Spring 2015

Effect of miRNA-138 Injected into the Medial Habenula on Nicotine Intake and Preference

Kelsey Fitzgerald
Kelsey.Fitzgerald@Colorado.EDU

Effect of miRNA-138 Injected into the Medial Habenula on Nicotine Intake and Preference

By:
Kelsey Fitzgerald
Department of Integrative Physiology, University of Colorado at Boulder

Defended April 2, 2015

Thesis Advisor:
Dr. Marissa Ehringer, Dept. of Integrative Physiology
Honors Council Representative:
Dr. David Sherwood, Dept. of Integrative Physiology
Defense Committee:
Dr. Ryan Bachtell, Dept. of Psychology

Abstract

Nicotine is one of the most commonly abused drugs in the world today and is associated with many adverse health outcomes, including death. Nicotine binds to nicotinic acetylcholine receptors (nAChRs) in the brain reward pathway, triggering the release of a number of neurotransmitters, including dopamine. The goal of the current study was to determine whether miR-138, a miRNA that targets and reduces β4 subunit gene expression, injected into the medial habenula would lead to changes in nicotine intake/preference. We hypothesized that miR-138 would down regulate β4 expression, leading to decreased aversion to nicotine and increased nicotine intake/preference. Male and female C57BL/6 mice were unilaterally injected with an adeno-associated virus containing either miR-138 or a scrambled control miRNA sequence. Mice were then given access to water and increasing concentrations of a nicotine solution. We measured overall fluid consumption as well as nicotine intake. Mice injected with the miR-138 showed a trend for reduced nicotine intake and consumption compared to controls. Preliminary results were not statistically significant, but suggest that miR-138 injected into the medial habenula does not affect nicotine intake/preference. The current results suggest that reducing the expression of β4 subunits in the medial habenula via miR-138 leads to reductions in nicotine reward-related behavior, which does not support our initial hypothesis.

Introduction

Tobacco use is the number one cause of preventable deaths in the United States, and each year an estimated 443,000 people die prematurely from smoking (CDC, 2011). Nicotine is the major psychoactive chemical in tobacco, and repeated exposure to nicotine often results in

nicotine dependence. Nicotine dependence includes many different features, where a primary component is the inability to stop using the substance (CDC, 2010). Nicotine induces physical and mood-altering effects that are rewarding to the user (depending on dose), which in turn perpetuates nicotine intake (Baker, 2012). Stopping use of the substance will result in physical withdrawal symptoms including anxiety, depression, and headaches (Baker, 2012). In the United States, nicotine dependence has a prevalence of 24.1% with the onset commonly occurring before the age of 25 and dependence continuing on well into the 40s (Breslau et al., 2001).

Nicotinic Acetylcholine Receptors

Nicotine binds to nicotinic acetylcholine receptors (nAChRs) in the brain. Nicotinic acetylcholine receptors are heteropentamer cholinergic receptors, consisting of five membrane proteins (subunits) that surround a pore in the middle, forming ligand-gated ion channels in the plasma membranes of neurons. Receptors are formed from a various combinations of α, β, Δ, and γ subunits (Albuquerque et al., 2009). In the brain only α2-α9 and β2-β4 subunits are expressed (Picciotto et al., 2000), and these subunits are free to interact, but not all subunit combinations will result in a nicotine binding site.

Of particular interest to the current project are the α3, α5 and β4 subunits because they are enriched in the habenulo-interpeduncular (Hb-IPN) midbrain pathway, a pathway implicated in the aversive effects of nicotine, and nicotine withdrawal (Antolin-Fontes et al., 2014). Further, the *CHRNA5-A3-B4* gene cluster encodes α5, α3 and β4 subunits and has been associated with vulnerability to tobacco dependence in human genetics studies (Bierut et al., 2008 and MacKillop et al., 2010).

β4 subunit and microRNAs involvement in nicotine dependence

This study focuses on the β4 subunit because the α5 subunit, although it co-assembles with α3β4, does not play a role in nicotine withdrawal and acute nicotine behaviors (Jackson et al., 2013). The β4 subunit, on the other hand, is highly involved in nicotine aversion (Baldwin, Alanis and Salas, 2011). For example, β4 knockout (KO) mice and α5 KO mice have fewer somatic signs of withdrawal compared to controls following chronic nicotine administration and attenuated withdrawal-induced hyperalgesia (Jackson et al., 2008 and Salas et al., 2009). Similarly, chronic nicotine treatment upregulates β4 gene expression in neurons of the Hb-IPN and infusion of the β4 nAChR antagonist SR16584 in the Hb-IPN elicited somatic signs of nicotine withdrawal (Zhao-Shea et al., 2013).

Along with the apparent role of the β4 subunit in nicotine withdrawal, recent findings also implicated β4 in aversive responses to nicotine. For example, overexpression of β4 in the medial habenula produced nicotine aversion in mice (Slimak et al., 2014). Further, voltage-clamp recoding showed that human β4 genetic variants that increased nicotine-induced current flow also increased aversion to nicotine in a two-bottle choice nicotine preference study. Variants that decreased nicotine-induced currents, on the other hand, also decreased aversion (Slimak et al., 2014). The role of β4 subunits in nicotine aversion and withdrawal implicated the subunit in nicotine addiction/dependence, and thus assessing how endogenous genetic regulatory mechanisms that may modulate nicotine reward is important to developing treatment options for nicotine dependence. One genetic regulatory mechanism that is gaining a lot of attention recently is a family of RNAs called microRNAs (miRNAs).

