Published in final edited form as:

Brain Behav Immun. 2011 June ; 25(Suppl 1): S165–S169. doi:10.1016/j.bbi.2011.03.002.

Minocycline Reduces Ethanol Drinking

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Keywords

C57Bl/6J mouse; therapy; voluntary consumption; neuroimmune; medications development

1. Introduction

The complex actions of alcohol and the lack of direct ligand-receptor pharmacology for ethanol provide challenges for development of effective pharmacotherapeutics for alcoholism. The purpose of our study was to investigate a new potential therapeutic pathway for effects on ethanol consumption. Given the recent growth in our understanding of alcohol–neuroimmune interactions, we hypothesized that altering the neuroimmune activity of brain glia may affect drinking. We elected to test our hypothesis using the immune modulatory antibiotic drug minocycline.

We utilized minocycline treatment because it is known to alter neuroimmune and cytokine expression in the brain (Fan et al., 2007; Jiang et al., 2009; Mishra and Basu, 2008; Popovic et al., 2002; Roulston et al., 2005; Wu et al., 2002), as well as peripherally, and neuroimmune networks are involved in drinking (Blednov et al., 2005; Blednov et al., 2011). In addition, there is a lack of more selective *in vivo* inhibitors of microglia and astroglia. Minocycline is a second generation tetracycline antibiotic that has immune modulatory actions independent of its bactericidal actions (e. g., Kielian et al., 2007; Kloppenburg et al., 1994; Sewell et al., 1996). As an immune modulator, minocycline can down-regulate expression of pro-inflammatory genes and mediators in both the central and peripheral immune systems (Henry et al., 2008; Homsy et al., 2009; Huang et al., 2009; Kloppenburg et al., 1996; Nikodemova et al., 2010). To test the effect of minocycline on alcohol intake, drinking in both male and female C57Bl/6J mice was measured using a free-choice voluntary drinking model. The results suggest that drugs that alter neuroimmune pathways may represent a new approach to developing additional therapies to treat alcoholism.

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Conflict of Interest Statement

All authors declare that there are no conflicts of interest.

2. Methods

2.1 Animal handling

Male and female C57BL/6J (B6) mice (70-day old; Jackson Laboratory, Bar Harbor, ME) were housed individually under standard humidity and temperature conditions, with lab chow and water *ad libitum*. Mice were housed under a reverse 12-h light/dark cycle (lights on at 2100 h and off at 0900 h). Special cage tops (designed in-house) were used to accommodate bottles in an inverted position. Mice and bottles were weighed daily at 0730 h. Mice were weighed every day to evaluate any effect of the drug administration (before, during and after) on body weight (as a parameter of health). All experimental procedures were carried out following protocols approved by the Texas Tech University Health Sciences Center Institutional Animal Care and Use Committee.

2.2. Drug Administration

Alcohol was administered to the mice as a 10% v/v solution in a 2-bottle choice experiment with water. Minocycline was administered as a solution of its salt form, minocycline HCl (Sigma Aldrich, St. Louis, MO, catalog no. 13614-98-7), dissolved in Phosphate Buffered Saline (PBS, pH 7.4), via an intraperitoneal (i.p.) injection at a dose of 50 mg/kg. A separate group of control mice received saline injections (10 ml/kg, i.p.). The minocycline dose was found effective in reducing damage and microglia activation in mouse models of neurodegenerative diseases (Wang et al., 2003; Wu et al., 2002). Injections were done daily during saline or minocycline administration at 0730 h, immediately after weighing the mice in order to prevent interference with dark phase activity. Stoppered 50 mL centrifugation tubes (BD Falcon brand, BD Biosciences, Franklin Lakes, NJ) with double ball bearing sipper tubes were used to dispense the alcohol and water.

2.3. Alcohol administration: A two-bottle free choice procedure of voluntary ethanol consumption

Mice had access to two fluid bottles (both water or water and ethanol) with a free choice to drink from either bottle. Intake of fluid was measured by weighing the bottles on a daily basis for the entire experimental period. The positions of the bottles were alternated daily after weighing to overcome the factor of any place or position preference in drinking. A within-subject experimental design was adapted where the ethanol intake before, during and after drug or saline administration was measured in the same mice.

