

# Evaluation of Chronic Oral Nicotine Treatment in Food Consumption, Body Weight and [125I] Epibatidine Binding in Adult Mice

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**Submission:** October 17, 2015; **Published:** November 25, 2015

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## Abstract

Despite its abuse potential, nicotine, acting on nicotinic acetylcholine receptor, has possible medicinal uses, in particular in treating neurodegenerative diseases. Therefore, animal models to evaluate exposure need to be characterized. Administration via drinking water is a stress-free route of administration but often results in low blood nicotine levels. Here we evaluated chronic exposure to low, medium and high concentrations of nicotine in drinking water. Three-month-old C57BL/6 male mice were treated for 23 days with 20, 120 or 300 µg/ml nicotine in 2% saccharin water, corresponding to 5, 30 and 55 mg/kg/d, respectively. Food intake and body weight were monitored, blood nicotine and cotinine levels, and 125I-epibatidine-binding sites were determined at day 23. Average blood cotinine levels of 11.7, 151.8 and 192.0 ng/ml were detected in mice receiving the low, medium and high dose, respectively. In contrast, nicotine was only consistently measured in the group receiving 300 µg/ml, with an average blood level of 6.2 ng/ml and was the only treatment group to exhibit significantly decreased food intake ( $p=0.005$ ) and body weight ( $p=0.043$ ), as well as increased 125I-epibatidine binding in cortex ( $p=0.055$ ) and hippocampus ( $p=0.019$ ). We evaluated possible effects of chronic nicotine (300µg/ml) exposure on anxiety-like behavior using the open field test. An anxiolytic effect was found compared to controls and there was no evidence for anxiogenic effects of chronic nicotine. Thus, a high concentration of nicotine in drinking water was necessary to achieve consistent blood nicotine levels in mice, which correlated with markers considered hallmarks of chronic nicotine treatment.

**Keywords:** Nicotine; nAChR; Hippocampus; Receptor Binding; Body Weight; Food Intake; Anxiety

**Abbreviations:** AD: Alzheimer's Disease; ANOVA: Analysis of Variance; DG: Dentate Gyrus; nAChR: Nicotinic Acetylcholine Receptor; ROD: Raw Optical Density.

## Introduction

The plant alkaloid nicotine interacts with neuronal nicotinic acetylcholine receptors (nAChRs), which are pentameric ligand gated cation channels that are either homomeric or heteromeric formed by different subunit combinations. Among the possible subunit combinations, heteromeric  $\alpha 4\beta 2$  nAChRs are the most widely expressed heteromeric nAChR subtype in the mammalian brain and exhibit high affinity for nicotine [1,2]. Nicotine, the major psychoactive ingredient in tobacco smoke [3], facilitates dopamine release and activates the reward pathway [4,5], and as such is considered an addictive drug, and strongly implicated in tobacco dependence [6,7]. However, nicotine may also have beneficial effects; the drug has shown cognitive enhancing, anxiolytic and neuro protective properties in animals and humans [8-10], may slow age-related neuronal

decline, and reduce neuronal degeneration in Alzheimer's or Parkinson's disease [11,12]. Neuro protective effects of nicotine are the result of nAChR activation; in particular, activation of heteromeric nAChRs seems to be important. Transgenic mice that lack the  $\beta 2$  subunit ( $\beta 2$  nAChR knockout (KO) mice) and thus do not express high affinity nAChRs, have reduced adult neurogenesis [13] and exhibit accelerated aging in cortical areas, underscoring the importance of heteromeric nAChRs to maintain a healthy mature brain [14,15]. Thus, medicinal use of nicotine or related nAChR agonists could have great beneficial effects for human health. Therefore, despite the known potential for abuse, nicotine and nicotinic receptor agonists are being evaluated for therapeutic uses [16-18]. Medicinal use of nicotine would often require long-term drug administration. To study effects of nicotine administration in adult animal models over several weeks or month, the route of nicotine administration

