Environmental Influences on the Sign Tracking of Ethanol: A Rodent Model of Alcohol Addiction

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Environmental influences on the sign tracking of ethanol.

A rodent model of alcohol addiction

by

John Casachahuá

A thesis submitted in partial fulfillment of the requirements for the degree of

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with a concentration in Behavioral Neuroscience

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Rodent models of alcoholism provide a method for exploring the factors that contribute to alcoholism. The rodent sign tracking procedure using a bottle (with ethanol or water) as the conditioned stimulus and a sugar pellet as the unconditioned stimulus has several components that appear related to drug use and abuse. In this study, the environmental influences of rearing condition and bacterial infection were explored as possible contributory factors to the abuse of alcohol. In Experiment 1, Sprague-Dawley rats reared in an enriched environment showed stronger acquisition of sign tracking behavior and consumed more ethanol than rats reared in a standard environment, but neither group developed a preference for ethanol. A negative-feature discrimination task revealed that the enriched- and standard-reared rats were not impulsive since they readily reduced sign tracking behavior on trials when the sugar pellet was omitted. Although, the enriched rats were more vulnerable to the effects of ethanol than the standard rats because they were sign tracking the bottle more, increased impulsivity does not adequately explain their “addiction to alcohol”. In Experiment 2, Long-Evans rats were trained in the sign tracking procedure with or without ethanol in the bottles as in the first experiment, but all rats were also given 24-hr access to ethanol in their home cage. Treatment with the bacterial endotoxin lipopolysaccharide (LPS) significantly increased the rats' preference for ethanol, nevertheless this greater liking for ethanol did not affect the sign-tracking of ethanol. Therefore the compulsive ethanol drinking in the Long-Evans rats, as in the Sprague Dawley rats in Experiment 1, appeared to be due to sign tracking procedure, rather than the rewarding properties of the ethanol. However, in contrast to the Sprague-Dawley rats the negative-feature discrimination task revealed substantial impulsivity of
sign tracking behavior in the Long-Evans rats. The results of both experiments suggest that environmental influences appear to have a profound impact on sign tracking performance and the responsiveness to ethanol but more research is needed to further evaluate the usefulness of the sign tracking of ethanol as an animal model of alcoholism and the underlying mechanisms that contribute to the alcoholic phenotype.
The influence of environmental experience on the sign tracking of ethanol: A rodent model of alcohol addiction

Alcohol, otherwise known as ethanol (EtOH), is frequently consumed for enjoyment and the reduction of social anxiety while in social situations (Enoch, 2006). Alcohol typically affects 5 main neurotransmitter systems in the brain. These five systems are the glutamate, GABA, dopamine, serotonin, and opioid systems. With glutamate, alcohol typically affects the NMDA receptor which binds this neurotransmitter. Alterations of this receptor during light drinking affect memory, but persistent heavy drinking will cause brain damage. GABA is partially responsible for the visible behavior effects of intoxication, and is integral in developing the tolerance of alcohol. Serotonin contributes to arousal and is responsible for consummatory behavior, which includes alcohol consumption. Dopamine and the opioid systems contribute to the pleasurable feeling of alcohol consumption and are found to increase during consumption while decreasing during withdrawal. Since the pleasurable feelings depart when the alcohol departs, this leads some people to abuse alcohol (Chastain, 2006).

Alcohol abuse and addiction have been found to typically develop while a person is in adolescence and later continue throughout adulthood (Enoch, 2006; Walker & Ehlers, 2009). There are three stages to the addiction cycle. These stages are the anticipation, binge drinking, and withdrawal stages. The anticipation stage is characterized by the fixation or sensitization toward a drug due to the intermittent presentation of the drug. The binge drinking stage occurs when the individual drinks to the point of intoxication due to dependence on the drug or due to other motivating...
pressures, like stress. The withdrawal stage is distinguished by a negative affect due to the body’s desire to re-experience the drug (Koob, 2000).

The introduction of alcohol to an individual for the sake of research would be a questionable practice, so rodent models are typically used to learn more about alcohol. Rodent models are often used because there are some common physiological elements they share with humans. The stages of addiction may be replicated in rat models to learn more about the underlying processes of alcohol addiction, provided that rats can overcome the aversive taste of alcohol (Koob, 2000). One promising model of alcohol addiction is the sign tracking model, which presents a good model of the anticipatory stage of alcohol addiction. In the sign tracking model, rats are trained to consume ethanol by pairing brief presentations of a bottle with food pellets. Sign tracking will be described in further detail later in this introduction.

Through research with animals and humans, many factors have been found to contribute to alcohol use and abuse. These factors include stress, genetics, behavioral (sensitization or impulsivity), and environmental factors like rearing conditions and exposure to potentially harmful substances. In human studies of adolescents, stress was found to diminish the reward system, affect the prefrontal cortex of the brain, and impair hippocampal development which in turn makes adolescents more responsive to addictive drugs. Three factors that contribute to the enhanced alcohol addiction of adolescents are the physiological changes within the prefrontal cortex during this time period which promotes risk taking behavior, neurobiological vulnerability, and the stress induced sensitization of the hypothalamic pituitary axis (HPA). (Andersen & Teicher, 2009; Enoch, 2006) Additionally, addictive drugs such as EtOH share neural mechanisms with
natural rewards. There is strong evidence that the pharmacologic effects of EtOH induce changes in the experience of rewarding stimuli, such as social and physical pleasure, to make these positive experiences feel more enjoyable (Tomie, Grimes, & Pohorecky, 2008).

Genetic factors responsible for alcohol abuse include the MET158 variant of the Catechol-o-methyl transferase (COMT) gene which was found to be linked with susceptibility to alcohol. However, an individual with the alcohol vulnerability COMT gene is not doomed to abuse alcohol, because the environment that a subject is raised in (rearing conditions) interacts with the potential to develop addiction. This interaction is affected by many neurotransmitters. Specifically, the neurotransmitter serotonin has been implicated in the control of impulsivity, which is one of the many behavioral factors that contribute to alcohol abuse. Impulsivity is described in further detail later in this introduction. Additionally, early environment's (rearing conditions) diverse impact on behavior is described in further detail later in this introduction.

Finally, immune system activation is a potential factor for alcohol addiction. Although there is not much research on the role of the immune system in alcohol addiction, several observations suggest a potential role of neuro-immune interactions in drug abuse. Research with humans has found that there is a high prevalence of HIV positive individuals that abuse drugs (Ferrando, 2001). Research with rats has found that HIV transgenic rats show a greater methamphetamine-induced behavior sensitization than control F344 rats. Although HIV-1 transgenic rats do not have HIV-1 infection, the HIV genes that have been inserted into the rat genome produce HIV proteins (e.g., gp 120) that affects immune system functioning (e.g., increased cytokine levels) which in
turn affects neuronal functioning. Greater sensitivity of HIV-1 Tg rats to methamphetamine may be due to the greater dopamine expression in the prefrontal cortex of the HIV rats (Liu, Chang, Vigorito, Kass, Li, & Chang, 2009). Research with alcohol preferring mice found that an intraperitoneal injection of 1 mg/kg of lipopolysaccharide (LPS) promoted higher alcohol consumption, with the effects lasting three months after the injection (Blednov, Benavidez, Geil, Perre, Morikawa, & Harris, 2011). LPS is a protein found in bacterial walls that when detected by the immune system activates an innate immune defense. The Blednov et al study suggests that a single immune system activation is sufficient to cause long term changes in neuronal functioning and subsequent EtOH consumption.

The purpose of the following experiments was to explore the effects of two environmental factors on the sign tracking of EtOH in rats: rearing condition (Experiment 1) and exposure to bacterial insult (Experiment 2). Additionally, modifications of the sign tracking procedure were introduced to further evaluate sign tracking as an animal model of compulsive alcohol use and abuse. Several studies suggest that like excessive alcohol use, sign tracking behavior is associated with impulsivity (Tomie et al, 2008). Thus, in the following experiments modifications of the sign tracking procedures were included as potential measures of impulsivity.

Sign Tracking, Incentive Sensitization, and Drug Abuse

Sign Tracking

Sign tracking procedures are characterized by the pairing of a conditioned stimulus (CS) with the prompt delivery of an appetitive (e.g. food) unconditioned
stimulus (US). These procedures represent a variation on the Pavlovian “classical” conditioning paradigm because the CS and the food US occur independent of the subjects’ behavior. After animals have learned to associate the CS with the US, conditioned responses (CR) of anticipatory behavior develop that are classified as goal tracking or sign tracking. Goal tracking, which is the typical response to a Pavlovian conditioning paradigm, refers to the animals’ use of the signal CS solely as a means of tracking the impending arrival of the reward US, with the anticipatory behavior being directed at the US. For example, goal tracking behavior is monitored by counting the number of breaks in an infrared beam that occur when the animal inserts its head in the food tray. Sign tracking is distinguished from goal tracking by the animals’ tendency to primarily track and direct its anticipatory behavior at the signal instead of the goal US (Robinson & Flagel, 2009). In sign tracking studies with birds, for example, investigators measure anticipatory pecks that birds direct at a key light CS. Rats will also show anticipatory approach and investigatory behaviors toward a light CS. Sign tracking behavior was originally erroneously called autoshaping by Brown and Jenkins (1968) because they believed that the behavioral fixation on the signal for food was due to superstitious (operant) conditioning. This superstitious conditioning implies that the animal fixated on the signal because its interactions with the signal seemed to produce the US, and satisfy a perceived operant behavioral requirement. Several sign tracking studies have demonstrated that the animal’s behavior will persist even when the USs are omitted on a substantial percentage of the trials, which suggests that the animal responding is not an operant response (Monterosso & Ainslie, 1999). The term “autoshaping” is more often referred to as sign tracking to reflect a more conceptually accurate representation of
its relationship to classical conditioning, i.e. sign CS predicting US. Unfortunately, some investigators continue to use the conceptually inaccurate term “autoshaping” when describing this procedure.

Sign tracking behavior can be manipulated to produce more profound CR in rats by using a signal that rats may interact with using their paws and teeth rather than a light that can only be observed by the rat. Replacing a light CS with a retractable lever CS, for example, causes many rats to direct their anticipatory behavior towards the lever. Some investigators have even added bars to the testing chambers. With the bar addition, the rats will direct their anticipatory (sign tracking) behavior of nosing, biting, or pressing to the bar. Often the rat will press the bar sufficiently enough to close a switch. With this modification, investigators will typically count the number of bar presses as an index of sign tracking behavior.

Sign tracking behaviors reveal that once the neutral signal gets associated with the positive stimulus of food the neutral signal gains its own motivational qualities or incentive salience for the animal. Not long after the neutral signal has gained incentive salience, most rats develop compulsive behaviors toward the signals. These compulsive-like behaviors, also known as incentive sensitization, which emerge through conditioning in a sign tracking procedure, can also occur in traditional operant or instrumental conditioning procedures (Robinson & Berridge, 2008).