MicroRNAs (miRNAs) are endogenous small (22 nucleotides) non-coding RNAs that target the 3'-untranslated region of mRNA to regulate gene expression post-transcriptionally. Over 1000 miRNAs have been identified in humans, and more than a third of all genes are regulated by miRNAs (Bartel et al., 2009). Since the discovery of miRNAs, a growing body of evidence implicates miRNAs in a number of neurological diseases, including addiction (Li and van der Vaart, 2011). MiRNAs have shown to play roles in the modification of the reward system in the brain that drives addiction due to drugs (Bali and Kenny, 2013). Relevant to the current project, nicotine can produce changes in miRNA expression, suggesting that nicotine can regulate miRNAs expression (Taki, Pan, and Zhang, 2014), and thus this change in miRNAs may facilitate changes in nAChR subunit expression, ultimately influencing the development of nicotine dependence/addiction. Recently, our lab showed that a specific miRNA, miR-138, leads to decreased gene expression using an in vitro luciferase assay targeting the 3'UTR of the human *CHRNB4* gene (Gallego, 2013), and is only expressed in brain tissue.

Purpose and Hypothesis

Given the importance of β4 receptor subunits in nicotine dependence/addiction, and the recent finding from our lab that miR-138 reduces gene expression of a vector containing the 3'UTR of *CHRNB4 in vitro*, we were interested to assess the effects of miR-138, injected unilaterally into the medial habenula, on two-bottle choice nicotine intake/preference. Because of the medial habenula's involvement in nicotine withdrawal (Zhao, Tapper, 2013), and the enrichment of β4 containing nAChRs in the medial habenula, we predicted that injecting miRNA-138 into the medial habenula would down-regulate β4 subunits and result in increased nicotine intake/preference.

Methods

Animals

Male and female C57BL/6J mice were purchased from Jackson Laboratories. Mice arrived at the Institute for Behavioral Genetics at 7 weeks of age and were housed 2-5 per cage during the 7-day acclimation period with *ad libitum* access to food and water. Following stereotaxic surgery (see below), mice were singly housed with *ad libitum* access to food and water and given a two-week period for miRNAs to have an effect. Experiments we performed on a 12:12 light cycle approved by the University of Colorado at Boulder Institutional Animal Care and Use Committee with the lights off at 1900 hours.

Adeno-Associated Viral vectors

Adeno-Associated Viral vectors were purchased from Vectors Biolabs (Philadelphia, PA) and were used for delivery of miR-138 into the brain. Procedure of administration is outlined in the surgery section below.

Nicotine Solutions

Nicotine solutions were made of free-base nicotine and diluted to the desired concentration of 25, 50, 100 and 200 ug/mL with tap water. The Nicotine was purchased from Sigma-Aldrich, St. Louis, MO.

Surgery

Mice (60 days old) were anesthetized with 100/10 mg/kg of Ketamine/Xylazine administered by IP injection. Duratears ophthalmic ointment (1 drop) was administered topically

to protect their corneas. Adeno-Associated Virus (AAV) was administered through microinjections under the approved IACUC protocol. An incision was made in the scalp and a burr hole in the skull, positioned to expose the injection site above the medial habenula The coordinates are X= 0.35, Y= -1.74, Z= 3.2 for males, and X= 0.35, Y= -1.50, Z= 3.2 for females. A Hamilton syringe needle (31 gauge), with AAV containing either miR-138 or a scrambled control miRNA, was injected into the medial habenula at a rate 0.1 mL/min totaling 0.5-1 mL. Green Fluorescent Protein (GFP) was injected along with the AAV and was used as a visible marker. Mice with either treatment received an injection of GFP to later verify the location of the injection into the MHb. 0.05-0.1 mg/kg of Buprenorphine and 100 mg/kg Penicillin (Twin-Pen) were administered through a subcutaneous injection to prevent infection following the procedure. The body temperature of the mice was maintained with a heating pad for the first 1-2 hours during recovery from anesthesia. The mice were monitored every 12 hours following surgery for the first 48 hours.

Nicotine Preference Study

Mice were given a nicotine two bottle choice paradigm and observed for 16 days as the concentration of the nicotine was increased. Following the 2-week period after AAV injection, standard water bottles were replaced with two test tubes fitted with drinking spouts (drinking tubes), one containing tap water and one containing increasing concentrations of nicotine (25 to 50, 100, and 200 ug/mL) diluted with tap water. The drinking amounts were observed by measuring the difference in volume over the 24 hour period. Nicotine concentrations were increased every four days. The side the nicotine was alternated every two days to avoid development of a side preference. The mice were weighed on the first day of each concentration increase (every four days) to ensure good health and to standardize the nicotine consumed by

body weight. Evaporation/leakage was accounted for using tubes placed in empty cages, and the mean volume loss was subtracted from the individual drinking values. Cages were changed every 7-days after drinking measurements were taken.

Statistical Analysis

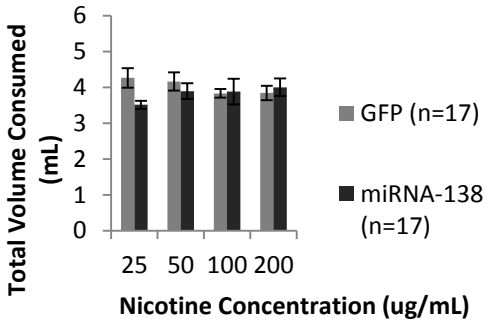
For all animal data, statistical analyses were performed using SPSS. Data (total volume, nicotine preference, and nicotine consumption) were analyzed using Analysis of Variance (ANOVA) with Treatment and Sex as the between subject factors, and nicotine concentration and day as the within subject factors. $P \leq 0.05$ were considered significant. Analysis of each dependent variable was done two ways for each item of collected data, the first being the collapsed across all four days per concentration, and the second way was by isolating days two and four per concentration (e.g. days 2 and 4 for 25ug/mL, days 6 and 8 for 50ug/mL , days 10 and 12 for 100ug/mL, and days 14 and 16 for 200ug/mL). We ran the analysis both ways because drinking behavior is influenced by stress, and by isolating days 2 and 4 of each concentration we reduced the variability in the behavioral data associated with the stress of handling the mice.