becomes very important. Voluntary oral consumption of nicotine via drinking water is non-invasive, stress-free and requires no additional animal handling. Therefore, this route of drug is suitable for long-term treatment in preclinical animal models, and is a viable alternative to daily injections or use of osmotic mini pumps. In addition, administration via drinking water results in nicotine fluctuations during a 24-hour period, which allows time for re-sensitization of desensitized nAChRs. On the other hand, oral nicotine administration results in poor bioavailability, which means that the fraction of administered nicotine reaching systemic circulation is lower compared to other routes, and results in low and more variable levels of nicotine in the brain. This is due to ion trapping of nicotine in the acidic milieu in the stomach [19], slow absorption through the gastrointestinal tract, and rapid first pass metabolism in the liver [20,21]. In mice in particular, nicotine is rapidly

metabolized, and strain specific metabolic rates for nicotine may differ [22,23]. On the other hand, nicotine can be absorbed directly through the buccal cavity where first pass metabolism is not a factor [19]. Together, these properties make it difficult to estimate effective doses of orally administered nicotine based solely on dose [24,25]. Two hallmarks of chronic nicotine exposure have been described for decades: an anorexic effect that results in decreased food consumption and body weight [26-29], and an increase in high affinity nAChR binding sites [30-33]. Together with determining blood nicotine and cotinine levels, we used these two hallmarks to evaluate central effects of chronic oral nicotine administration in adult C57BL/6 mice. In addition, there are reports that chronic nicotine exposure affects anxiety in humans and in animal models [34-38]. Therefore, we also evaluated the effects of chronic nicotine administration on anxiety-like behavior.

## Materials and Methods

### Animals and Drug Administration

**Table 1:** Nicotine concentrations, calculated as free base, in 2% saccharin drinking water for each treatment group over the course of the three week treatment period.

Treatment day	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8-23
Dose ( $\mu\text{g/ml}$ )								
Control	0	0	0	0	0	0	0	0
Low	20	20	20	20	20	20	20	20
Medium	20	40	60	80	100	100	120	120
High	20	40	80	120	160	200	250	300

Young adult male C57BL/6 mice were obtained from a breeding colony established at Texas A&M University's Laboratory Animal Research and Resource facility from mice originally obtained from the Jackson Laboratory, Bar Harbor, ME. At three months of age, mice were individually caged and randomly assigned to different dosing groups ( $n = 5$ ). Then all mice received 2% saccharin (saccharin sodium salt hydrate, >98%) (Sigma, St. Louis, MO, USA) in their drinking water starting two weeks prior to actual dosing with nicotine to establish their individual daily drinking volume. (-) -Nicotine (hydrogen tartrate) (Sigma) was administered in 2% saccharin containing water. Four nicotine concentrations (given as free base) were tested: control - 0  $\mu\text{g/ml}$  (0 mg/kg/day), low dose (LD) - 20  $\mu\text{g/ml}$ , medium dose (MD)- 120  $\mu\text{g/ml}$  and high dose (HD)- 300  $\mu\text{g/ml}$  resulting in approximately 5, 30 and 55 mg/kg/day free base nicotine, respectively, calculated based on average liquid consumption and body weight. Animals in the MD-and HD-groups initially received 20  $\mu\text{g/ml}$  of nicotine on the first day of treatment and the concentrations were gradually increased until the final concentrations were reached on day 7 and day 8 of treatment, respectively (Table 1). Individual water consumption was not significantly affected until a concentration of 200  $\mu\text{g/}$

ml was reached; at 300  $\mu\text{g/ml}$  nicotine, water intake was 86.1% (77.7 - 98.08 %) of the initial individual consumption levels ( $p=0.018$ ). All mice were treated until day 23. Access to food and drinking solutions was ad libitum. Food intake and body weight were assessed every three days, starting from day 1 to day 19, and normalized to the first measurement of each individual animal. On days 20 to 22, the mice were subjected to behavioral testing, and then euthanized on day 23. All procedures were approved by the Texas A&M University Animal Use Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996).

