

Targeting the  $\beta 4$  subunit of nAChRs: miR-138 expressed in the medial habenula of male mice  
reduces nicotine consumption and preference

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## **ABSTRACT**

Nicotine addiction is the most common form of addiction in the United States. Nicotinic acetylcholine receptors (nAChRs) are the major binding site for nicotine, which are ligand-gated ion channels consisting of combinations of five alpha and/or beta subunits that form the receptor pore. Previous research suggests that expression of the  $\beta 4$  nAChR subunit is involved in nicotine aversion. Further, the  $\beta 4$  subunit is expressed at relatively high levels in the medial habenula, and the expression of the gene coding for  $\beta 4$  can be decreased by miR-138, a small non-coding microRNA (miRNA) that naturally regulates gene expression. Mice were injected with a GFP-labeled adeno-associated virus containing either miR-138 or a scrambled-miRNA control. Next using a two-bottle choice protocol, nicotine and tastant drinking were measured and brains were collected to assess injection site location. Among mice with confirmed injection in the medial habenula, consumption and preference of nicotine as well as quinine was significantly lower in miR-138 treated mice than scrambled controls. This research will provide a greater understanding of the biological mechanisms of  $\beta 4$  on nicotine addiction and gives rise to potential treatments for nicotine dependence.

## **INTRODUCTION**

Tobacco use and consequently nicotine consumption is a serious public health issue. Each year tobacco use accounts for six million deaths in the world (WHO, 2016), and 480,000 deaths in the United States (CDC, 2015) making it the leading cause of preventable death in both the U.S. and the world. Additionally, smokers have life expectancies at least ten years shorter as compared to non-smokers (Jha et al. 2014). Not only does tobacco have a profound cost to human life, it also is a detrimental economic cost to our society. According to the CDC, smoking costs the U.S. \$96 billion each year due to direct medical costs and \$97 billion each year due to productivity loss from premature death (2008). Numerous public health efforts have tried to reduce the incidence of smoking tobacco, and increase the rate of successfully quitting. However, due to the biological consequences of nicotine use, successfully quitting does not come easily. Accordingly, 68% of National Health Interview Survey respondents in 2015 expressed a desire to quit smoking, however, only 7.4% were able to (Babb, 2017). Therefore, the biological mechanisms of nicotine dependence require extensive research, and should give rise to possible routes of treatment in the future.

## **BACKGROUND**

### *Properties of Nicotine*

The principle addictive component of tobacco, nicotine, stimulates neuronal nicotinic acetylcholine receptors (nAChRs) and initiates both rewarding and aversive signaling mechanisms in the central nervous system. To date, most research has investigated the intricacies and mechanisms of how nicotine intake activates dopamine reward areas in the mesolimbic pathway such as the ventral tegmental area and nucleus accumbens. For instance,

$\alpha 4\beta 2$  nAChRs within the ventral tegmental area are required for activation of dopaminergic neurons related to the reward in nicotine dependence (McGranahan et al. 2011). This finding supports the use of varenicline (Chantix) as a smoking cessation drug because it targets  $\alpha 4\beta 2$  receptors. However, this drug has considerable side effects that limit the adherence of the treatment. Importantly, nicotine does not only stimulate reward pathways in the brain but also aversive signaling pathways, which play an important role in determining nicotine intake (Riley, 2011). Therefore, the role of aversive mechanisms mediating nicotine intake must also be considered.

### *Nicotine Aversion*

Despite its rewarding effects, nicotine is particularly noxious. For instance in humans, first-time smokers have reported symptoms of nausea and dizziness, and those that report these negative subjective effects have a lower likelihood of developing nicotine dependence (DiFranza et al. 2007). It is harder to assess aversive behaviors in mice, but several behavioral tests can serve as a valid measure. For instance, one test to assess nicotine aversion compares the consumption of nicotine when given a choice to consume a non-aversive substance. When consumption of nicotine consists of <50% of the total consumption, it is considered to be behaviorally aversive. Another valid test includes a conditioned place aversion test in which mice are exposed to an environment where there are two distinct areas of different sensory stimuli that are paired with either saline or nicotine. After a paired conditioning period, the time spent in each distinct area is analyzed to assess aversion to nicotine. In its most simple sense it is believed that nicotine aversion essentially operates by activating nAChRs, except among other factors, the receptor subunits and brain regions all differ.

### *Subunits of Nicotinic Acetylcholine Receptors*

Nicotinic acetylcholine receptors are pentameric and consist of various combinations of eleven possible subunits ( $\alpha 2$ -  $\alpha 7$ ,  $\alpha 9$ ,  $\alpha 10$ ,  $\beta 2$ -4). The subunit composition and stoichiometry determines the functional properties of these nAChRs (Albuquerque et al. 2009). Individual and combinations of nAChR subunits have been extensively researched. Importantly, three of these subunits ( $\alpha 5\beta 4\alpha 3$ ) are transcribed as a cluster of genes in both humans and mice. In human genetic studies, this gene cluster has been associated with smoking related behaviors (Berrettini et al. 2008). Specifically, a single nucleotide polymorphism in the gene encoding  $\alpha 5$ , which is highly conserved across species, results in a functional change that is associated with greater risk for nicotine dependence (Bierut et al. 2008). From mouse studies, this gene cluster is believed to play a role in nicotine aversion, consequently increasing the vulnerability to nicotine dependence by reducing the aversive effects of nicotine (Fowler and Kenny, 2013). Considering the genetic link of  $\alpha 5\beta 4\alpha 3$  and their role in nicotine dependence, studies have focused efforts on manipulating individual subunits and assessing behavioral responses. For example,  $\alpha 5$  knock out mice had significantly higher self-administration of nicotine as compared to wild type (Fowler et al. 2011). There is less research about manipulating  $\alpha 3$  expression in mice because manipulations lead to renal complications resulting in high mortality weeks after birth (Xu et al. 1999). In summary, combinations of particular nAChR subunits mediate the receptor's function and the cluster of  $\alpha 5\beta 4\alpha 3$  subunits is especially important for nicotine consumption and preference.