Total volume consumed was measured as the overall consumption of nicotine and water. Nicotine preference was calculated as the percent of nicotine consumed of the total volume. Nicotine Consumption was measured by the amount of nicotine consumed at the concentration and then standardized by each animal's body weight. Significant interactions uncovered from the omnibus ANOVA were followed up with lower-order ANOVAs and *post hoc* t-tests where appropriate.

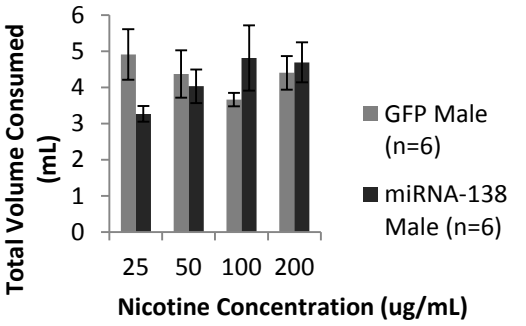
Results

Total Volume Consumed Results

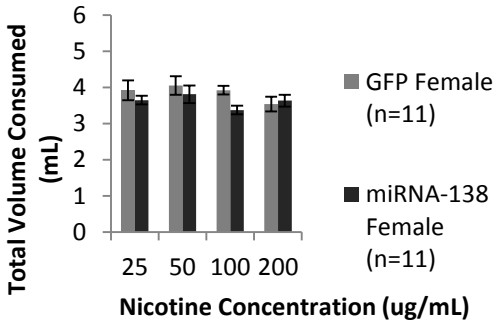
Intake averaged across concentration



A)



B)



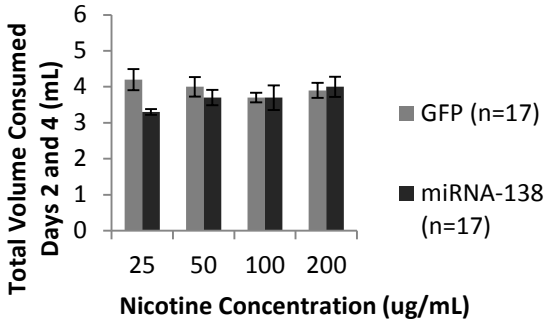
C)

Figure 1: The total volume of both nicotine and water consumed for the four different nicotine concentrations tested. Data are shown collapsed by sex (A), and split into males (B) and females (C).

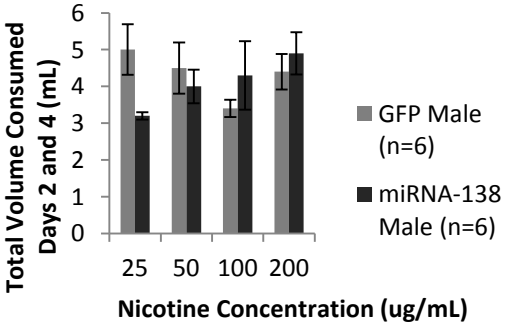
Figure 1 shows the amount of total volume consumed (mL) for each experimental group, collapsed across concentration days. Three-way ANOVA (Concentration x Sex x Treatment) of total volume indicated a main effect of sex only [$F(1,30)=9.7$, $p<0.01$)], showing that males drank more total fluid than females. The main effect of treatment did not reach significance ($p=0.276$). Analyses also uncovered a Concentration x Sex x Treatment interaction [$F(3,90)=3.8$,

p=0.012], but follow-up analyses within sex indicated no significance [Female: F(1,20)=2.3, p=0.148; Male: F(1,10)= 0.14, p=0.716)]

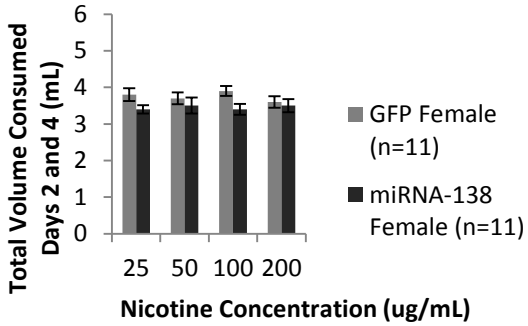
Intake averaged across days 2 & 4 of each concentration



A)



B)



C)

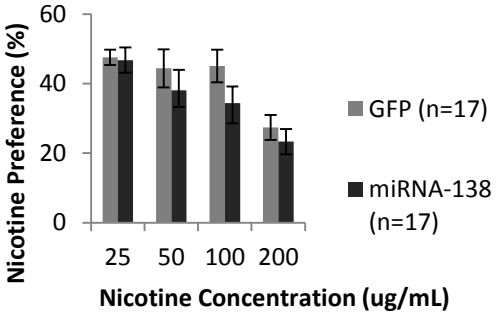
Figure 2: The total volume of both nicotine and water consumed for the four different nicotine concentrations tested averaged across days 2 and 4. Data are shown collapsed by sex (A), and split into males (B) and females (C).

Figure 2 shows the amount of total volume consumed (mL) for each experimental group, collapsed across concentration days 2 and 4. Three-way ANOVA (Concentration x Sex x Treatment) of total volume indicated a main effect of sex only [$F(1,30)=11.6$, $p<0.01$)], showing that males drank more total fluid than females. The main effect of treatment did not reach significance ($p=0.175$). Analyses also uncovered a Concentration x Sex x Treatment interaction

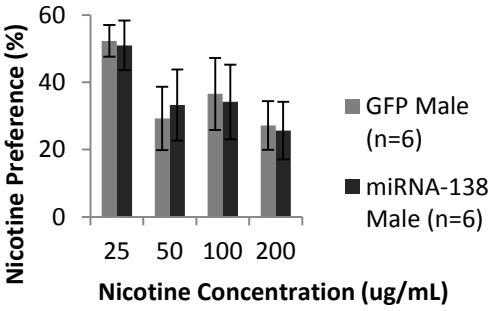
[F(3,90)=3.4, p=0.021], but follow-up analyses within sex indicated no significance [Female: F(1,20)=3.3, p=0.085; Male: F(1,10)= 0.299, p=0.596)]

