DMD Fast Forward. Published on March 25, 2013 as doi:10.1124/dmd.112.050765

Title Page

Nicotine Kinetics in Zebra Finches *in vivo* and *in vitro*.

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Nicotine kinetics in zebra finches.

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No. text pages: 32

No. tables: 0

No. figures: 5

No. references: 54

No. words

Abstract: 244

Introduction: 561

Discussion: 1418

List of Abbreviations: AUC: area under the concentration curve, CYP: cytochrome P450, HPLC: high performance liquid chromatography.

DMD Fast Forward. Published on March 25, 2013 as DOI: 10.1124/dmd.112.050765
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Abstract

Nicotine enhances cognitive performance, and in the zebra finch *(Taeniopygia guttata),* which is a well-established model of cognition, the effect of nicotine on song production has been reported. Nicotine and cotinine plasma levels were assessed *in vivo* after s.c. injection of 0.18 mg/kg nicotine, a dose that elicits changes in song production. The half-life of nicotine elimination was 33 min, and levels were undetectable by 4h. Average plasma nicotine over 2 hours was 32 ng/ml, similar to levels seen in human smokers and rat models of nicotine behavior. Nicotine brain levels were 30 and 14 ng/g at 1 and 2 h after treatment. To understand the potential for drug interactions and regulation of nicotine metabolism in zebra finches, we characterized *in vitro* nicotine metabolism and the hepatic enzyme involved. In humans, cytochrome P450 (CYP) 2A6 metabolizes nicotine to cotinine, and CYP2A-like activity and protein have been reported in some birds. Zebra finch liver microsomes metabolized nicotine and bupropion (CYP2B substrate) but not coumarin (CYP2A substrate). Nicotine was metabolized to cotinine with a K_m of 96 µM and V_{max} of 56 pmol/min/mg. Nicotine and bupropion metabolism were inhibited by C-8-xanthate (specific CYP2B inhibitor) but not CYP2A-specific inhibitors, and hepatic levels of CYP2B-like but not CYP2A-like proteins correlated with nicotine ($r = 0.52$, $p = 0.04$) and bupropion metabolism ($r = 0.81$, $p < 0.001$), suggesting CYP2B-mediation of nicotine metabolism as seen in rats. These results will facilitate further investigation of nicotine's effects in zebra finches.

Introduction

Nicotine is a psychoactive alkaloid that is able to enhance cognition in humans. This property has prompted investigation into therapies acting through the nicotinic acetylcholine receptor system for impaired cognition in humans (Levin et al., 2006; Taly et al., 2009). The zebra finch *(Taeniopygia guttata)* is a well-established model of cognition, learning and memory, and development of complex behaviour patterns having strong analogies to similar processes in humans (Gale and Perkel, 2010). As such, this animal model is suitable for investigating cognitive deficits associated with neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Adult males communicate vocally, and young males learn their song through mimicry and practice. Zebra finches are closed-end learners and by the end of the unary sensitive period for song learning, a single, complex highly stereotyped song is learned. The learning of the song resembles speech acquisition in humans (Bolhuis et al., 2012). The neural circuitry involved in song learning is regulated in part by inputs from the dopaminergic system and through nicotinic acetylcholine receptors (Salgado-Commissariat et al., 2004; Lovell et al., 2008; Kubikova and Kostal, 2010). The effect of nicotine in the zebra finch model was reported recently for the first time (Cappendijk et al., 2010). Both song production and locomotion demonstrated a dose-dependent response to nicotine. Acute nicotine treatment affected song production, and prolonged nicotine treatment appeared to cause sensitization. The effects of nicotine were more evident during the first and second hours after treatment than at later times after treatment. However, the *in vivo*

disposition of nicotine and its metabolites, and the characteristics of hepatic nicotine metabolism are unknown in zebra finches.

In most mammals that have been studied, nicotine is metabolized primarily to cotinine, which is further metabolized to *trans* 3'-hydroxycotinine (Nwosu and Crooks, 1988; Matta et al., 2007). When nicotine is administered *in vivo,* either experimentally or through smoking, it is cleared from the plasma rapidly, with half-lives ranging from approximately 8 minutes in mice to 2 hours in humans, whereas cotinine is cleared more slowly, with half-lives ranging from approximately 30 minutes in mice to 16 hours in humans (Siu and Tyndale, 2007; Benowitz et al., 2013). In humans, monkeys and mice, the metabolism of nicotine to cotinine and further to *trans* 3'-hydroxycotinine is mediated by CYP2A enzymes (Nakajima et al., 1996; Messina et al., 1997; Siu and Tyndale, 2007), whereas in rats the metabolism of nicotine to cotinine is mediated by a CYP2B enzyme, and *trans* 3'-hydroxycotinine is a minor metabolite, essentially undetectable in plasma (Nwosu and Crooks, 1988; Nakayama et al., 1993). The livers of domestic fowl express a CYP2A-like protein and can metabolize coumarin (Diaz et al., 2010; Murcia et al., 2011), a substrate of human CYP2A6 (Pelkonen et al., 2000), however nicotine metabolism has not been investigated in any birds including zebra finches.

This study of the pharmacokinetics and hepatic metabolism of nicotine in zebra finches was undertaken as it is important to understand the relationship between nicotine dose and plasma levels of nicotine and its metabolites, not only to give a better understanding of the behavioural response to nicotine in finches, but also to enable

comparison with other animal models. In addition, it is useful to characterize *in vitro* nicotine metabolism in an animal model to avoid potential drug interactions during *in vivo* experiments, and to understand how enzyme regulation may alter nicotine metabolism and subsequent behaviors.