#### *$\beta 4$ subunit*

To date, the role of  $\beta 4$  has not been emphasized to the same extent as the  $\alpha 5$  subunit. However, recent studies have provided a greater understanding on  $\beta 4$  expression and nicotine intake. For instance, Frahm and colleagues found that overexpression of  $\beta 4$  in mice increased aversion to nicotine and as a result mice with greater number of  $\beta 4$  containing receptors

consumed less nicotine than controls (2011). This study utilized several well-designed behavioral tests to assess preference and aversion, which supports the role of  $\beta 4$  in aversion to nicotine. Essentially, the  $\beta 4$  subunit regulates sensitivity to the aversive effects of nicotine (Picciotto and Kenny, 2013). In addition to the role of  $\beta 4$  with nicotine aversion, this subunit is expressed only in certain areas of the brain and is enriched in the medial habenula (Salas et al. 2009). Taken together,  $\beta 4$  is implicated in sensitivity to nicotine aversion and is particularly enriched in one area, the medial habenula.

### *Medial Habenula*

The habenula is a small brain structure in the epithalamus, which is a part of the diencephalon. Anatomically, the habenula is separated into the lateral habenula and the medial habenula (MHb) because of the different connectivity to other brain regions. Accordingly, the MHb receives input from the septum (Herkenham and Nauta, 1977) and projects to the interpeduncular nucleus (IPN) (Herkenham and Nauta, 1979). Importantly, this MHb-IPN pathway is unique in its high expression of the  $\alpha 5\beta 4\alpha 3$  cluster (Antolin-Fontes et al. 2014). This trend is also true of the MHb. For instance, at least 90% of MHb neurons express  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\beta 2$ , and  $\beta 4$  subunits (Sheffield et al. 2000). The role of the medial habenula (MHb) in nicotine addiction has been outlined in several rodent studies of nicotinic receptors (Velasquez et al. 2014). This can be explained by its involvement in nicotine aversion (Lee et al. 2015). Specifically, this has been determined by studies elucidating the roles of the  $\alpha 5$  and  $\beta 4$  subunits (Fowler et al. 2011; Frahm et al. 2011). Taken together, manipulating the expression of  $\beta 4$  in the MHb *in vivo* affects nicotine aversion and consequently nicotine consumption. One of the most biologically relevant methods to decrease gene expression is the use of microRNAs.

### *MicroRNAs: function and treatment*

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression by targeting messenger RNA (mRNA) and function primarily to decrease its protein product. More specifically, miRNAs bind to complementary regions of the 3' untranslated regions of mRNA, which can decrease expression by destabilizing mRNA, degrading mRNA, or blocking translation. However, miRNAs can also function to increase expression of a gene in some instances (Vasudevan et al. 2007). Given their versatile function, techniques to introduce or inhibit specific miRNAs are being developed as viable treatments for a variety of diseases (Broderick and Zamore, 2011). Despite some advances, no study has explored the role of miRNAs regulating nicotine consumption behavior.

### *Hypothesis/Predictions*

Prior research from our lab showed that a specific miRNA, miR-138, likely decreases the expression of the gene encoding  $\beta 4$  using a luciferase reporter gene assay (Gallego et al. 2013). This result gave rise to the possibility of manipulating expression of the  $\beta 4$  subunit *in vivo*. Accordingly, we can inject miR-138 into the MHB of mice to assess nicotine consumption using a mouse model. Given the potential role of miRNAs in the treatment of nicotine dependence, this study will provide valuable insight towards the biological function of miR-138 on nicotine intake and preference. We expect an increase in voluntary nicotine consumption after miR-138 injection due to a decrease in nicotine aversion related to  $\beta 4$ .

## **METHODS**

### *Animals*

C57BL/6 breeder mice were purchased from the Jackson laboratory, and were mated at BioFrontiers for producing experimental mice. A total of 19 male mice were used in the

experiment (10 injected with miR-138, and 9 injected with a scrambled miRNA control). However, during brain sectioning (explained below), it was found that one of the mice had severe hydrocephaly and consequently was not included in data analysis. Prior to testing, mice were group housed and provided food and water ad libitum.

#### *Adeno-Associated Viral Vector Injection*

At 60 days old the mice were anesthetized with a Ketamine/Xylazine/Acepromazine (100/10/0.5mg/kg) and had intracranial injections with an adeno-associated viral (AAV) vector containing either miR-138 or a scrambled control miRNA into the MHb. Both AAVs also carried a reporter gene (Green Florescent Protein) in order to identify the injection site location later in the experiment. AAVs were purchased from Vector Biolabs (Philadelphia, PA). AAVs were administered with a 10 uL Hamilton syringe and a thin 31-gauge metal needle. Injections consisted of a total volume of 0.5 uL AAV volume at a rate of 0.1uL/min. Injections were set to the MHb from the predetermined stereotaxic coordinates  $x= 0.35$ ,  $y= -1.74$ , and  $z= 0.32$ .