Nicotine Preference Results

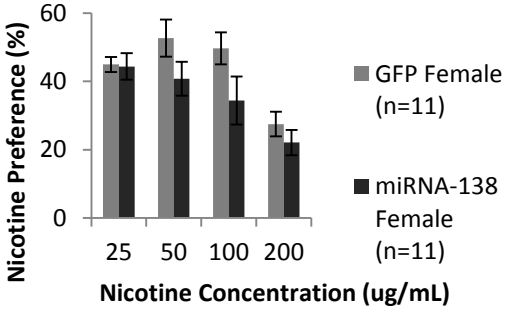
Preference averaged across concentration



A)



B)



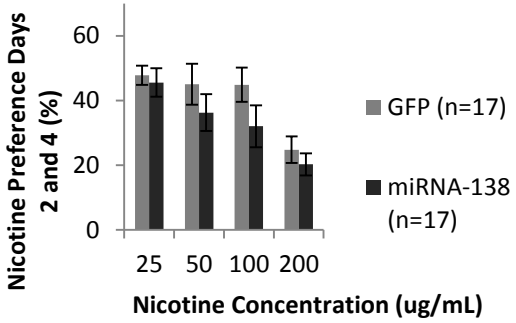
C)

Figure 3: Nicotine Preference for the four different nicotine concentrations tested. Data are shown collapsed by sex (A), and split into males (B) and females (C).

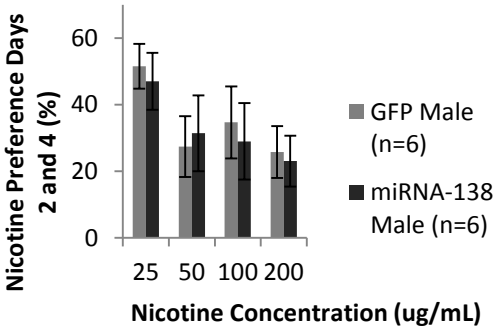
Figure 3 shows the nicotine preference (%) represented as a percentage of the total volume for each experimental group. Three-way ANOVA (Concentration x Sex x Treatment) of nicotine preference indicated a main effect of concentration only [F(3,90)=12.2, p<0.0001], showing that there was a higher preference at lower concentrations. The main effect of treatment did not reach significance (p=0.343). Analyses also uncovered a Concentration x Sex interaction

[F(3,90)=3.4, p=0.021]. Follow-up analyses comparing Sex at each concentration indicated the interaction was due to a significant main effect of Sex at the 50 ug/mL concentration only [F(1,32)=4.6, p=0.04].

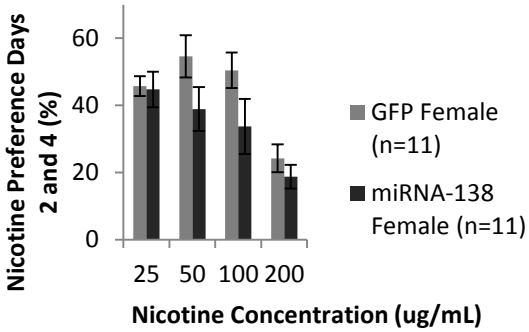
Preference averaged across days 2 & 4 of each concentration



A)



B)



C)

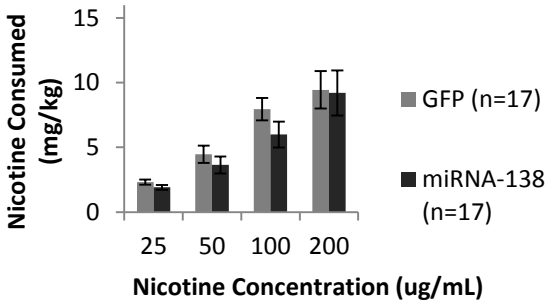
Figure 4: Nicotine Preference for only days two and four for the four different nicotine concentrations tested. Data are shown collapsed by sex (A), and split into males (B) and females (C).

Figure 4 shows the nicotine preference for days 2 and 4 (%) for each experimental group. Three-way ANOVA (Concentration x Sex x Treatment) of nicotine preference indicated a main effect of concentration only [F(3,90)=10.5, p<0.0001], showing that there was a higher preference at lower concentrations. The main effect of treatment did not reach significance (p=0.24). Analyses also uncovered a Concentration x Sex interaction [F(3,90)=2.8, p=0.043].

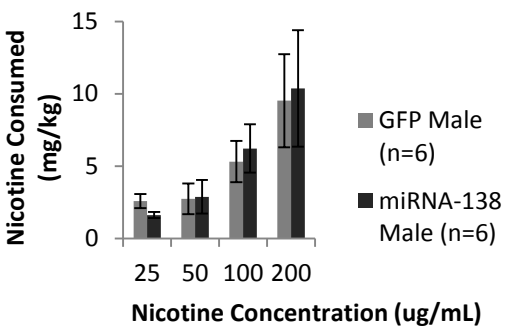
Follow-up analyses within sex indicated no significance for male mice, and for female mice there was a main effect of concentration [$F(3,63)=14.4$, $p<0.001$]

Nicotine Consumption Results

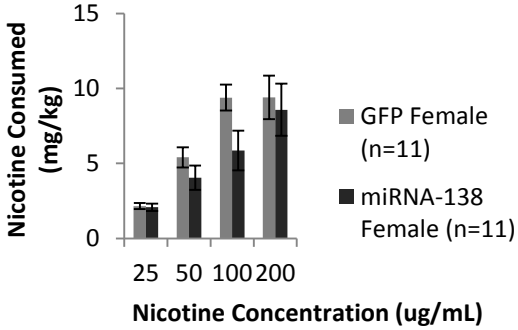
Consumption averaged across concentration



A)



B)



C)

Figure 5: Nicotine Consumed for the four different nicotine concentrations tested. Data are shown collapsed by sex (A), and split into males (B) and females (C).