Materials and Methods

Animals: Adult male zebra finches purchased from Acadiana Aviaries, LA, were group housed 3-4 per cage in the aviary room for a minimum of two weeks. One week before testing, the animals were transferred to single housing conditions. Housing and testing conditions were kept constant at 26 ± 2ºC, 14 h light:10 h dark, lights on at 8.00 a.m., with food and water ad libitum throughout the full length of the experiment. All procedures were approved by the Animal Care and Use Committee at Florida State University, in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Reagents: The ZORBAX Bonus –RP and ZORBAX SB C18 HPLC columns were from Agilent Technologies Inc., Mississauga, Canada; Isolute HM-N columns were from Biotage LLC, Charlotte, NC. Bupropion, hydroxybupropion and human lymphoblast-expressed CYP2A6 and CYP2B6 were from BD Biosciences, Mississauga, Canada; 7-hydroxycoumarin was from Chem Service, West Chester, PA; monoclonal anti-beta-actin antibody (A3853), nicotine bitartrate, cotinine, coumarin, 4-hydroxycoumarin, timolol maleate, 8-methoxypsoralen, pilocarpine hydrochloride, quinidine and ketoconazole were from Sigma-Aldrich Canada Ltd., Oakville, Canada; 5-methyl-

cotinine and C-8-xanthate were from Toronto Research Chemicals, Toronto, Canada. Polyclonal rabbit anti-CYP2B1 antibody (AB10079) and goat anti-rabbit-HRP (AP187P) secondary antibody were from EMD Millipore, Temecula, CA; polyclonal mouse anti-CYP2A6 antibody (SAB1400063) was from Sigma-Aldrich, St Louis, MO; nitrocellulose membrane was from Pall Canada Ltd., Mississauga, Canada; Pierce ECL western blotting substrate and goat anti-mouse-HRP secondary antibody (31430) were from Thermo Scientific, Rockford, IL; autoradiographic film was from Ultident Scientific, St-Laurent, Canada. All other reagents were from standard suppliers. Human liver was obtained from the Biocentre in Basel, Switzerland (Meier et al., 1983) from a male individual with average K_m and V_{max} for nicotine metabolism (Al Koudsi et al., 2010).

***In vivo* nicotine pharmacokinetics:** Zebra finches (n=24) were treated with 0.18 mg nicotine base/kg s.c. as a sterile solution of nicotine bitartrate in saline at pH 7.4. This dose was chosen based on a previous study where it was found to have the strongest effect on song production (Cappendijk et al., 2010). Blood samples were taken at two time points from each animal, the first from the paw vein, the second at sacrifice by heart puncture under equithesin anaesthesia. Brains and livers were harvested at sacrifice and stored at -80 ºC. Sampling times were staggered to provide samples at 30 (n=10), 45 (n=8), 60 (n=8), 90 (n=10), 120 (n=8) and 240 min (n=4). Plasma samples were deconjugated in 0.2 M acetate buffer pH 5.0 with β-glucuronidase at a final concentration of 15 mg/ml, overnight at 37ºC. Samples were prepared by the solid phase extraction procedure and analyzed by HPLC as previously described (Siu et al., 2006), with a modified mobile phase of citric acid buffer:acetonitrile mixture (100:5.5

v/v). The citric acid buffer consisted of 0.034 M citric acid and 0.034 M potassium phosphate monobasic, 3.3 mM heptane sulfonic acid and 0.5% triethylamine, adjusted to pH 5.5. The limits of detection for nicotine, cotinine and *trans* 3'-hydroxycotinine were 5 ng/ml (11 nM), 12.5 ng/ml (71 nM) and 10 ng/ml (52 nM), respectively.

Nicotine and cotinine levels in brain: Individual brains, collected at 1 and 2 h after nicotine administration, were homogenized in 3 volumes (w/v) of ice cold saline; 65 µg 5-methyl-cotinine was added as the internal standard, and the mixture centrifuged at 3000 rcf for 10 min. The supernatant was prepared by the solid phase extraction procedure, but without prior deconjugation, and analyzed by HPLC as above. Nicotine and cotinine calibration curves were prepared by adding nicotine and cotinine (0-1000 ng/g brain tissue) to brain homogenate (0.166 g/ml) from untreated animals with 5-methyl-cotinine, and the homogenate then prepared for HPLC as above.

***In vitro* nicotine metabolism**: Microsomal membranes were prepared from individual livers as described previously, and membranes suspended in 1.15% aqueous potassium chloride and cytosol (supernatant) were stored at -80 ºC until use (Siu et al., 2006). Linear conditions were established by incubating a mixture of pooled liver microsomes at protein concentrations of 0, 0.5, 0.75 and 1.0 mg/ml, at nicotine concentrations of 60 and 480 µM, for 10, 15, 20 and 40 min. Various concentrations of cytosolic protein (a source of aldehyde oxidase required after the CYP-mediated conversion of nicotine to an iminium ion, to convert the iminium ion to cotinine), 25, 50, 75 and 100 µg, were incubated with 0.75 mg/ml microsomal protein for 20 min at 480 µM nicotine. The final linear incubation conditions were 0.5 mg/ml microsomal protein in

50 mM Tris-hydrochloric acid buffer, pH 7.4, 75 µg cytosolic protein, pre-warmed at 37 ºC for 2 min, followed by addition of 1 mM NADPH and nicotine in a final volume of 0.5 ml to initiate the reaction, then incubation for 20 min. Reactions were stopped by adding 100 µl 20% aqueous sodium carbonate. Samples were prepared for HPLC by liquid-liquid extraction (Schoedel et al., 2003) by addition of 65 µg 5-methyl-cotinine as the internal standard, 50 µl 10 N sodium hydroxide and 4 ml dichloromethane. Mixtures were shaken for 10 min, centrifuged at 3000 rcf for 10 min, and the organic layer with 25 µl 6 N hydrochloric acid was evaporated under a stream of nitrogen at 37 ºC. The residue was reconstituted with 105 µl distilled water and 90 µl analyzed by HPLC as above. Kinetic parameters of nicotine C-oxidation to cotinine by bird liver microsomes were determined in triplicate by incubating pooled microsomes with various concentrations of nicotine (0 – 960 µM), and microsomes from each individual birds were assessed at 45, 90 and 960 µM nicotine. For correlations, the velocity of cotinine formation by individual liver microsomes was determined with 1 mM nicotine, approximate nicotine concentration at V_{max} (10 x K_m of 96 µM determined from pooled microsomes).