### Open Field Test

To test locomotor, exploratory and anxiety behavior, on day 20 of nicotine treatment, mice were placed individually in an open field (length  $\times$  width  $\times$  height: 20  $\times$  20  $\times$  30 cm) (Versa max animal activity monitor and analyzer, AccuScan Instruments, Columbus, OH, USA) equipped with vertical and horizontal infrared beam sensors. The apparatus was constructed using transparent Plexiglas, located in a quiet room, and environmental cues including lighting and experimenter were kept constant

during testing. Mice were acclimated to the experimental room for 5 minutes, and then placed in the open field for 30 minutes. Activity was recorded every minute. The open field chamber was cleaned with 50% ethanol between mice. Horizontal activity, total distance, movement time, rest time, stereotype counts, stereotype time, margin distance, margin time, center distance, center time, rearing activity, and rearing time were automatically recorded.

### Tissue preparation

All mice were anesthetized using isoflurane (IsoFlo, North Chicago, IL, USA) and decapitated on day 23 in the morning. Approximately 1 ml of trunk blood was collected from each mouse, kept on ice, and centrifuged at 4°C at 3000 rpm for 20 minutes. One hundred to 300 µl of serum was collected from each mouse and sent to Bio analytical Core Laboratory Service Center (Department of Pharmaceutics, School of Pharmacy, Virginia Commonwealth University, Richmond, VA, USA) for analysis of nicotine and cotinine levels. The brains were rapidly dissected, frozen on powdered dry ice, and stored at -80°C.

### [125I]-Epibatidine Binding

Radioactive, iodinated [125I]-epibatidine ligand was used to evaluate the relative levels of heteromeric nAChR binding sites. Receptor autoradiography was performed as previously described [39]; briefly, tissue sections were warmed to room temperature (RT) and pre-incubated 5 minutes in Tris-HCl buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, pH 7.4). Then sections were incubated with 0.4 nM [125I]-epibatidine (PerkinElmer Life Science NEX358, Boston, MA, USA, specific activity: 2200 Ci/mmol) for 1 hour at RT. For non-specific binding, sections were incubated with the presence

of 400 µM (-)-nicotine hydrogen tartrate. Sections were washed in ice-cold Tris-HCl buffer twice for 1 minute each, followed by 10 seconds in cold ddH<sub>2</sub>O, and dried under air stream for 1 hour. Sections were dried at RT over night before exposure to BioMax MR Film (Kodak, Rochester, NY, USA). After 1-day of exposure, films were developed in D19 Kodak developer for 4 minutes, rinsed in water, fixed in Kodak Rapid Fixer for 5 minutes, and air-dried.

### Data Analyses

Quantitative analysis of auto radiograms was done using a PC-based image analysis system, MCID basic (InterFocus Imaging Ltd, Haverhill, Suffolk, UK). Receptor binding levels were measured as raw optical density (ROD) in the hippocampus (combined measures in the molecular layer of the dentate gyrus (DG) and the stratum lacunosum molecular of the CA1 in hippocampus proper), thalamus (the post thalamic nucleus and the ventral posteromedial thalamic nucleus), and cerebral cortex, specifically layers IV-V of the visual and somato sensory cortex. All anatomical structures were determined according to the Mouse Brain Library atlas. SPSS 14.0 was used for statistical analysis. Significance was defined as  $p \leq 0.05$ . For food intake and body weight changes, repeated measurements were used for between-subject effects after nicotine reached the highest dose in all groups (day 8), and Student's t-tests were used to determine significant differences at each time point between nicotine and control groups. For receptor binding, one-way analysis of variance (ANOVA) was used and Tukey HSD was performed as a post hoc test when needed. For open field test, Student's t-test was used to compare nicotine and control groups. All data are presented as average  $\pm$  standard error the mean (SEM).

## Results

### Blood Nicotine and Cotinine Levels

**Table 2:** Blood nicotine and cotinine levels in mice treated for 23 days with low, medium and high nicotine doses in the drinking water containing 2% saccharin. Average and range blood serum levels for nicotine and cotinine as ng/ml on day 23 of treatment (n=5). In parentheses are the calculated concentrations in nM and µM for nicotine and cotinine, respectively. Detection limit for nicotine was 2ng/ml, for cotinine 1 ng/ml. Effects on food intake, body weight and receptor binding are summarized: =, no change; ↓ - decreased; ↑ - increased in comparison to control mice.