Figure 5 shows the nicotine consumed (mg/kg) for each experimental group. Three-way ANOVA (Concentration x Sex x Treatment) of nicotine preference indicated a main effect of concentration only [$F(3,90)=22.5$, $p<0.01$], showing that more nicotine per body weight was consumed at higher concentrations. The main effect of treatment did not reach significance ($p=0.493$). There was no initial significance between the treatment groups, so follow-up analysis was not run on the separated sexes.

Consumption averaged across days 2 & 4 of each concentration

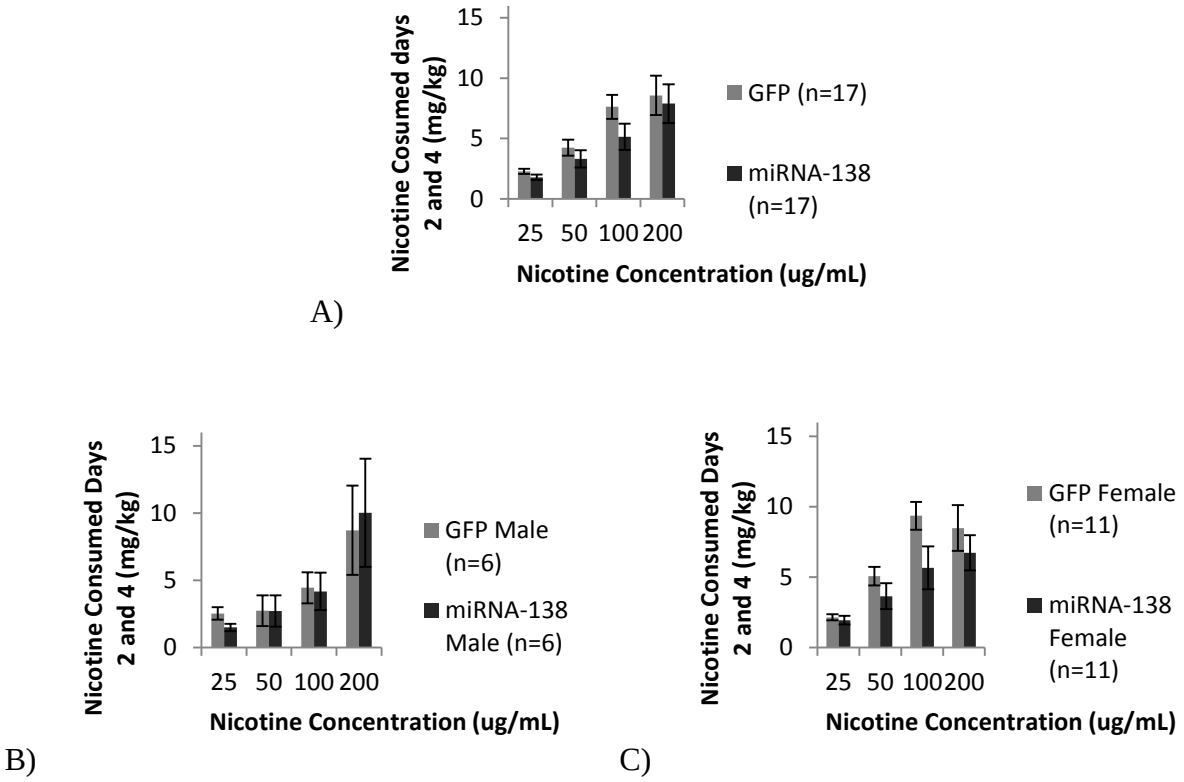
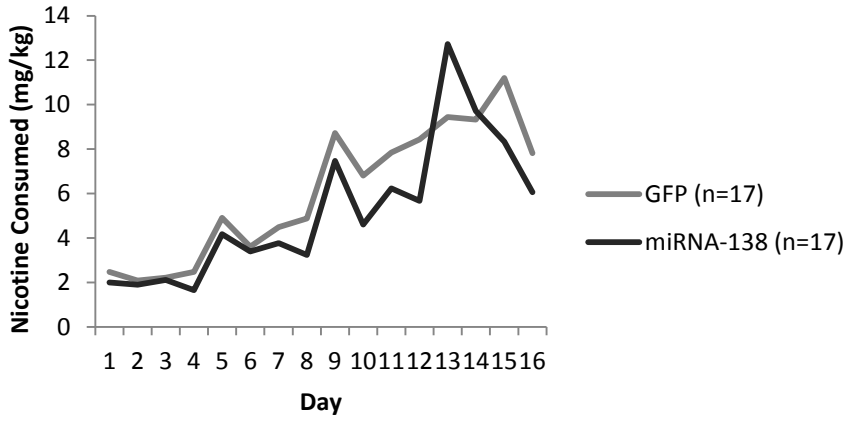


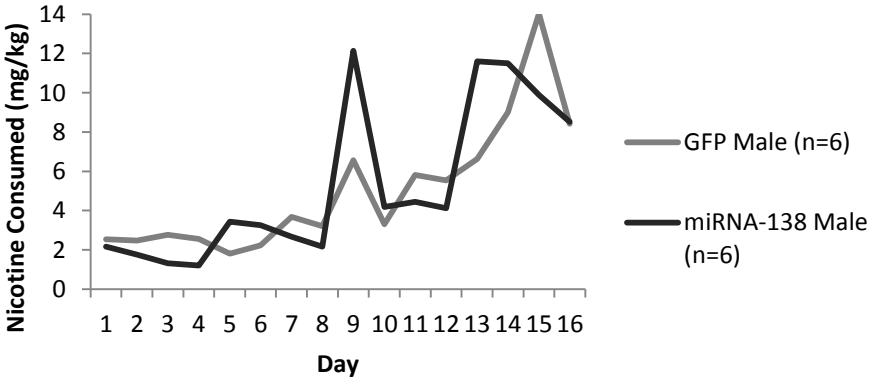
Figure 6: Nicotine Consumed for only days two and four for the four different nicotine concentrations tested. Data are shown collapsed by sex (A), and split into males (B) and females (C).