#### *Nicotine Drinking Testing*

Following three weeks of recovery from injections, behavioral testing was conducted. This consisted of assessing nicotine preference and tastant preference using a standard two bottle free choice procedure (see Kamens et al. 2010). Nicotine solutions were made by diluting free base nicotine with tap water to gain the desired concentration. Free base nicotine was purchased from Sigma-Aldrich (St. Louis, MO). To initiate the nicotine preference test, mice were first individually housed with access to one bottle for water for one week. Following this one-week acclimation phase, mice were presented with the choice of two bottles. For the first four days, water was present in both bottles. Next, one of the bottles with water was switched with a 25 ug/mL nicotine solution. Every four days following this, the nicotine was present and



concentration was increased (50, 100, 200 ug/mL). To avoid development side preference, the side the nicotine tube was on was alternated every two days and mice were weighed. Lastly, two cages without mice followed the same bottle handling procedures in order to estimate evaporation and leakage.

### *Tastant Drinking Testing*

Following a two-week washout period, the same mice followed a tastant drinking protocol. A similar two bottle choice procedure was used to assess preference to saccharine (a sweet tastant) and quinine (a bitter tastant). Saccharine and quinine hemisulfate were both purchased from Sigma-Aldrich (St. Louis, MO). Both tastant solutions were diluted with tap water to achieve the desired concentration. After another four day acclimation period, saccharin concentration increased after 4 days from .033% to .066% and similarly quinine from .03 mM to .06 mM.

### *Confirmation of injection into medial habenula*

To confirm the injection of miR-138 into the correct location, mice were perfused and their brains collected. Mice were deeply anesthetized with Ketamine/Xylazine/Acepromazine (100/10/0.5mg/kg) and transcardially perfused with 1X phosphate buffered saline followed by 4% paraformaldehyde (PFA). Brains were immediately removed and postfixed in 4% PFA solution for 24 hours. Following this, brains were transferred into solution containing 1X PBS (10mL) and sucrose (3g) and stored in refrigerator. At a later date, brains were flash-frozen in isopentane and stored in an -80°C freezer to be sectioned at a later time. Later, ~100 coronal sections surrounding the region of the MHb to the IPN at 30-micron width were taken in a -20°C cryostat. Approximately, ten to twelve sections were placed on each microscope slide, to assess

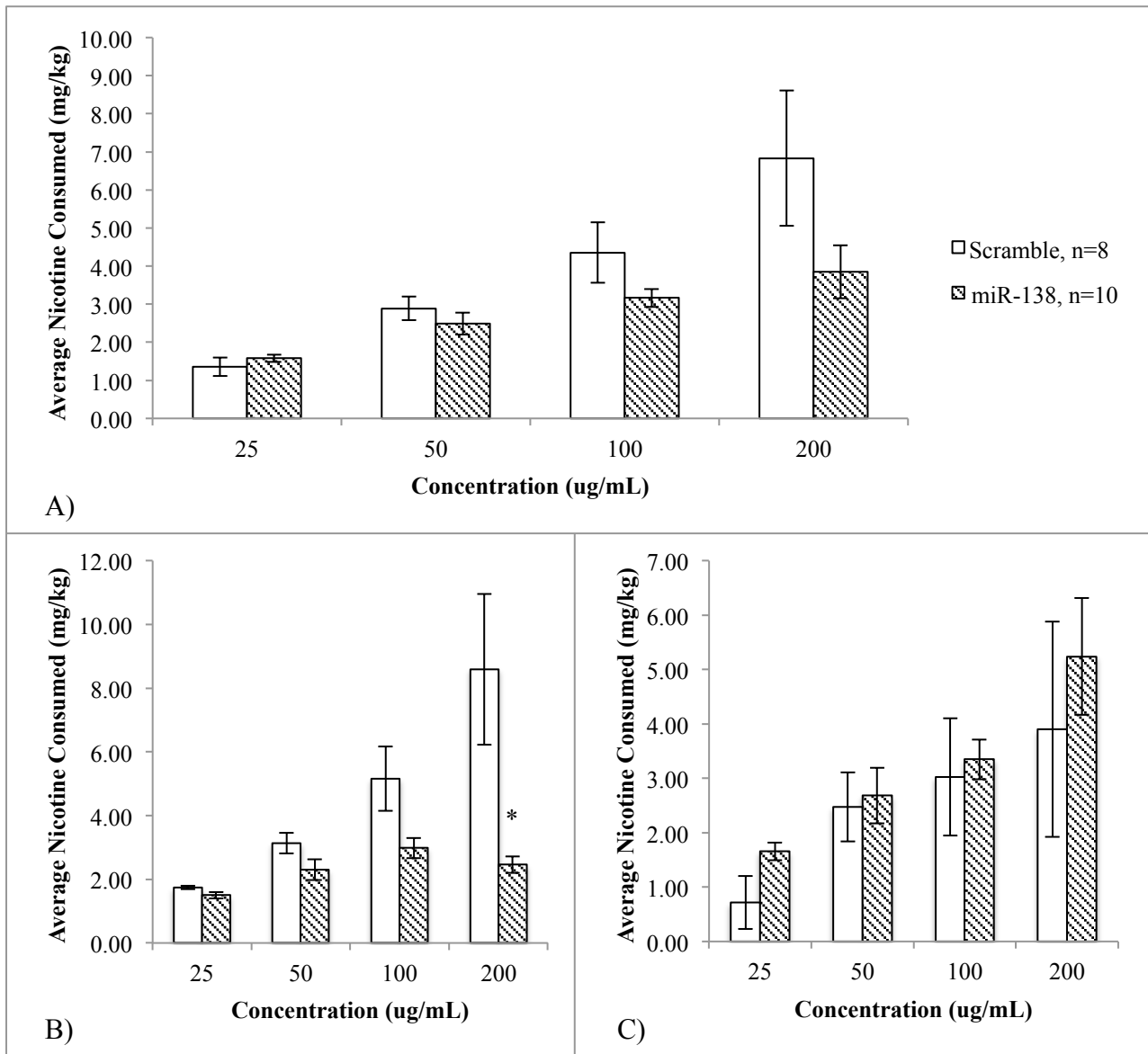
if GFP expression was localized only in the MHb and the assessment was done blind to the assignment of treatment groups.

