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Activation and Suppression of the Innate Immune System: Effects on Alcohol Intake

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by

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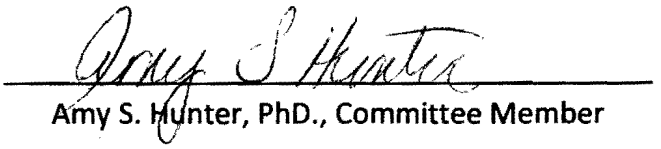

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Abstract

Prior research indicates that immune system activation with a single dose of the bacterial toxin lipopolysaccharides (LPS) causes increased ethanol (EtOH) intake in rodents even three months after an injection. This result suggests that immune system activation may induce a long-term change in behavior. Naltrexone is known to be a partial antagonist on the same toll-like receptors of immune cells that are targeted by LPS. This drug presumably would nullify the LPS effect. In this study, we attempted to replicate the LPS-induced increase in EtOH consumption in rats and investigated the impact of repeated naltrexone treatment on the effects of LPS on subsequent EtOH intake. The animals received one injection of LPS (or saline). For two weeks before and two weeks after LPS treatment, the animals received daily naltrexone or saline injections. LPS did not reliably increase EtOH intake as in previous studies, therefore an intermittent drinking schedule was introduced to further increase drinking rates in all groups. Interestingly, the results of the intermittent home-cage exposure to EtOH and subsequent individual short-term preference tests indicate that the LPS and naltrexone have an additive effect as the group with both treatments drank the most EtOH.

Activation and Suppression of the Innate Immune System: Effects on Alcohol Intake

Recently, the interplay between the immune and nervous systems has been of great interest to researchers. The role of the immune system on behavior is larger than previously imagined. Neuroimmune pharmacology is a young field created as a convergence of neuroscience, immunology and pharmacology (Freilich & Izeku, 2011). The immune system is implicated in many neurological and psychiatric diseases such as autoimmune diseases of the brain, neurodegenerative disorders, mood disorders, and drug abuse (Freilich & Izeku, 2011). It seems likely that the introduction of, and subsequent immune response to, one foreign body (e.g. microbes) will affect the response to another foreign body (e.g. alcohol). Because of the relationship to the nervous system, this response may have behavioral implications. In order to better understand the interplay between the nervous system and the immune system, it is helpful to describe some of the basic functions of the immune system.

Basic Functions of the Immune System

There are several components to the immune system, but the one most relevant to the current discussion is the role of the immune system in identifying foreign particles entering the cells of the body (cell-mediated immunity). The cells of the immune system that play this role are called T cells or T lymphocytes. The T cells destroy the microbes themselves or individual cells infected with the microbes. How does a T- cell know that a cell is infected with a microbe? For this cell-mediated immunity to work it is necessary for a piece of the foreign protein on the surface of the microbe (i.e., the antigen) to be removed and inserted into the outer membrane of the infected cell itself. The infected cell can then be recognized by T cells for destruction.

Normal T cells are naïve and need to be informed that the microbes are present in the body; that is they need to be induced and mobilized with other cells for an efficient attack. Several cell types in the class of cells called *antigen-presenting cells* (APC) have the task of quickly notifying the T cells of the presence of the microbes. An APC captures the invading microbe and after inserting the antigen into its own surface membrane it travels to the lymphoid organs where it presents the antigen to the naïve T cells to induce an immune response. Some T cells do the killing and some help with the mobilization. The T cells that do the killing of infected cells are *Cytotoxic T cells*. There are also *Helper T cells* which when presented with an antigen from an APC release signals (small proteins called *cytokines*) to mobilize the T cells and several other cells (e.g., macrophages & B lymphocytes) for an effective defensive action (Abbas & Lichtman, 2005).

Beta-2-microglobulin (B2m) is a protein in the class of molecules called *major histocompatibility complex* (MHC). MHC molecules are found on every cell of the body with nuclei (not red blood cells and platelets because they do not have nuclei). MHC is necessary for APC to present the antigen to Cytotoxic T cells because T cells can only recognize foreign protein fragments that have been bound to MHC molecules. By knocking out the B2m gene the MHC – antigen complex is disrupted, so the Cytotoxic T cells do not "see" the infected cells (Abbas & Lichtman, 2005).

Cathepsin S (CTSS) is an enzyme found in lysosomes that breaks down other proteins (a protease). CTSS helps create the antigen by breaking down a protein from the foreign microbe that can be incorporated in the MHC of APC. Without the ability to create this protein fragment

an antigen cannot be presented to the Cytotoxic T cells for induction of an immune response. Cathepsin F (CTSF) is another lysosomal protease with similar functions as the other cathepsins (Abbas & Lichtman, 2005).

Interleukin 1 receptor antagonist (Il-1ra) is a *cytokine*. A cytokine is a small protein that immune cells secrete to communicate with each other (essentially a hormonal communication system for immune cells). The Helper T cells release cytokines to mobilize the immune response. Examples of cytokine classes are the *interleukins* and the *interferons*. A member of the interleukin cytokines is *Interleukin-1* (IL-1). IL-1 (there are an alpha and beta versions) is responsible for the production of inflammation and fever, so it is classified as a *pro-inflammatory cytokine* (there are also anti-inflammatory cytokines). It is primarily the macrophages that release IL-1. The IL-1ra therefore antagonizes the pro-inflammatory effects of IL-1 and most likely is involved in regulating the effects of IL-1. Therefore by knocking out the gene for IL-1ra the regulation of IL-1 is most likely disrupted. Interleukin 6 (IL-6) is a cytokine that has both pro-inflammatory and anti-inflammatory properties. IL-6 is released by T cells and macrophages. IL-6 also plays an important role in producing and regulating fever (Abbas & Lichtman, 2005).

Innate and adaptive immunity. The body's cell-mediated defense against microbes described so far is called *adaptive immunity* and it is contrasted with *innate immunity*. Adaptive immunity occurs in response to infection and adapts (hence the name) to repeated infection by increasing in magnitude and by improving its defensive capabilities with subsequent infections by mechanisms not discussed here. Innate immunity is the immediate

defensive reaction to microbes even before infection has set in (i.e., the first line of defense). Even if the infection repeats, the innate immune response does not change; it is always the same in magnitude and in its defensive capability. While innate immunity evolved first and only reacts to microbes, adaptive immunity evolved later and reacts to microbes as well as non-microbial substances (e.g., cancer cells). It is the adaptive immune system that is the likely culprit in autoimmune diseases, for example. Therefore it would seem that the effect of LPS on EtOH consumption would be mediated through effects on innate immunity rather than adaptive immunity. However, the innate and adaptive immune responses have evolved into a bi-directional integrative defensive system. One of the strategies of the adaptive immune system to combat microbes is to activate and enhance the innate immune response (for example, when Helper T cells release cytokines to activate phagocytes). Also while the innate immune response provides the first defensive response to microbes it also serves as a "warning" to activate the adaptive immune response which then prepares for possible subsequent infections. Therefore although LPS directly activates the innate immune system, it also indirectly affects the adaptive immune response which in turn may affect the nervous system and subsequent behavior (Abbas & Lichtman, 2005).

Toll-like receptors. The CD14 molecule is a receptor protein that is expressed in the cells of the innate immune system that identifies microbial pathogens. It is this receptor that binds with (and therefore identifies) the LPS molecule located on the surface of bacteria. However, the CD14 receptor cannot work alone, it is really a co-receptor. To detect LPS the CD 14 receptor must be activated as well as the *Toll-like receptor TLR 4* and the *MD2* receptor. A CD 14 knockout mouse would be missing one of the co-receptors so it would not be able to detect LPS

and therefore an innate immune response to bacteria would not occur and the adaptive immune response would not be activated (Abbas & Lichtman, 2005).

In previous studies, researchers activated the immune system of rodents using lipopolysaccharides (LPS) and observed changes in subsequent ethanol (EtOH) consumption (Blednov et al., 2011; Casachahua, 2011). LPS are large molecules found on the outside of gram-negative bacterial cells and are recognized by the immune system (Raetz & Whitfield, 2002; Rosenfeld & Shai, 2006). The immune system activates in the presence of LPS alone (Rosenfeld & Shai, 2006). Creating an immune response in this manner is preferable to bacterial infection because there is no actual infection, merely an immune response as though there was one.