The concept of incentive sensitization is explored in an addiction model in which a distinction is made between drug liking (the high) and drug wanting, i.e., the craving (Robinson & Berridge, 2008). This model parallels the finding that over the course of
developing addiction and with repeated exposure, there is a marked increase in drug wanting while there is either no change or a small decrease in drug liking. This may be due to different neural mechanisms being responsible for the two components of drug reward, and because repeated use causes a sensitization of the “wanting” system but no sensitization or even tolerance in the “liking” system. It is theorized that the mesolimbic dopamine system can be sensitized by repeated administration of many abused drugs and that this neural circuit may be more important in drug wanting than in drug liking (Robinson & Berridge, 2008).

Research indicates that individual differences in the tendency to sign-track (focused anticipatory behavior) are connected with the different tendencies to attribute incentive salience to distinct reward-related cues (Flagel, Watson, Akil, & Robinson, 2008). This suggests that sign-trackers are prone to a form of plasticity (addictive phenotype) that may contribute to the development of addiction (Robinson and Berridge, 2000, 2001; Saunders & Robinson, 2010), which in turn parallels the finding that drug abusers are individuals predisposed to develop pathological levels of incentive salience attributable to reward-related cues (Tomie et al, 2008).

Within a different exploration of compulsive behavior, Tomie (1996) introduced the concept of “Cue and Manipulandum” (CAM). Cue refers to the CS or the predictive object, and manipulandum refers to an interactive object. Essentially, CAM represents an alternative method of describing incentive sensitization. In the typical operant conditioning experiment the subject is required to act on a manipulandum to obtain a reward. The reward (and cues associated with it) is usually located at a distance from the manipulandum. CAM occurs when the experimenter puts a reward cue very near or on an
object that must be manipulated during an instrumental response. This close spatial relationship between the manipulandum and reward cue facilitates a compulsive response toward the manipulated object. The compulsive and excessive behaviors persist even though they serve only to delay or prevent the delivery of reward. Tomie found that although the operant procedure required that the subject simply make a response then retrieve the reward, the close proximity of reward cues with the manipulandum induced sign tracking of the manipulandum which interfered with the simple operant requirement. This finding indicates that the sign-tracking CR performance is not under strict voluntary control. Furthermore, Tomie’s findings suggest that the animals’ maladaptive behavior in the CAM situation is due to conditioning and not poor self-regulation (Tomie, 1996).

Tomie (1995) suggests that the exaggerated responses to objects can also be found in humans that consume drugs (a reward) using only one method of administration (like an alcoholic to a beer glass) or when the object that administers the drug is directly related to the drug’s reinforcing effects (like the consumption of a drug in pill form). These compulsive behaviors are also acknowledged by other addiction researchers as being reminiscent of the fixated behavior that drug addicts exhibit toward their desired paraphernalia of administration. Additionally, addiction researchers suggest that these behaviors are typically activated by subjective emotional or motivational states that contribute to the impulse use of the drug, which in turn enhances the likelihood of drug consumption (Tomie et al, 2008).

Tomie suggested that the sign tracking procedure can be modified to more closely model the acquisition of compulsive behaviors directed toward drug-delivering paraphernalia in humans by replacing the retractable lever in the sign tracking procedure
with a bottle. Thus, a rat sign tracking a bottle will lick at the spout and therefore self administer any drug contained within the bottle.

Tomie’s (2005) study found that repeated intermittent presentations (sign tracking procedures) of an ethanol sipper tube induced more ethanol intake than did continuous access to the EtOH sipper tube. Also more gross motor activity was found in an intermittency condition than in a continuous access condition, which is perhaps indicative of higher levels of arousal. Therefore, one factor causing excessive responding in sign tracking is the experience with repeated insertions and retractions of the sipper tube which induces a state of arousal or sensitization, increasing the likelihood that an active rat would contact and drink EtOH from a sipper tube. Tomie also found that although random presentations of the bottle and food US do generate sign tracking behavior, paired bottle-US presentations produce significantly greater sign tracking. This indicates that behavior directed toward the bottle increases further when the bottle becomes a signal for the US. Thus, EtOH intake in the sign tracking procedure appears to be due to intermittency-induced arousal plus Pavlovian CS-directed responding (Tomie, Gittleman, Dranoff, & Pohorecky, 2005; Krank, 2003).

Thus, the sign tracking procedure using a bottle as the CS has three components that appear related to drug use and abuse. First, individuals prone to drug abuse (addictive phenotype) are more likely to respond to the intermittent presentations of the bottle, resulting in compulsive responding toward the bottle that approximates addictive behavior. Second, the presence of a Pavlovian relationship between the bottle CS and food US attaches incentive salience to the bottle signaling the reward US further increasing bottle-directed behavior. Finally, when the bottle contains a drug, the
compulsive behavioral interaction with the bottle signal may further contribute to the maintenance of the compulsive behavior since the interaction with the bottle results in administration of a drug (e.g. alcohol).

Behavioral Sensitization and Drug Abuse

Another way that sign tracking behavior is related to drug abuse is through behavioral sensitization. Behavioral sensitization is demonstrated as an increase in the locomotor-stimulating effects of a drug, such as amphetamine, after repeated exposure to a consistent drug dose. The increased sensitivity to the drug with repeated experience is believed to be a determinant factor of addictive behavior in rats and humans, and may be a result of direct changes in the circuitry of the brain. Neuroimaging studies describe prefrontal activity alterations and striatal activity alterations resulting from behavioral sensitization. It is believed that altered prefrontal activity, as evidenced by problems with emotional stress regulation and inhibitory control, along with heightened striatal responses to addicted drug and drug-related salient stimuli perpetuate habitual drug seeking (Li & Sinha, 2008; Feil et al, 2010). Sign tracking responses and the psychomotor activation syndrome appear to be similar behavior because both behavior types are skeletal-motor responses. Skeletal motor responses include forward locomotion actions as well as directed approaches that include contact and manipulation responses, which culminate in consummatory-like responses, such as gnawing, licking, sniffing, chewing, and swallowing (Tomie et al, 2008). Thus the increase of sign tracking behavior as a result of repeated exposure to paired CSs and USs may be related to the increase in drug induced behavior (sensitization) as a result of repeated drug exposure.
Evidence of a relationship between sign-tracking and psychomotor sensitization has also been reported. In rats, sensitization has been shown with many stimulant drugs (e.g., cocaine) as well as with morphine. Although it has been more difficult to demonstrate behavior sensitization with EtOH, cross sensitization has been shown between EtOH and morphine (Nestby et al., 1997; Herz, 1997). Cross sensitization is the experience in which an individual is initially sensitized to one substance (morphine) that consequently sensitizes the individual to a different substance (EtOH). This is usually due to a relationship between substances, such as similar neurobiological effects. The cross-sensitization between EtOH and morphine may be mediated by a common interaction on the opioid system. Evidence that there is a “cross-sensitization” between sign tracking and stimulant induced sensitization comes from a study reporting that rats that develop predominant sign-tracking behavior show an enhanced tendency to exhibit psychomotor sensitization to cocaine, when compared to rats that develop predominant goal-tracking behavior (Flagel et al., 2008).

Previous studies of the nucleus accumbens core (NAC) of the brain demonstrated that the crucial structure for sign tracking is the same structure that is implicated in drug relapses within addiction. Flagel et al suggested that sign-trackers are susceptible to a form of plasticity that may contribute to the development of addiction. In support of this, Flagel et al also reported that predominant sign trackers exhibited higher levels of D1 mRNA in the NAC relative to predominant goal-trackers after the first day of training with sign-tracking procedures (Flagel, Watson, Robinson, & Akil, 2007), but after 5 days of training, sign-trackers showed dulled dopaminergic expression patterns relative to goal trackers, including lower levels of tyrosine hydroxylase, dopamine transporter, and
dopamine D2 mRNA relative to goal-trackers (Flagel et al., 2007). These data are consistent with the hypothesis that behavioral changes induced by sign-tracking procedures are related to changes in the dopaminergic system, in a manner well-known by addiction researchers. Furthermore, levels of the D1 receptor was found to be integral for sign track learning (Dalley et al, 2005) and levels of the D2 receptor have been associated with increased reports of "drug-liking" in humans (Volkow et al., 2002).

**Impulsivity and Drug Abuse**

Impulsivity is closely related to drug use and abuse, both as a contributor to use and as a result of use. Impulsivity has been used to refer to a wide range of seemingly unrelated maladaptive or inappropriate behaviors including the inability to wait, difficulty in withholding responses, excessive presence of non-rewarded responses, and insensitivity to negative or delayed consequences of responding. As a trait, impulsivity is a risk factor for drug experimentation, problematic drug use, and contributes to the inability to refrain from drug use. Brief fluctuations in decision-making or inhibition may have especially negative consequences for drug users who are trying to abstain from drug use, because momentary lapses in control or inhibition could increase the risk of drug use. Extended exposure to a drug may also result in impaired inhibitory capacity, which may be due to long-term neurological damage from chronic drug use (de Wit, 2009).

Drug addiction has specifically been related to impulsivity by studies reporting that rats that are intolerant of reward delays subsequently self-administer more EtOH than do delay tolerant rats (Poulos, Le, & Parker, 1995; Poulos, Parker, & Le, 1998). Poulos et al have shown that rats, exhibiting intolerance to reward delay by choosing small
immediate rewards over larger delayed rewards, subsequently consumed more EtOH than rats that were less delay-intolerant. Their work revealed that impulsivity and EtOH drinking are linked phenomena (Poulos, Parker, & Le, 1997), and provides support for the hypothesis that rats that perform more sign-tracking CRs tend to be more impulsive and drink more EtOH (Tomie et al, 2008).

Impulsivity’s link to sign tracking was tested by Tomie through the use of a delay-discounting (impulsive choice) test. Impulsivity was tested by using a two-choice lever-press operant procedure. In this procedure, the rat had a choice between two levers that could be pressed. The left lever would be readily available and if pressed would generate an immediate small reward of one pellet, while the right lever would be available less frequently but if pushed would generate a three to five pellet reward. Rats that demonstrated prior predominant sign tracking behavior were more impulsive-like and would respond to both levers, while goal trackers primarily responded on only one lever. Additionally, during sign tracking sessions the adaptive (impulsive) group acquired sign tracking faster, and with more CR than the rigid strategy group. Impulsivity was also reported after injections of dopamine agonist-like compounds such as cocaine, amphetamine, and methamphetamine (Tomie, Aguado, Pohorecky, & Benjamin, 1998).

There have not been too many studies on the strain differences in impulsivity, but one study did explore how Lewis (LEW) and Fischer (F344) rat strains differ on a number of physiological characteristics, such as hypothalamic–pituitary–adrenal (HPA) axis activity, as well as on behavioral tasks, including the sign tracking task (Kearns, Gomez-Serrano, Weiss, & Riley, 2006). Since sign tracking has been linked to HPA axis functioning, impulsivity and drug taking, Kearns et al compared LEW and F344 rats on
their rate of attainment and presentation of the sign tracking responses. Rats were trained on a negative automaintenance procedure. In the negative automaintenance procedure, the rat was first trained on the sign tracking procedure. Later, the sign tracking procedure was changed so that the sign tracking responses toward the manipulandum (interactive object) were then punished by the cancellation of the food pellet delivery. While sign tracking behaviors were diminished in the negative automaintenance procedure, they were usually not eradicated entirely (Monterosso & Ainslie, 1999). The animals that were affected the least by the “punishment” were seen as being more impulsive. While there were not significant differences between strains within the negative automaintenance procedure, LEW rats did acquire the sign tracking response faster and performed the sign tracking response at a superior rate to the F344 rats. This is consistent with existing research that indicates that LEW rats behave more impulsively, are more sensitive to the rewarding effects of drugs, and more readily self-administer drugs of abuse than F344 rats. These findings also indicate that the HPA axis may have a modulatory impact on sign tracking behavior.