### *Data Analysis*

For all data, two-way ANOVAs (concentration x miRNA treatment) were run to compare mean consumption and preference of nicotine, saccharine, or quinine. Average consumption was expressed with the units mg/kg with the equation ((daily final-initial volume of treated solution(mL) \* concentration of (nicotine, saccharine, or quinine) in solution (ug/mL) \* (1mg/1000ug))/ body weight in kilograms. Preference (%) was equated to (volume of treated solution consumed/ total volume consumed \* 100). Average consumption/preference for each of the concentrations (4 days) were used as the dependent variable.

## RESULTS

### Nicotine Consumption



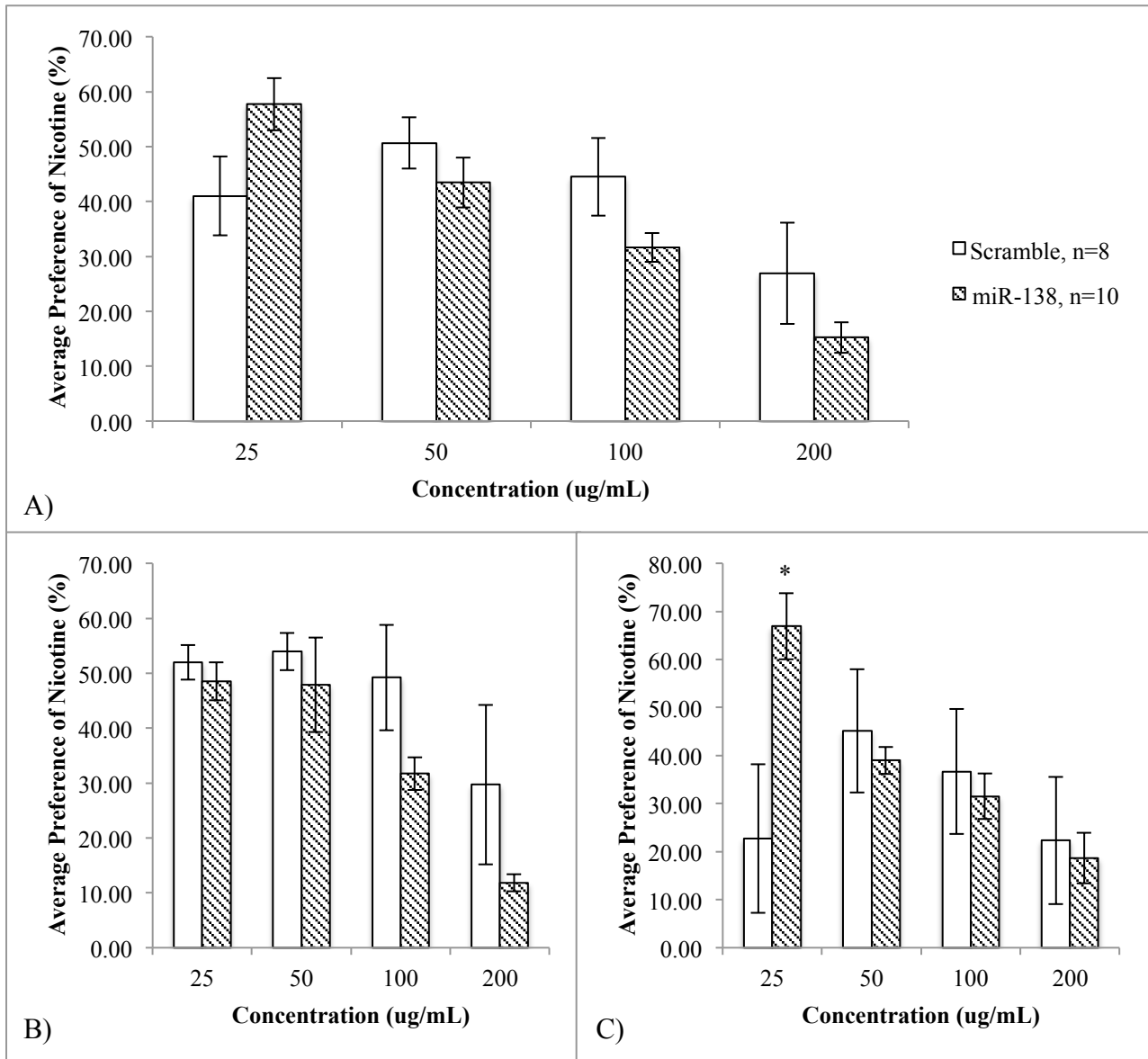
**Figure 1:** Average nicotine consumed (mg/kg) in C57BL/6 mice injected with either a scrambled or miR-138 AAV. Data are shown as aggregated data (A), and also split by confirmed injection in the MHb (n=10) (B) and confirmed injection outside of the MHb (n=8) (C). Error bars represent the standard error of each mean. \* indicates statistical significance between groups within a certain concentration using a *post-hoc* Tukey test following a two-way ANOVA (Concentration x Treatment Group) of consumption.