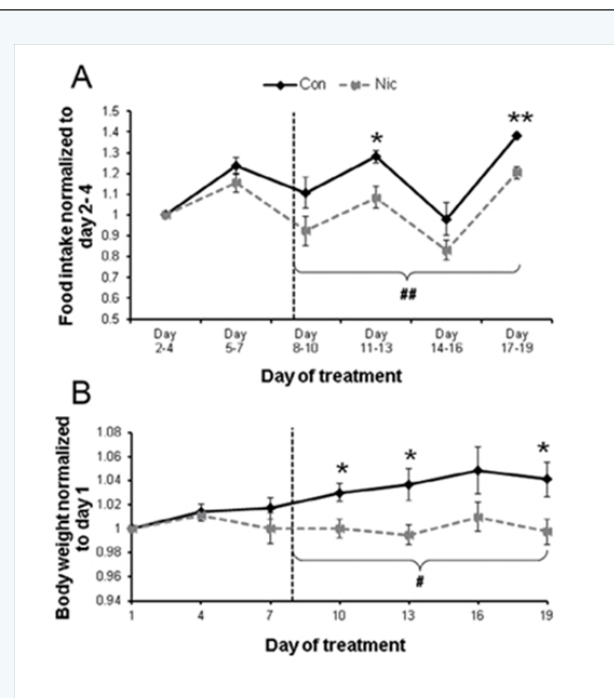
	Low	Medium	High
<b>Treatment dose:</b>	<b>20 µg/ml</b>	<b>120 µg/ml</b>	<b>300 µg/ml</b>
Blood nicotine in ng/ml	0	0	6.2 (38.3 nM) 0 - 21.1 ( $\leq$ 130 nM)
Blood cotinine in ng/ml	11.7 ng/ml 3.2--25.0	151.8 ng/ml 2.0-586.9	192.0 (1.1 µM) 41.4 - 578.1 (0.2 - 3.3 µM)
Food intake	0	=	↓
Body weight	=	=	↓
Receptor binding	=	=	↑

Nicotine or cotinine were not detected in the serum of control mice (< 2 ng/ml and < 1 ng/ml, respectively). In the LD- and MD- group, 4 out of 5 mice had undetectable levels of nicotine (< 2 ng/ml) in blood serum, with one mouse in the LD group with 2.9 ng/ml and one mouse in the MD group with 19.7 ng/ml nicotine. Cotinine levels ranged from 3.2 to 25.0 ng/ml with an average of 11.7 ng/ml in the LD group (20 µg/ml), and from 2.0 to 586.9 ng/ml with an average of 151.8 ng/ml in the MD group (120 µg/ml) (Table 2). In the HD group (300 µg/ml) four out of five mice had detectable blood nicotine levels ranging from 2.1 to 21.1 ng/ml with an average of 6.2 ng/ml and cotinine concentrations had a range of 41.4 to 578.1 ng/ml and an average of 192 ng/ml. There were no differences in food intake and body weight between mice in the control, LD- and MD-groups (food intake: repeated measurements, between-subject effect,  $p = 0.52$ ; body weight: repeated measurements, between-subject effect,  $p = 0.88$ ). In addition, numbers of nAChR receptor binding sites detected with receptor autoradiography using 125I-epibatidine binding, showed no significant difference between brain sections derived from mice in the control versus LD- or MD-groups in cortex, hippocampus or thalamus (one-way ANOVA; cortex:  $p = 0.63$ ; hippocampus:  $p = 0.56$ ; thalamus:  $p = 0.45$ ). Thus, low and medium doses of nicotine administered via drinking water, did not result in consistent blood nicotine levels and did not affect food intake, body weight or nicotinic receptor binding (Table 2). Only the highest dose of nicotine tested in this study resulted in significant differences in these measures, which we will describe in more detail.