Blednov and colleagues (2011) performed one intraperitoneal injection of LPS (1 mg/kg) in EtOH-preferring mice, and after allowing a recovery period, tested EtOH preference. Using a two-bottle test, they slowly increased the percentage of EtOH in one of the bottles. The mice that had been injected with LPS preferred EtOH significantly more than the controls. This effect was seen even three months after the single dose of LPS, indicating that immune system activation had long term influence on later behavior. Casachahua (2011) attempted to replicate this finding using Long-Evans rats in order to see if the effect observed in mice also occurs in rats; however, he also included a measure of sign-tracking bottles containing EtOH. Although no effect of LPS treatment was found on the sign tracking of EtOH, like the previous study with mice, he found an increase in EtOH preference in the rats that had been injected with LPS. As with Blednov and colleagues (2011), Casachahua (2011) found that this effect lasted for several months after the injection.

The previous studies indicated that activating the immune system increases voluntary EtOH intake. More recent research has also shown that a depressed immune system leads to lowered EtOH intake. Blednov et al (2012) tested 6 different immune knockout mice. The knock-out technique is a procedure used in molecular biology that target specific genes and remove them to create a new strain. The mice used by Blednov et al had beta-2-microglobulin, cathepsin S, cathepsin F, interleukin 1 receptor antagonist, CD14, or interleukin 6 genes knocked out. All of these genes play a large role in immune system activation and function (see below). They then gave the mice three tests – a 24-hour two bottle test, a limited access two bottle test (present for 3 hours during the dark cycle), and limited access to EtOH only (2-4 hours during the light cycle). The authors found that knockout mice preferred EtOH less in the 24-hour two bottle test than did the non-knockout mice. The other tests showed differences in preference for specific gene knock outs. There were no consumption differences of saccharin or quinine, revealing that the immune system gene knockouts affected EtOH drinking specifically, and not drinking behavior in general. These immune knockout mice provide compelling evidence that the immune system is integrally involved in EtOH consumption.

Purpose of the Current Experiment

The current experiment serves several purposes. The first is to attempt to replicate the findings of Casachahua (2011) and Blednov and colleagues (2011) that indicate LPS increases EtOH intake in rodents. The second goal of this study is to determine if blocking the Toll-Like receptor 4 with naltrexone will moderate this effect either partially or completely.

The method of this experiment aligns with those used in previous studies. Rats were injected with LPS in order to create an innate immune response. They were then tested with a 24-hour two bottle choice test with a gradual increase in EtOH percentage. Since the animals were caged in pairs, a limited two bottle choice test for the individual rats was added. However, in order to subdue the immune system response in some rats without completely knocking out any of the co-receptors needed to detect LPS, pre- and post- treatment of naltrexone was used. Interestingly, recent studies indicate that some drugs long known to act as competitive antagonists of opioid receptors also block the TLR4 receptor (Hutchinson et al., 2010). Naltrexone is a competitive antagonist of the μ- and κ- opioid receptors (Lee et al., 1988) and, like the related drug naloxone, appears to block the TLR 4 receptor (Hutchinson et al., 2008).

However, there are some potential confounds when using naltrexone in this study that were worked around. The first is that the drug reduces the palatability of many different substances. By measuring facial responses to different tastes, Ferraro III, Hill, Kaczmarek, Coonfield and Kiefer (2002) found that naltrexone created more aversive reactions to sucrose, sodium chloride, quinine, and EtOH. In fact many studies have found that naltrexone reduces palatability and intake of EtOH, even at low doses (Parkes & Sinclair, 2000; Lankford & Myers, 1996; Zalewska-Kaszbuska, Gorska, Dyr & Czamecka, 2008; Coonfield et al., 2002). If the rats do not like the taste of EtOH because of naltrexone's properties, then they will not drink it for that reason, not because of the suppression of their immune system.

Naltrexone also reduces caloric intake in general. In both human and rat studies, participants given naltrexone ate less especially if previously given access to the substance

(Kanarek, Mathes, Heisler, Lima & Monfared, 1997; MacDonald, Billington & Levine, 2003; Yeomans & Gray, 1996; Yeomans & Gray 1997; Lowy & Yim, 1981). Human participants found that their appetite was smaller and their enjoyment of food was reduced when taking naltrexone. These participants also ate significantly less than the placebo group (Yeomans & Gray, 1996; Yeomans & Gray, 1997). However, one study found that naltrexone treated rats had higher food intake; this may be an anomaly though (De Tomasi & Juarez, 2011). Since EtOH has a high caloric content, if naltrexone causes loss of appetite and decreased caloric intake, then reduced consumption of EtOH may not be caused by immune system suppression.

Possibly of the most concern is that naltrexone is used as a treatment for alcoholism. In fact, that is one of its main clinical uses. Since naltrexone decreases EtOH intake presumably because of competitive antagonism through the actions on opiate neurons, then using the drug to study EtOH intake caused by altered immune function could complicate interpretation of the results.

The potential confounding effects of naltrexone are either co-extensive with the administration of the drug or terminate shortly after its last administration (Kanarek et al., 1997; MacDonald, Billington & Levine, 2003; Yeomans & Gray, 1996; Yeomans & Gray 1997; Lowy & Yim, 1981; Parkes & Sinclair, 2000; Lankford & Myers, 1996; Zalewska-Kaszbuska et al., 2008; Coonfield et al., 2002; Ferrero III et al., 2002). This is the key to avoiding those effects. Naltrexone was given in this experiment for two weeks before and after LPS treatment but not during the subsequent part of the experiment when the rats are drinking EtOH. The TLR4 receptor should be occupied by the naltrexone before the LPS injection, so that the immediate

immune activation is reduced. Naltrexone, however, has a fairly short half-life in the body. While naltrexone may be effective in humans for as long as 108 hours after ingestion, rats have a higher metabolism rate of the chemical (Lee et al., 1988). Gonzalez and Brogden (1988) found that the half-life in rats was only between 2.7 and 3.7 hours after injection. Therefore while we were unable to block the Toll-like receptor during the entire period of exposure to LPS it is of interest to determine if a chronic, but partial blockage of TLR4 receptors reduces LPS-induced increase in EtOH consumption. At least one study reported that daily pretreatment with the opiate antagonist naloxone reduced the inflammatory effects of LPS (i.e. naloxone had anti-inflammatory effects) (Liu et al., 2002).

As discussed earlier, naltrexone decreases EtOH consumption in a variety of ways. However, naltrexone is not needed once the LPS is out of the rats' bodies. Therefore, naltrexone was discontinued before EtOH preference testing began. Since naltrexone has such a short half-life in the rat, there should be no naltrexone affecting the study once it preference testing began. Thus, any observed effects of naltrexone treatment on subsequent EtOH consumption will have been a result of long-lasting effects on the cells of immune or neural systems with receptors targeted by naltrexone.

Method

Subjects

The subjects were 30 male, approximately 40 days old, Long Evans rats from Harlan Laboratories, Inc. (Indianapolis), raised in pairs within shoebox cages. These rats were given food and water ad libitum and were maintained on a 12 hour light-dark cycle, with the light turning on at 8 am. Forty-six days after the beginning of this experiment, one of the rats had to be separated from his cage-mate because of sudden weight-loss, mostly likely resulting from aggressive dominance from its cage mate. He subsequently regained weight and his data have been included. This experiment was approved by Seton Hall University's Institutional Animal Care and Use Committee. All guidelines for the care and use of rats set by the United States Public Health Service have been firmly followed.

Drugs

Naltrexone Treatment. Fourteen days prior to and twelve days after the LPS injection, 16 rats were injected intraperitoneally (IP) with 5.0 (first six days) to 7.0 (remaining twenty days) mg/kg of naltrexone (from Sigma Aldrich, St. Louis, MO) dissolved in saline, while the other 14 were injected with the equivalent amount of saline every day at the same time (approximately 12 PM). Naltrexone dosing varies greatly in the literature. Chronic or repeated naltrexone doses vary from 1.0 mg/kg/day to 10 mg/kg/day (Sanchis-Segura, Pastor & Aragon, 2004; De Tomasi & Juarez, 2011). Therefore, we used a middle dose of 5mg/kg/day. The lower dose was used initially to make sure that naltrexone did not disrupt normal eating and drinking and was increased to 7mg/kg/day dose for the remainder of treatment when the data confirmed that feeding and drinking remained at normal levels.