Graph 1A shows average nicotine consumed (mg/kg) in n=18 C57BL/6 mice administered either a scrambled (n=8) or miR-138 (n=10) AAV. Overall, nicotine consumption increased as the concentration increased in both groups. A two-way ANOVA indicated a main effect of both concentration [F(3,64)=10.176; P < .00001] and treatment [F(1,64)=4.831; P < .05] on nicotine consumption. However, no interaction effect [F(3,64)=1.974] on nicotine consumption was found, so no *post-hoc* analysis was conducted.

Graph 1B displays average nicotine consumed (mg/kg) in n=10 C57BL/6 mice with confirmed injection of either a scrambled (n=5) or miR-138 (n=5) AAV in the MHb. Generally, this graph shows fairly constant nicotine consumption among miR-138 injected mice as opposed to marked increase of the scramble control with increasing concentration. A two-way ANOVA indicated main effects of both concentration [F(3,32)=6.526; P < .01] and treatment [F(1,32)=12.563; P < .01] on nicotine consumption among confirmed hits. Further, an interaction effect was found [F(3,32)=4.012; P < .05]. Follow-up analyses of treatment within each concentration indicated a significant difference between treatment groups at the 200 ug/mL concentration only (p=.0013).

Graph 1C shows average nicotine consumed (mg/kg) in n=8 C57BL/6 mice with confirmed injection of either a scrambled (n=3) or miR-138 (n=5) AAV outside of the MHb. This graph shows a general increase in nicotine consumption among both groups as concentration increases. A two-way ANOVA indicated only a main effect of concentration [F(3,24)=5.941; P < .01]. Accordingly, there were not any significant differences in nicotine consumption between groups at different concentrations.

Nicotine Preference



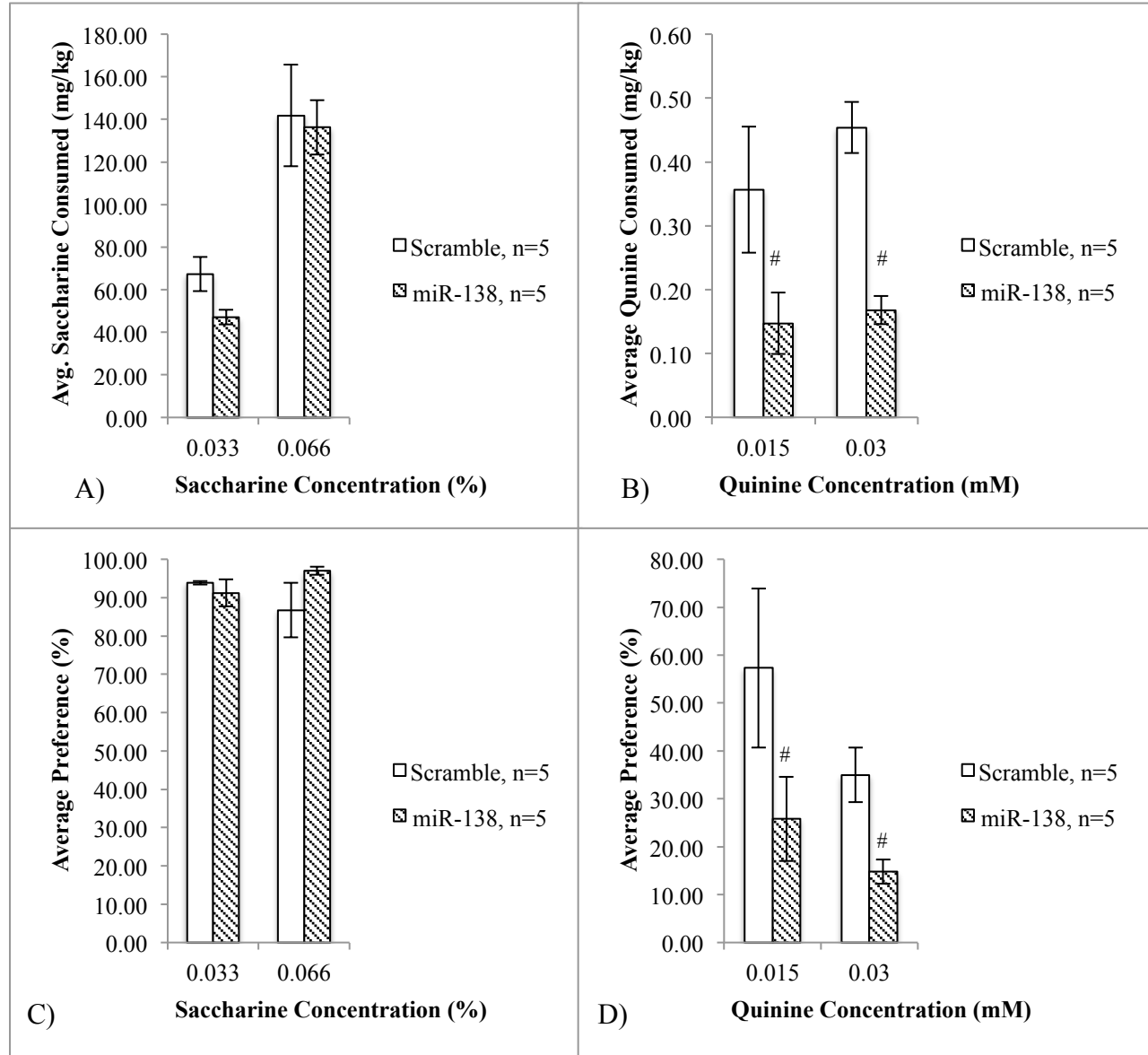
**Figure 2:** Average preference of nicotine (%) in C57BL/6 mice injected with either a scrambled or miR-138 AAV. Data are shown as aggregated data (A), and also split by confirmed injection in the MHb (n=10) (B) and confirmed injection outside of the MHb (n=8) (C). Error bars represent the standard error of each mean. \* indicates statistical significance between groups within a certain concentration using a *post-hoc* Tukey test following a two-way ANOVA (Concentration x Treatment Group) of consumption.