### Food Intake and Body Weight

Food intake at the first measurement time point was similar in control- and HD-groups (control:  $3.61 \pm 0.14$  g/day; HD-group:  $3.75 \pm 0.07$  g/day). Subsequent measurements were normalized to the first measurement for each individual mouse, and comparisons were made between groups starting from day 8, when the high dose of 300 µg/ml nicotine in the drinking water was reached (Table 1). After day 8, the average daily food intake was significantly lower in mice in the HD-group compared to control-group (control:  $4.30 \pm 0.26$  g/day; HD-group:  $3.79 \pm 0.13$  g/day; repeated measurements, between-subject effect,  $p = 0.005$ ) (Figure 1A). Overall, mice in the HD-group decreased their food intake by about 12% compared to control mice. Body weights on the first day of treatment were identical between groups (control:  $26.59 \pm 1.00$  g; HD-group:  $26.39 \pm 0.70$  g), and subsequent measurements were normalized to the initial weight of each individual animal. Body weights in control mice increased continuously, over the treatment period and mice gained, on average,  $0.16 \pm 0.04$  g/day (Figure 1B). In contrast, after day 8, when the full-dose of nicotine was reached in the HD-group, mice exhibited a slight loss in body weight of  $0.02 \pm 0.07$  g/day. This resulted in increased body weight in control mice but not in mice from the HD-group on day 19 compared to their individual weight on day 1 (Student's t-test, control:

$p = 0.02$ ; nicotine:  $p = 0.814$ ), and subsequently a 4% decrease in body weight between treatment groups on day 19 (control:  $27.59 \pm 0.83$  g; HD-group:  $26.39 \pm 0.51$  g; body weight, repeated measurements, between-subject effect,  $p = 0.043$ ).



**Figure 1:** Nicotine treatment (300 µg/ml) decreased food intake and lowered body weight.

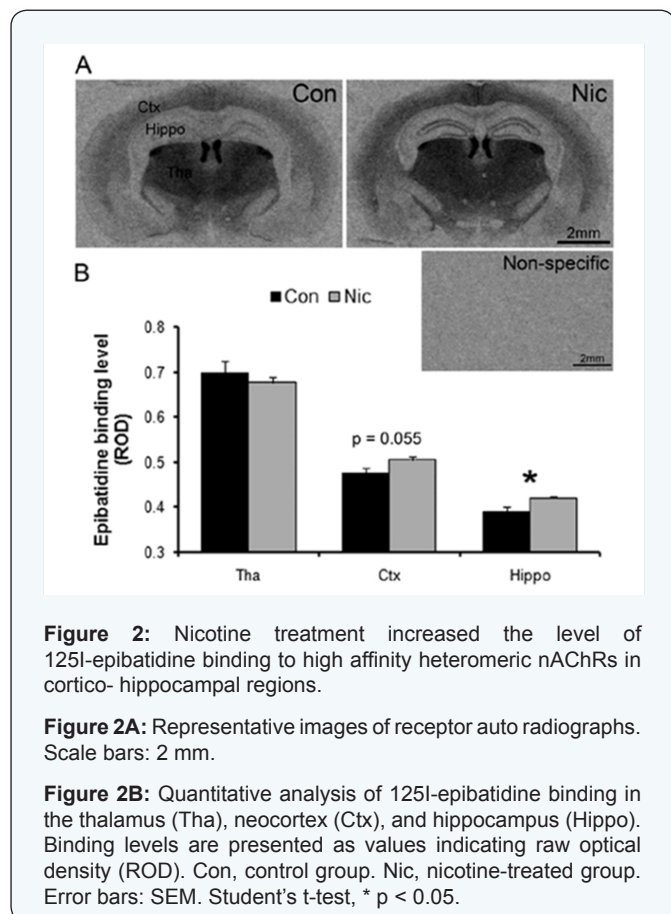
**Figure 1A:** For each mouse, food intake was normalized to their individual food intake on Days 2-4. Measurements were taken every three days until Day 19.

**Figure 1B:** Body weights were normalized to the individual bodyweight of each mouse on Day 1. Measurements were taken every three days until Day 19. Statistics: repeated measurements, between-subject effect, #  $p < 0.05$ ; ##  $p < 0.01$ . Student's t-test, compared between control and nicotine groups, \*  $p < 0.05$ , \*\*  $p < 0.01$ . The dashed vertical line indicates the day the full dose of nicotine was reached (Day 8). Con, control group; Nic, nicotine-treated group. Error bars: standard error of the mean (SEM).