***In vitro* coumarin metabolism:** Liver microsomes (0.5 mg/ml in 50 mM Tris-hydrochloric acid buffer pH 7.4 prewarmed at 37 ºC for 2 min) were incubated with 1, 5 and 50 µM coumarin, which are approximately 2x, 10x and 100x K_m for formation of 7-hydroxycoumarin by human liver microsomes (van Lersel et al., 1994), added with 1 mM NADPH in a final volume of 500 µl, and the reaction stopped after 30 min with 100 µl 20% aqueous sodium bicarbonate. Human liver microsomes (0.5 mg/ml) and human

lymphoblast cDNA-expressed expressed CYP2A6 (10 pmol) were used as positive controls. A 100 µl aliquot of the incubation mixture with 25 ng 4-hydroxycoumarin as the internal standard was prepared and separated by HPLC as previously described (Ho et al., 2008); the limits of quantification were 5 ng/ml (34 nM) for coumarin and 25 ng/ml (154 nM) for 7-hydroxycoumarin.

***In vitro* bupropion metabolism:** Liver microsomes from individual birds, and human liver microsomes as a positive control, (0.5 mg/ml in 50 mM Tris-hydrochloric acid buffer pH 7.4 prewarmed at 37 ºC for 2 min) were incubated with 100 µM bupropion which is the approximate K_m for formation of hydroxybupropion by human liver microsomes (Hesse et al., 2000), added with 1 mM NADPH in a final volume of 500 µl, and the reaction stopped after 20 min with 100 µl 20% aqueous sodium bicarbonate. A 0.5 ml aliquot of the incubation mixture was prepared for HPLC (Loboz et al., 2005) by adding 1.5 µg timolol maleate as the internal standard, and extracting with 0.5 ml 0.5 M carbonate buffer pH 10.8 and 5 ml 1.5% isoamyl alcohol in n-heptane, vortexing, shaking 10 min and centrifuging at 3,500 rcf for 15 min. The organic layer was added to 100 µl 0.1 M hydrochloric acid, dried under a stream of nitrogen at 37 ºC, and reconstituted with 105 µl mobile phase (methanol and 0.05 M phosphate buffer pH 5.8, 45:55 v/v). A 90 µl aliquot was separated on a ZORBAX Bonus-RP column (5 µm, 250 x 4.6 mm) at a flow rate of 1 ml/min and UV detection at 214 nm. Limits of quantification were 10 ng/ml (approximately 40 nM) for both bupropion and hydroxybupropion.

Immunoblotting: To determine the linear range of the assays, a range of protein concentrations (0 – 50 µg) of bird liver microsomes were separated by SDS

polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, then immunoblotted for CYP2B and CYP2A proteins. Blots were stained with Ponceau S, and also probed with anti β-actin antibody to assess equal protein loading. Blots were blocked for 1 h with 1% skim milk in Tris buffered saline with 0.1% triton X-100 and 0.5% bovine serum albumin (TBST) then probed for 1 h with either polyclonal mouse anti-human CYP2A6 (1 in 1000) or polyclonal rabbit anti-rat CYP2B1 (1 in 3000) in TBST, followed by anti-mouse (1 in 5000) or anti-rabbit (1 in 4000) horse radish peroxidase-conjugated secondary antibody in TBST. Proteins were visualized by electrochemical detection, CYP protein band density was expressed relative to β-actin band density; each sample was assayed at least three times. For CYP2A and CYP2B assays, 25 µg and 10 µg of liver microsomes, respectively, were determined to be in the middle of the linear range and were used as loading amounts for all subsequent assays.

Inhibition of *in vitro* nicotine and bupropion metabolism: In all inhibitor experiments, pooled bird liver microsomes (0.5 mg/ml) were pre-warmed in 50 mM Tris-hydrochloric acid buffer pH 7.4 and cytosol for 2 min before addition of NADPH and inhibitor or vehicle control. After 15 min, nicotine or bupropion was added at 30, 90 or 360 µM in a final volume of 500 µl and the reaction stopped after a further 20 min by addition of 100 µl 20% aqueous sodium bicarbonate. Samples underwent liquid-liquid extraction and were analyzed for nicotine and cotinine by HPLC as described above. Inhibitors were used at approximately 1x, 5x and 10x K_i established for human liver microsomes. Inhibitors used were 8-methoxypsoralen (CYP2A6 and CYP2B1, 1, 5 and 10 µM, (Koenigs and Trager, 1998a; Zhang et al., 2001)), pilocarpine (CYP2A6, 4 and

40 µM, (Bourrie et al., 1996)), coumarin (CYP2A6, 2.5 and 25 µM, (Messina et al., 1997)) ,C-8-xanthate (CYP2B6, 1, 5 and 10 µM, (Yanev et al., 1999)), quinidine (CYP2D6, 0.05 and 5 µM, (Bourrie et al., 1996)) and ketoconazole (CYP3A4, 0.002 and 0.2 µM, (Bourrie et al., 1996)).

