provides evidence that the zebra finch can be used as an innovative animal model in nicotine research with unlimited potential, not only in respect to cognition, but also in studies examining nicotine dependence and addictive mechanisms.

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