### Nicotinic receptor autoradiography

Nicotinic receptor autoradiography using 125I-epibatidine, was performed to evaluate nicotinic receptor binding sites after chronic treatment to validate nicotine exposure in the brain [40]. Non-specific binding was negligible, and the specific binding pattern was similar to those previously reported with strong binding in the thalamus, medial habenula, and fasciculus retroflexus, moderate binding in cortex and low levels of binding in the hippocampus [41,42] (Figure 2A). In the hippocampus, relative 125I-epibatidine binding, measured in stratum lacunosum moleculare of the CA1 and in the molecular layer of the dentate gyrus, was significantly higher in brain slices from mice in the HD-group compared to control mice (ROD control:  $0.39 \pm 0.010$ ; nicotine:  $0.42 \pm 0.003$ ; Student's t-test,  $p = 0.019$ )

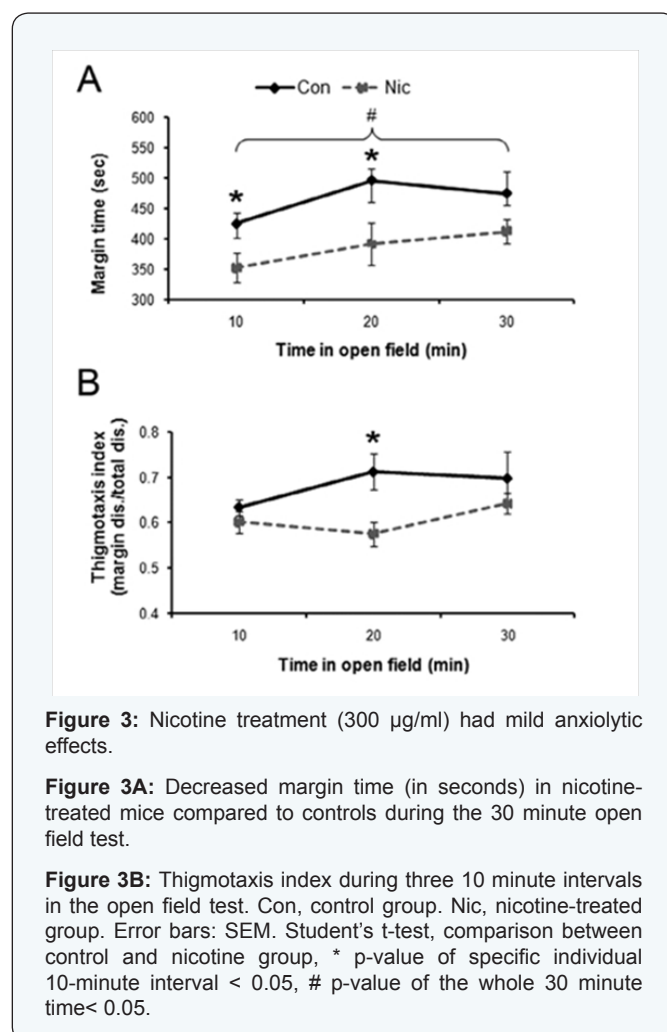
(Figure 2B). In the cortex, relative 125I-epibatidine binding level was slightly higher in mice from the HD-group compared to control mice (control:  $0.48 \pm 0.011$ ; nicotine:  $0.50 \pm 0.007$ ; Student's t-test,  $p = 0.055$ ) (Figure 2B). In contrast, in the thalamus, relative 125I-epibatidine binding was not affected by high dose nicotine treatment (control:  $0.70 \pm 0.024$ ; HD-group:  $0.68 \pm 0.011$ ,  $p = 0.435$ ) (Figure 2B).



### Effects on anxiety like behavior

Nicotine, when used for medicinal purposes, should not have anxiogenic effects. Therefore, we used the open field test to evaluate anxiety-like behavior in mice from control- and HD-group. On day 20 of treatment, locomotor activity, exploratory- and anxiety-like behaviors were assessed in the open field test. Of the 12 parameters measured (see material and method section), 10 parameters showed no significant differences (Student's t-test,  $p > 0.05$ ) indicating that locomotor activity and exploratory behaviors were neither impaired nor augmented in the HD-group compared to control. Only margin and center time, two parameters that indicate anxiety levels, were significantly affected. Control mice spent 78% of the time at the margins, significantly longer than mice from the HD-group, which spent only 64% of the time at the margins (30 minutes overall, Student's t-test,  $p = 0.025$ ) (Figure 3A). When activity was analyzed in 10 minute intervals, control mice spent significantly more time in