LPS Treatment. After an initial phase of training to drink in test cages (see procedure) and after 2 weeks of daily treatment with naltrexone or saline, 16 rats (8 treated with Naltrexone, and 8 treated with saline) were injected IP with 1.0 ml/mg/kg of LPS (from Salmonella enterica, Cat#L65 11, Sigma, St. Louis, MO) dissolved in saline, while the other 14 rats (8 treated with Naltrexone, and 6 treated with saline) were injected with the equivalent amount of saline. These groups were equated in terms of body weight and how much they drank during the training in the test cages. LPS treatment induces malaise and elevated body temperature lasting up to 24 hrs, thus injections were aligned with rat pairing (i.e., each cage-mate received the same injection treatment). This injection occurred immediately after the naltrexone injection that day. The rats were given nineteen days of recovery time prior to the introduction of EtOH solution. Additionally, rat bodyweights were recorded from two weeks prior to injection and the following 15 days. The behavior of the rats was monitored for indications of malaise as well as body weight changes to confirm that there was a response to LPS.

Procedure

The timeline of the procedure is listed in Table 1.

Initial training in the test cages. In order for the rats to learn to drink in the individual tests later in the experiment, they were trained using Polycose solutions. Prior to the LPS injection, the rats were adapted to suspended stainless steel mesh cages (20.3 cm x 20.3 cm x 22.9 cm) and trained to drink in these test cages. They were placed in the cages for 20 minutes a day for four consecutive days and given a highly preferred Polycose (8%) solution in one bottle and water in another (the bottles consist of 100 ml plastic graduated cylinders). The

position of the bottles was alternated each day. The intakes were determined by weighing the bottles before and after the test session. As the time between this training and the short term EtOH preference tests was several months, reminder training sessions were given roughly four weeks before these EtOH preference tests were administered. For the reminder sessions, a 4% Polycose solution was used. These cages were also used for short-term preference tests for EtOH after EtOH was introduced in the home cage.

Home cage (24 hr) exposure to ethanol (EtOH). Beginning twenty days after the LPS injection, each cage had both EtOH and water bottles present at all times in the home cages. The EtOH bottle began at 1% and was increased gradually by 2% with at least 2 days at each concentration up to 13% (as per Blednov et al, 2011). However, at 7% EtOH concentration, all groups suddenly decreased in EtOH preference. The preference did not recover when the concentration was decreased. Previous studies indicated that EtOH intake in rodents can be increased if EtOH is provided on the home cages intermittently (Rosenwasser et al., 2012). Three-day-a-week intermittent EtOH availability was then introduced. The EtOH bottle was introduced Monday, Wednesday, and Friday at approximately noon and removed Tuesday, Thursday, and Saturday at approximately noon. EtOH preference increased with this schedule and the gradual increase of concentration was resumed. Preference began to decline at 13% EtOH, so the concentration was kept at this level for the remainder of the experiment while the rats were given individual short-term two-bottle preference tests. The position of both bottles was switched each day to account for location preference. The consumption from both bottles in each cage were measured daily by weighing each bottle.

Short term EtOH preference tests. Although the rats sharing a cage were in the same LPS (LPS or saline) and naltrexone (naltrexone or saline) conditions, it was not possible to know the amount of EtOH consumed by the individual rats. Therefore twice a week, each animal was tested individually for their EtOH preference in the test cages after the concentration in the home cages was maintained at 13%. Each animal was placed in test cages with water and 5%-18% EtOH concentration presented in two bottles for 20 minutes, after which the bottles were weighed to determine the quantity of EtOH consumed. The bottle location was switched the next test day. These tests took place after the rats had not had access to EtOH for 24 hours. Each concentration of EtOH (5%, 8%, 12%, 15%, and 18%) was tested twice to account for possible side preference. This range of concentrations allowed for a better estimation of each animal's consumption of, and preference for, EtOH.

Table 1. Procedural Timeline

Experimental Day(s)	Procedure
1-4	Polycose (8%) training sessions
3-8	Naltrexone (5mg/kg) or saline daily IP injections
9-28	Naltrexone (7mg/kg) or saline daily IP injections
17	LPS (1mg/kg) IP injection
36-57	24/7 home-cage access to EtOH (1%-7%) (Phase 1)
50-51; 57-58	Polycose (4%) training sessions
58-123	3-day-a-week home-cage intermittent EtOH availability (5%-13%) (Phase 2)
86 and 88	Short-term individual tests at 5% EtOH
93 and 98	Short-term individual tests at 8% EtOH

102 and 107	Short-term individual tests at 12% EtOH
114 and 116	Short-term individual tests at 15% EtOH
121 and 123	Shot-term individual tests at 18% EtOH

Data Analysis

Bodyweight, EtOH preference, and EtOH intake in grams were the main dependent variables of interest. The bodyweight data were analyzed only on the LPS groups. Two mixed design ANOVAs were used to analyze the data. The data for the before and after LPS were analyzed with a naltrexone (Yes or No) x days (3) mixed methods ANOVA using the average of the 3 days prior to LPS administration as the before group. The data for the recovery from LPS were analyzed with a naltrexone (Yes or No) x days (4) mixed methods ANOVA. Naltrexone was used as the between groups factor days as the within groups factor. The primary independent variables for each of the EtOH and Polycose intake tests were naltrexone treatment, LPS treatment, and concentration. Naltrexone treatment and LPS treatment were the between-groups factors. Concentration was the within-groups factor.

Results

Immediate LPS effectiveness

Weight-change after LPS injection. Figure 1 shows body weight change after treatment with LPS compared to the average bodyweight of the three days prior to the LPS injection. A mixed two way ANOVA revealed a significant main effect for days, $F(2,28)=18.271$, $p<.001$, $\eta^2=.566$. Post-hoc Bonferroni-corrected paired-samples *t*-tests revealed that the animals weighed significantly more the three days prior to the injection ($M=286.75$, $SD=3.86$) than the day after ($M=274.75$, $SD=3.69$), $t(15)=5.07$, $p=.001$, and two days after ($M=273.438$, $SD=4.18$) the injection, $t(15)=4.41$, $p=.002$. and two days after ($M=273.438$, $SD=4.18$) the injection. There was no effect of naltrexone, $F(1,14)=.043$, $p=.839$, $\eta^2=.008$.