Measures of impulsivity

Impulsivity is a multi-dimensional construct, with various impulsivity measures reflecting separate underlying processes. One process is impulsive choice which is measured by the delay discounting procedure that measures impulsive choice and behavior disinhibition as described above (de Wit, 2009). Another process includes impulsive response-inhibition, such as responding on a schedule which measures the inability to withhold a response (e.g. Differential Reinforcement of Low Rates procedure) (de Wit, 2009; Monterosso & Ainslie, 1999). An additional impulsive process is
Impulsive action, which is measured in the negative automaintenance procedure also described previously (Killeen, 2003). A different impulsive process is non-discriminated appetitive conditioning, which is measured by conditioned locomotor activity that demonstrates behavior disinhibition (de Wit, 2009; Winstanley et al., 2004). In non-discriminated appetitive conditioning, rats are fed at the same time each day and their locomotor activity is assessed. Typically, an increase in activity is found to be present prior to the expected delivery of food which represents a lack of behavioral inhibition. This increase in activity is due to the association between the specific time of day and the food delivery (Winstanley et al., 2004). In each of these paradigms, impulsivity is implicitly or explicitly associated with the effect delay has on the value of reward (Monterosso & Ainslie, 1999).

In the present experiments we evaluated the negative-feature discrimination procedure as a potential measure of impulsivity. In this procedure, a target conditioned stimulus (bottle) is paired with a food US as usual, but in the presence of a negative-feature stimulus (a light, smell, or sound) the bottle CS is not followed by the food US. The ability to use the negative-feature to predict that the US will not occur is known as negative-feature discrimination. This task is used in this study as an impulsivity metric to investigate whether there are differences in the acquisition of negative-feature discrimination between sign tracking rats with different environmental experience. If sign tracking rats in one condition are more impulsive than sign tracking rats in another condition, they may show poorer acquisition of the negative-feature discrimination.
Environmental Influence of Rearing Condition

Flagel et al (2010) have noted that rats selectively bred for high responsivity to environmental novelty are almost exclusively sign-trackers in appetitive conditioning procedures and rats selectively bred for low responsivity to environmental novelty are almost exclusively goal-trackers. When these rats were used in sign-tracking procedures with a cocaine US, the same results were found. The high-responders toward novelty all acquired predominant sign-tracking CR performance, while none of the low responders did so. Thus, the high responsivity phenotype exhibits predominant sign tracking in procedures employing either food US or cocaine US, while the low responsivity phenotype does not exhibit sign-tracking to signals for either food US or cocaine US.

Since high responsivity toward environmental novelty is typical behavior of rats raised in enriched housing conditions, the investigation of rearing conditions on sign tracking behavior in the presence of EtOH presents an exciting avenue of exploration. The two main rearing conditions are the standard rearing condition and the enriched rearing condition. The idealized standard rearing condition of rats often consists of the inclusion of two rats in a cage with no other stimulation at their disposal, while the idealized enriched environment rearing condition might consist of the housing of several rats (typically 4 or 5) in larger than average sized cages that contain various stimulating items such as running wheels, tunnels and small toys which may be altered on a regular basis. Studies of enriched environments have demonstrated that the enriched condition brings on various neurobiological and behavioral modifications which may have an impact on drug sensitivity and addiction (Laviola, Hannan, Macri, Solinas, & Jaber, 2008).
Findings from studies on environmental enrichment suggest that this condition might act on precise brain regions that handle responses to novelty or conflict (such as the hippocampus, amygdala, and the cingulate). Additionally, environmental stimulation, especially applied throughout adolescent development, adjusts the neurobehavioral systems as is evident in learning, memory and defensive responses (Laviola et al, 2008). The behavioral modifications include, amongst other things, the decrease of anxiety-like behavior. This adjustment change highlights the continued plasticity of the systems mediating emotion beyond the age of weaning and demonstrates the importance of an animal’s physical environment (Holmes, le Guisquet, Vogel, Millstein, Leman, & Belzung, 2005). This type of adjustment plasticity might be the reason why environmental interventions protect against the effects of genetic and/or acquired vulnerabilities (Laviola et al, 2008).

Previous drug research with rats has shown that rats reared in an enriched condition are more sensitive to the acute effects of amphetamine (dopamine agonist) than rats reared in an isolated condition (Green et al, 2010). Yet, enriched condition rats self-administer less amphetamine than isolated condition rats (Brenes & Fornaguera, 2008), which contrasts the results of an experiment with voluntary EtOH intake that indicated that enriched animals consumed greater amounts of EtOH than isolated animals within a two bottle (EtOH vs. water) preference task (Rockman, Gibson, & Benarroch, 1989). In an effort to corroborate the different accounts, one study used cocaine to further explore the environmental enrichment behavioral phenotype. For this study, enriched condition and isolated condition rats were studied with a cocaine conditioned place preference (CPP) behavior test while cocaine self-administration was measured. Enriched condition
rats exhibited less cocaine self-administration, despite showing enhanced cocaine CPP. It appears that this is because the enriched condition rats exhibit a protective phenotypic plasticity against addiction (Green et al., 2010). Nevertheless, this effect is paradoxical because enriched rats are more sensitive to the locomotor-activating, dopamine-releasing, and rewarding effects of drugs. Therefore, environmental enrichment seems to diminish addiction liability without decreasing drug sensitivity (Green et al., 2010). Essentially, rats would be expected to show the sensitization towards drugs during use (as measured by sign tracking), without the addictive preference for drugs (as measured by self-administration procedures such as the previously mentioned preference task).
Experiment 1

Rearing Conditions

Sign tracking behavior is believed to be strongest in rats with a high propensity of assigning incentive salience to stimuli associated with rewards, as is typically found in the addictive phenotype (Tomie et al., 2008). Thus, this experiment sought to assess the effect of rearing condition (enriched vs. standard) on sign tracking of a bottle filled with EtOH or with water. The observation that an enriched environment changes sign tracking performance suggests that rearing conditions modulate the addictive phenotype (Laviola et al., 2008). Since groups that differ in susceptibility to sign tracking also differ in measures in impulsivity (Tomie et al., 2008), we tested the animals in a negative-feature discrimination task as a potential measure of impulsivity. More impulsive rats are expected to show poorer discrimination than less impulsive rats because discrimination tasks require that rats learn to inhibit conditioned responding (i.e., licking the water bottle) on days when the bottle is not followed by the food US.

Moreover, by comparing sign tracking of EtOH with sign tracking of water it is possible to determine if the additional consumption of the addictive drug EtOH while sign tracking further enhances sign tracking behavior. For example, it is possible that rats drinking EtOH will show greater sign tracking than the rats drinking water because the EtOH has become rewarding and has motivated the rats to consume more EtOH. In addition to looking for greater sign tracking in EtOH-exposed rats, we also took advantage of the negative-feature discrimination procedure to evaluate the rewarding properties of ethanol. If the rats drinking ethanol find the ethanol to be rewarding then
they should not show discrimination because the solution in the bottle is motivating their drinking, not just the bottle as a signal for the food pellet US. Thus rats sign tracking water should show discrimination, but not rats sign tracking ethanol if the EtOH is itself rewarding. To evaluate the effectiveness of this strategy, some rats were given a highly preferred Polycose solution in the bottle they were tracking. It is well known that rats find Polycose highly rewarding. Therefore rats sign tracking a Polycose solution should not show discrimination since drinking from the bottle is motivated by the Polycose and not just the food pellet US.

Method

Subjects

The subjects were 17 male Sprague-Dawley rats from Harlan (Indianapolis) that were born on November 3, 2009 and were previously used in other experiments. All rats within this experiment were previously used in fear conditioning and morphine conditioned place preference experiments. All rats had experienced morphine treatment in the previous experiment, thus it was not necessary to counterbalance rats when assigned to the present experiment. Eight rats had been housed in enriched environments in groups of four since rats were 6 weeks of age. These enriched environments consisted of weekly toy rotation and 15 minute rodent handling. The other seven standard rats were housed in pairs within shoebox cages. These housing conditions were maintained throughout the experiment except the last 2 weeks, when the enriched environment rats were transferred in pairs to shoe box cages to free the enrichment cages for other experiments. All rats were maintained on a 12 hour light-dark cycle, with the light
turning on at 8 am. All rats were given water and food *ad libitum*, with one exception. Standard rats experienced a 7 day food deprivation via daily 1 hour food access during the first 7 days of EtOH’s introduction into the sign tracking paradigm. One standard rat was dropped from experiment prior to EtOH introduction to reduce running time of experiment. This experiment was approved by Seton Hall’s Institutional Animal Care and Use Committee. All guidelines for the care and use of rats set by the United States Public Health Service were firmly followed.

**Apparatus**

**Sign Tracking Chambers**

Rats were trained in four standard (23 x 18 x 23.5 cm) operant conditioning chambers that were modified to accommodate a retractable bottle. The four testing chambers were constructed similarly, but there were some differences. All chambers had cue lights and a lever that were located on the same metal wall as the food tray, but they were not used for these experiments. Additionally, there were speakers located between the two pairs of sign tracking cages that provided background white noise for these experiments. All equipment was controlled by programs written in MedPC (Med Associates Inc., St. Albans, Vermont).

Chambers 1 and 2 have cue lights located on the top left of one of the metal walls 10 cm above the grid floor. The lever is located in the middle of the same metal wall as the cue lights 9.5 cm above the grid floor. The food trays are approximately 4.3 cm x 4.3 cm, and are located in the middle of the same metal wall 2.5 cm above the grid floor. Chambers 3 and 4 have cue lights located on the top left
of one of the metal walls 8.5 cms above the grid floor, and 2.5 cms above the food tray. The lever is located in the middle of the same metal wall as the cue lights 9 cms above the grid floor. The food trays are approximately 5 cm x 5 cm, and are located in the left (2.5 cm away from plastic wall) of the same metal wall 1 cm above the grid floor. All four chambers were installed with a retractable bottle mechanism from Med Associates on the plastic wall closest to the food tray. A hole in the plastic wall that received the bottle sipper tube was approximately 2.5 cm from the grid floor. The bottle was retracted between trials. During CS presentation, the bottle was advanced so that the sipper tube was flush with the plastic wall so that the rat could lick the sipper tube but not touch it with its paw. This permitted the monitoring of lick rates. The rats' approach to the US in the food tray (i.e., head pokes) were recorded with infrared sensors from Med Associates that are attached to the clear sides of the food tray. Lickometers from Med Associates that were connected to the bottle sipper tubes and also to the grid floor were used to monitor licks.