the margin area compared to mice from the HD-group during the first and second 10 minute intervals (first interval: control:  $425 \pm 17$  sec, HD-group:  $352 \pm 24$  sec, Student's t-test,  $p = 0.037$ ; second interval: control:  $496 \pm 19$  sec, HD-group:  $392 \pm 35$  sec, Student's t-test,  $p = 0.031$ ). Group differences diminished during the last ten minutes when mice in the HD-group increased the time spent at the chamber's margins (control:  $475 \pm 36$  sec; HD-group:  $411 \pm 20$  sec) (Figure 3A). These results suggested that chronic nicotine had no anxiogenic effects, but to further assess this possibility, we calculated the thigmotaxis index (margin distance / total distance), which has been suggested to be an index of anxiety in mice [43]. The thigmotaxis index was slightly higher in control mice compared to mice from the HD-group, with significant differences during the second 10 minute interval (control:  $0.71 \pm 0.04$ ; HD-group:  $0.59 \pm 0.03$ ; Student's t-test,  $p = 0.021$ ) (Figure 3B), which also suggested reduced anxiety in mice treated with the high dose of nicotine.



### Discussion

The goal of this study was to determine an oral dose of nicotine that is well tolerated by mice, and induces central effects typically associated with chronic nicotine. In this study

we show that voluntary oral consumption of nicotine-containing drinking water can result insignificant decreases in food intake and subsequently body weight, and increased nicotinic receptor binding, measures which are considered hallmarks of chronic nicotine exposure. However, these effects were only seen with the highest dose of nicotine (300 µg/ml) administered in the drinking water, which was also the only dose that resulted consistently in measurable blood nicotine levels in the adult male mice used in this study. We used three different concentrations of nicotine in the drinking water (20, 120 and 300 µg/ml) and measured the resulting blood nicotine and cotinine levels after three weeks of exposure. All three doses were well tolerated by the mice with no obvious negative effects. The low and medium doses (20 and 120 µg/ml) resulted in undetectable blood nicotine concentrations in 4 out of 5 mice. Only mice treated with the highest dose had measurable blood nicotine levels in most animals (4 out of 5 mice) with an average concentration of 6.2 ng/ml. Blood nicotine concentrations of up to 21.1 ng/ml were measured, which are comparable to concentrations seen in people using nicotine replacement products, smoking a cigarette or using smokeless tobacco products such as chewing tobacco or snuff [19]. However, variability in the HD groups was high because of the rapid degradation of nicotine in mice. With increasing nicotine concentrations in the drinking water, we saw increases in blood cotinine levels, the major metabolite of nicotine. However, at all three doses, blood cotinine levels exhibited high variability, which seems to be characteristic for the oral administration route. At 300µg/ml, blood cotinine concentrations ranged from 41.4-578.1 ng/ml with an average of 192 ng/ml. These levels are comparable to findings from other groups, which have shown that in mice treated with nicotine via drinking water (200 to 500 µg/ml), plasma cotinine levels from 652 to 1450 ng/ml were found [44,45]. The large variability of plasma cotinine levels between and within studies is likely due to the relatively short half-life of cotinine in mice (20-40 minutes) compared to other species (rats 5-6 hours and human 19 hours) [24,25,46,47]. Thus, blood cotinine concentration may not be a reliable surrogate measure of blood nicotine levels in mice, but does indicate recent nicotine consumption. Over the years, a number of studies have shown that chronic nicotine reduces body weight and/or suppresses food intake in humans and laboratory animals [48-52]. In neonates, even very low doses of nicotine reduces body weight gain [53], and people using nicotine replacement therapy during smoking cessation, also exhibit reduced weight gain [54,55]. In this study, an anorexic effect of nicotine was only observed in the HD-group, whereas in the LD-group and MD-group, there was no effect on either food intake or body weight. This is consistent with other studies which used the oral route of nicotine administration and reported that chronic treatment with lower nicotine concentrations via drinking water does not affect weight gain or food intake [56]. Furthermore, nicotine's anorexic effect did not start until treatment day 8, when the highest dose of nicotine was reached. This result correlated with more consistently measurable blood nicotine levels in

