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Ethanol Induced Sign-tracking in Swiss Mice

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Abstract
Rationale It is thought that alcohol addiction is influenced by environmental cues. One way this relationship is built is through Pavlovian learning, in which the alcohol is repeatedly paired with an environmental cue. Sign-tracking is a type of behavior that exhibits a Pavlovian learned association.
Objectives Our experiment studies induced sign-tracking using ethanol and a light visual cue (conditioned stimulus or CS).
Methods In this study, one set of mice was given ethanol through intraperitoneal (IP) injections before being placed in an apparatus with a spatially isolated light visual cue. A control group was also placed in an apparatus with the light visual cue, but was not given ethanol IP injections until an hour after the trial (in the home cage). Following these conditioning trials, the mice were given a series of preference tests, where the visual cue was present (CS+) and both groups of mice received saline IP injections prior to the test.
Results Throughout the conditioning trials, there was no distinction between groups for the time spent on the light side of the apparatus. However, when examining the nose poke counts per trial, the paired group poked more in the CS+ hole during preference test 2 compared to the CS- hole.
Conclusions The paired group of mice did learn the association, and it can be argued that sign-tracking was observed because the mice showed a preference for the CS+ in the nose poke count of preference test 1.

Introduction
It has been thought that alcoholism can be influenced by environmental cues. With alcoholics, cues such as the smell of alcohol or a bar sign are repeatedly paired with alcohol use (Pickering & Liljequist, 2003). It is thought that these cues are present before drug consumption, and they are present regardless of how the drug is administered (Uslaner, Acerbo, Jones, & Robinson, 2006). Over time these cues may become associated with the consumption of alcohol. For example the idea that when a person frequently drinks alcohol at a specific bar, that the next time that person is in that bar, he or she will want to drink.

One way that this relationship is created is through Pavlovian learning, through which alcohol is repeatedly paired with an environmental cue. Through this association, it is believed that the cue may acquire incentive salience (Uslaner et al., 2006). Incentive salience is the idea that an object can seem to stand out amongst other objects and becomes a kind of “motivational magnet”. This is thought to be gained through Pavlovian learning.

Sign-tracking is an observed behavior that can be used to study a Pavlovian learned association. Sign-tracking refers to the behavior of an animal that moves into close proximity to a cue or signal (Brooks, Tomie, & Zito, 1989). This behavior is a phenomenon where once an animal has learned the association between a cue and a drug, the animal will then approach the cue. Sign-tracking is a behavioral response that demonstrates that a cue can gain incentive salience (Uslaner et al., 2006). The phenomenon of sign-tracking was first reported by Brown & Jenkins in 1968.
What is interesting about sign-tracking is that there is no experimentally designed reason for the animal to approach the cue. There is no reward for approaching the cue, and approach to the cue is not required to receive the drug reward. Previous studies have shown that animals approach a cue even if moving towards the cue leads the animal farther away from the reward (Uslaner et al., 2006).

As mentioned above, the first sign-tracking study, then referred to as autoshaping, was performed in 1968 by Brown and Jenkins. In their study, pigeons were placed in an operant chamber with a response key and a food magazine. Throughout the trial, the key would illuminate for 8 seconds, and then the food magazine would be presented for 4 seconds. If the pigeon pecked at the key while illuminated, the food magazine would immediately be presented. However, key pecking was not necessary for the food to be presented (Brooks et al., 1989). What they found was that the pigeons began to peck at the illuminated key.

There have not been many successful studies that show sign-tracking using a drug reward. A study by Uslaner in 2006 exhibited cocaine induced sign-tracking to an illuminated lever. Sprague-Dawley rats were given intravenous infusions of cocaine when an illuminated lever was presented for 8 seconds in an operant chamber. The cocaine was administered regardless of the rat’s behavior. The number of approaches to the lever was observed and recorded. The rats in this experiment increased over trials in number of approaches to the lever when paired with cocaine.

A study done by Cunningham & Patel in 2007 showed ethanol induced sign-tracking in mice to a visual cue of a star located on the floor. In this experiment, mice were given IP injections of ethanol right before being placed in a box with the distinct star visual cue. On alternate days, the mice were given saline IP injections right before being placed in the box without the visual cue. After 6 trials, the mice were given two tests. Both tests had the visual cue present, but in one test the mice received ethanol IP injections, and in the other they received saline IP injections. This experiment was conducted on two different types of mice, NZB/B1NJ and DBA/2J. The mean activity and left time was recorded during each trial and test. Over 6 conditioning trials, NZB/B1NJ mice spent increasingly more time on the side of the floor with the star during ethanol trials. The DBA/2J mice did not show a preference for either side of the box during ethanol conditioning trials. The tests revealed that the NZB/B1NJ mice preferred the star side of the box when given either ethanol or saline IP injections. The DBA/2J mice only showed a preference for the star side of the box during the saline IP injection test.