Statistical and pharmacokinetic analyses: *In vivo* kinetic parameters were determined using PK Functions for Microsoft Excel (J.I.Usansky, A.Desia, D.Tan-Liu, Department of Pharmacokinetics and Drug Metabolism, Allergon, Irvine, CA). *In vivo* $AUC_{0\text{-}t}$ was determined using the linear trapezoid rule, $AUC_{0\text{-}inf}$ was determined as above and estimation by log-linear decline, elimination half-life was determined from the terminal slope. *In vitro* kinetic parameters were determined with GraphPad Prism, confirmed by Eadie-Hofstee analysis, and Pearson's correlation coefficients (r) were considered significant if $p<0.05$. Digital images of immunoblots were analyzed with MCID software (Interfocus Imaging Ltd, Linton, UK).

Results

Nicotine pharmacokinetics

Nicotine and cotinine, but not *trans* 3'-hydroxycotinine, were detected in plasma (Fig. 1). The nicotine $AUC_{30\text{-}240}$ was 38 ng.h/ml, with an estimated $AUC_{0\text{-}inf}$ of 67 ng.h/ml, and $AUC_{0\text{-}120}$ of 64 ng.h/ml. Thus, over the first two hours, the average plasma nicotine levels were 32 ng/ml, decreasing to below limits of quantification (5 ng/ml) between 2 and 4 h. Nicotine was eliminated with a half-life of 33 min calculated from the averaged time point data from all zebra finches, consistent with an average half-life of 32 ± 18 min (mean ± S.D., n = 24) calculated from two time points per finch. The nicotine elimination constant was 1.3 h^{-1}, the rate of clearance was 0.04 L/h, and volume of distribution for nicotine was 0.03 L. Cotinine levels peaked at approximately 2 h, and the calculated $AUC_{0\text{-}480}$ was 137 ng.h/ml, resulting in average plasma cotinine levels of 26 ng/ml over 4 h. Both nicotine and cotinine were detected in brain at 1 h and 2 h after nicotine injection, times at which behavioral changes in song production were previously detected (Cappendijk et al., 2010). At 1 and 2 h respectively, the average brain nicotine levels (mean ± SD, n=2) were 30 ± 6 and 14 ± 4 ng/g, and the brain to plasma ratios were 2.7 ± 0.4 and 2.1 ± 0.0. At 1 and 2 h respectively, cotinine levels were 29 ± 7 and 40 ± 10 ng/g and the brain to plasma ratios were 0.8 ± 0.1 and 0.7 ± 0.2.

***In vitro* hepatic nicotine, coumarin and bupropion metabolism**

Pooled finch liver microsomes metabolized nicotine to cotinine with a K_m of 96 ± 7 µM, a V_{max} of 56 ± 2 pmol/min/mg, and V_{max}/K_m of 0.59 ± 0.03 (mean ± SE of three

experiments, Fig. 2). There was considerable variation among liver microsomes from individual finches for both the Km and Vmax (Fig 2, inset). No *trans* 3'-hydroxycotinine was detected. When finch liver microsomes were incubated with coumarin, there was no detectable formation of 7-hydroxycoumarin (CYP2A-mediated metabolite). However, 7-hydroxycoumarin was formed with velocities of 171 pmol/min/mg by human liver microsomes and 2.8 pmol/min/pmol by cDNA-expressed human CYP2A6. When incubated with bupropion, finch liver microsomes (n = 16) formed hydroxybupropion (CYP2B-mediated metabolite) with an average velocity of 405 ± 88 pmol/min/mg (mean ± SD), and human liver microsomes produced hydroxybupropion with a velocity of 645 pmol/min/mg.

Immunoblotting for CYP2A and CYP2B enzymes

Immunoblotting with CYP2A and CYP2B antibodies detected two distinct proteins in zebra finch liver microsomes with estimated molecular weights of 47 and 55 kDa for CYP2A- and CYP2B-like proteins respectively. Since these antibodies were raised against mammalian rather than bird CYPs, we refer to them as CYP2A- and CYP2B-like proteins for descriptive purposes based on their immunoreactive properties *per se*. Antibodies detected their respective positive controls, cDNA-expressed human CYP2A6 and rat CYP2B1, and there was no cross-reactivity detected between the two (data not shown). CYP2A- and CYP2B-like proteins were detected at variable levels among individual finch liver microsomes and they co-migrated with cDNA-expressed human CYP2A6 and rat CYP2B1 proteins respectively (Fig. 3A).

There was no correlation between the expression levels of CYP2A and CYP2B proteins (r = 0.38, p = 0.15). Cotinine formation correlated with CYP2B protein levels (r = 0.52, p = 0.04 Fig. 3B), but not with CYP2A protein levels (r = 0.11, p = 0.70, Fig. 3C). Hydroxybupropion formation correlated with CYP2B protein levels (r = 0.81, p < 0.001, Fig. 3D) but not with CYP2A protein levels (r = 0.27, p = 0.32). In addition, cotinine formation and hydroxybupropion formation were highly correlated among individual bird livers (r = 0.76, p<0.001, Fig. 3E).

