Nicotine Kinetics in Zebra Finches In Vivo and In Vitro

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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 effects of nicotine on song production have been reported. Nicotine and cotinine plasma levels were assessed in vivo after subcutaneous injection of 0.16 mg/kg nicotine, a dose that elicits changes in song production. The half-life of nicotine elimination was 33 minutes, and levels were undetectable by 4 hours. 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 1 and 2 hours after treatment. To understand the potential for drug interactions and the regulation of nicotine metabolism in zebra finches, we characterized in vitro nicotine metabolism and the hepatic enzyme involved. In humans, cytochrome P450 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 (a CYP2B substrate) but not coumarin (a CYP2A substrate). Nicotine was metabolized to cotinine with a Michaelis-Menten constant (Km) of 81 μM and a Vmax of 56 pmol/min per milligram. Nicotine and bupropion metabolism was inhibited by C-8-xanthate (a specific CYP2B inhibitor) but not by 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, memory, and the development of complex behavior 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 male zebra finches 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 (Bohlhus 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 CYP2B 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...
nicotine, cotinine, and homogenate was 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°C until use (Siu et al., 2006). Linear conditions were established by incubating a mixture of pooled liver microsomes at protein concentrations of 0.5, 0.75, and 1.0 mg/ml, at nicotine concentrations of 60 and 480 μM for 10, 15, 20, and 40 minutes. Various concentrations of cytosolic protein (a source of aldehyde oxidase required after the cytochrome-mediated conversion of nicotine to an aminion ion, to convert the aminion ion to cotinine), 25, 50, 75, and 100 μg, were incubated with 0.75 mg/ml microsomal protein for 20 minutes 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, prewarmed at 37°C for 2 minutes, followed by the addition of 1 mM NADPH and nicotine in a final volume of 0.5 ml to initiate the reaction and then incubated for 20 minutes. Reactions were stopped by adding 100 μl of 20% aqueous sodium carbonate. Samples were prepared for HPLC by liquid–liquid extraction (Schoedel et al., 2003) by the addition of 65 μg of 5-methyl-cotinine as the internal standard, 50 μl of 10 N sodium hydroxide, and 4 ml of dichloromethane. Mixtures were shaken for 10 minutes and centrifuged at 3000 rcf for 10 minutes, and the organic layer with 25 μl 6 N hydrochloric acid was evaporated under a stream of nitrogen at 37°C. The residue was reconstituted with 105 μl of distilled water and 90 μl was analyzed by HPLC as described. 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 bird 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 $V_{max}$ ($10 \times K_m$ 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°C for 2 minutes) were incubated with 1, 5, and 50 μM coumarin, which are approximately 2×, 10×, and 100× $K_m$ for formation of 7-hydroxycoumarin by human liver microsomes (van Iersel et al., 1994), added with 1 mM NADPH in a final volume of 500 μl, and the reaction stopped after 30 minutes 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 Vivo Buproprion 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°C for 2 minutes) were incubated with 100 μM bupropion, which is the approximate $K_m$ for the formation of hydroxybupropion by human liver microsomes (Hesse et al., 2000), added with 1 mM NADPH in a final volume of 500 μl; the reaction was stopped after 20 minutes with 100 μl 20% aqueous sodium bicarbonate. A 0.5-ml aliquot of the incubation mixture was prepared and separated by HPLC as previously described (Ho et al., 2008) by adding 1.5 μg of timolol maleate as the internal standard and with 0.5 ml 0.5 M carbonate buffer, pH 10.8, and 5 ml of 1.5% isomyl alcohol in n-heptane, vortexing, shaking for 10 minutes, and centrifuging at 3500 rcf for 15 minutes. The organic layer was added to 100 μl of 0.1 M hydrochloric acid, dried under a stream of nitrogen at 37°C, 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 × 4.6 mm) at a flow rate of 1 ml/min and UV detection at 214 nm.
Results

Nicotine Pharmacokinetics. Nicotine and cotinine, but not trans-3'-hydroxycotinine, were detected in plasma (Fig. 1). The nicotine AUC$_{30-240}$ was 38 ng.h/ml, with an estimated AUC$_{0-Inf}$ of 67 ng.h/ml, and AUC$_{0-120}$ of 64 ng.h/ml. Thus, over the first 2 hours, the average plasma nicotine levels were 32 ng/ml, decreasing to below limits of quantification (5 ng/ml) at between 2 and 4 hours. Nicotine was eliminated with a half-life of 33 minutes, calculated from the averaged time point data from all zebra finches, consistent with an average half-life of 32 ± 18 minutes (mean ± S.D., n = 24), calculated from two time points per finch. The nicotine elimination constant was 1.3 hour$^{-1}$, the rate of clearance was 0.04 l/h, and the volume of distribution for nicotine was 0.03l. Cotinine levels peaked at approximately 2 hours, and the calculated AUC$_{0-480}$ was 137 ng.h/ml, resulting in average plasma cotinine levels of 26 ng/ml over 4 hours. Both nicotine and cotinine were detected in the brain at 1 hour and 2 hours after nicotine injection, times at which behavioral changes in song production were previously detected (Cappendijk et al., 2010). At 1 and 2 hours, respectively, the average brain nicotine levels (mean ± S.D, 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 hours, 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 ± S.E. of three experiments, Fig. 2). There was considerable variation among liver microsomes from individual finches for both the $K_m$ and $V_{max}$ (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 (CYP2B2-mediated metabolite) with an average velocity of 405 ± 88 pmol/min per milligram (mean ± S.D.), and human liver microsomes produced hydroxybupropion with a velocity of 645 pmol/min per milligram.