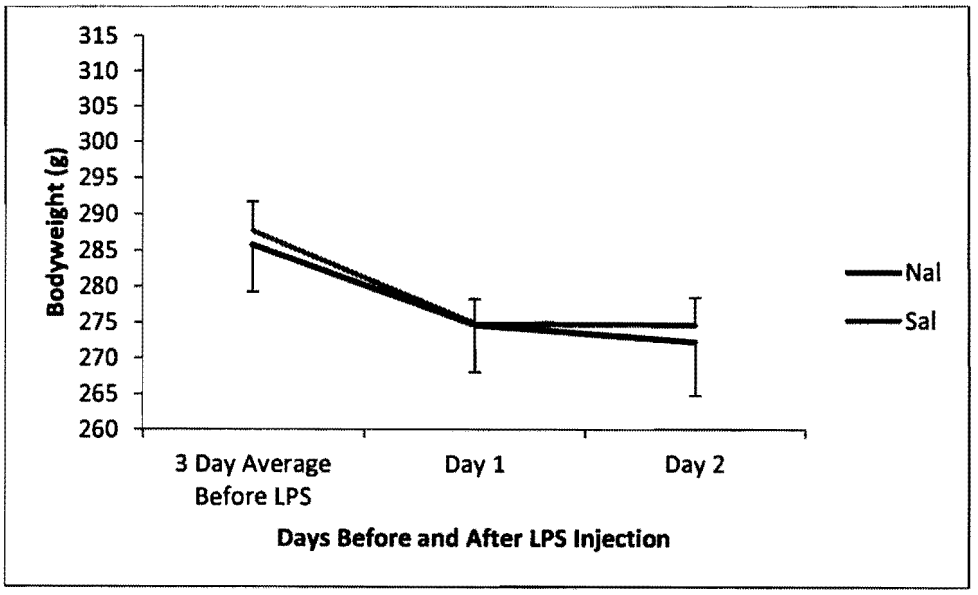

Figure 1. Bodyweight changes before and after LPS injection

Weight recovery after LPS injection. The mean bodyweights for the first four days after the LPS injection are shown in Figure 2. A significant main effect of days, $F(2,28)=24.763$, $p<.001$, $\eta^2=.740$, confirmed that body weights were recovering. However, a Day (4) x Drug treatment (naltrexone or saline) mixed ANOVA revealed a significant Day X Naltrexone interaction, $F(3,42)=3.41$, $p=.026$, $\eta^2=.196$, indicating that the naltrexone-treated animals showed a greater recovery of body weight following LPS treatment compared to saline-treated controls. Post-hoc Bonferroni-corrected dependent-samples *t*-tests revealed that the rats treated with naltrexone weighed significantly more on the third day after LPS treatment compared to the first day, $t(7)=8.142$, $p<.001$, but the rats treated with saline did not, $t(7)=1.568$, $p=.161$. However, an independent-samples *t*-test revealed that the naltrexone and saline groups were not significantly different from each other by five days after the LPS injection, $t(14)=.727$, $p=.479$.

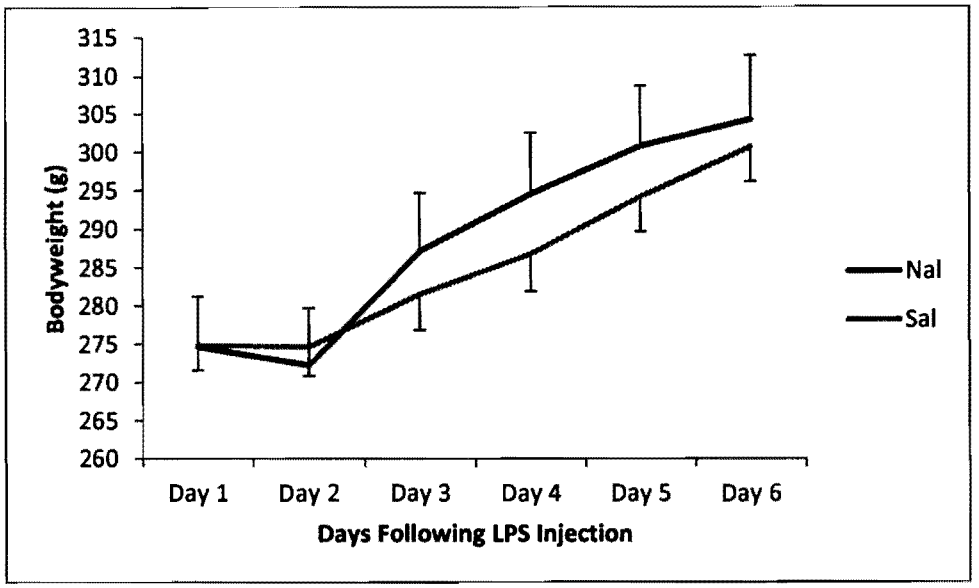

Figure 2. Bodyweight change in days following LPS injection

Home cage EtOH consumption

Phase 1 Continuous EtOH Access - EtOH preference. Preference scores were calculated by dividing the grams of liquid consumed from the EtOH bottle by the total grams of liquid consumed in both the EtOH bottle and the water bottle and multiplying by 100. As can be seen in Figure 3, all groups consumed more EtOH than water at the two lowest EtOH concentrations, but this preference was no longer present at the 7% concentration for any group. A mixed 2(Naltrexone: Yes, No) x 2(LPS: Yes, No) x 4(Concentration: 1%, 3%, 5%, 7%) ANOVA was performed on the percent preference for EtOH. This ANOVA revealed a significant main effect for concentration, $F(3,33)=16.22$, $p<.001$, $\eta^2=.596$. Post-hoc Bonferroni-corrected paired-samples *t*-tests revealed that the preference for 7% ($M=43.74$, $SD=23.97$) was significantly less than for 1% ($M=77.78$, $SD=11.94$), $t(14)=-4.65$, $p<.001$, 3% ($M=81.05$, $SD=8.58$), $t(14)=-6.29$,

p<.001, and 5% (M=70.97, SD=22.57), t(14)=4.17, p=.001. These tests also revealed that the preference for 3% was more than for 5%, t(14)=2.12, p=.052, but this was merely a trend. There was no Naltrexone X LPS X Concentration interaction, F(3, 33)=.465, p=.709. All other interactions also failed to be significant.

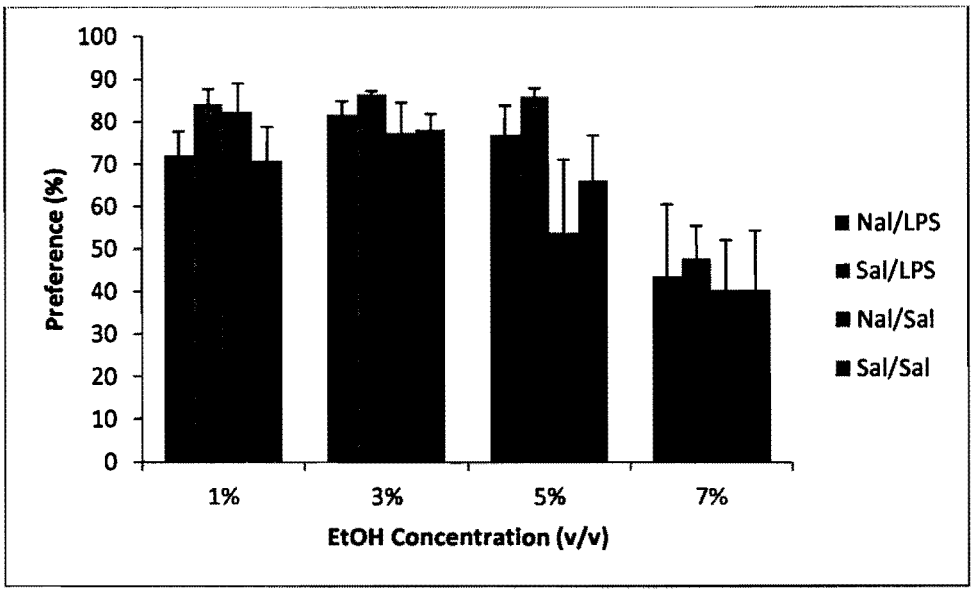

Figure 3. Preference for EtOH bottle during phase 1

Phase 1 Continuous EtOH Access - EtOH grams consumed. Grams of EtOH consumed were calculated by multiplying the grams of liquid consumed from the EtOH bottle by the weight of EtOH per gram at each concentration (Figure 4). A mixed 2(Naltrexone: Yes, No) x 2(LPS: Yes, No) x 4(Concentration: 1%, 3%, 5%, 7%) ANOVA was performed on the grams of EtOH consumed by the animals. This ANOVA revealed a significant main effect for concentration, F(3,33)=21.25, p<.001, η^2=.659. Post-hoc Bonferroni-corrected paired-samples t-tests revealed that the grams consumed at 1% (M=.47, SD=.07) was significantly less than for

3% (*M*=1.54, *SD*=.18), *t*(14)=-26.57, *p*<.001, 5% (*M*=2.08, *SD*=.72), *t*(14)=-9.81, *p*<.001, and 7% (*M*=1.79, *SD*=.99), *t*(14)=-5.03, *p*<.001. These tests also revealed that the grams consumed at 3% was significantly less than at 5%, *t*(14)=-3.26, *p*=.006. There was no Naltrexone X LPS X Concentration interaction, *F*(3, 33)=.142, *p*=.934.