**Holding Cages**

Each day, prior to testing in the sign tracking chambers the rats were placed in suspended stainless steel mesh cages (20.3 cm x 20.3 cm x 22.9 cm) in the sign tracking room for a waiting period of about 5 minutes. These cages were also used for acceptance and preference tests by mounting one (acceptance tests) or two (preference tests) bottles to the front of the mesh cages.
Procedure

Rats were weighed daily and tested 5 days a week, Monday to Friday, during the early afternoon. The rats were tested in squads of four. The rats were carried to the testing room and placed in the holding cages for approximately 5 minutes before being transferred to the sign tracking chambers. The bottles used in the sign tracking procedures were weighed before and after a session to determine the rats' intake in grams. The start of a session was signaled by the onset of a white noise. At the end of a session, the white noise was turned off and the rats were returned to their home cages.

Phase 1 - Adaptation and magazine training

In order to adapt the rats to the chambers, the rats were placed in the chambers for 15 minutes with five 45 mg sucrose pellets (P.J. Noyes Company, Lancaster, PA.) in their food trays. If all of the pellets were not consumed, the rats would be exposed to a day of magazine training. In the magazine training, the rats would be placed in their chambers for 15 minutes with pellets being dispersed after each minute. This magazine training would train the rat to associate the magazine's clicking with the presentation of food. If the rats were having trouble making the associations, the rats would be exposed to another day of magazine training. The rats received 2 days of adaptation training before being introduced to sign track training.

Phase 2 - Induction of sign tracking and goal tracking

All rats were initially exposed to 10 days of sign track training with water in the bottle. During training, the bottle (CS) was presented for 10 seconds followed immediately by the disbursement of a 45 mg sucrose pellet (US). After an intertrial
interval (ITI) of 60 seconds, the CS-US was presented again for a total of 30 trials. Since the standard-housed rats took longer to develop sign tracking behavior, they experienced 10 additional days of sign track training with water (total of 20 days) before being switched to EtOH.

Phase 3 – Introduction of Ethanol

In the next phase water was replaced with EtOH for 4 rats in the enriched condition and 4 rats in the standard condition. The other half of the enriched- and standard-housed rats would continue with water to serve as controls. Because it was unclear how sign tracking performance would proceed, and we were interested in getting the EtOH rats to consume as much EtOH as possible, the four most proficient sign trackers were given EtOH and the remaining rats were given water. EtOH started at 1% concentration, and gradually increased to 9% concentration in one to three day increments dependent on rat performance. The enriched rats reached 9% concentration, while the standard rats stopped at 6% concentration. The enriched rats were then reduced to 6% concentration for direct comparisons of 7 days of sign tracking performance. During this phase some additional minor manipulations were introduced as pilot tests of dishabituation (4 days) and spontaneous recovery (4 days). Dishabituation tests consisted of a single presentation of a stimulus change (e.g. room lights off) prior to the 23rd trial of a session. Spontaneous recovery involved testing the animals twice in the same day with varying delay intervals between tests. These manipulations did not affect sign tracking performance and will not be reported in this thesis.
Acceptance and Preference Tests

After the completion of Phase 3 all rats were tested in one bottle, 20 minute acceptance tests within the holding cages. To adapt the rats to drinking in these cages they were given several days to drink a highly preferred Polycose solution from 100 ml plastic graduated cylinders (results will not be reported) followed by 1 day with 3% EtOH solution, 1 day with 6% EtOH solution, and 1 day with 9% EtOH solution. The acceptance tests were followed by 4 days of 20 minute, two-bottle preference tests. The preference test assesses the rats’ choice and consumption of either a water solution or an EtOH solution. Greater preference for EtOH suggests that ethanol has gained rewarding value. There were 2 days with 3% EtOH solution followed by 2 days with 6% EtOH solution. The position of the bottle with EtOH was reversed across days.

Phase 4 – Negative-Feature Discrimination

A negative-feature discrimination task was introduced as a potential measure of the differences in impulsivity between the different rat conditions and as a second measure of the rewarding properties that may have accrued to the EtOH. The sign tracking procedure was continued during this phase, but with two changes made to the procedure. First, pellets were omitted on half of the days and a cue (the “negative-feature”) would be added to signal the absence of the pellet US. Second, the trials were reduced from 30 trials to 15 trials in order to limit the possibility of behavior extinction. On the days of food omission, an odor stimulus was added to signal the omission of food. This odor stimulus was a vanilla dryer sheet that was placed in the tray below the grid floor. Days with food are designated A+, while days without food are designated A−.
The days of food omission were chosen randomly, with no more than two consecutive days with the same condition for a total of 10 days. Specifically, the sequence of days was: A+, AB-, A+, AB-, A+, AB-, A+, AB-, AR-, and A+. Additionally, the bottle liquids were changed to be 1 of 4 possible combinations. The four possible solutions were 5% Polycose solution, 7% EtOH and 5% Polycose mixture, 7% EtOH, or water.

Data analysis

The primary independent variables in each phase are the housing condition (enriched vs. standard housing condition) and the days of training, with housing condition as a between-subjects factor and days as a within-subjects factor. When EtOH was in the bottles the type of Solution in the bottle (EtOH or water) was a between-subjects factor, and the EtOH Concentration (days at ethanol concentration vs. same combination of days with water) was a within-subjects factor. During negative-feature discrimination training (Phase 4) an additional independent variable was responding in the presence (A+) and absence (AB-) of the negative-feature (within-subjects). The dependent variables were licks and milliliters of solution consumed for measures of sign tracking, while head pokes was the dependent variable for measures of goal tracking. The dependent variables for each phase were analyzed by separate ANOVAs followed by post hoc comparisons using SPSS. The Phase 2 and Phase 3 data were analyzed as Rearing Condition (2) x Days mixed factorial ANOVA. In Phase 3 and 4 (when EtOH is introduced) the data were analyzed with a Rearing Condition (2) x Ethanol Concentration (6 or 9) x Solution (2) mixed ANOVA. The negative-feature discrimination data were analyzed with a Rearing Condition (2) x Days x Negative-feature (2) ANOVA. Additional ANOVAs were calculated as needed.
Results and Discussion

Starting with the first 10 days of sign tracking acquisition with water, the enriched rearing condition had begun to show an impact in sign tracking acquisition. As seen in Figure 1, the sign tracking performance as demonstrated by licks (or approaches to CS) show performance differences which began at Day 5 of training. A Rearing Condition (2) x Days (10) mixed factorial ANOVA revealed a significant interaction of Days x Rearing Condition, $F(9,135) = 8.023, p < .001$. Thus, the findings suggest that enriched rats acquired and demonstrated more pronounced sign tracking behavior than standard rats.

Concurrently, with sign tracking acquisition the rats also showed evidence of classical conditioning as demonstrated by head pokes into the food tray during the presentation of the bottle CS as shown in Figure 2. This conditioning is seen by comparing head poking 10 seconds prior to CS period (Pre-CS), during the CS, and the 10 second period following the CS (Post-CS). Evidence that the bottle CS was associated
with the food pellet US is indicated by greater responding during the CS compared to the Pre-CS period. Typically conditioned responding continues into the Post-CS period before declining later in the ITI. A Rearing Condition (2) x Time Period (3) x Days (10) mixed factorial ANOVA revealed a significant interaction, $F(18,270) = 3.072, p < .001$. This interaction was evaluated with t-tests for further interpretation.

T-tests revealed no significant differences between the Pre-CS and CS head pokes on Day 1 with the enriched, $t(7) = -2.317, p > .05$ or standard, $t(7) = 4.085, p > .05$ rat groups. But, by Day 2 the CS head pokes was significantly greater than Pre-CS head pokes with the enriched, $t(7) = -2.768, p < .05$ and standard, $t(7) = -3.022, p < .05$ rat groups indicating conditioned head poking. With repeated days, head poking in the CS declined in the enriched rats, but not the standard rats. By Day 10 the head pokes no longer differed between the Pre-CS and the CS periods in the enriched rats, $t(7) = 1.231, p > .05$ but continued to differ in the standard rats, $t(7) = 4.277, p < .05$. This decline in the enriched rats was due to the much greater increase in sign tracking in the enriched rats compared to the standard rats.
Figure 3 shows the mean consumption of EtOH or water in milliliters for the rats raised in both rearing conditions in Phase 3 when the EtOH rats were receiving gradually increasing concentrations of EtOH. This figure shows EtOH concentrations as blocks, which are composed of the mean intakes on the days with the same EtOH concentration. This concentration is then compared to the same combination of days as the controls that consumed water. The EtOH concentration is confounded with days of training in the sign tracking procedures since with each increasing concentration the rats had more experience in the sign tracking procedure. Nevertheless, to facilitate the presentation of the data the days were averaged by EtOH concentration. Because the standard rats received fewer days of training in this phase and therefore received only up to 6% EtOH, only the first 6 concentrations were analyzed in a Rearing Condition (2) x Solution (2) x Concentration (6) mixed factorial ANOVA. The analysis found an interaction of Concentration x Rearing Condition, $F(5, 60) = 2.836$, $p < .05$, and an interaction of...
Concentration x Solution, $F(5, 60) = 6.354, p < .001$. The difference between the standard rats tracking EtOH and the standard rats tracking EtOH was pronounced early in Phase 3 when EtOH was 1%, $t(6) = 2.835, p < .05$, but was no longer significant at the end of the phase when the EtOH rats were drinking 6% EtOH, $t(6) = -.012, p > .05$. This lack of difference was due to the rats sign tracking water (which were initially poor sign trackers) increasing their sign-tracking behavior with repeated training. The difference when EtOH was 1%, $t(6) = 2.470, p < .05$, also tended to decline between the enriched rats tracking EtOH and the enriched rats tracking water, and by 9% these groups no longer differed, $t(6) = -.689, p > .05$. This analysis suggests that the observed differences between the EtOH drinking and water drinking groups was due to strength of sign tracking performance and not influenced by the availability of EtOH. Essentially, consumption rates were higher in the enriched rats than the standard rat, and EtOH consumption was higher than water consumption in both conditions. However, this does not indicate that the EtOH sign tracking rats experienced EtOH as rewarding. Therefore, we decided to introduce another manipulation within the discrimination task to look for a hint of reward.
Figure 3. Phase 3 - The Introduction of Ethanol. Mean solution consumed by groups sign tracking water or gradually increasing concentrations of EtOH. Intakes were averaged across days with the same EtOH concentration available for the EtOH groups.

Figure 4 shows the mean EtOH consumption as grams of EtOH consumed per kilogram of body weight. This figure shows only the rats that received EtOH during sign track training. For analysis, by controlling for body weight and removing water sign trackers it is possible to see that enriched rats consumed more EtOH relative to body weight than standard housed rats. A Rearing Condition (2) x Concentration (6) mixed factorial ANOVA supports this finding with an interaction of Concentration x Rearing Condition, F (5, 30) = 6.072, \( p < .05 \). Additionally, there was no difference in consumption over the 7% to 9% EtOH concentrations in the enriched rats as revealed by a one way repeated measures ANOVA, F (2, 6) = .253, \( p > .05 \). This means that EtOH concentrations of 7- 9% do not appear to further increase mean consumption of EtOH with enriched rats. These findings suggest that rearing in enriched environments generate
more pronounced sign tracking behavior which in turn generated higher EtOH consumption thereby increasing vulnerability to EtOH.