2.3.1 Experimental procedure—After a day of acclimation to the experimental room and reverse light/dark cycle, the minocycline-treated mice were offered two bottles with plain tap water for four days (adaptation period / water phase, D1 to D4, in Table 1). Following adaptation, one of the two bottles of water was replaced with 10% v/v ethanol solution and fluid intake was recorded (pre-minocycline phase, D5 to D8, Table 1). Keeping all the experimental conditions the same as in the pre-minocycline phase, mice were injected with minocycline (50 mg/kg) based on body weight. The mice were then allowed access to water and ethanol solutions. Body weights were recorded and fluid intake was measured to evaluate the effect of the drug on drinking (minocycline 1 phase, D9 to D12, Table 1). To evaluate effects on drinking post-minocycline treatment, minocycline administration was discontinued and the fluid (ethanol and water) intake was measured again in the absence of minocycline (post-minocycline 1 phase, D13 to D16, Table 1). Further, to test if any response to the minocycline was reproducible, the minocycline administration was reinstated identically to the minocycline 1 phase and mice were tested for water and ethanol intake for four days (minocycline 2 phase, D17 to D20, Table 1). The mice were again taken off minocycline and the effect of drug removal on fluid intake was measured (post-minocycline 2 phase, D21 to D24, Table 1). After acclimation, control mice were offered water or 10% v/v

v ethanol for 4 days, followed by daily saline injections and access to water and ethanol solutions to access the pre- and post-saline effect on consumption.

2.4 Data Analysis

For all experiments, water and ethanol intake was calculated as fluid consumption in g/kg body weight over 24 h. Preference for ethanol was calculated in percentage as (mL of ethanol/ mL of ethanol + mL of water) $\times$ 100. Outliers in the data were identified and removed if the value was greater or lesser than 2 standard deviations from the group mean. Individual intake was measured as the average of each 4-day period (see Table 1). Saline data were analyzed by paired t-test. Minocycline data were analyzed by repeated measures one-way ANOVA to determine significant overall treatment (drug, no drug) effect on intake. The Tukey's *post hoc* test was used to determine significant differences between individual phases. GraphPad Prism 3.0 (GraphPad Prism Software Inc., San Diego, CA, USA) was used for statistical analysis. Significant differences were set as $p < 0.05$.

3. Results

3.1 Minocycline decreased ethanol intake in B6 mice

Saline treatment had no significant effect on ethanol intake in both male [$n=10$, $t = 1.054$, $p = 0.315$] and female [$n=10$, $t = 1.685$, $p = 0.126$] B6 mice (Figure 1). On the other hand, minocycline treatment had a significant overall effect on ethanol intake in both male [$n=14$, $F(4, 69) = 19.52$, $p < 0.0001$] and female [$n=13$, $F(4, 64) = 10.06$, $p < 0.0001$] B6 mice as determined using a repeated measures one-way ANOVA. After finding that there was a significant overall treatment effect of minocycline, comparisons between individual treatment phases were done. The first minocycline administration caused a significant reduction in ethanol intake compared to pre-minocycline levels for male [$F(1, 13) = 6.697$, $p < 0.0001$] and female [$F(1, 12) = 4.955$, $p < 0.001$] mice (Figure 1). Upon cessation of the minocycline administration, the ethanol intake increased significantly (minocycline 1 vs. post-minocycline 1 phase) in male [$F(1, 13) = 11.63$, $p < 0.0001$] and female [$F(1, 12) = 7.364$, $p < 0.0001$] mice essentially back to pre-minocycline levels. To assess whether the minocycline impact on ethanol consumption was reproducible, minocycline administration was reinstated again for four days (Table 1). Similar to the first exposure, administration of minocycline again significantly decreased ethanol intake (post-minocycline 1 vs. minocycline 2 phase) in male [$F(1, 13) = 7.077$, $p < 0.0001$] and female [$F(1, 12) = 6.459$, $p < 0.0001$] mice. As occurred following the minocycline-1 phase, removal of drug caused a significant increase in ethanol intake (minocycline 2 vs. post-minocycline 2 phase) in male [$F(1, 13) = 4.357$, $p < 0.01$] and female [$F(1, 12) = 4.733$, $p < 0.01$] mice (Figure 1).