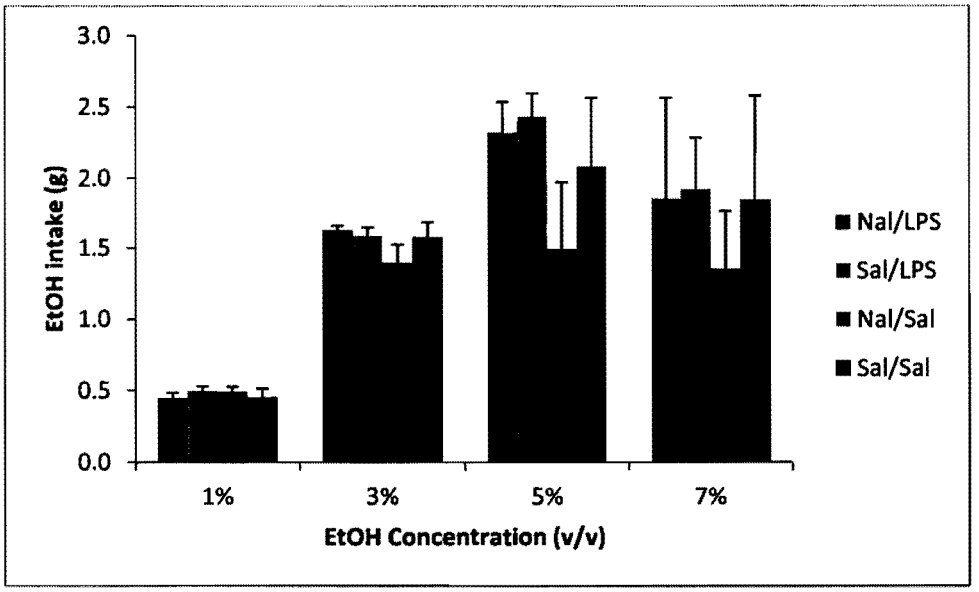

Figure 4. Grams of EtOH consumed during Phase 1

Phase 2 Intermittent EtOH Access - EtOH preference. Because the consumption of EtOH consumption dropped unexpectedly at the 7% EtOH concentration, an intermittent schedule of EtOH was introduced to induce greater EtOH consumption in the home cages. As can be seen in Figure 5, preference for EtOH generally increased at all but the highest concentration. A mixed 2(Naltrexone: Yes, No) x 2 (LPS: Yes, No) x 5 (Concentration: 5%, 7%, 9%, 11%, 13%) ANOVA was performed on the percent preference scores. This ANOVA revealed a significant main effect for concentration, *F*(4,48)=16.22, *p*<.001, η^2=.642. Post-hoc Bonferroni-corrected paired-samples

t-tests revealed that the preference for 13% (*M*=50.22, *SD*=14.77) was significantly less than for 5% (*M*=66.88, *SD*=20.77), *t*(15)=-3.13, *p*=.007, 7% (*M*=81.41, *SD*=8.99), *t*(15)=9.08, *p*<.001, and 9% (*M*=68.88, *SD*=16.97), *t*(15)=5.56, *p*<.001, and 11% (*M*=64.32, *SD*=12.88), *t*(15)=7.34, *p*<.001. These tests also revealed that the preference for 7% was significantly more than for 5%, *t*(15)=3.697, *p*=.002, 9%, *t*(15)=4.82, *p*<.001, and 11%, *t*(15)=7.195, *p*<.001. Additionally, the preference for 9% was significantly more than for 11%, *t*(15)=2.35, *p*=.033. Further, there is a trend of a concentration x naltrexone interaction, *F*(4,48)=2.37, *p*<.066, η^2=.165, suggesting that naltrexone had a tendency to increase preference for EtOH at the highest EtOH concentrations. This naltrexone effect appears to have been mostly due to the Nal/LPS group, however the Naltrexone X LPS X Concentration interaction failed to reach statistical significance, *F*(4, 48) =.176, *p*=.950. No other interactions were significant.

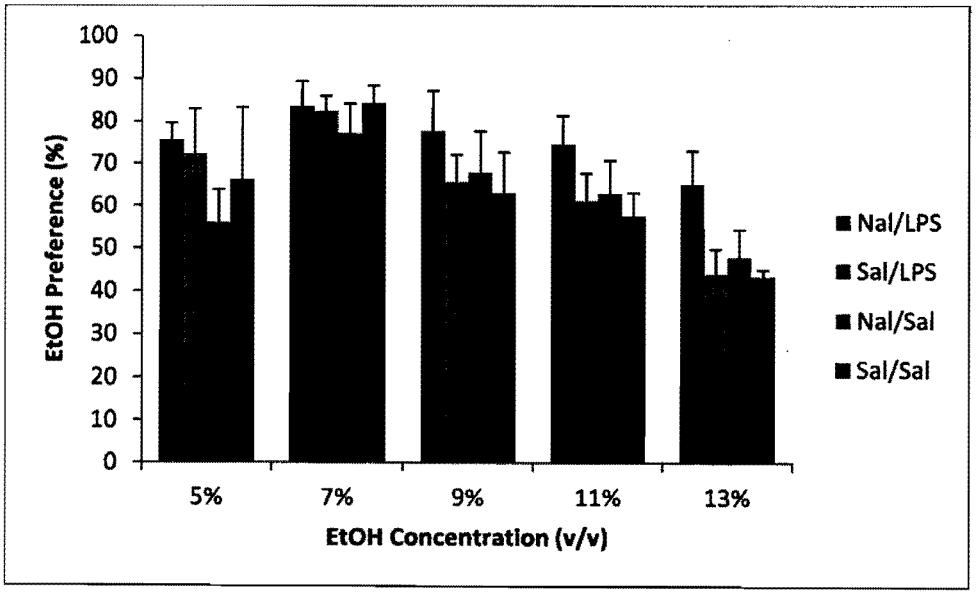

Figure 5. Preference for EtOH bottle during Phase 2

Phase 2 Intermittent EtOH Access - EtOH grams consumed. Figure 6 replots the preference data in terms of the mean grams of EtOH consumed. A mixed 2 (Naltrexone: Yes, No) x 2 (LPS: Yes, No) x 5 (Concentration: 5%, 7%, 9%, 11%, 13%) ANOVA was performed on the grams of EtOH consumed. This ANOVA revealed a significant Naltrexone x LPS x Concentration interaction, $F(4,48)=2.63$, $p=.045$, $\eta^2=.180$. Additional 2 (Naltrexone: Yes, No) x 2 (LPS: Yes, No) between-groups ANOVAs were conducted on each EtOH concentration to identify the nature of the interaction. Significant naltrexone x LPS interactions occurred at 7%, $F(1, 12)=4.73$, $p=.050$, $\eta^2=.283$, 11%, $F(1,12)=5.12$, $p=.043$, $\eta^2=.298$, and 13%, $F(1,12)=10.75$, $p=.007$, $\eta^2=.472$. Pairwise comparisons reveal that at 7%, the Nal/LPS group ($M=3.98$, $SD=.90$) consumed significantly more grams of EtOH than the Nal/Sal group ($M=2.74$, $SD=.65$) (mean difference = 1.23, $p=.044$). However, Sal/Sal group ($M=4.25$, $SD=.73$) consumed more than the Nal/Sal group ($M=2.74$, $SD=.65$) (mean difference = 1.50, $p=.027$). The interaction at 9% was trending towards significance, $F(1,12)=3.55$, $p=.084$, $\eta^2=.228$. Pairwise comparisons reveal that the Nal/LPS group ($M=4.75$, $SD=1.52$) consumed more grams of EtOH than the Nal/Sal group ($M=2.93$, $SD=.77$) (mean difference = 1.82, $p=.047$). Pairwise comparisons reveal that at 11%, the Nal/LPS group ($M=5.69$, $SD=1.32$) consumed significantly more grams of EtOH than the Nal/Sal group ($M=3.56$, $SD=.47$) (mean difference = 2.12, $p=.012$). There is also a trend that the Nal/Sal group ($M=5.69$, $SD=1.32$) consumed more EtOH than the Sal/LPS group ($M=4.32$, $SD=1.13$) (mean difference = 1.36, $p=.096$). Pairwise comparisons reveal that at 13%, the Nal/LPS group ($M=5.28$, $SD=1.53$) consumed significantly more grams of EtOH than the Nal/Sal group ($M=2.55$, $SD=.62$) (mean difference = 2.73, $p=.001$). Additionally, the Nal/LPS group ($M=5.28$, $SD=1.53$) consumed more than the Sal/LPS group ($M=2.99$, $SD=.75$) (mean difference = 2.29, $p=.007$). A significant

Naltrexone x Concentration interaction was also revealed, $F(4,48)=3.15$, $p=.022$. Additionally, a significant main effect for concentration was found, $F(4,48)=40.08$, $p<.001$.