Rats

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<th>EtOH Concentration (%)</th>
<th>1.0</th>
<th>2.0</th>
<th>3.0</th>
<th>4.0</th>
<th>5.0</th>
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<th>7.0</th>
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<tr>
<td>Mean EtOH Consumed (g/kg)</td>
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Figure 4. Phase 3 - The Introduction of Ethanol. EtOH intake expressed as grams of EtOH consumed per kg of body weight

Enriched rats were found to drink more EtOH, and at higher concentrations than standard rats within the sign tracking procedures. Nevertheless, this finding does not translate to mean that enriched rats are addicted to EtOH. The data suggests that the sign tracking procedure was generating the drinking behavior and the addition of EtOH did not affect drinking behavior. In order to determine whether or not EtOH had become at all reinforcing to these rats, EtOH was provided outside of the sign tracking procedure.

A one bottle acceptance test was used as a preliminary procedure to determine if the rats voluntarily accept the solution. Within this test, the greater intake means the greater acceptance of solution. This experiment used acceptance tests with 3%, 6%, and 9% EtOH solutions. This procedure was then followed by 3% and 6% EtOH solution...
preference tests. The two bottle preference test was introduced to assess drug seeking behavior which is associated with addiction. Rats that find EtOH to be rewarding will seek the EtOH and drink more of it over water. Figure 5 shows the results of the acceptance (top) and preference tests (bottom) that occurred at the end of Phase 3. A mixed factorial ANOVA of Rearing Condition (2) x Training Solution (2) x Concentration (3) revealed an interaction of Concentration x Solution within the acceptance tests, $F(2, 24) = 4.597, p < .05$. This interaction was due to the enriched rats that sign tracked EtOH showing a greater preference for 6% than the other groups, but no group differences at other concentrations. Additionally, there was an effect of concentration, $F(2, 24) = 10.818, p < .001$. This supports the overall declining trend seen in Figure 5 of decreasing consumption within the higher concentrations.

The preference tests were calculated as percent EtOH consumed using the formula:

$$\frac{\text{mls of EtOH}}{\text{mls of EtOH} + \text{mls of Water}} \times 100$$

A score of 50% indicates no preference for EtOH, a score above 50% indicates a preference for EtOH, and a score less than 50% indicates a preference for water. The graphs (see Figure 5) show that the groups generally demonstrated no preference for EtOH. Although the figure suggests a preference for 3% EtOH in the standard rats, a mixed factorial ANOVA of Rearing Condition (2) x Ethanol Concentration (2) calculated
on the preference data revealed a non-significant interaction of Concentration x Rearing Condition, $F(1,12) = 1.76, p > .05$. All other interactions and main effects were also not significant. The results from the acceptance tests indicate that despite the considerable consumption of EtOH during the sign tracking procedure, the EtOH did not become sufficiently rewarding to establish a preference for EtOH. The fact that the enriched rats drinking EtOH while sign tracking drank more of the 6% EtOH than the other groups during the acceptance test may reflect some habituation to the aversive taste quality of EtOH, since these animals consumed the most EtOH during Phase 3 of sign tracking (see Figure 3).
Two-Bottle Pdemwe Test

Standard

- Enriched

3% EtOH
6% EtOH

**Figure 5.** One-bottle acceptance tests (top graph) and two-bottle preference tests (bottom graph) following the end of Phase 3.

Thus, the accumulated evidence suggests that because the enriched rats are better sign trackers, they consume more EtOH. However, because the enriched rats showed no preference for EtOH compared to the standard rats, there is no evidence that enriched rats are addicted to EtOH. While this data does not provide a complete picture of addiction, the data does suggest a lack of drug seeking behavior within the two bottle preference task.

Because the enriched rats consume more ethanol and water than standard rats within the sign tracking procedures, it is possible that the enriched rats are engaging in more impulsive responding toward the bottle. For that reason, a negative-feature discrimination procedure was introduced as a potential impulsivity metric. Within the negative-feature discrimination, impulsive temperament might be demonstrated in two potential ways. First, as in the previous test phases higher responding in the enriched than
controls on A+ days in which the negative-feature (the vanilla odor) is not present and they receive a US, would suggest an impulsive temperament. Second, impulsivity might also be demonstrated by slower acquisition of discrimination learning. That is, impulsive rats should have greater difficulty learning to withhold responding despite non-reinforcement (AB-).

It is possible to use the negative-feature discrimination task to further evaluate the rewarding quality of EtOH. It may be that the two bottle test was not sufficiently sensitive to detect the rewarding properties of EtOH. If the EtOH became rewarding to the rats sign tracking EtOH they should also fail to show discrimination learning because the solution in the bottle is motivating their drinking, not just the bottle as a signal for the food pellet US. Thus rats sign tracking water should show discrimination since water is not reinforcing to non-thirsty rats, but sign tracking EtOH should not show discrimination if the EtOH is itself rewarding. To evaluate the effectiveness of this strategy, Polycose was added to the bottles of half the rats sign tracking water (Polycose) and half of the rats sign tracking 7% EtOH (EtOH-Polycose), the remaining half of the original group continued to receive water or EtOH. Therefore, rats sign tracking a Polycose solution should not show discrimination since drinking from the bottle is motivated by the Polycose and not just the food pellet US.

Based on an initial analysis, rats exposed to the EtOH and water solutions responded similarly within negative-feature discrimination tests. There was no apparent EtOH effect or interaction of Polycose and EtOH, but analysis is limited to the low numbers of rats per condition (N=4). Thus, the rats were divided into two groups for further analysis. The rats sign tracking EtOH or water were combined to form the Group...
Non-Polycose and the rats exposed to the Polycose solutions were combined to form the Group Polycose. The negative-feature discrimination was analyzed as a mixed factor ANOVA of Group (Polycose: Non-Polycose) x Rearing Condition (enriched vs. standard) x Discriminative Stimulus (SD) (A+ vs. AB-) x Days (5). Figure 6 depicts the responding (sign tracking licks) within the negative-feature discrimination tests. The ANOVA showed an interaction of Days x SD x Rearing Condition, $F(4, 48) = 3.417, p < .05$. The enriched and standard groups sign tracking Polycose did not show an effect of discrimination, $F(1, 6) = 66.887, p > .05$, which supports the argument that when a rewarding solution is in the bottles the rats will not show discrimination. It does not matter that the Polycose group is not getting a US on AB- trials, they drink because they like what is in the bottle. Additionally, the Non-Polycose rats did show an effect of discrimination, $F(1, 6) = 37.434, p < .05$, and they showed it very quickly. This means 2 things. First, the Non-Polycose group does not find EtOH or water rewarding, which confirms the preference tests with regards to EtOH. Second, enriched rats are not impulsive. Even though they are sign tracking at very high levels, the discrimination task suggests that the enriched rats are not impulsive. Additionally, the enriched rats' discrimination was better with the Non-Polycose solution than the standard rats, suggesting that they may be less impulsive than the standard rats.

Rats in the Polycose groups showed the highest overall responding, with greater responding demonstrated on the days in which the bottle preceded the sugar pellet. Additionally, standard Polycose drinking rats showed the highest responses, with enriched EtOH responders generating higher responses than standard EtOH responders. The negative-feature discrimination findings suggest three interpretations. First,
discrimination was learned by the standard and enriched rats in the Non-Polycose group. Second, the findings support the rewarding properties of Polycose. Third, there was higher overall responding and better discrimination with enriched rats in the negative-feature discrimination tests.

To sum up these findings from Experiment 1, enriched rats showed greater acquisition of sign tracking and thus consumed more ethanol than standard rats. Nevertheless, the consumption of EtOH during sign tracking did not establish a preference for EtOH in either housing group. Negative-feature discrimination tests revealed that the enriched rats were not impulsive since they readily reduced responding when the sugar pellet reward was not presented on AB-trials. Good discrimination performance also confirmed that the EtOH and water were not reinforcing during sign tracking, since the Polycose conditions demonstrated that when a rewarding solution is in the bottle, rats do not display discrimination training. Therefore, although the enriched rats were more vulnerable to the effects of EtOH than the standard rats because they were sign tracking more, increased impulsivity as measured by the discrimination task and an "addiction to alcohol" does not adequately explain their drinking behavior.
Figure 6. Phase 4 – Negative-feature Discrimination task. A+ denotes the trials in which the bottle is followed by the sugar pellet. AB- denotes the trials in which the bottle is not followed by the sugar pellet.
Experiment 2

**Environmental Activation of the Immune System**

In the past two decades researchers from several fields of study have discovered that the nervous system and the immune system interact intimately in response to foreign substances entering the body including viruses, bacteria, and drugs of abuse. This discovery has led to a new interdisciplinary field called **Neuroimmune Pharmacology** (Ikuzu & Gandelman, 2008). Thus the neuro-immune response to drugs of abuse may share characteristics similar to the neuro-immune response to bacterial infection, suggesting that immune system activation by one foreign invader (e.g., bacteria) may affect the subsequent neuro-immune response to another foreign invader (e.g., alcohol). Since the nervous system is involved, some of these response alterations may be behavioral. One way to investigate activation of the immune system is to expose subjects to lipopolysaccharides (LPS) rather than to actual bacteria.

LPS are large molecules consisting of a lipid endotoxin and a polysaccharide that are found in the outer membrane of gram-negative bacteria (Raetz & Whitfield, 2002). LPS serve as a physical barrier that provides bacteria protection from its surroundings and is recognized by the immune system as a marker for the detection of bacterial pathogen invasion. LPS is responsible for the development of inflammatory responses and in extreme cases, endotoxic shock (Rosenfeld & Shai, 2006). In essence, LPS act as endotoxins that elicit strong immune reactions in animals.

LPS stimulates production of inflammatory cytokines in the brain and blood serum. Cytokines are small proteins, peptides, or glycoproteins that are secreted by cells.
of the immune system that are used extensively in cellular communication including tumor necrosis alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) (Staikos, Malellari & Chang, 2008).

LPS have been found to cause acute sickness in rats with such features as hyperthermia, reduced food intake, or inactivity. Exposure to LPS may have a long-term impact on the nervous system which may generate nervous system pathology and behavioral changes and in turn produce enhanced susceptibility to drugs of abuse. Rodent models could accommodate a better understanding of these immune-nervous system interactions. In Blednov et al’s (2011) LPS study with mice they found that exposure to LPS caused alcohol-prefering mice to drink more ethanol as long as 3 months after a single injection. Experiment 2 examines if this effect of LPS on subsequent alcohol intake is also observed in rats that were not selectively bred to prefer alcohol. However, there are several substantial differences between the Blednov et al study and the present experiment. Whereas Blednov measured the preference for EtOH in 24-hour two-bottle tests, in the sign tracking procedure the rats are exposed to small volumes of EtOH in brief daily sessions. Tomie et al (2004) and the results of Experiment 1 show that although rats will consume EtOH while sign tracking, they do not develop a preference for alcohol as measured by separate two-bottle tests. Thus, although the sign tracking procedure induces alcohol consumption, the short term daily exposure to EtOH is not sufficient to induce a preference for alcohol over water. Therefore, in Experiment 2 EtOH was introduced in the home cage to provide 24-hour access. This addition to the experimental procedure permitted an evaluation of the effects of LPS on compulsive ethanol consumption in the sign tracking procedure. on 24-hour two-bottle preference
tests in the home cage, and in short-term two-bottle preference tests in a test cage. Also, rat strain was changed to Long Evans rats because these rats are suggested to be better sign trackers and are the exclusive strain used in Tomie’s studies (Tomie, 2008).