Inhibition of *in vitro* nicotine and bupropion metabolism

The most effective inhibitors of nicotine and bupropion metabolism by bird liver microsomes were C-8-xanthate (CYP2B inhibitor) and 8-methoxypsoralen (CYP2B and CYP2A inhibitor) (Fig. 4). C-8-Xanthate, at approximately K_i for human and rat CYP2B, inhibited nicotine metabolism by 60% and bupropion metabolism by 67% (close to the expected 50% based on using a substrate concentration of approximately Km, Fig. 4). The percent inhibition of nicotine metabolism by each inhibitor correlated with the percent inhibition of bupropion metabolism by each inhibitor at both 90 µM (r = 0.96, p < 0.001) and at 360 µM (r = 0.87, p = 0.02) substrate concentrations. For C-8-xanthate the approximate K_is were 4 µM and 2 µM for nicotine and bupropion, respectively (Fig 5A, C) similar to that reported for rat and human CYP2B (Yanev et al., 1999), and from Cornish Bowden plots (not shown), C-8-xanthate showed characteristics of a mixed inhibitor. 8-Methoxypsoralen, at approximately K_i for human and rat CYP2B, inhibited nicotine metabolism by 30% and bupropion metabolism by 33% (Fig. 4), with approximate K_is of 2 µM and 8 µM for nicotine and bupropion respectively (Fig 5B, D),

similar to that reported for rat CYP2B1 and human CYP2A6 (Koenigs et al., 1997; Koenigs and Trager, 1998b; Koenigs and Trager, 1998a). From Cornish-Bowden plots (not shown), 8-methoxypsoralen showed characteristics of a competitive inhibitor. There was less than 10% inhibition by other CYP inhibitors at approximately K_i for their respective human isoforms (Fig. 4).

Discussion

Animal models are frequently used to study nicotine pharmacology and associated behaviors (Matta et al., 2007), and this includes studies in birds. For example, nicotine can affect song production in zebra finches (Cappendijk et al., 2010), can improve performance in a sustained attention task in pigeons (Lemmonds et al., 2002; Lemmonds and Wenger, 2003), and produce conditioned place preference in Japanese quail (Bolin et al., 2012). However, plasma and brain levels of nicotine and metabolites were not assessed in these studies, and nicotine metabolism has not been investigated in any species of bird. This is the first characterization of *in vivo* nicotine pharmacokinetics and *in vitro* nicotine metabolism in zebra finches.

The nicotine dose (0.18 mg base/kg) used here, which was previously shown to have significant effects on song production and locomotion in zebra finches (Cappendijk et al., 2010), resulted in nicotine plasma levels of 32 ng/ml which is within the range (10–50 ng/ml) seen in human smokers (Benowitz et al., 2009), and similar to plasma levels following nicotine replacement therapy including the nicotine patch (10-20 ng/ml) and gum (5-15 ng/ml) (Benowitz et al., 2009). These plasma levels are also similar to those seen in rat nicotine behavioral studies. In a rat nicotine self-administration study, plasma nicotine levels were 53 ng/ml immediately after rats intravenous self-administered 7 infusions of 0.02 mg/kg i.v. (total 0.14 mg/kg) over 14 minutes (Donny et al., 2000). Plasma nicotine levels were 73 ng/ml in rats after 7 days of 3 mg nicotine/kg/day delivered by s.c. infusion pump, which was sufficient to produce mecamylamine-precipitated withdrawal (Shram et al., 2008). The effect of nicotine on zebra finch song

production was seen primarily during the first hour after nicotine injection (0.18 mg/kg) (Cappendijk et al., 2010) a time when nicotine was detected at 30.3 ng/g in brain, and nicotine brain to plasma ratios of approximately 3 were observed, comparable to those seen in the self-administration study in rats described previously (Donny et al., 2000). These brain nicotine levels are also comparable to those seen in rats 1 h after s.c. nicotine injection at 99 ng/g for a dose 2.5 time higher (0.46 mg/kg dose) (Crooks et al., 1997). These observations indicate that in zebra finches behaviorally relevant doses of nicotine produce plasma nicotine levels similar to those seen in human smokers, and brain and plasma levels similar to those seen in other animal models. Therefore, it is likely that in birds there is a similar interaction between nicotine drug levels and drug targets mediating the behaviours, presumably nicotinic receptors, to that observed in humans and other animal models.

Zebra finch liver microsomes metabolized nicotine to cotinine *in vitro* with a relatively low affinity (K_m of 96 µM), similar to the affinity seen in rat (K_m of 134 µM) (Tyndale, unpublished observations), compared to humans, monkeys and mice (K_ms of 65, 24 and 11 µM respectively) (Messina et al., 1997; Schoedel et al., 2003; Siu and Tyndale, 2007). The V_{max} was also relatively low (56 pmol/min/mg) in zebra finches; this was more similar to the V_{max} in rats (126 pmol/min/mg), than to the higher V_{max} seen in humans, monkeys and mice (V_{max} of 1100, 3400 and 600 pmol/min/mg, respectively). The intrinsic clearance of 0.6 L/min/kg is similar to that seen in rat (0.9 L/min/kg), however, it did not predict the short half-life of 33 min observed for nicotine *in vivo* likely due to a number of unknown factors affecting nicotine plasma clearance in zebra

finches; these could include higher hepatic blood flow or clearance via other metabolic pathways. Fowl have twice the hepatic blood flow of mammals, 4.4 compared to 1.2-1.9 l/min/kg in mice, rats, monkeys and humans (Boxenbaum, 1980; Beers et al., 1992). Some other parameters that could possibly affect nicotine clearance were not very different in zebra finches compared to mice, rats, monkeys and humans, such as liver as percentage of body weight (2% in finches compared to 2-5% in other species) (Boxenbaum, 1980), and blood volume relative to body weight (69 ml/kg in finches compared to 60-72 ml/kg in other species) (Diehl et al., 2001; Williams et al., 2012).