Figure 6 shows the nicotine consumed for days 2 and 4 (mg/kg) for each experimental group. Three-way ANOVA (Concentration x Sex x Treatment) of nicotine consumption indicated a main effect of concentration only [$F(3,90)=16.3$, $p<0.001$], showing that there was more nicotine consumed per body weight at higher concentrations. The main effect of treatment did not reach significance ($p=0.34$). The collapsed data showed no significance between treatments, so further analyses were not run on the separate sexes.

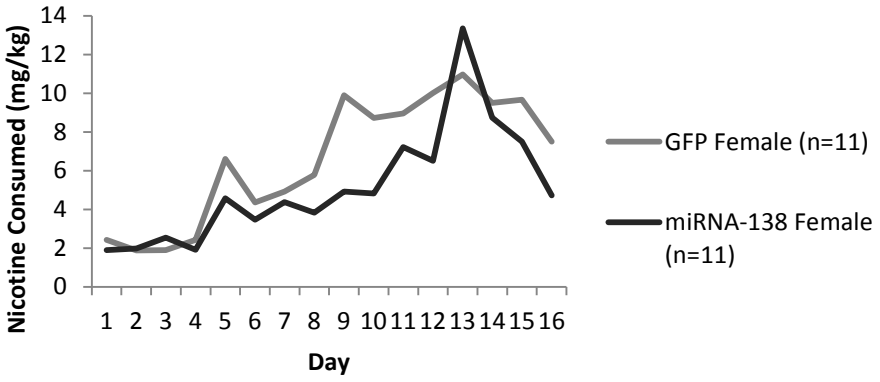
Consumption per day across the concentrations



A)



B)



C)

Figure 7: Nicotine Consumed per day. Data are shown collapsed by sex (A), and split into males (B) and females (C).

Figure 7 shows the nicotine consumed per individual day (mg/kg) for each experimental group. Three-way ANOVA (Concentration x Sex x Treatment) of nicotine preference indicated a main effect of concentration [$F(3,90)=22.5$, $p<0.01$], showing that more nicotine was consumed per body weight at higher concentrations. These results are the same for the averaged data across the concentrations days. Follow up analyses did not reach significance.

Discussion

The purpose of the current study was to assess the involvement of β4 subunits in nicotine reward-related behaviors by using miR-138, which may be able to reduce the expression of β4 nAChR subunits. We hypothesized that the down regulation of β4 subunits would result in an increase in nicotine intake/preference. MiR-138 is predicted to bind to β4 mRNA (Gallego, 2013), resulting in decreased expression of the β4 subunit protein. The β4 subunit plays a role in nicotine aversion (Baldwin, Alanis and Salas, 2011), and when down regulating the β4 subunit we expected to see a decrease in aversion and consequently an increase in nicotine intake/preference. Our preliminary results suggest that miR-138 injected into the medial habenula leads to reduced consumption of nicotine, which did not support our hypothesis.

Findings

During our analysis we observed a decrease in overall consumption of total volume for the miR-138 female mice (Figure 1, graph C). We also saw a decrease in nicotine consumption by the female miR-138 mice (Figure 5, graph C). Visually both show different consumptions than the control mice, but when looked at together we cannot say that the decrease in nicotine consumption for the miR-138 females mice is relevant when there was a general decrease in total

volume for the miR-138 females. Therefore we cannot say the effect was specific to nicotine without further preference analysis.

Although our statistical analyses found no statistical significance between treatment groups, there was evidence for differences within certain concentrations. Results showed that more nicotine was consumed per body weight at higher concentrations (Figure 5). This is consistent with previous work showing nicotine induces the reward pathway at higher doses (Fowler and Kenny, 2011) which may result in more consumption at the higher concentrations. For nicotine preference we observed that there was an increase in preference at lower concentrations (Figure 3). The mice tended to drink more of the diluted concentrations and less as the concentration increased. This agrees with the idea that more volume at a lower concentration is necessary to achieve desired levels of nicotine to activate the reward pathway (Baker et al., 2012).

Sex differences also played a role in consumption and preference. Studies support our findings that sex plays a role in total volume consumption and nicotine consumption that is adjusted for body weight (Klein et al., 2004). Males consumed more volume overall than females (Figure 1), but did not consume more nicotine. For nicotine preference (Figure 4) the males showed no significance while the females had a statistical significant result between treatments. Males also had more variance in their consumption while females had a more consistent trend from day to day. Visual inspection of Figure 7 suggests that the observed interaction effect is due to males reducing intake of the 100 ug/mL nicotine concentration (days 9-12 in graph B) while females show consistent intake across the 100 ug/mL concentration days (days 9-12 graph C).

Based on previous studies (Chester et al., 2006), we anticipated that stress due to changing the bottles might alter the drinking behaviors. However, similarities visually and in our analyses between Figures 3 and 4 and Figures 5 and 6 shows that this stress did not affect drinking behaviors for both nicotine preference and nicotine consumption. The graphs isolating days two and four show the same results with similar values as the graphs representing all days collapsed. This indicates that the stress involved in changing bottles on days one and three did not affect the amount of nicotine consumed.

We hypothesized that by potentially down regulating the β4 nAChRs with miR-138 we would see an increase in nicotine intake/preference. Surprisingly, our results suggest an opposite effect. Although not reaching statistical significance, when analyzed separately by sex, there is modest evidence that the miR-138-injected mice consume less nicotine. It remains unclear what the underlying mechanisms contributing to this effect may be. One possibility is the overall alteration of nAChR stoichiometry upon miR-138 injection (Taki, Pan, and Zhang, 2014). Altering the configuration of the receptor may lead to a decrease in binding capability for the nicotine. Without nicotine binding, the mice would not experience the release of dopamine and the effects of the reward pathway that the nAChRs trigger (Antolin-Fontes et al., 2014).