Method

Subjects

The subjects were 24 male, 40 day old, Long Evans rats from Harlan (Indianapolis) raised in pairs within shoebox cages. These rats were given food and water ad libitum. These rats were maintained on a 12 hour light-dark cycle, with the light turning on at 8 am. This experiment was approved by Seton Hall’s Institutional Animal Care and Use Committee. All guidelines for the care and use of rats set by the United States Public Health Service were firmly followed.

Apparatus

This experiment used the same apparatus as Experiment 1, with the following modification. For the negative-feature discrimination task, a buzzer sound was used as the signal for non-reward instead of the vanilla dryer sheets used in Experiment 1. A Piezo-buzzer (RadioShack 273-0066) was mounted on the top of the ceiling of all four chambers.

Procedure

LPS Treatment

At the age of 55 days, 12 rats were injected intraperitoneally with 1 ml/kg/kg of LPS (from Salmonella enterica, Cat#L6511, Sigma, St. Louis, MO) dissolved in saline.
while the other 12 rats were injected with the equivalent amount of saline. Injections were aligned with rat pairing (each cage-mate received the same injection treatment) to minimize confounds, and for ease of measurement. The rats were given 1 week of recovery time prior to the progression of adaptation training. Additionally, rat body weights were recorded from 1 day prior to injection and the following 15 days.

**Phase 1 – Adaptation and magazine training**

This procedure was the same as Experiment 1. Rats within this experiment experienced 2 days of adaptation before continuing to Phase 2.

**Phase 2 - Induction of sign tracking and goal tracking**

This procedure was the same as Experiment 1. The rats experienced 9 days of sign tracking training prior to Phase 3.

**Phase 3 – Introduction of Ethanol**

Water was replaced with EtOH for 7 rats in the LPS injected condition and 7 rats in the saline injected condition. The other 10 sign tracking rats continued with water to serve as controls. With the exception of two pairs (one LPS-treated pair and one saline-treated pair), the rats were housed with a partner that drank the same solution within the sign tracking chamber. EtOH started at 1% concentration, and worked up to 10% concentration in one to three days increments dependent on rat performance. This procedure continued for 58 days. Concurrently, beginning on Day 28 and continuing for the duration of the sign tracking of EtOH procedures a second bottle which contained EtOH was introduced into the rats’ home cage which followed a similar concentration
progression as the sign track training. The EtOH and water bottle position were alternated daily. This was followed by 5 days of 20 minute preference tests within the holding cages. There were 3 days with 6% EtOH solution followed by 2 days with 9% ethanol solution with the left/right position of the bottles alternated across days. The same bottles as Experiment 1 were used. Thus, the procedural sequence for this phase was: 30 days of sign tracking with ethanol, 28 days with ethanol in the testing chamber and home cage, 3 days at 6% EtOH solution preference tests, and 2 days at 9% EtOH solution preference tests.

Phase 4 – Negative-Feature Discrimination and Exposure Training

There were several changes made to the negative-feature discrimination procedure that was used in Experiment 1, for the purpose of exploring alternative methods of administration. In the previous experiment, the reinforced (A+) and the non-reinforced (AB-) trials were given on alternating days, with the same trial type within a day. In the present experiment, the two types of discrimination trials were given in the same day. The A+ and AB- trials would occur in Trial blocks, with the AB- block starting a session on a random half of the negative-feature training days. This negative-feature discrimination task was run for 9 days with a 10% ethanol solution in the bottles of the EtOH groups and water in the other groups. The negative-feature training was followed by 4 days of extinction training in which the bottle would appear each trial within its typical schedule, but without the pairing of the sucrose pellet. Home cage EtOH bottle remained available for only the first 5 days of negative-feature training. Thus, the negative-feature discrimination procedure sequence was 5 days of training with
concurrent home cage EtOH followed by 4 days of training without home cage EtOH and then 4 days of extinction training.

Data Analysis

The primary independent variables in each phase were the LPS treatment (LPS or saline) and days of training. The dependent variables were licks and milliliters of solution consumed for measures of sign tracking, while the dependent variable were head pokes for measures of goal tracking. The dependent variables for each phase were analyzed by separate ANOVAs followed by post hoc comparisons. The Phase 2 and Phase 3 dependent variables were analyzed with an immune system Condition (2) x Days or Concentration (10) x Solution (2) mixed factor ANOVA. Immune system Condition and Solution are between subject variables and Days or EtOH Concentrations (i.e., days at EtOH concentration vs. same combination of days with water) are within subject variables. Additional ANOVAs as described in Experiment 1 will be conducted, except that LPS Treatment replaces rearing condition as the primary independent variable.

Results and Discussion

Body weights of LPS treated and saline treated rats were recorded from one day prior to injection, to 2 weeks after injection to assess the effects of LPS on subsequent body weight change. A mixed factorial ANOVA of Injection Condition (2) x Days (2) revealed only an effect of days on the body weight change from the day prior and the day of injections, $F(1, 22) = 6.822, p < .05$. However, a mixed factorial ANOVA of Injection Condition (2) x Days after injection (11) revealed an interaction between Days x Injection Condition, $F(10, 220) = 3.137, p < .001$. As evident in Figure 7, the LPS
injection resulted in lower mean body weight change that continued for two weeks, with the weight changes being approximately the same by the end of the two weeks. Thus LPS was effective in inducing weight change, and a presumed acute illness as a result of immune system activation.

Classical conditioning was demonstrated by head pokes to the food tray during the presentation of sucrose pellets, as seen in Figure 8. As a reminder, this conditioning is seen by comparing the time point of 10 seconds of head poking prior to CS (Pre-CS), to the head poking during CS, and the 10 seconds of head poking following the CS (Post-CS). An Injection Condition (2) x time Period (3) x Days (9) mixed factorial ANOVA revealed a significant interaction between Period x Days, $F(16, 352) = 2.399, p < .05$. Additionally, there was an effect of Period [$F(2, 44) = 13.771, p < .001$], a non-
significant three-way interaction [$F(16,352) = 1.161, p>.05$], and non-significant main effect of injection condition [$F(2, 44) = .186, p>.05$]. These results indicate that classical conditioning does not appear to be affected by LPS injections.

Figure 8. Phase 2 - Acquisition of Head poking

Sign tracking acquisition is shown in Figure 9. There were no significant differences between sign tracking acquisition performance with water between the
Injection Conditions, $F(8, 160) = 1.079, p > .05$. There was only an effect of sign tracking performance over Days, $F(8,160) = 3.661, p < .05$, confirming the acquisition of sign tracking in both groups.

Figure 9. Phase 2 - Acquisition of Sign Tracking with Water in the Bottle

An Injection Condition (2) x Solution (2) x Concentration blocks (10) mixed factorial ANOVA on the sign tracking data during the introduction of EtOH (Phase 3) revealed significant interactions of EtOH concentration by Injection Condition, $F(9, 180) = 2.758, p < .05$, and of Solution x EtOH Concentration, $F(9,180) = 2.064, p < .05$. Yet, the expected three way interaction failed to be significant, $F(9,180) = 2.37, p > .05$. Thus, interpretation of these data is complicated with continued “improvement” in sign tracking performance. Figure 10 shows the injection condition by EtOH concentration interaction, with the 4 groups on separate plots. EtOH concentration blocks are composed of the days
that the rats received a given EtOH concentration (1%-10%). These concentrations were compared to the same combination of days as the groups that consumed water. Inspection of the graph suggests that EtOH may have increased sign tracking in the saline-treated rats, but not the LPS-treated rats. Note that although the LPS-treated rats showed similar lick rates at all concentrations, the mean licks of the saline-treated rats were increasing in the beginning of Phase 3 when the EtOH concentrations were low, which most likely reflects increased sign tracking with practice.

Although Tomie (2008) found that the addition of EtOH to the bottle can increase sign tracking in Long Evans rats, it seems unlikely to explain the apparent difference between the EtOH and water drinking saline-treated rats because the difference was observed at the very beginning of this phase, when the EtOH concentrations were very low and unlikely to produce significant pharmacological effects.
Figure 10. Phase 3- The Introduction of Ethanol. Mean licks by groups sign tracking water or gradually increasing concentrations of EtOH. Licks were averaged across days with the same EtOH concentration available for the EtOH groups.

To further evaluate the data from Phase 3, Solution (2) x Concentration blocks (10) mixed factorial ANOVAs were conducted on the LPS- and Saline-treated rats separately. For the LPS-treated groups there was no effect of EtOH Concentration, $F(9, 90) = 1.53, p > .05$, or Solution consumed, $F(1, 10) = .016, p > .05$, and there was no interaction between these two factors, $F(9, 90) = 0.118, p > .05$. These results indicate that sign tracking performance of the LPS-treated rats remained stable throughout this phase, and the gradual introduction of EtOH failed to further increase sign-tracking behavior. For the saline-treated groups the results are a little more complex. A significant main effect of Concentration blocks, $F(9, 90) = 4.428, p < .001$, and a non-significant Concentration block x Solution interaction, $F(9, 90) = 1.645, p > .05$, revealed that sign tracking performance of saline-treated rats increased significantly in this phase regardless
of the solution consumed. Moreover, the main effect of Solution failed to be significant. 

$$F(1, 16) = 1.324, p > .05.$$ Thus, as with the LPS rats there is not sufficient evidence that adding EtOH to the bottle significantly increased sign tracking performance of the saline-treated animals. It may be that there is a subtle EtOH effect but it did not reach significance because of the small number of subjects per group. This possibility is supported by the significant effect of Solution by ethanol Concentration, 

$$F(9, 180) = 2.064, p < .05,$$ mentioned above when the data was analyzed as an Injection condition (2) x Solution (2) x Concentration blocks (10) mixed factorial ANOVA. Figure 11 shows this interaction. Note that greater licking is apparent in the rats sign tracking EtOH than the rats sign tracking water at the moderately high concentrations (i.e., 6, 7 & 8%), but also note that this group difference at these concentrations is mostly due to a decreased licking in the rats sign-tracking water rather than due to increased licking in the rats sign-tracking EtOH. Moreover, at the highest concentrations (9% & 10%) the lick rates of the rats sign-tracking EtOH decreased to the levels of the rats sign-tracking water.