Graph 2A shows average nicotine preference (%) in n=18 C57BL/6 mice administered either a scrambled (n=8) or miR-138 (n=10) AAV. Overall, nicotine preference decreased as the concentration is increased throughout time in both groups. Specifically, a two-way ANOVA indicated only a main effect of concentration [ $F(3,64)=11.151$ ;  $P < .00001$ ] on nicotine preference.

Graph 2B displays average nicotine preference (%) in n=10 C57BL/6 mice with confirmed injection of either a scrambled (n=5) or miR-138 (n=5) AAV in the MHb. Generally, this graph shows a decrease in nicotine preference in miR-138 treated mice as compared to controls with increasing concentration. Specifically, a two-way ANOVA indicated a main effect of both concentration [ $F(3,32)=7.495$ ;  $P < .001$ ] and treatment [ $F(1,32)=4.772$ ;  $P < .05$ ] on nicotine preference among confirmed hits. However, no interaction effect was found [ $F(3,32)=.538$ ].

Graph 2C shows average nicotine preference (%) in n=8 C57BL/6 mice with confirmed injection of either a scrambled (n=3) or miR-138 (n=5) AAV outside of the MHb. Although average preference values at 25 ug/mL are uncharacteristic, this graph shows a general decrease in nicotine preference among both groups as concentration increases. A two-way ANOVA indicated a main effect of concentration [ $F(3,24)=4.804$ ;  $P < .01$ ] and an interaction effect [ $F(3,24)=4.134$ ,  $P < .05$ ]. Further Tukey *post-hoc* analysis indicated that there was a significant difference ( $p=.0236$ ) in nicotine preference at 25 ug/mL.

Tastant Results



**Figure 3:** Average tastant consumed (mg/kg) and preference in C57BL/6 mice injected with either a scrambled or miR-138 AAV. All data shown are confirmed hits of the MHb (n=10). The figure is separated by saccharine (A,C) and quinine (B,D) as well as consumption (A,B) and preference (C,D). Error bars represent the standard error of each mean. # indicates a main effect between treatment groups at all concentrations using a two-way ANOVA (Concentration x Treatment Group) of consumption.

Graph 3A shows average saccharine consumption (mg/kg) in n=10 C57BL/6 with confirmed injection of either a scrambled (n=5) or miR-138 (n=5) AAV in the MHb. This graph shows that saccharine consumption increases with increasing concentration in both groups. A two-way ANOVA indicated only a main effect of concentration  $F(1,16)=32.961$ ;  $P < .0001$ ].

Graph 3B shows average quinine consumption (mg/kg) in n=10 C57BL/6 with confirmed injection of either a scrambled (n=5) or miR-138 (n=5) AAV in the MHb. The graph shows a treatment difference of quinine consumption. A two-way ANOVA indicated a main effect of treatment [ $F(1,16)=17.270$ ;  $P < .001$ ].

Graph 3C shows average saccharine preference (mg/kg) in n=10 C57BL/6 with confirmed injection of either a scrambled (n=5) or miR-138 (n=5) AAV in the MHb. The graph shows that both groups preferred saccharine at both concentrations. A two-way ANOVA indicated no differences in saccharine consumption at either concentration or group.

Graph 3D shows average quinine preference (mg/kg) in n=10 C57BL/6 with confirmed injection of either a scrambled (n=5) or miR-138 (n=5) AAV in the MHb. The graph closely resembles Graph 3B, in respect to the difference between groups. A two-way ANOVA indicated a main effect of treatment [ $F(1,16)=6.819$ ;  $P < .05$ ].



## DISCUSSION

The purpose of this experiment was to assess if miR-138, injected into the MHb, affects nicotine consumption and preference in mice, presumably by changing  $\beta 4$  expression. Previous research has found that transgenic mice with overexpression of the  $\beta 4$  subunit gene show a strong aversion to nicotine (Frahm et al. 2011). We predicted that reduced expression of the  $\beta 4$  subunit gene in mice would lead to a decrease in nicotine aversion and consequently show greater nicotine consumption and preference. Prior work in our lab found that miR-138 likely decreases the expression of the gene encoding  $\beta 4$  *in vitro* and we therefore hypothesized that miR-138 injection to the MHb would increase nicotine consumption and preference. However, during our analysis, we found that injection of miR-138 actually decreased nicotine consumption and preference.