Fig. 1. In vivo plasma nicotine and cotinine levels. Zebra finches were injected with 0.18 mg/kg nicotine base subcutaneously, and two plasma samples were taken from each bird (n = 48) at various times up to 4 hours after injection. Data shown are mean ± S.D. plasma nicotine and cotinine levels in ng/ml (n = 4 − 10 birds per time point).

Fig. 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 per milligram; mean ± S.E. 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 S.D.
**Immunoblotting for CYP2A and CYP2B Enzymes.** Immunoblotting with CYP2A and CYP2B antibodies detected two distinct proteins in zebra finch liver microsomes of estimated 47 and 55 kDa for CYP2A- and CYP2B-like proteins, respectively. Because these antibodies were raised against mammalian rather than bird cytochromes, 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 (unpublished data). CYP2A- and CYP2B-like proteins were detected at variable levels among individual finch liver microsomes, and they comigrated with cDNA-expressed human CYP2A6 and rat CYP2B1 proteins respectively (Fig. 3A).

No correlation was found 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 $K_m$, 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 $\mu$M ($r = 0.96; P < 0.001$) and 360 $\mu$M ($r = 0.87; P = 0.02$) substrate concentrations. For C-8-xanthate, the approximate $K_i$s were 4 and 2 $\mu$M for nicotine and bupropion, respectively (Fig. 5, A and 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 $K_i$s of 2 and 8 $\mu$M 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, 1998a,b). From Cornish-Bowden plots (not shown), 8-methoxypsoralen showed characteristics of a competitive inhibitor. There was less than 10% inhibition by other cytochrome 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), improve performance in a sustained attention task in pigeons (Lemonds et al., 2002; Lemonds 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 is similar to plasma levels after 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 intravenously self-administered seven infusions of 0.02 mg/kg (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/kg of nicotine daily delivered by subcutaneous 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 the 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 hour after subcutaneous nicotine injection at 99 mg/kg for a dose 2.5 times greater (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 an interaction between nicotine drug levels and drug targets mediating the behaviors, presumably nicotinic receptors, that is similar 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 $\mu$M), similar to the affinity seen in rat ($K_m$ of 134 $\mu$M) (Tyndale, unpublished observations), compared with humans, monkeys, and mice ($K_m$ of 65, 24, and 11 $\mu$M, respectively) (Messina et al., 1997; Schoedel et al., 2003; Siu and Tyndale, 2007). The $V_{max}$ was also relatively low (56 pmol/min per milligram) in zebra finches; this was more similar to the $V_{max}$ in rats (126 pmol/min per milligram) than to the higher $V_{max}$ seen in humans, monkeys, and mice ($V_{max}$ of 1100, 3400, and 600 pmol/min per milligram, respectively). The intrinsic clearance of 0.6 l/min per kilogram is similar to that seen in rats (0.9 l/min per kilogram), however, it did not predict the short half-life of 33 minutes observed for nicotine in vivo, which was 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 with 1.2–1.9 l/min per kilogram in mice, rats, monkeys, and humans (Boxenbaum, 1980; Beers et al., 1992). Some other parameters that could affect nicotine clearance were not so different in zebra finches compared with mice, rats, monkeys, and humans, such as liver as percentage of body weight (2% in finches compared with 2–5% in other species) (Boxenbaum, 1980) and blood volume relative to body weight (69 ml/kg in finches compared with 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 hours 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 the 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, in which 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); 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-xantheate (Yaney et al., 1999), and hepatic CYP2B-like protein levels correlated significantly with both in vitro cotinine and hydroxybupropion formation. Although 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 have been 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 isoforms are most similar to zebra finch CYP2C45 and CYP2H1, with the isoforms 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 chickens, turkeys, ducks, and quails, 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 Neovaves, which includes the songbirds (Nam et al., 2010). It remains to be seen whether 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 the behavioral effects of nicotine, as seen in rats, cotinine formation is mediated by an enzyme more CYP2B-like than CYP2A-like.

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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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