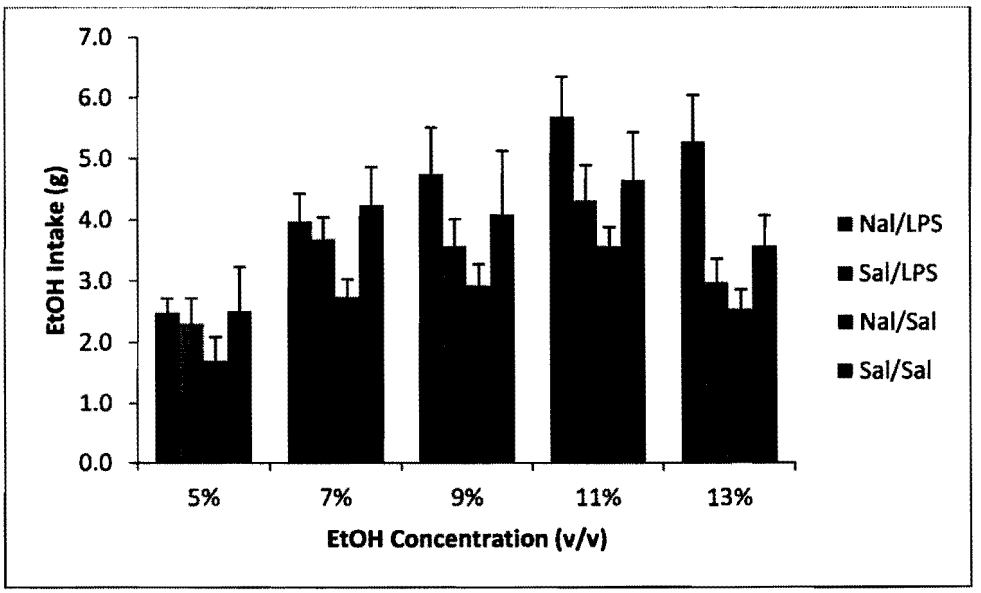

Figure 6. Grams of EtOH consumed during Phase 2

Individual short-term two-bottle preference tests.

Polycose preference. Before initiating preference tests with EtOH, all groups were trained with two concentrations of Polycose versus water. A 2 (Naltrexone: Yes, No) x 2 (LPS: Yes, No) x 2 (Concentration: 4%, 8%) mixed ANOVA was performed on percent preference for Polycose. A significant Naltrexone X Concentration interaction was revealed, $F(1,26)=6.023$, $p=.021$, $\eta^2=.188$. Post-hoc independent-samples t-tests revealed a trend that the naltrexone animals preferred the 8% Polycose solution less than the 4% solution, $t(15)=-2.11$, $p=.052$, whereas, the saline group did not differ in preference, $t(15)=1.312$, $p=.212$. However, post-hoc

analyses did not reveal significant group differences. There was no Naltrexone X LPS X Concentration interaction, $F(1,26)=1.82$, $p=.189$.

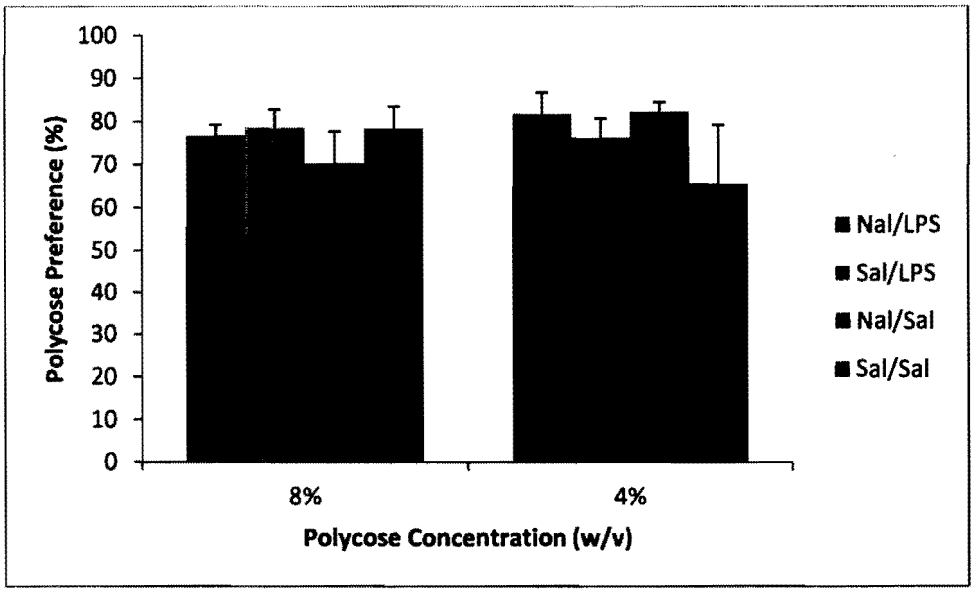

Figure 7. Preference for the bottle containing Polycose

EtOH preference. A mixed 2 (Naltrexone: yes, no) x 2 (LPS: yes, no) x 3 (Concentration: 5%, 8%, and 12%) ANOVA was performed on percent preference for the bottle containing EtOH and revealed no concentration effect, $F(2, 52)=0.167$, $p=.847$. A mixed 2 (Naltrexone: yes, no) x 2 (LPS: yes, no) x 2 (Concentration: 15% and 18%) ANOVA was performed on percent preference for the bottle containing EtOH and revealed no concentration effect, $F(1,26)=1.689$, $p=.205$. Therefore, the 5 concentrations (5%, 8%, 12%, 15%, and 18%) were collapsed into two groups: "Low" for concentrations below the home cage concentration of 13% and "High" for concentrations above the home cage concentration. These preference scores are plotted in figure 8. A mixed 2 (Naltrexone: yes, no) x 2 (LPS: yes, no) x 2 (Concentration: low, high) ANOVA

was performed on percent preference for the bottle containing EtOH. A main effect for concentration was revealed, $F(1, 26)=19.199$, $p<.001$, $\eta^2=.425$. There was a significantly greater preference for the "low" concentration ($M=74.82$, $SD=12.00$) than the "high" concentration ($M=59.03$, $SD=16.81$). There was no Naltrexone X LPS X Concentration interaction, $F(1,26)=.010$, $p=.922$. No other interactions were significant.