It is clear that the compulsive drinking in the sign tracking procedure is driven primarily by the Pavlovian conditioning schedule and the effects of EtOH and LPS-treatment on this compulsive drinking is at best a subtle one.
To further explore the differences in EtOH consumption among LPS- and Salinetreated rats, both injection conditions were compared on milliliters consumed in grams (1mL = 1g) per kilogram of body weight (g/kg) as seen in Figure 12. However, when LPS and saline EtOH sign trackers were compared there were no significant differences in consumption \([F(9,108) = .677, p > .05]\) as tested in a mixed factorial ANOVA of injection condition (2) by concentration block (10). However, concentrations had an effect on milliliters consumed, \(F(9,108) = 14.876, p < .001\).
To further explore the relationship of bacterial infection experience (LPS injection) with subsequent EtOH consumption, ethanol was made available in the best ethanol sign tracker rats’ home cages. The rats lived in pairs so it was not possible to know what each individual rat consumed. However, with the exception of two pairs, all rats were housed with rats that had the same injection and drank the same solution while sign tracking. The 2 pairs that drank different solutions were not included in this analysis. Additionally, 2 rats had to be separated and lived alone. Thus, the preferences plotted are based on the averages of LPS-treated and Saline-treated cages that housed one or two rats. This is not ideal, but it is still informative and we also did the short-term 2-bottle tests with the individual rats to confirm these results. The statistics were calculated using the mean preference for each concentration rather than days. As a reminder,

Figure 12. Phase 3 – The Introduction of Ethanol. EtOH intake expressed as grams of EtOH consumed per kg of body weight.
preference is calculated as percent EtOH consumed using the formula: \( \frac{\text{mls of EtOH}}{\text{mls of EtOH} + \text{mls of water}} \times 100 \). A mixed factorial ANOVA of Condition (2) \( \times \) Concentration (10) was performed on the preference for EtOH. There was an interaction that fell short of significance, \( F(9, 90) = 1.836, p = .07 \). However, there was an effect of Concentration, \( F(9, 90) = 8.415, p < .001 \), reflecting an increasing preference for EtOH with increasing EtOH concentration. Inspection of Figure 13 shows that the animals treated with LPS clearly developed a greater preference for EtOH (at the highest doses of EtOH) than the saline-treated animals. When an independent t-test was done on the final concentration (10%) at the end of this phase, the LPS-treated rats showed a significantly greater preference for EtOH, \( t(6) = 2.475, p < .05 \).

![Figure 13. Phase 3: The Introduction of Ethanol. Percent EtOH consumed when EtOH and water were constantly available in the home cage. 4% EtOH concentration was not administered.](image-url)

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At the end of the sign track training with EtOH, all rats were individually tested in two-bottle preference tests with 6% and then 9% EtOH solutions. A mixed factorial ANOVA of Treatment Condition (2) x prior Solution experience (2) by EtOH Concentration (2) within the preference tests revealed a significant interaction of Concentration x Treatment Condition, $F(1,20) = 6.764, p < .05$. There was not a significant interaction of Solution x Concentration $F(1, 20) = .685, p > .05$. This was further supported by One Way ANOVAs of Treatment Condition (2) x prior Solution experience (2), revealing a LPS treatment effect with the 6 % EtOH preference test, $F(1, 20) = 8.577, p < .05$. Also, the LPS treatment effect was also found with the 9% EtOH preference test, $F(1, 20) = 24.429, p < .001$. These effects can be seen within Figure 14. Thus, LPS treated rats show a higher preference for EtOH when compared to saline treated rats, regardless of solution experience during sign track training.

![Figure 14. Two-bottle preference tests following the end of Phase 3](image-url)
The body of evidence suggests that although LPS injection does not affect compulsive drinking of EtOH during sign tracking, LPS caused a greater preference for EtOH that was induced by daily home-cage exposure to EtOH compared to saline injected rats.

Sign tracking has been associated with impulsivity. For that reason, the negative-feature discrimination task was used to look for indications of impulsivity in the Long Evans rats, and to determine if EtOH and LPS-treatment further increased impulsivity. Within the negative-feature discrimination, impulsive temperament might be demonstrated in two potential ways. First, higher responding than controls on trials on which the discriminative stimulus (buzzer noise) is not presented would suggest an impulsive temperament. Second, impulsivity might also be demonstrated through a higher responding that continues over trials, without regard for discriminative stimulus presence.

To tease apart the effect of EtOH on the LPS and the saline treated rats, EtOH was kept available within the rats' home cage for the first 5 days of negative-feature training, and then removed for the following days. This negative-feature discrimination task was run for 9 days with a 10% EtOH solution or water in the bottle. The negative-feature training was followed by 4 days of extinction training in which the bottle would appear each trial within its typical schedule, but without the pairing of the sucrose pellet.

In the negative-feature discrimination task, as rats learn the discrimination they should demonstrate progressive inhibition of responses to the AB- trials because the reinforcement will not occur in the presence of the negative-feature (R), but they should
continue to respond on A+ trials. Rats that are impulsive should have a difficult time inhibiting responses during AB- trials and the rats may not show discrimination learning. Mean licks were analyzed with a mixed factor ANOVA with Treatment Condition (2) x bottle Solution (2) by Discriminate Stimulus (SD) presented (2) x mean licks during trials over Days (9). There was an interaction of Days x SD, $F(8,160) = 2.992$, $p < .05$, and SD x Solution, $F(1, 20) = 4.686$, $p < .05$. However, the higher order interactions with treatment condition were not significant. These effects can be seen within Figure 15.

Examination of Figure 15 reveals that responding during AB- did not decrease systematically over trials in any of the 4 groups. Therefore, there is not good evidence of negative-feature discrimination learning in any group. This means that either the Long-Evans rats are very impulsive or that there was a problem with the buzzer and/or the discrimination task that prevented the rats from learning the task. We can address this later possibility with the head poking data. But, why was there a significant SD x Days interaction? The difference between A+ and AB- first occurred on Day 5 when the home-cage EtOH was withdrawn and the difference was due to more responding to A+, rather than less responding to AB-. Also, this difference was greater in the animals sign tracking EtOH as indicated by the SD x Solution interaction. But the difference between A+ and AB- did not persist, and was not observed during the last 2 days of discrimination training. Clearly, the Long-Evans rats showed much poorer discrimination than the enriched Sprague Dawley rats in Experiment 1. This suggests that the Long-Evans rats may be more impulsive than the enriched Sprague Dawley rats.
To analyze the extinction data, the results of the last 4 days of paired stimulus were compared against the 4 days of extinction training to determine whether or not extinction was being learned by the rats. Analysis of the extinction data with a mixed factor ANOVA of Treatment Condition (2) x bottle Solution (2) x Phase (2) x Days (4) yielded only an interaction of Phase x Days, \( F(1, 60) = 3.316, p < .05 \), but rather than licking decreasing over days of extinction, licking on Day 4 was significantly greater than on Day 1 \( (p < .05) \). Since the main effect of Phase was not significant, \( F(1, 20) = 0.248, p > .05 \), this effect was due to greater responding during extinction, rather than reduced responding. Thus, there was no evidence of extinction.

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**Figure 15.** Phase 4. Negative-feature Discrimination task. A+ denotes the trials in which the bottle is followed by the sugar pellet. AB- denotes the trials in which the bottle is not followed by the sugar pellet. On Day 5, EtOH was removed from the rats' home cages.
Therefore, the results of the lick responses during negative-feature training suggest several interpretations. EtOH appears to have had some effect on sign tracking (lick responses), especially when the home EtOH bottles (Day 5) were withdrawn and the rats were potentially in withdrawal. Discrimination learning does not appear to be shown with lick responses (see Figure 15). The data suggests that rats in the sign tracking procedure, whether they are drinking EtOH or water, were showing poor discrimination and little extinction and therefore all groups appear impulsive, especially when compared to the Sprague Dawley rats who showed very nice discrimination when they were drinking water or EtOH (not the Polycose drinkers).

Since discrimination learning was not clearly demonstrated with lick responses during negative-feature training, it is possible that the use of the buzzer as the negative-feature and/or the within-session procedure was not sufficient to support discrimination learning. To evaluate this hypothesis, mean head pokes were examined to determine how the head poking behavior was modulated by the negative-feature training. If the discrimination task was not sufficient to support discrimination learning then head poking should also show poor discrimination learning. A mixed factor ANOVA was run on Treatment Condition (2) x Solution (2) x Discriminative Stimulus (SD) presence (2) x Days (9). There was an interaction of Days x SD, $F(8,160) = 7.153, p < .001$. The Treatment Condition did not significantly interact with the other factors. As seen in Figure 16, head poking during AB-trials was generally lower than during A+ trials and decreased over days in all four groups. Therefore, discrimination was shown with the head poking (goal tracking) behavior.
Additionally, the results of the last 4 days of paired stimulus (bottle with sugar pellet) were then compared against the 4 days of extinction training to determine whether or not extinction was being learned by the rats. A mixed factor ANOVA was run on Treatment Condition (2) x bottle Solution (2) x training Phase (2) x Days (4). There was a significant main effect of Phase, $F(1, 20) = 81.869, p < .001$, which confirmed that head poking extinguished when the food pellets were no longer administered. Thus, the failure to discriminate was specific to sign-tracking behavior, further supporting the hypothesis that sign-tracking behavior (but not goal tracking behavior) in Long Evans rats reflects impulsive responding.

![Figure 16](image)

**Figure 16. Phase 4 - Negative-feature Discrimination task.** $A^+$ denotes the trials in which the bottle is followed by the sugar pellet. $A^-$ denotes the trials in which the bottle is not followed by the sugar pellet. On Day 5, EtOH was removed from the rats' home cages.
To sum up the findings of Experiment 2, LPS injection resulted in lower mean body weight change that continued for two weeks, but the weight changes were approximately the same by the end of the two weeks which suggests that the LPS injection had induced acute illness. LPS does not appear to affect classical conditioning. There were not significant differences between both treatment groups sign tracking acquisition performance with water, and the gradual introduction of ethanol failed to further increase sign-tracking behavior. Therefore, compulsive drinking in the sign tracking procedure is driven primarily by the Pavlovian conditioning schedule. EtOH was made available in the ethanol sign tracker rats' home cages, and all rats were individually tested in two-bottle preference tests with 6% and then 9% EtOH solutions. The LPS treated rats show a higher preference for EtOH when compared to saline treated rats. Thus, the body of evidence suggests that although LPS injection does not affect compulsive drinking of EtOH during sign tracking, LPS caused a greater preference for ethanol that was induced by daily home-cage exposure to EtOH compared to saline injected rats.

The results of the negative-feature training found that EtOH appears to have had some effect on sign tracking, especially when the home cage EtOH bottles were removed and the rats were potentially in withdrawal. Discrimination learning does not appear to be shown with lick responses, but by head poke responses. The data suggests that the Long Evans rats in the sign tracking procedure showed little extinction and therefore appear impulsive when compared to the Sprague Dawley rats in the Non-Polyoxyne group. Thus, the failure to discriminate suggests that sign-tracking behavior (but not goal tracking behavior) in Long Evans rats reflects impulsive responding.
General Discussion

The sign tracking procedure using a bottle as the CS has three components that appear related to drug use and abuse. First, subjects prone to drug abuse (addictive phenotype) are more likely to respond to the intermittent presentations of the bottle, resulting in compulsive responding toward the bottle that approximates addictive behavior. Second, the presence of a Pavlovian relationship between the bottle CS and food US attaches incentive salience to the bottle signaling the reward US further increasing bottle-directed behavior. Finally, when the bottle contains a drug the compulsive behavioral interaction with the bottle signal may further contribute to the maintenance of the compulsive behavior since the interaction with the bottle results in administration of a drug (e.g., alcohol). Perhaps, sign tracking might be a type of behavioral sensitization generated by the Pavlovian schedule and intermittency effects.