Limitations

It is important to note that miRNAs have multiple mRNA targets and are often a part of transcriptional regulatory loops, and thus we cannot rule out the possibility that miR-138 has transcript targets other than the β4 subunit. MiRNAs may also result in subtle phenotypes making it difficult to distinguish behavior solely caused by the nicotine and not due to the microRNA (Coolen and Bally-Cuif, 2009).

Future directions

The next step in this study is to add animals to the subgroups and to confirm that our microinjections into the medial habenula are in fact in the medial habenula. Brains from all mice have been removed postmortem and will be sliced for immunohistochemical verification of the virus injection site. Data from animals where the medial habenula was missed will be removed from analysis.

A tanstant study will also be run in a separate cohort of mice to ensure that miR-138 does not affect nicotine consumption/preference based on taste alone. Mice will again be injected with miR-138 or a control and then tested for saccharin (sweet) and quinine (bitter) preference.

Interestingly, miRNA-138 has also been associated with learning and memory. A recent paper showed that miR-138 injected into the hippocampus resulted in improved short term memory in mice (Tarto et al.,2013). The medial habenula is also implicated with learning and memory, as it is the site where learned information is stored. Future studies should focus on examining the role of miR-138 in drug reward memories, as learning and memory plays an important role in maintaining nicotine dependence (Gould et al., 2006).

Summary and Conclusions

In summary, we observed an overall decrease in nicotine consumption and preference within the miRNA-138 treated mice. Our findings did not support our initial hypothesis that we would see an increase in nicotine preference. However, the fact that our preliminary results show an effect of the miRNA is very interesting so future work aimed at understanding the mechanisms will be important.

Acknowledgements

I want to thank my honors thesis advisor Dr. Marissa Ehringer, and also the postdoctoral fellow I worked with, Dr. Matthew Powers. I want to thank my committee, Dr. Ryan Bachtell and Dr. David Sherwood.

References

Albuquerque, E. X., Pereira, E. F. R., Alkondon, M., & Rogers, S. W. (2009). Mammalian Nicotinic Acetylcholine Receptors: From Structure to Function.*Physiological Reviews*, *89*(1), 73–120. doi:10.1152/physrev.00015.2008

Antolin-Fontes B, Ables JL, Corlich A, Ibanez-Tallon I. (2014). The habenulo-interpeduncular pathway in nicotine aversion and withdrawal. *Neuropharmacology.* Science Direct

Baker, T. B., Breslau, N., Covey, L., & Shiffman, S. (2012). DSM Criteria for Tobacco Use Disorder and Tobacco Withdrawal: A Critique and Proposed Revisions for DSM-5. *Addiction (Abingdon, England)*, *107*(2), 263–275. doi:10.1111/j.1360-0443.2011.03657.x

Baldwin, P. R., Alanis, R., & Salas, R. (2011). The Role of the Habenula in Nicotine Addiction. *Journal of Addiction Research & Therapy*, *Suppl 1*(2), 002. doi:10.4172/2155-6105.S1-002

Bali, P., & Kenny, P. J. (2013). MicroRNAs and Drug Addiction. *Frontiers in Genetics*, *4*, 43. doi:10.3389/fgene.2013.00043

Bartel, D. P. (2009). MicroRNA Target Recognition and Regulatory Functions.*Cell*, *136*(2), 215–233. doi:10.1016/j.cell.2009.01.002

Bierut LJ, Stitzel JA, Wang JC, Hinrichs AL, Grucza RA, Xuei X, et al. Variants in Nicotinic Receptors and Risk for Nicotine Dependence. Am. J. Psychiatry. 2008; 165:11163-1171. [PubMed: 18519524]

Breslau N, Johnson EO, Hiripi E, Kessler R. Nicotine Dependence in the United States: Prevalence, Trends, and Smoking Persistence. *Arch Gen Psychiatry.*2001;58(9):810-816. doi:10.1001/archpsyc.58.9.810.

Broms U., Silventoinen K., Madden P. A., Heath A. C., Kaprio J. (2006). Genetic architecture of smoking behavior: a study of Finnish adult twins. *Twin Res. Hum. Genet.* 9 64–72 10.1375/twin.9.1.64 [PubMed]

Centers for Disease Control and Prevention (US); National Center for Chronic Disease Prevention and Health Promotion (US); Office on Smoking and Health (US). How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General. Atlanta (GA): Centers for Disease Control and Prevention (US); 2010. 4, Nicotine Addiction: Past and Present.Available from: http://www.ncbi.nlm.nih.gov/books/NBK53018/

Center for Disease Control and Prevention. Targeting the Nation's Leading Killer At A Glance 2011, USA, MMWR Morb Mortal Wkly Rep. 2011

Chester JA, Paula Barrenha G, DeMaria A, Finegan A. (2006) Different Effects of Stress on Alcohol Drinking Behaviour in Male and Female Mice Selectively Bred for High Alcohol Preference. Alcohol and Alcoholism Journal, 41:44-53; DOI: 10.1093/alcalc/agh242

Coolen M, Bally-Cuif L. (2009) MicroRNAs in brain development and physiology. *Neurobiology*. Science Direct 19:461-470

Fowler, C. D., & Kenny, P. J. (2011). Intravenous nicotine self-administration and cue-induced reinstatement in mice: Effects of nicotine dose, rate of drug infusion and prior instrumental training. *Neuropharmacology*, *61*(4), 687–698. doi:10.1016/j.neuropharm.2011.05.012

Frahm S, Slimak MA, Ferrarese L, Santos-Torres J, Antolin-Fontes B, et al. Aversion to nicotine is regulated by the balanced activity of beta4 and alpha5 nicotinic receptor subunits in the medial habenula. Neuron. 2011;70:522–535. [PubMed]