3.2 Minocycline affects water intake in asexually dimorphic manner

Water intake was measured in the mice over each 24 h period and the values were averaged over each experimental phase. Saline injections did not significantly affect average water intake in male or female mice (data not show). However, a repeated measures one-way ANOVA revealed there was an overall significant treatment effect of minocycline on water intake in the male mice [$n=14$, $F(4, 69) = 4.325$, $p < 0.001$] but not in the female mice [$n=13$, $F(4, 64) = 1.474$, $p = \text{ns}$]. More specifically, while post-hoc analysis revealed that there was no effect of minocycline on water intake in males during the first phase of drug administration (pre-minocycline vs. minocycline 1 phase) [$F(1, 13) = 1.259$, $p = \text{ns}$], there were significant differences between the pre-minocycline vs. minocycline 2 phase [$F(1, 13) = 4.278$, $p < 0.01$] and the pre-minocycline vs. post-minocycline 2 phase [$F(1, 13) = 4.932$, $p < 0.001$]. In females there was no significant change in average water consumption during the minocycline 1 phase (Figure 2).

3.3 Minocycline treatment affects preference for ethanol in B6 mice

Repeated measures one-way ANOVA revealed an overall significant main effect of treatment on preference for ethanol in the male mice [$n=14$, $F(4, 69) = 3.433$, $p < 0.01$] and female mice [$n=13$, $F(4, 64) = 2.906$, $p < 0.01$] (data not shown). Post-hoc analysis revealed that only the minocycline 1 vs. post-minocycline 2 comparison was significantly different in both male [$F(1, 13) = 4.405$, $p < 0.01$] and female [$F(1, 12) = 4.519$, $p < 0.01$] mice. All other comparisons were not statistically different.

3.4 Minocycline affects total daily fluid intake

Total fluid consumption (water plus ethanol) was computed for each day of the study for the male and female mice. Not surprisingly, there was no effect of saline injection, but the minocycline effect on total fluid intake mirrored its effect on ethanol intake, consistent with the high ethanol preference (>80% on average) of the mice in this study (data not shown).

3.5 Effect of minocycline treatment on body weight of B6 mice

To evaluate any effects of minocycline on general health of the mice in terms of food intake or caloric utilization, body weights were measured daily. Body weights remained stable during the study (see Figure 1). However, statistical analysis on average body weights for each phase revealed a significant overall effect of treatment only in the male mice [$n=14$, $F(4, 69) = 4.064$, $p = 0.0061$]. Post-hoc analysis found a small, but significant reduction in body weight (<3% change) between Pre-minocycline and Minocycline 1 phases ($p < 0.01$) and Pre-minocycline and Post-minocycline 1 phases ($p < 0.05$). In contrast, minocycline treatment did not affect body weight of female mice as assessed by repeated measures one-way ANOVA.

4. Discussion

Our study is the first to our knowledge to examine the effect of a neuroimmune modulator drug on alcohol drinking. The model system chosen for study was the C57Bl/6J (B6) mouse, a well characterized high alcohol drinking inbred strain. We examined effects in both male and female mice for possible gender-selective actions. The rationale for our study was that alcohol intake was found to be altered in mice lacking selective genetic components of the neuroimmune system (Blednov et al., 2005; Blednov et al., 2011), thus suggesting that neuroimmune interactions may be involved in drinking behavior of normal mice. The immune modulator chosen for study was minocycline because of its known action in the CNS. The within-subject experimental design allowed us to examine potential post-treatment effects and whether the efficacy of the drug in reducing ethanol intake remained the same or if previous exposure changed the response, possibly due to pharmacological tolerance or desensitization to the drug. A single 50 mg/kg dose was chosen for our initial study given the efficacy of this dose in mice (Fan et al., 2007; Kielian et al., 2007; Wang et al., 2003).

Minocycline administration caused a significant reduction in ethanol intake in both male and female B6 mice. The effect was selective in the female mice, with treatment having no significant effects on water intake or body weight. In contrast, minocycline treatment had additional effects on male B6 mice, including a slight reduction in body weight during the first exposure and changes in water intake later in the procedure. The reason for these side-effects in males is currently unclear. They do not, however, contribute to the reduction in ethanol drinking observed during the minocycline treatment. The reduction in ethanol drinking by minocycline treatment was reproducible upon repeated administration separated by four days of drinking, indicating an apparent lack of drug tolerance or desensitization.

The effect was also reversible upon termination of drug administration suggesting a direct action of minocycline and no carry-over activity.

The reduction in ethanol intake by minocycline treatment was modest. However, since only a single dose was given once a day it would be premature to conclude that the drug has low efficacy until additional doses and treatment regimens are tested. In addition, the 24 h free access may not be the optimal paradigm and others could be tested.