In Experiment 1, enriched rats showed stronger acquisition of sign tracking and consumed more ethanol than standard rats within the sign tracking procedure, but not during the preference tests. The negative-feature discrimination task was used to measure the ability to inhibit responding; difficulty inhibiting responding is indicative of impulsivity. The negative-feature discrimination tests revealed that the enriched rats were not impulsive since they readily reduced responding when the sugar pellet was not presented on AB trials. If the effect of enrichment is to increase exploratory behavior, then this altered behavioral profile would have been sufficient to increase sign tracking. The results of the negative-feature discrimination tests with the Sprague Dawley rats suggest that robust sign tracking does not require that the animals be impulsive. Nevertheless, the enriched rats were more vulnerable to the effects of EtOH than the
standard rats because they were sign tracking more. This may seem counter-intuitive because “enrichment” is seen as a positive effect, yet these animals are now displaying more “compulsive” behavior which is typically seen as a negative effect since compulsions are stereotyped and excessive behavior. The licking of the bottle is not a requirement to get the sugar pellets so the behavior is excessive and because the compulsive action resulted in the consumption of EtOH in some animals, those rats were at greater risk of drug abuse. Although the enriched rats appear to have a stronger vulnerability to EtOH within the sign tracking procedure, the rats appear to have otherwise reduced addictive propensities (i.e., little impulsivity and no increased preference for ethanol). Perhaps, this is due to a protective effect or due to tolerance mechanisms being modulated. However, a complex phenomenon such as alcohol addiction has many contributing factors. Increased tendency to take in alcohol when in a schedule that induces compulsive intake might be a contributory factor to addiction, but is not sufficient to produce an addiction. Perhaps if the daily sign track sessions were longer and the enriched rats were exposed for many more months, they may be more likely to develop an addiction.

The compulsive drinking of the Long Evans rats in Experiment 2 also appears to be controlled primarily by the sign tracking procedure, since similar behavior was observed in the group that sign tracked EtOH or water. The failure to observe discrimination of sign tracking in the negative-feature discrimination task suggests that the Long Evans rats are impulsive. One possibility is that the noise discriminative stimulus may have not been sufficiently noticeable to support discrimination learning. However this is unlikely since the rats did show discrimination of goal tracking. The negative-feature
discrimination results of Experiments 1 and 2, therefore, suggest that the Long Evans rats are more impulsive than the Sprague Dawley rats.

There is some debate concerning the construct of impulsivity. One study found that lesions of the Serotonin (5-HT) system in the brain increased all aspects of impulsivity which suggested that impulsivity is a unitary construct; at least in terms of its regulation by the serotonergic system (Winstanley, Dalley, Theobald, & Robbins, 2004). However, data from human volunteers with tryptophan depletion (which presumably altered serotonin brain functioning) found increases in impulsive actions, but not in impulsive choices. These data suggest that behavioral inhibition, rather than impulsive decision-making, is more sensitive to alterations in the serotonergic system. The use of the negative-feature discrimination task in the present experiments reflects a measure of behavioral inhibition.

In Experiment 1 the Sprague Dawley rats drinking EtOH while track-matching the bottle did not show a preference for EtOH. Perhaps there was not sufficient consumption of EtOH in the brief daily tests to establish a preference. Thus in Experiment 2 ethanol exposure was increased by providing 24-hour access to gradually increasing concentrations of EtOH in the home cage. Moreover, the LPS injections resulted in a much stronger preference for EtOH. Yet despite the greater preference for EtOH the sign tracking of EtOH was not increased in the LPS-treated rats compared to the rats sign tracking EtOH but treated with saline.

The finding that the activation of the immune system functioning with LPS increases preference for EtOH extends Blednov et al.'s (2011) finding in mice to rats.
suggests that immune system regulation impacts on alcohol consumption. It is unknown if LPS would have increased EtOH preference without the additional home cage exposure to EtOH because we did not compare Long-Evans rats given home cage EtOH with Long Evans rats not given home cage EtOH.

Bacterial infection (LPS) may induce long-term alterations in cytokine responses to peripheral infection in adulthood. This altered immune reaction may also influence cognitive processes such as the post-training consolidation (memory organizing) processes of memory storage (Bilbo, Levkoff, Mahoney, Watkins, Rudy, & Maier, 2005). Neonatal exposure to LPS increases hypothalamic–pituitary–adrenal (HPA, neuroendocrine system that influences sign tracking, responses) reactions to stress, decreases in natural killer cell activity and impairs tumor immunity, decreases susceptibility to inflammation, and attenuates fever in response to a subsequent challenge in adult rats. One study found that rats infected with Escherichia coli (E. coli, LPS being one of its features) as neonates displayed impaired memory for a recently explored context in adulthood (Bilbo et al., 2005). However, this impairment was only observed in rats that received LPS immediately after context exposure. This is in line with research that has found that cytokines (such as, interleukin IL-1β) released in the course of an immune response have significant influences on memory.

IL-1 receptors are distributed throughout the brain, with the highest density in the hippocampus which makes it vulnerable to immune-related adjustments that may lead to memory impairments. Furthermore, IL-1β is induced following long-term potentiation induction in the hippocampus, and IL-1β is required for its maintenance. As such, IL-1β appears to be required for normal memory processes, and any alterations in IL-1β.
signaling, such as may occur as a result of infection during development, will likely have significant consequences for these processes throughout life (Bilbo et al., 2005). Since sign tracking and drug use are also acquired (learned) behaviors, LPS exposure was thought to influence the development of these behaviors. However, within this study LPS did not impair sign tracking performance when compared to saline controls, and discrimination learning was found with head poke responses.

LPS has been found to induce opioid sensitivity, specifically with beta-endorphins (Knigge et al., 1994). Beta-Endorphins are involved in alcohol consumption and dependence, as measured with knockout mice in a two-bottle preference test (EtOH vs. water) (Racz et al., 2008). Pregnant female Sprague-Dawley rats were injected with a few injections of LPS which resulted in the male offspring showing a preference for alcohol in a two-bottle preference test (EtOH vs. water) (Liu, Lee, Yee, Brees, Poland, & Pochnick, 2004). Additionally, heavy alcohol drinkers show lower beta-endorphins levels than moderate or light drinkers (Racz et al., 2008).

However, other studies have shown that LPS exposure leads to low basal dopamine levels in the nucleus accumbens (NA) which has been associated with high ethanol preference and consumption in rats. Moreover, profound NA dopamine release has been reported in rats withdrawn from repeated EtOH exposure. Additionally, the ethanol-dependent rats consume EtOH until NA dopamine levels are restored to control. Reduced dopamine release has also been reported in detoxified alcoholics (Blednov et al., 2011).

Perhaps, LPS negatively impacts the opioids within the dopaminergic reward circuit. Enhanced dopamine release may be needed to activate opioids, and consequently
excessive consumption might be the result of opioid-deficiency induced EtOH tolerance. However, alternative theories focus on how LPS acts on peripheral tissues, macrophages, and liver Kupffer cells which may in turn stimulate the beginnings of alcoholic liver disease (Qin, He, Hanca, Pluzarev, Hong, & Crews, 2008). Thus, the EtOH tolerance might be opioid induced, and/or peripherally induced.

The findings of this study appear to support the incentive sensitization theory of addiction. When incentive salience leads to the administration of a drug, this effect is typically referred to as incentive sensitization. Robinson and Berridge (2008) describe the key feature of this addiction model as the distinction between drug liking (the high) and drug wanting (craving), which is in line with the findings that over the course of developing addiction and with repeated exposure, there is a marked increase in drug wanting while there is either no change or a decrease in drug liking. This disparity is believed to be due to different neural mechanisms being responsible for the two components of drug reward, and because repeated use causes a sensitization of the “wanting” system but no sensitization or even tolerance in the “liking” system. It is theorized that the dopamine reward circuit can be sensitized by repeated administration of abused drugs, and that this neural circuit may be more important in drug wanting than in drug liking (Meyer & Quenzer, 2005). Although Berridge and Robinson emphasize drug-induced sensitization effects, the sign-tracking studies suggest that intermittent presentations of rewards and Pavlovian associations can produce “wanting” effects in the form of compulsions responding. Enrichment factors in Experiment 1 show an effect of wanting (sensitization effect in the sign tracking procedure) but no change in “liking” (no preference for ethanol), whereas Experiment 2 shows that LPS injection does not
substantially increase wanting (during sign tracking) but increases liking, e.g., stronger preference and more impulsive sign tracking behavior in the discrimination task.

The findings of the present experiments support much of the prior neurobiological experiments. Increases in compulsive-like responding are mediated by increased dopaminergic activity are consistent with studies showing correlations between high levels of sign-tracking performance and high tissue levels of dopamine and its metabolite DOPAC in the nucleus accumbens. Evidence suggests that impulsivity contributes to the loss of control in drug-taking and that sign tracking CRs mediate symptoms of drug abuse. Drug abuse researchers have noted conspicuous similarities between behaviors elicited by Pavlovian sign tracking procedures and prominent symptoms of drug abuse. This connection is seen with sign tracking CR performance being poorly controlled, exhibiting spontaneous recovery (Durlach, 1986) and long-term retention. These effects strongly resemble relapse. The effects of sign-tracking on corticosterone levels and activation of dopamine pathways resemble the neurobiological effects of abused drugs. Lastly, the neurobiological profile of subjects susceptible to sign tracking resembles the pathophysiological profile of vulnerability to drug abuse, which means that vulnerability to sign tracking predicts vulnerability to impulsive responding and alcohol self-administration (Tomie et al., 2008). Taken together, these findings suggest that sign tracking, impulsivity, and drug abuse may be related phenomena (Tomie, Aguado, Polacrecky, & Benjamin, 1998).
Future directions of the sign tracking model of addiction may include the exploration of the underlying mechanisms that have been theorized to contribute to this rodent model of alcoholism. But before these experiments are completed it is helpful to confirm some of the findings of the present study. For example, to confirm impulsivity differences among strain it would be a good idea to compare Sprague Dawley and Long-Evans rats in the same experiment with the same negative-feature discrimination task. It also would help to compare groups in home-cage ethanol exposure versus no home-cage ethanol exposure to better determine how greater overall exposure to ethanol affects sign-tracking for ethanol. The brief exposures in the daily sessions may not be enough to produce strong ethanol dependence; for dependence it may be necessary for rats to experience withdrawal effects that drive more EtOH drinking. Experiment 2 suggested that withdrawal from EtOH may increase sign tracking for ethanol (remember that there was an increase on A+ trials of the discrimination task on at least a couple of days). This result suggests that the sign tracking model may be a way to look at withdrawal effects of EtOH on subsequent ethanol consumption.

There are many contributing factors to alcoholism. The rodent sign tracking model of alcohol addiction is primarily designed to look at the sensitizing and positive reinforcing effects (e.g. preference tests) of EtOH, but EtOH abuse may also be driven by negative reinforcement, i.e., drinking to prevent the aversive withdrawal effects. Thus, adding systematic periods of withdrawal and looking at its impact on the sign tracking of EtOH may be informative. Finally, although EtOH was the focus of these experiments, other drugs can be investigated by mixing them with the water bottles. EtOH has been observed to produce sensitization in mice, but it has been very difficult to demonstrate
EtOH-induced sensitization in rats. Perhaps cocaine and methamphetamine may be useful since these drugs, like sign tracking, have been shown to produce sensitization effects in rats.


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