the HD-group, and suggests that nicotine consumed through the drinking water can exert central anorexic effects. However, decreases in body weight and food intake have not consistently been reported with chronic oral nicotine administration [57], or were only seen after prolonged treatment [45]. It is possible that sufficient blood nicotine levels were not reached for long enough time periods to trigger an anorexic effect [57], or were only reached after the highest dose was administered [45]. Another hallmark for the central effects of chronic nicotine exposure is an increase in heteromeric high affinity nicotinic binding sites in the brain, which have also been observed in oral nicotine administration models using mice [33,57,58]. Using receptor autoradiography, we determined that only the highest treatment dose of nicotine increased 125I-epibatidine binding to heteromeric nAChRs in cortical and hippocampal regions. In contrast, low and medium doses had no effect, although, cotinine measurements indicated recent consumption of nicotine. The receptor binding results correlate well with the lack of consistently detectable blood nicotine concentrations and lack of an anorexic effect in the LD- and MD-groups, and suggest that nicotine has to be chronically present at sufficient levels in order to up regulate nicotinic binding sites. Taken together, nicotine and cotinine blood levels, food intake, body weight, and receptor binding results indicate that nicotine consumed via the drinking solution, is effective in reaching relevant levels of nicotine in the mouse brain comparable to those seen in human smokers or patients using nicotine replacement medications. However, due to the rapid metabolism of nicotine in mice, relatively high levels of nicotine doses need to be consumed to achieve levels that reliably induce central effects. Chronic exposure to nicotine can affect anxiety levels in humans and animals, but the issue is complex. Most human smokers experience lower anxiety levels than non-smokers when challenged with stress, and increased anxiety levels during smoking cessation, suggesting that chronic nicotine exposure can reduce anxiety in humans [34-38]. However, in rodents, nicotine's effects on anxiety are less consistent. Chronic or acute nicotine administration can result in either an anxiolytic or anxiogenic response, depending on several confounding factors such as age, dose, route of administration, sex and species [37,38]. Nicotine or other nAChR agonists given chronically for medicinal purpose should not induce anxiety. Very few studies have evaluated the effects of chronic nicotine exposure administered via drinking water on behavioral measures. In one such study, female but not male mice exhibited increased anxiety-like behavior after chronic oral nicotine administration [59], whereas a sex independent anxiogenic effect was found in wild type mice in another study [60]. Therefore, we evaluated anxiety-like behavior in the HD-group using the open field test. In this study, locomotor and exploratory activities were not changed, but two parameters, margin time and thigmotaxic index, were lower in mice from the HD-group compared to controls, indicating a trend towards reduced anxiety levels. This indicates that, chronic oral nicotine treatment with a dose sufficient to up regulate heteromeric

nAChR binding sites and to affect body weight, did not increase anxiety-like behavior in adult male mice, but might have mild anxiolytic effects. These findings are consistent with results found in adult male mice after chronic oral administration of nicotine, which also did not result in increased anxiety-like behavior [59], but contrast with results from a study which used a lower dose of nicotine [60]. The reasons for the discrepancies between these studies are not clear.

### Conclusion

Oral self-administration of nicotine via drinking water provides a stress-free route of chronic drug treatment, but requires relatively high doses to reach biological active levels of nicotine in the mouse brain. Three weeks of nicotine consumption decreased food intake and body weight, and increased expression of high affinity nicotine binding sites, hallmarks of central effects of chronic exposure to nicotine. Furthermore, chronic voluntary oral consumption of nicotine at levels high enough to exhibit central effects of chronic nicotine exposure, and did not have anxiogenic effects, making this route and this dose suitable for long-term treatment of adult mice.

### Conflict of Interest

The authors declare that there are no conflicts of interest.

### Acknowledgement

The open access publishing fees for this article have been covered by the Texas A&M University Online Access to Knowledge (OAK) Fund, supported by the University Libraries and the Office of the Vice President for Research.

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