In most mammals cotinine is further metabolized by CYP2A to *trans* 3'-hydroxycotinine, and this is a major metabolite in humans and mice (Matta et al., 2007; Siu and Tyndale, 2007). However, as observed in rat plasma (Kyerematen et al., 1988; Nwosu and Crooks, 1988), this metabolite was undetectable in zebra finch plasma up to 4 h after nicotine administration, and when nicotine was metabolized by liver microsomes *in vitro*. Based on the plasma *trans* 3'-hydroxycotinine to cotinine ratios of 0.2-0.3 in human, monkey and mouse after a single nicotine dose and measured after cotinine has peaked in the plasma (Dempsey et al., 2004; Siu and Tyndale, 2007; Ferguson et al., 2012), this metabolite should have been detectable by 2 to 4 hours post-nicotine. The similarity between zebra finch and rat *in vitro* nicotine metabolism (i.e. K_m and V_{max}) and the lack of detectable *trans* 3'-hydroxycotinine formation *in vivo* and *in vitro*, suggest that zebra finches may use enzyme(s), and resulting metabolic pathways, which are more similar to rats than to humans, monkeys and mice.

In rats, the formation of cotinine from nicotine is mediated by CYP2B enzymes (Hammond et al., 1991; Nakayama et al., 1993), unlike in humans, monkeys and mice where this pathway is mediated by CYP2A (Messina et al., 1997; Schoedel et al., 2003; Siu and Tyndale, 2007). Both CYP2A- and CYP2B-like proteins were detected in zebra finch liver microsomes. However *in vitro* there was no detectable metabolism of coumarin to 7-hydroxycoumarin, a reaction mediated in mammals specifically by CYP2A (Pelkonen et al., 2000; Siu and Tyndale, 2007; Ferguson et al., 2012), the formation of cotinine from nicotine was not inhibited by the CYP2A-specific inhibitor pilocarpine (Bourrie et al., 1996) or the CYP2A-specific substrate coumarin (Messina et al., 1997), and finally the hepatic CYP2A-like protein levels did not correlate with *in vitro* cotinine formation. In contrast, there was detectable metabolism of bupropion to hydroxybupropion, a reaction mediated in mammals specifically by CYP2B (Faucette et al., 2000; Richert et al., 2009). Both hydroxybupropion and cotinine formation were potently inhibited by the CYP2B-specific inhibitor C-8-xanthate (Yanev et al., 1999), and hepatic CYP2B-like protein levels correlated significantly with both *in vitro* cotinine and hydroxybupropion formation. While it is unknown whether the probe substrates and inhibitors used in this study show the same selectivity in humans and zebra finch, and thus the conclusions should be taken with caution, the findings are consistent with the other lines of evidence from this study suggesting that in zebra finches, as seen in rats, cotinine formation is mediated by an enzyme more CYP2B-like than CYP2A-like.

The zebra finch genome has been sequenced, and the derived amino acid sequences of CYP enzymes reported (Warren et al., 2010). The CYP2 family in birds is less

diverse than in mammals and orthologs of CYP2A and CYP2B have not been identified in zebra finches or other birds (the evolutionary ancestors of mammals and birds diverged more than 300 million years ago) (Nam et al., 2010; Kubota et al., 2011). Sequence comparisons suggest that mammalian CYP2A and CYP2B isozymes are most similar to zebra finch CYP2C45 and CYP2H1, with the isozymes sharing approximately 50% amino acid sequence identity. CYP2H1 is the best characterized subfamily in birds, and in chickens, CYP2H1 and CYP2H2 are inducible by phenobarbital, and are thought to resemble rat CYP2B1 and CYP2B2, respectively (Walker, 1998). The CYP2A-like enzymes vary among birds; zebra finches, unlike chicken, turkey, duck and quail, do not metabolize coumarin to 7-hydroxycoumarin *in vitro* (Murcia et al., 2011). This variation is consistent with the divergence over 100 million years ago between the Galloanserae, which includes fowl, ducks and geese, and the Neoaves, which includes the songbirds (Nam et al., 2010). It remains to be seen if in the Galloanserae, the formation of cotinine from nicotine is mediated by a CYP2A-like enzyme, similar to humans, monkeys and mice.

In conclusion, in zebra finches the dose of nicotine that produces behavioral effects results in plasma and brain levels similar to those seen in smokers and other animal models. Nicotine is metabolized to cotinine *in vivo* by a zebra finch CYP isoform that may be related to CYP2B, similar to rats, rather than to CYP2A as in humans, monkeys and mice. This may be important in future studies where nicotine is combined with other drugs (e.g. CYP2B-metabolized bupropion) for understanding potential drug interactions and their consequences on the behavioral effects of nicotine.

Acknowledgements

The authors wish to thank David Smith B.Sc. and Steven Lo MSc. for their technical assistance.

Authorship contributions:

Participated in research design: Cappendijk, Miksys, Tyndale

Conducted experiments: Zhao, Perry, Cappendijk, Miksys

Performed data analysis: Miksys, Tyndale

Wrote or contributed to the writing of the manuscript: Cappendijk, Miksys, Tyndale

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Footnotes

This work was supported by the Canadian Institutes of Health Research [MOP 97751, MOP86471], a Canada Research Chair, the Campbell Family Mental Health Research Institute, Centre for Addiction and Mental Health, and the Canada Foundation for Innovation [#20289 and #16014], the CAMH Foundation and the Ontario Ministry of Research and Innovation and the James and Esther King Biomedical Research Program [06-NIR02].

Rachel F. Tyndale has participated in one day advisory meetings for Novartis and McNeil.

Parts of this study were presented previously at the Society for Research on Nicotine and Tobacco Annual Meeting, Houston, TX, March 13-16, 2012.

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Figure Legends

Figure 1. *In vivo* plasma nicotine and cotinine levels.

Zebra finches were injected with 0.18 mg/kg nicotine base s.c., and two plasma samples taken per bird (n = 48) at various times up to 4 h after injection. Data shown are mean + SD plasma nicotine and cotinine levels in ng/ml (n = 4-10 birds per time point).