### *Discussion of Nicotine Consumption and Preference Results*

Figure 1A and 2A show that nicotine consumption and preference in all mice depend on concentration. Accordingly, nicotine consumption and preference both showed main effects of concentration. This result is not surprising, as we would expect a dose-dependent effect. Further, nicotine consumption also had a main effect of treatment. This means that throughout the study miR-138 treated mice consumed significantly less nicotine than controls. Given that nicotine preference was not found to be significantly different, this would suggest that total volume consumed might be a confounding variable. However, evaluating just these data would not address our hypothesis concerning the proposed role of  $\beta 4$  in the MHb. A more accurate interpretation of the results would be to analyze just the confirmed hits of the MHb and these are shown in Figure 1B and 2B. During data analysis, the confirmed hits of the MHb, like the aggregated data, displayed a main effect of concentration. However, using only the confirmed

hits the analysis indicated a significant difference in nicotine consumption and preference between treatment groups. This suggests that miR-138 injected into the MHb affects nicotine-drinking behavior possibly via changes in expression of the  $\beta 4$  subunit. However, these results do not support our initial hypothesis and there are some possible explanations why.

It is possible that miR-138 may have had a different *in vivo* role than *in vitro* role as we observed previously. Our previous work indicated that miR-138 likely decreases  $\beta 4$  expression *in vitro*, however it is possible that miR-138 actually increased  $\beta 4$  expression *in vivo*.

Accordingly, some miRNAs can actually increase gene expression in response to different cell conditions (Valinezhad Orang et al. 2014). For instance in humans, a miRNA (miR-206) promoted expression of a protein (KLF4) in normal mammary epithelial cells, but in breast cancer cells miR-206 repressed the expression of KLF4 (Lin et al. 2011). Notably, these molecular mechanisms of the change from repression to activation or vice versa are only characterized in a few different conditions and tissue types. This suggests that either the current understanding of the differential role of miRNAs in gene expression is limited or that this special function is isolated to a few different circumstances. At this point, the possible role of miR-138 in increasing vs. repressing  $\beta 4$  expression during nicotine exposure must be considered.

Although, miRNAs primarily function to decrease expression of proteins, depending on the cell condition, miR-138 may have actually increased  $\beta 4$  expression in the medial habenula.

Another possibility is that both over expression and under expression of  $\beta 4$  lead to decreased nicotine consumption and preference in mice. This could be explained by the change in stoichiometry of the nAChRs within the MHb. For instance, one study has shown that changing the expression of  $\alpha 4$  and  $\beta 2$  subunits of nAChR changes  $\alpha 4\beta 2$  receptor stoichiometry and sensitivity (Zwart and Vijverberg, 1998). Therefore, changing the expression of nAChR

subunits can change the ratio of subunits present in the receptor, and the decreased expression of one subunit can affect receptor assembly and/or degradation (Gotti et al. 2006). In regards to our interest in  $\beta 4$ , researchers found that  $\beta 4$  is rate-limiting for the assembly of  $\alpha 3\beta 4$  and competes with  $\alpha 5$  to form  $\alpha 5\beta 4\alpha 3$  receptors (Frahm et al. 2011). Additionally, under expression of  $\alpha 5$  leads to increased consumption of nicotine (Fowler et al 2011). Taken together, the decrease in  $\beta 4$  might have an indirect effect on nicotine consumption by allowing  $\alpha 5$  to account for a greater percentage of the subunits within  $\alpha 5\beta 4\alpha 3$  receptors. If this were the case, we would expect that a higher relative percentage of  $\alpha 5$  subunits within nAChRs would lead to decreased nicotine consumption, which we observed. Using this rationale, we must speculate that the expression or relative percentage of  $\alpha 5$  in  $\alpha 5\beta 4\alpha 3$  receptors is essentially the “gatekeeper” of nicotine aversion in this pathway. Meaning that if  $\alpha 5$  is expressed at all in the MHb, there will be aversion to nicotine and consequently less consumption. Thus, altering the ratio of the  $\beta 4$  subunit within nAChRs that are essential for controlling nicotine intake may have similar behavioral consequences from both under and over expression of  $\beta 4$ .

Alternatively, our rationale for the hypothesis may have been improper. Despite the role of aversion in the MHb-IPN pathway, it is possible that nicotine affected nAChR subunits in this pathway in a rewarding way instead of an aversive way. In a recent study, using a different nicotine intake paradigm,  $\beta 4$  knock out mice administered less intravenous nicotine at all concentrations as compared to wild type mice and had higher basal extracellular levels of dopamine in the nucleus accumbens (Harrington et al. 2016). This finding emphasizes both the rewarding and aversive effects of nicotine. Conceivably, the  $\beta 4$  knockout led to an increased sensitivity to nicotine, meaning it would take less nicotine to elicit a biological response. Further, while  $\beta 4$  is the principle subunit within the MHb, there are several other subunits

present that can also modulate response to nicotine (Shih et al. 2014). In relation to our study, miR-138 may have directly or indirectly affected another receptor subunit in the medial habenula to have the effect found. Alternatively, miR-138 may have diffused into other brain regions such as the lateral habenula, which is implicated with drug reward as opposed to the medial habenula role with aversion. In summary, the likely decrease of  $\beta 4$  from miR-138 may have instead affected the reward pathways by increasing sensitivity to nicotine to consequently reduce nicotine consumption.