The underlying mechanism whereby minocycline reduced ethanol intake requires further study. Minocycline readily enters the brain and has been found to exert effects *in vivo* through actions on microglia (Fan et al., 2007; Hayakawa et al., 2008; Mishra and Basu, 2008; Roulston et al., 2005), thus microglia are considered a target cell that mediates minocycline action in the brain. However, whether minocycline reduces ethanol drinking through effects on microglia, other brain cells, or via peripheral immune cells is not known. It is also possible that minocycline may impact other factors that influence drinking. For example, minocycline may affect taste and reduce ethanol consumption to the extent that the mice drink because they like the flavor of 10% ethanol. While there is not data to indicate minocycline affects taste, the possibility cannot be ruled out. Chen et al., (2009) reported transient suppression of locomotor activity following 40 mg/kg minocycline which may affect the ability to consume ethanol. However, the 24 h availability of the ethanol, the 5 h interval between minocycline injection and the period of peak fluid consumption during the dark cycle, and unpublished data indicating minocycline reduces ethanol drinking when administered 20 h before exposure to ethanol, suggest a more direct action of minocycline on drinking.

In summary, a 50 mg/kg dose of minocycline was found to reduce ethanol intake with little effect on water or body weight change. Although the degree of alcohol consumption reduction was modest, other minocycline doses and exposure regimens may cause greater reductions. Whether the effect is specific to ethanol is unknown. It will be necessary to test the effect of minocycline on other rewarding substances. However, minocycline was recently used to treat a case of methamphetamine use disorder (Tanibuchi et al., 2010). Minocycline may represent an additional tool in the treatment of alcoholism, whether as a potential therapeutic agent itself, or as a chemical scaffold to build improved therapeutic agents.

Acknowledgments

We thank Kaitlin Dye for technical support on this study.

Supported by NIH grant U01AA13475 (SEB)

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Figure 1. Minocycline, but not saline, reduces ethanol intake in male (A) and female (B) C57Bl/6J mice. The bars represent average ethanol consumption in g/kg over the four days of measurement as mean $\pm$ SEM. Saline injection had no effect on ethanol intake ($p > 0.05$, $n=10$ per group). Repeated measures one-way ANOVA revealed a significant minocycline treatment effect for the male ($p < 0.0001$, $n=14$) and female mice ($p < 0.0001$, $n=13$). Tukey's post-hoc analysis revealed significant differences among experimental phases as depicted. (***) $p < 0.0001$, (**) $p < 0.001$, and (*) $p < 0.01$). Inserts: Body weight data (mean $\pm$ SD) during experimentation with minocycline is shown about each graph for male and female mice.

Figure 2.

Effects of minocycline on water intake in male (A) and female (B) C57Bl/6J mice. The bars represent average water consumption in g/kg over the four days represented as mean $\pm$ SEM for $n=14$ for the male and $n=13$ for female mice for the different experimental phases denoted on the abscissa. Repeated measures one-way ANOVA revealed a significant treatment effect only for the male ($p < 0.001$) mice whereas treatment had no significant effect on female mice ($p = ns$). Tukey's post-hoc analysis among experimental phases in male mice revealed statistically significant difference as depicted. (** $p < 0.001$, and * $p < 0.01$).

Schematic representation of the experimental design: Pre- indicates intake before drug administration and post- indicates intake after drug administration. Minocycline 1 and 2 indicate 1st and 2nd rounds of minocycline injections and respective post-minocycline intake. Water is plain tap water and EtOH is 10% v/v ethanol solution in tap water. Minocycline was administered intra-peritoneally according to body weight before replacing the water and alcohol bottles. The weights of bottles and mice were recorded daily. Freshly filled water and ethanol solution bottles were used for each phase of the study.

Table 1

Days of study	D1 to D4	D5 to D8	D9 to D12	D13 to D16	D17 to D20	D21 to D24
Days of study	Water	Pre-minocycline	Minocycline 1	Post-minocycline 1	Minocycline 2	Post-minocycline 2
Solutions offered	Water + Water	Water + EtOH	Water + EtOH	Water + EtOH	Water + EtOH	Water + EtOH
Drug administered	None	None	Minocycline (50 mg/kg)	None	Minocycline (50 mg/kg)	None