Gallego, X., Cox, R. J., Laughlin, J. R., Stitzel, J. A., & Ehringer, M. A. (2013). Alternative *CHRNB4* 3′-UTRs Mediate the Allelic Effects of SNP rs1948 on Gene Expression. *PLoS ONE*, *8*(5), e63699. doi:10.1371/journal.pone.0063699

Gallego X, Ruiz-Medina J, Valverde O, Molas S, Robles N, Sabrià J, et al. (2012) Transgenic over expression of nicotinic receptor alpha 5, alpha 3, and beta 4 subunit genes reduces ethanol intake in mice. Alcohol 46, 205–215. 10.1016/j.alcohol.2011.11.005 [PubMed]

Gould, T. J. (2006). Nicotine and Hippocampus-Dependent Learning: Implications for Addiction. *Molecular Neurobiology, 34*(2), 93–107. doi:10.1385/MN:34:2:93

Hendrickson, L. M., Guildford, M. J., & Tapper, A. R. (2013). Neuronal Nicotinic Acetylcholine Receptors: Common Molecular Substrates of Nicotine and Alcohol Dependence. *Frontiers in Psychiatry*, *4*, 29. doi:10.3389/fpsyt.2013.00029

Jackson KJ, Marks MJ, Vann RE, Chen X, Gamage TF, Warner JA, et al. The role of α5 nicotinic acetylcholine receptors in the behavioral and pharmacological effects of nicotine in mice. J. Pharmacol. Exp. Ther. 2010; 334:137-146. [PubMed: 20400469]

Jackson KJ, Martin BR, Changeux JP, Damaj MI. Differential role of nicotinic acetylcholine receptor subunits in physical and affective nicotine withdrawal signs. J. Pharmacol. Exp. Ther. 2008; 325:302-312. [PubMed: 18184829]

Jackson, K. J., Sanjakdar, S. S., Muldoon, P. P., McIntosh, J. M., & Damaj, M. I. (2013). The α3β4* nicotinic acetylcholine receptor subtype mediates nicotine reward and physical nicotine withdrawal signs independently of the α5 subunit in the mouse. *Neuropharmacology*, *70*, 228–235. doi:10.1016/j.neuropharm.2013.01.017

Kamens, HM, Andersen, J and Picciotto, MR (2010) Modulation of ethanol consumption by genetic and pharmacological manipulation of nicotinic acetylcholine receptors in mice. Psychopharmacology (Berl) 208, 613-626. [PubMed]

Kenny, P. J. (2014). Epigenetics, microRNA, and addiction. *Dialogues in Clinical Neuroscience*, *16*(3), 335–344.

Klein LC, Stine MM, Vandenbergh DJ, Whetzel CA, Kamens HM. (2004). Sex differences in voluntary oral nicotine consumption by adolescent mice: a dose-response experiment. *Pharmacology Biochemistry and Behavior.* ScienceDirect. 78:13-25

Lee H, Kang MK, Chung J, Noh J (2015) Repeated nicotine exposure in adolescent rats: Reduction of medial habenular activity and augmentation of nicotine preference. *Physiology & Behavior.* Science Direct 138:345-350

Li MD, van der Vaart AD. (2011) MicroRNAs in addiction: adaptation's middlemen? *Mol. Psychiatry* 16 1159–1168 10.1038/mp.2011.58 [PubMed]

MacKillop, J., Obasi, E., Amlung, M. T., McGeary, J. E., & Knopik, V. S. (2010). The Role of Genetics in Nicotine Dependence: Mapping the Pathways from Genome to Syndrome. *Current Cardiovascular Risk Reports*, *4*(6), 446–453. doi:10.1007/s12170-010-0132-6

Picciotto MR, Caldarone BJ, King SL, Zachariou V (2000). Nicotinic Receptors in the Brain: Links between Molecular Biology and Behavior. Nature. 2000;22, 451-465. doi: 10.1016/S0893-133X(99)00146-3

Picciotto MR, Zoli M, Rimondini R, Lena C, Marubio LM, Pich EM, Fuxe K, Changeux JP. Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. Nature. 1998;391(6663):173–177.[PubMed]

Salas, R., Sturm, R., Boulter, J., & De Biasi, M. (2009). Nicotinic Receptors in the Habenulo-Interpeduncular System Are Necessary for Nicotine Withdrawal in Mice. *The Journal of Neuroscience*, *29*(10), 3014–3018. doi:10.1523/JNEUROSCI.4934-08.2009

Schnoll, R. A., & Leone, F. T. (2011). Biomarkers to optimize the treatment of nicotine dependence. *Biomarkers in Medicine*, *5*(6), 745–761. doi:10.2217/bmm.11.91

Ślimak, M. A., Ables, J. L., Frahm, S., Antolin-Fontes, B., Santos-Torres, J., Moretti, M., … Ibañez-Tallon, I. (2014). Habenular expression of rare missense variants of the β4 nicotinic receptor subunit alters nicotine consumption.*Frontiers in Human Neuroscience*, *8*, 12. doi:10.3389/fnhum.2014.00012

TATRO, E. T., Risbrough, V., Soontornniyomkij, B., Young, J., Shumaker, S., Jeste, D. V., & Achim, C. L. (2013). Short-term recognition memory correlates with regional CNS expression of microRNA-138 in mice. *The American Journal of Geriatric Psychiatry : Official Journal of the American Association for Geriatric Psychiatry*, *21*(5), 461–473. doi:10.1016/j.jagp.2012.09.005.

Zhao-Shea, R., Liu, L., Pang, X., Gardner, P. D., & Tapper, A. R. (2013). Activation of GABAergic neurons in the interpeduncular nucleus triggers physical nicotine withdrawal symptoms. *Current Biology : CB*, *23*(23), 10.1016/j.cub.2013.09.041. doi:10.1016/j.cub.2013.09.041