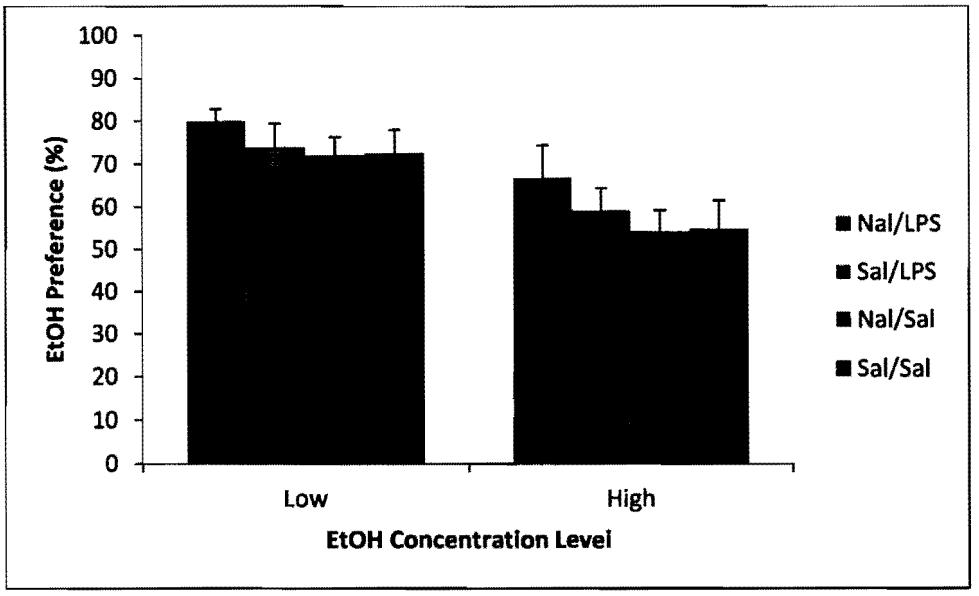

Figure 8. Preference for the bottle containing EtOH during individual tests

EtOH grams consumed. A mixed 2 (Naltrexone: yes, no) x 2 (LPS: yes, no) x 3 (Concentration: 5%, 8%, and 12%) ANOVA was performed on grams of EtOH and revealed no concentration effect, $F(2, 52)=.682$, $p=.510$. A mixed 2 (Naltrexone: yes, no) x 2 (LPS: yes, no) x 2 (Concentration: 15% and 18%) ANOVA was performed on grams of EtOH and revealed no concentration effect, $F(1,26)=.021$, $p=.887$. Therefore, 5 concentrations (5%, 8%, 12%, 15%, and 18%) were collapsed into two groups: "Low" for concentrations below the home cage

concentration of 13% and "High" for concentrations above the home cage concentration. The grams of EtOH consumed are plotted in figure 9. A mixed 2 (Naltrexone: yes, no) x 2 (LPS: yes, no) x 2 (Concentration: low, high) ANOVA was performed on the grams of EtOH consumed. This ANOVA revealed a significant concentration X LPS interaction, $F(1,26)=4.306$, $p=.048$, $\eta^2=.142$. Post-hoc independent samples t-tests revealed that the animals which received LPS (Low: $M=.32$, $SD=.12$; High: $M=.49$, $SD=.16$) consumed significantly more grams of EtOH than the animals which received saline (Low: $M=.29$, $SD=.12$; High: $M=.38$, $SD=.12$) only at high concentrations, $t(28)=2.15$, $p=.040$. There was a significant main effect of concentration, $F(1, 26)=35.492$, $p<.001$. There was no Naltrexone x LPS x Concentration interaction, $F(4,26)=.005$, $p=.943$.

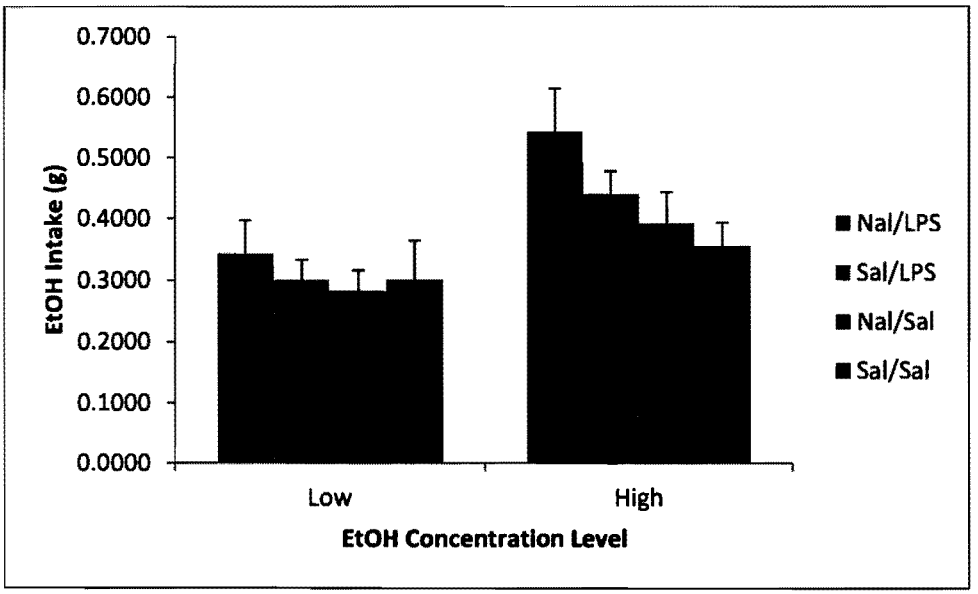

Figure 9. Grams of EtOH consumed during individual tests

Discussion

In 2011, Blednov et al showed that a single injection of LPS increased the intake and preference of EtOH in mice bred to be EtOH-preferring. Casachahua (2011) found this same effect in Long-Evans rats not bred specifically for EtOH preference, but this study also addressed other questions not focused on EtOH intake and preference and included sign-tracking of EtOH in daily sessions. The present study attempted to replicate the LPS-induced increase of EtOH intake and extend the study by examining the effects of Naltrexone on the LPS effect. In the present study we did not observe an LPS-induced increase in home-cage EtOH preference over water. While initially there was a strong preference by all groups for the EtOH bottle at lower EtOH concentrations, at 7% EtOH this preference drastically decreased in all groups. When the EtOH concentration was reduced as low as 3%, there was no preference recovery. It is unclear why the rats in the present study (regardless of LPS treatment) consumed less EtOH in the home cages compared to the Casachahua study. One contributing factor may be that all of the rats in the previous study had daily experience in a sign tracking procedure where a bottle CS was paired with sucrose pellet UCSs and half of the rats had the additional experience of consuming various concentrations of EtOH from the bottle CS.

In an attempt to induce EtOH intake again the intermittent availability of EtOH was introduced. Previous studies have shown that the intermittent access to EtOH increases EtOH intake in rodents (Rosenwasser et al., 2012; Simms et al, 2008). In fact Simms and colleagues (2008) found that Long-Evans rats consumed more EtOH and had a higher preference for a 20% EtOH solution when on a three-day-a-week intermittent schedule than for continuous access to 10% or 20% EtOH. In 2010, Simms and colleagues found that Long-Evans rats acquire operant

conditioned responding with a 20% EtOH reinforcer if tested while home-cage EtOH was provided on an intermittent schedule but not on a daily schedule. Interestingly the intermittency effect is not seen solely in EtOH intake, but sucrose as well (Martin & Timofeeva, 2010). By changing the schedule from daily EtOH exposure to a three-day-a-week schedule EtOH intakes increased again and a preference for EtOH was re-established. Although the original studies as well as some of the intermittent schedule studies had animals consuming high levels of EtOH at least up to an 18% concentration (Blednov et al., 2011; Casachahua, 2011; Simms et al., 2008; Simms et al., 2010), most of the animals in the current study stopped displaying a preference for the EtOH bottle at 13%. Therefore, the concentration was maintained at this level while individual two-bottle preference tests were conducted for the remainder of the experiment.

Previous literature does not delve into the mechanisms behind why intermittency increases EtOH consumption. However, learning theory suggests several possibilities. Habituation processes, for example, may modulate intake during intermittent access to EtOH. According to Rankin and colleagues (2009) habituation is a decrease in a behavioral response to a stimulus resulting from the repeated exposure to the stimulus. Although habituation is traditionally thought of as an experimental paradigm for the study of non-associative learning, it occurs in many situations outside of habituation procedures, even during motivated behaviors such as feeding and operant responding (Rankin et al., 2009). The novelty of the EtOH induced increased consumption at the lowest concentrations, habituation could explain why the animals reduced responding to the EtOH bottle when presented continuously. Habituation has several other characteristics associated with it. Another characteristic of

habituation is spontaneous recovery which occurs after the response habituates, if the stimulus is withheld, the response recovers when it is later reintroduced (Rankin et al., 2009). By only having access to the EtOH bottle three days a week, the rats began responding to the EtOH bottle again each time it was reintroduced. When the animals began drinking from the EtOH bottle to which they had become habituated they were exhibiting spontaneous recovery.