Figure 2. *In vitro* nicotine metabolism to cotinine.

The average K_m was 96 ± 7 µM and the average V_{max} was 56 ± 2 pmol/min/mg; mean ± SE of three independent experiments using pooled liver microsomes, n=8. Inset table shows these parameters for the same eight individual birds analyzed separately with the mean and SD.

Figure 3. Hepatic CYP2A-like and CYP2B-like protein and correlations with metabolism.

Zebra finch hepatic protein levels were assessed by immunoblotting with polyclonal antibodies against human CYP2A6 and rat CYP2B1 (A). Zebra finch hepatic immunoreactive proteins co-migrated with cDNA-expressed human CYP2A6 and rat CYP2B1 (2A, 2B, arrows), and expression levels varied among different individual birds (n=16). Cotinine formation (nmol/min/mg) correlated with hepatic CYP2B-like protein (arbitrary optical density units relative to beta-actin levels) (B), but not with hepatic CYP2A-like protein (arbitrary optical density units relative to beta-actin levels) (C). Bupropion metabolism (nmol/min/mg) correlated with hepatic CYP2B-like protein (D) and with cotinine formation (E).

Figure 4. Inhibition of nicotine and bupropion metabolism *in vitro*.

Nicotine (A) and bupropion (B) metabolism at 90 µM (approximate K_m) by zebra finch hepatic microsomes was assessed with chemical CYP inhibitors, each at concentrations of 1 and 10 x K_i against a specific CYP. Data is reported as % of cotinine (A) or hydroxybupropion (B) formation relative to vehicle control. C-8-xanthate (CYP2B) and 8-methoxypsoralen (CYP2B and CYP2A) were the most effective inhibitors, compared to quinidine (CYP2D), coumarin (CYP2A), pilocarpine (CYP2A) and ketoconazole (CYP3A).

Figure 5. Dixon plots of inhibition of nicotine and bupropion metabolism *in vitro*.

Nicotine and bupropion metabolism by zebra finch liver microsomes were assessed at three substrate concentrations (30, 90 and 360 µM), and three concentrations of the inhibitors C-8-xanthate and 8-methoxypsoralen (1, 5 and 10 µM). The K_i was derived from Dixon plots of data for C-8-xanthate against nicotine, 4 µM (A), 8-methoxypsoralen against nicotine, 2 µM (B), C-8-xanthate against bupropion, 2 µM (C), and 8-methoxypsoralen against bupropion, 8 µM (D) (arrows, ordinate 1/velocity of cotinine or hydroxybupropion formation, min.mg/nmol).

Figure 1

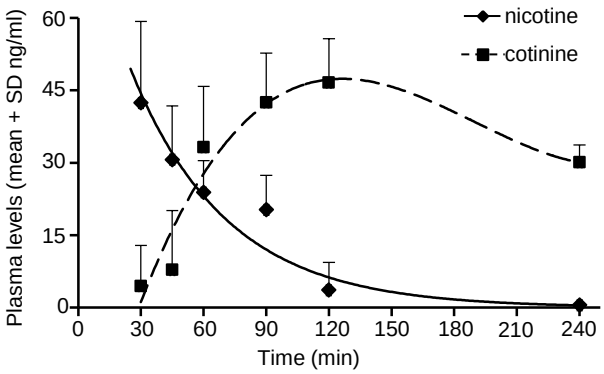

nicotine
cotinine
Plasma levels (mean + SD ng/ml)
60
45
30
15
0
0
30
60
90
120
150
180
210
240
Time (min)

Figure 2

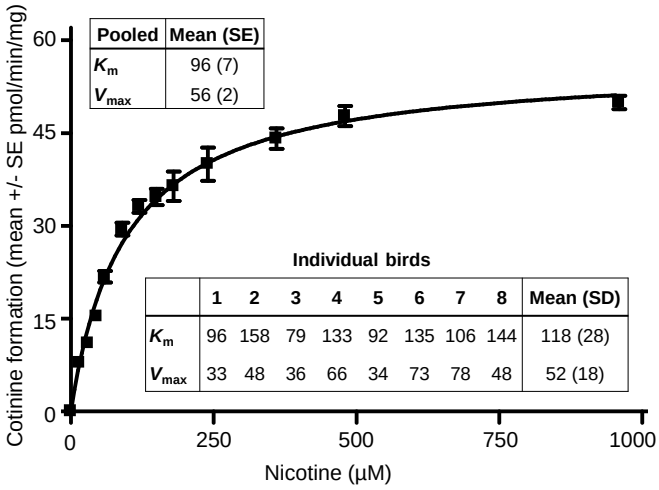

Pooled
Mean (SE)
K_m
96 (7)
V_{max}
56 (2)
Individual birds
1 2 3 4 5 6 7 8 Mean (SD)
K_m 96 158 79 133 92 135 106 144 118 (28)
V_{max} 33 48 36 66 34 73 78 48 52 (18)
Cotinine formation (mean +/- SE pmol/min/mg)
0
15
30
45
60
0
250
500
750
1000
Nicotine (µM)