#### *Discussion of Tastant Consumption and Preference Results*

Based on the current tastant results, we cannot conclude that the observed decrease in nicotine consumption and preference is exclusively due to the proposed role of miR-138 on nicotine aversion. Possibly, miR-138 affected nAChRs involved in bitter taste pathways instead. Because there was a main effect of treatment on quinine consumption and preference, as seen in Figure 3B and 3D, miR-138 may have affected general taste instead of anything specific to nicotine. However, as seen in Figure 3A and 3C, saccharine consumption and preference did not significantly differ between treatment groups, suggesting that miR-138's effect is specific to bitter tasting solutions. There are a number of potential explanations for the effect of miR-138 on both nicotine and quinine consumption.

One possibility is that the *in vivo* role of miR-138 is on taste pathways. Accordingly, nicotine can activate nicotine-specific taste pathways with nAChRs as well as activate taste pathways common to other bitter tastants (Oliveira-Maia et al. 2009). Therefore, since nicotine has its own taste pathway independent of bitter tastants like quinine, miR-138 could have functioned to decrease the expression of a nAChR subunit within this independent taste pathway. This could only be the case if miR-138 diffused out the MHb, because the MHb is not implicated

with taste. Accordingly, if miR-138 acted on a nAChR subunit somewhere within this nicotine-independent taste pathway and modulated function, the taste of nicotine would be perceived differently in miR-138 treated mice and control mice. We might speculate that this perceived difference in nicotine taste between treatment groups may explain the observed difference in nicotine consumption and preference. While this may not be a likely case, because we confirmed AAV injection and localization within the MHb by visualizing GFP in brain slices, this specific role of miR-138 must be considered.

In addition to nicotine-specific taste pathways, nicotine activates responses common to other bitter tastants. This finding may explain the difference in quinine consumption and preference between treatment groups. Since we ran a nicotine preference test prior to the tastant study, there are a few considerable confounding effects. It is possible that the miR-138 mice developed a conditioned taste aversion to nicotine. Accordingly, quinine would activate a common bitter taste pathway as nicotine, and thus miR-138 mice would associate these similar tastes with the existing aversion to nicotine. Due to this, the decreased consumption of quinine several days later may just be an associated extension from the bitter taste from high concentrations of nicotine at the end of the nicotine preference test. Taken together, the possible roles of miR-138 on taste should be addressed when assessing nicotine and quinine consumption.

### *Strengths*

In addition to just the nicotine drinking study, we ran a control tastant study and confirmed the location of the injection site. These additional tests provided valuable insight towards understanding the role of miR-138 in the MHb and its role with nicotine consumption and preference. Interestingly, by separating “hits” from “misses” each of the nicotine behaviors showed a greater effect between treatment groups.

Additionally, to date no other study has explored the role of miRNAs on nicotine consumption and preference. Therefore, this study displays innovation and explores the efficacy of using miRNAs as a method to modulate drug-seeking behavior.

### *Limitations*

This study came with limitations. First, we did not confirm nicotine aversion with subsequent behavioral tests. We assessed nicotine aversion by assessing nicotine preference through a two bottle choice protocol, however subsequent tests to confirm aversion are warranted. Additionally, since our rationale was to indirectly change aversion to nicotine specific to the proposed role of  $\beta 4$  in the MHB, it would be essential to confirm that a difference in nicotine consumption was due to  $\beta 4$  expression levels rather than other factors like taste.

The *in vivo* role of miR-138 in the brain should be considered. The rationale behind the experiment is that miR-138 likely decreases the expression of the gene encoding  $\beta 4$  *in vitro*, however miR-138 has numerous *in vivo* effects. Among other functions in the body, miR-138 is involved in dendritic spine morphogenesis in the rat hypothalamus (Siegal et al. 2009), but it has also been shown to suppress axon regeneration (Liu et al. 2013). Potentially, miR-138 is involved in other neural networks that are involved in aversion. Ultimately, with the current protocol we can confirm that miR-138 was injected into the MHB but cannot assess if miR-138 is directly affecting the expression of the  $\beta 4$  subunit.

### *Future Directions*

Considering the importance of aversion to nicotine in this study, we should run additional tests to confirm that miR-138 is specifically involved in aversion to nicotine. Therefore, in order to ensure that aversion to nicotine is mediating the observed behavioral outcomes, we could additionally test for aversion with either the conditioned place aversion or conditioned taste

aversion paradigms. This would require running another replication of this experiment with scramble and miR-138 injected mice following the conditioned place aversion or conditioned taste aversion protocols.

In order to limit the potential confounding variables from running the nicotine preference test prior to the tastant test, we could run another replication of this experiment with naïve mice that only receive the tastant test instead of both behavioral tests. Or, we could run another replication of this experiment but run the tastant test first. This way we could more directly address the effect miR-138 has on general taste compared to its effect on nicotine consumption and preference.

To address if miR-138 is indeed affecting  $\beta 4$  expression *in vivo*, we will conduct an *in situ* hybridization test. This will ensure that miR-138 binds to the mRNA transcript of the  $\beta 4$  subunit and that it is localized in the region. If the results from this additional test confirm that miR-138 decreases expression of  $\beta 4$  *in vivo* then we have more confidence in our current nicotine consumption and preference results.

### *Conclusion*

This project evaluated the effect miR-138, injected into the MHB, has on nicotine consumption and preference. Considering confirmed hits of the MHB, our analyses revealed nicotine consumption and preference among miR-138 mice are significantly lower than scrambled-miRNA control mice. However, since these treated mice also consumed significantly less quinine than controls, we must run further experiments to confirm that the psychopharmacological properties of nicotine are mediating our observed behavioral data instead of other confounding factors like taste. If we can confirm this, miR-138 may inform future

treatment option for nicotine dependence assuming advancements to the delivery and localization of this genetic therapy.

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