Additionally, the absence of the EtOH bottle may have allowed the rats to recover from negative effects of EtOH which would decrease their intake time. Acute intoxicating effects of EtOH in rats include loss of righting reflex, loss of coordination, and sedation (Caldwell et al., 2006),effects that may prevent the animals from consuming EtOH at least at the highest concentrations used. This would lead to decreased consumption of EtOH while intoxicated. However, if the animals were given time to recover from these effects, the next time the EtOH bottle was presented, the animals would be able to reach and consume from the bottle.

Our attempt to increase EtOH intake by introducing an intermittent home-cage exposure to EtOH was successful; however, once the rats were consuming the EtOH, an interesting pattern emerged that differed from previous studies. While Blednov et al. (2011) and Casachahua (2011) found that rodents injected with a single dose of LPS drank significantly more than controls, the present study found that only the group treated with LPS and naltrexone showed increase in intake of grams of EtOH during an intermittent EtOH availability schedule. Casachahua (2011) used a sign-tracking procedure that this study did not utilize. Perhaps this task prompted the LPS animals to drink more in their home cages. However, the group of rats that received both naltrexone and LPS consumed significantly more EtOH than the

other three groups. Interestingly, of the animals who received LPS, those that also received naltrexone recovered faster from its effects as indicated from the recovery of body weight after LPS treatment. This is not surprising. Lin et al (2005) found that naltrexone pretreatment prevented sepsis in rats given a large dose of LPS (10 mg/kg). The rats which had been pretreated had significant recovery of many of the effects including bradycardia, vasoconstriction, and hypotension within six hours of administration; whereas, the control animals did not. Wang et al (2005) found that post-treatment with naltrexone prevented liver malfunction in mice after receiving an injection of LPS and D-galactosamine, a hormone which damages the liver. Given these protective and recovery promoting effects, it makes sense that the rats receiving naltrexone had a faster recovery rate, as measured by body weight. The results of the present study suggest that naltrexone also affected the impact of LPS on subsequent EtOH intake.

We had hypothesized that the naltrexone would possibly decrease the effect that LPS had on EtOH drinking. LPS binds to the TLR4 on immune cells, while naltrexone blocks these same receptors. The recovery effects of naltrexone could be a result of this partial blocking of the TLR4 (Hutchinson et al., 2008). Because this is the same receptor to which LPS binds, chronically blocking it could lead to a smaller immune response and faster recovery time (Waetz & Wittfield, 2002). Just as we saw this faster recovery in bodyweight after LPS treatment, presumably due to blocking the TLR4, we expected a reduced LPS effect on EtOH intake and preference. However, we found the opposite; naltrexone interacted with LPS to increase EtOH drinking in these animals. Naltrexone is not a selective antagonist of the TLR4; it also antagonizes the opioid system (Lee et al., 1988). It may be that somehow the chronic

antagonism of the opioid receptors in the brain along with the TLR4 in the immune system interacted to create the increase in EtOH intake seen in these animals. We cannot rule out that direct effects on the opiate receptors of the brain are at least partially responsible for the results found in this study.

It is important to note that a LPS-induced increase in EtOH drinking was only seen in the grams of EtOH consumed, not the preference of EtOH. This means that while these animals consumed more EtOH, they also consumed more water. Greater absolute intake in EtOH without a significant preference for EtOH may suggest that the some other mechanism is driving EtOH intake other than a preference for EtOH over water. It is possible, for example, that the initial "preference" for EtOH at the low concentrations is due to an increase in EtOH intake unrelated to palatability (e.g., due to the novelty of the taste) but that with further experience an increase in a true hedonic preference for the EtOH develops over water that is observed even at the highest EtOH concentrations. Whereas a LPS-induced increase in intake and preference was achieved in the previous study (Casachahua, 2011), a significant increase in absolute intake of EtOH was observed in the present study without a significant increase in EtOH preference over water. It is possible, for example, that the rats which consumed more EtOH in this study were also drinking more water to counteract some effects of EtOH (e.g., washing the mouth of the taste of EtOH).

An important distinction in consumption is that of liking as opposed to wanting. Liking in this study was measured as the preference of EtOH over water; whereas, grams of EtOH consumed was the measure of wanting. Liking may be caused by the taste or subjective effects

of the substance (that is, the palatability of the substance). Wanting, however, may develop from a multitude of motivational factors such as caloric value, intermittent availability, or social interaction (Berridge, 1996). Interestingly, liking and wanting have different pathways in the brain. The preference for a substance is controlled by the opioid system; whereas, wanting is mediated by the mesotelencephalic dopamine pathway (Berridge, 1996). Therefore, any long-lasting effect of naltrexone on the opioid system would likely have decreased preference (liking) rather than increase the intake of grams of EtOH (wanting). This makes it more likely that the results seen in this study can be attributed to the combination of effects on the TLR4 from LPS and naltrexone.

The short-term individual tests were generally consistent with the pattern of results. The graphs of these data show an apparent naltrexone x LPS interaction, suggesting that LPS resulted in greater EtOH intake in Naltrexone-treated rats than in Saline-treated rats; however, this interaction fell short of significance. One of the issues is a lack of power; each group only had a maximum of eight rats. Nevertheless a significant LPS x EtOH concentration interaction suggests that overall LPS did have an effect on EtOH intake, although not as robust as in the prior study. However, this effect was only seen at concentrations higher than the home cage concentration of 13%.

It is possible that LPS has a weaker effect on EtOH intake in rodents not bred for EtOH preference, such as Long-Evans rats, compared to EtOH-preferring mice. Perhaps to have an LPS effect there must be an initial high rate of EtOH intake as seen in the Blednov et al (2011) EtOH-preferring mice, or in rats with experience in the home cage and outside of the home cage, as

in Casachahua (2011), or in rats exposed to intermittent EtOH presentations. Of these three possibilities, the first procedure appears to be the most effective.

Blednov et al (2011) found that different genetic background of mice produced different magnitudes of the LPS effect. They found the greatest difference in the strain bred to consume high amounts of EtOH. Blednov et al (2011) also suggest that the effect of LPS on EtOH intake is due to modulation of the TLR4. This is at least partially validated by Blednov et al (2012) finding that CD14 knockout mice had very low EtOH intake because CD14 is a co-receptor of TLR4 (Abbas & Lichtman, 2005). Blednov et al (2011) suggest that because LPS cannot pass the blood-brain barrier, the cytokines released from the peripheral immune cells affect the central nervous system, leading to neuroinflammation. Long-term neuroinflammation is the most likely cause of the increased EtOH consumption after LPS treatment. However, the results of the present study suggests that antagonism of the TLR4 receptor on subsequent EtOH consumption is complex, since repeated naltrexone treatment before and after an LPS injection appears to have enhanced a weak LPS-induced increase in EtOH consumption.

This study leads to many possible future directions. Future studies should manipulate the effects of environment changes on the LPS effect to understand why the results of this study varied from Casachahua (2011). This study would attempt to replicate the present findings but also manipulate experiences outside of the home cage. This can be achieved by adding a group that is trained to sign-track a bottle with or without EtOH, while another group has no such experience. This study only examined individual EtOH preference and consumption after the home-cage concentration was maintained at 13%. Another change would be to give

the short-term preference tests repeatedly from the beginning of the study. This would also determine if increases in the absolute intakes of EtOH appear before the development of a preference or if preferences emerge before or simultaneous with increased intakes. That is, it would be of interest to determine if immune system activation by LPS increases EtOH consumption, which in turn results in the development of an EtOH preference, which then may contribute to a path towards EtOH dependence.

In order to determine if the opioid system is involved in the escalation of intake by the rats receiving both naltrexone and LPS, a future study should use dextro-naltrexone instead of the standard levo-naltrexone. D-naltrexone is the mirror image of l-naltrexone, which causes interesting effects (Hutchinson et al., 2008). It is not used frequently because, unlike its left-handed brother, it does not antagonize the opioid system (Chatterjie, Sechzer, Lieberman & Alexander, 1998). Therefore, it was not thought to be effective for use in treatment for narcotics or alcohol. This would prevent the decrease in caloric intake and the decreased palatability from being an issue in this study. However, it still binds to the TLR4 in the same manner as l-naltrexone does (Hutchinson et al., 2008). This means that it would still suppress the immune system.

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