Figure 3

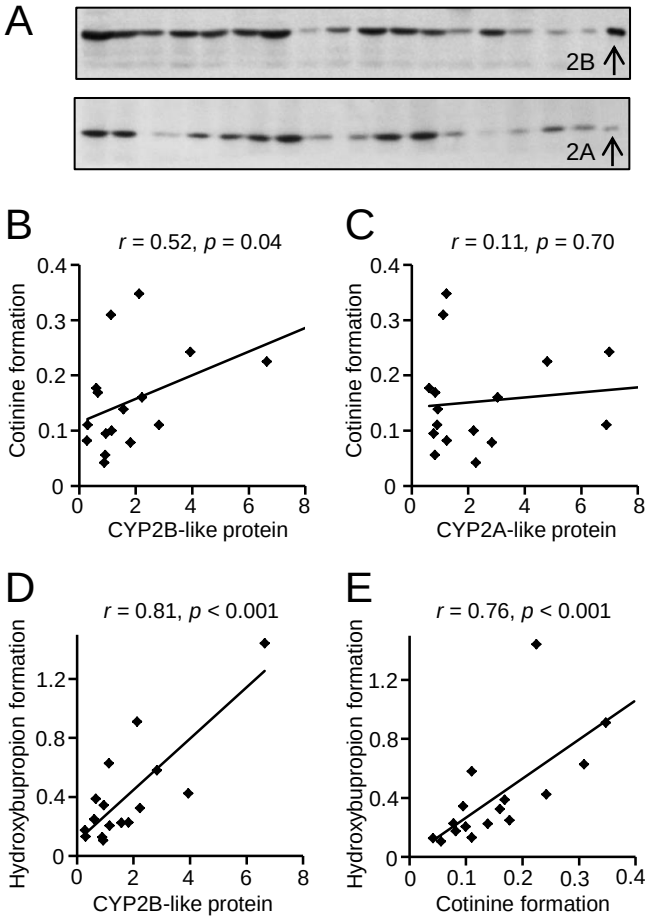

A
2B
2A
B
r = 0.52, p = 0.04
Cotinine formation
CYP2B-like protein
C
r = 0.11, p = 0.70
Cotinine formation
CYP2A-like protein
D
r = 0.81, p < 0.001
Hydroxybupropion formation
CYP2B-like protein
E
r = 0.76, p < 0.001
Hydroxybupropion formation
Cotinine formation

Figure 4

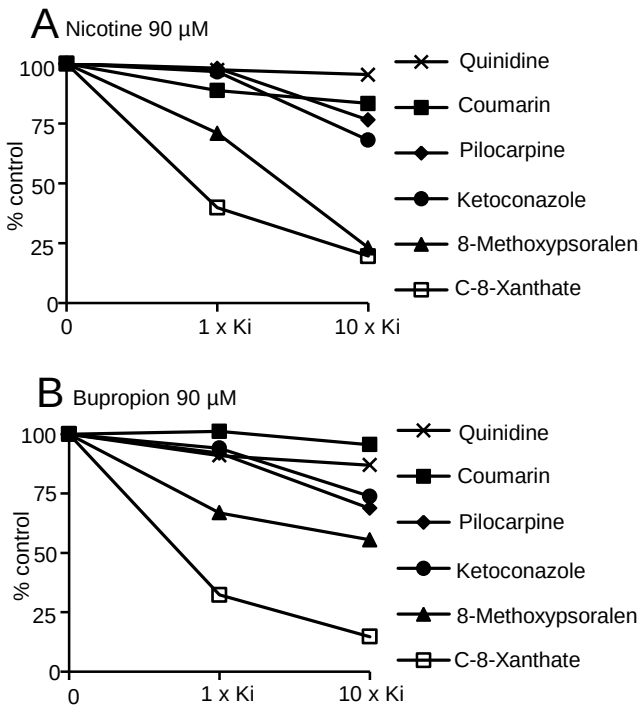
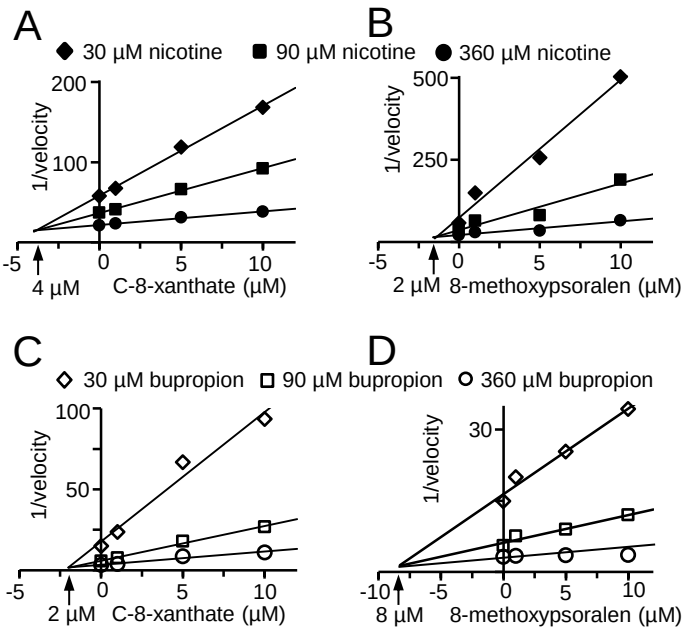

A Nicotine 90 µM
100
75
50
25
0
% control
0
1 x Ki
10 x Ki
Quinidine
Coumarin
Pilocarpine
Ketoconazole
8-Methoxypsoralen
C-8-Xanthate
B Bupropion 90 µM
100
75
50
25
0
% control
0
1 x Ki
10 x Ki
Quinidine
Coumarin
Pilocarpine
Ketoconazole
8-Methoxypsoralen
C-8-Xanthate

Figure 5


A
30 µM nicotine
90 µM nicotine
360 µM nicotine
1/velocity
200
100
-5
0
5
10
4 µM
C-8-xanthate (µM)
B
1/velocity
500
250
-5
0
5
10
2 µM
8-methoxypsoralen (µM)
C
30 µM bupropion
90 µM bupropion
360 µM bupropion
1/velocity
100
50
-5
0
5
10
2 µM
C-8-xanthate (µM)
D
1/velocity
30
-10
-5
0
5
10
8 µM
8-methoxypsoralen (µM)