Our experiment examines whether Swiss-Webster mice will exhibit sign-tracking when given IP injections of ethanol, which are then paired with a light visual cue. This experiment sets up an environment where the cue is present each time the mouse is exposed to ethanol. During conditioning trials when the visual cue is present, ethanol will be given regardless of the animal’s behavior. This study is similar to the one done by Cunningham & Patel in 2007, but uses a different type of visual cue. It also will look for sign-tracking behavior in a different strain of mice, Swiss mice. Swiss mice are capable of learning a conditioned place preference, based on previous studies (Risinger & Oakes 1996).
Cunningham & Patel (2007) observed sign-tracking through increasing amounts of time that the mice spent on the star side of the box, and therefore what we expect to see in our sign-tracking study is that the paired group of Swiss mice will spend more time in proximity to the visual light cue. What this would mean is that the paired group did learn the association between the drug and the cue. Because the paired group spent more time next to the cue, then it can be said that the paired group liked the ethanol experience. This behavior indicates its usefulness as a model of craving.

**Materials and methods**

**Subjects**

Forty-eight male Swiss Webster mice, 8 weeks of age, were separated into 12 squads of 4 mice (6 squads for each paired and unpaired experiment). Mice were housed 4 to a cage made of polycarbonate with cob bedding and were placed in a ventilated Thoren rack. The mice had continuous access to food and water. The animal room was on a 12-hr light-dark cycle (lights on at 0700). This experiment followed the National Institutes of Health (NIH) “Principles of Laboratory Animal Care.”

**Apparatus**

Individual squads were run in 4 rectangular conditioning boxes (30x15x15). The walls of the boxes were acrylic with mesh flooring and no lid. Six infrared emitter/detector pairs were mounted 2.2cm above the floor at 5cm intervals. These emitter/detector pairs were used to determine location (left vs. right side) and general activity. Time spent on each side of the box and infrared beam breaks were recorded by a computer. Each conditioning box was enclosed in an individual ventilated, light- and sound-attenuating chamber (Coulbourn Model E10-20), which was illuminated by a 10cm “Mini Moon Lite” (AmerTac Model Mo. 73060, 3 VDC). The “Mini Moon Lite” was attached to the back wall of the chamber with Velcro. The light from the “Mini Moon Lite” was diffused by 20-lb (75g/m²) white paper (92 brightness) that was taped to the outside of the acrylic back wall of the conditioning box. A camera was attached to the ceiling of each chamber, and recorded the activity that took place in each conditioning box.

Each box had 2 nose poke holes located 1 inch from the floor on both the right and left wall. The visual cue was a light positioned in one of the two nose poke holes. The bulbs for the light cue were #47 6.3 volts, and received 5 volts during the experiment. In between squads, the inside walls and mesh flooring were wiped with a wet sponge to distribute animal odors.

**Procedure**

The experiment was broken into 3 different sessions: a pretest (day 1), conditioning trials (days 2-13), and preference tests (day 14). Each session consisted of the same procedure. One hour before the trials were to be run, the mice were brought down to room 721 and allowed to habituate to the room while in their home cages. Right before the trial, each mouse was weighed and given an intraperitoneal (IP) injection and immediately placed into the center of the conditioning apparatus. Each trial duration was 10min. After the trial the mice were removed and returned to the home cage. One hour after the last squad of mice returned to the home cage, each mouse was weighed and given an IP injection.

**Pretest**
During the pretest, each mouse was given an IP injection of saline before being placed in the apparatus. All of the mice were exposed to the light cue while in the apparatus. Half of the mice received the light in the right nose poke hole, and the other half received the light in the left nose poke hole. Each mouse was given a post IP injection of saline one hour later in the home cage.

**Conditioning**
The mice were divided into two groups, a paired group and an unpaired group. Within these groups, half of the mice received the light in the right nose poke hole during conditioning trials, and the other half received the light in the left nose poke hole. Conditioning trials consisted of alternating days when the light cue was present in the apparatus (CS+ day) and days when the light cue was not present in the apparatus (CS- days). On CS+ days, the paired group received an IP injection of ethanol before being placed in the apparatus, and the unpaired group received an IP injection of saline before entering the apparatus. Post IP injections given in the home cage on CS+ days were saline for the paired group, and ethanol for the unpaired group. The post IP injections of ethanol insured that each mouse received the same amount of ethanol per CS+ day. On CS- days, both the paired and unpaired groups received IP injections of saline before being placed in the apparatus and one hour later in the home cage. Six conditioning trials were conducted.

**Preference test**
Preference test 1 followed the same procedure as the pretest. All mice were exposed to the light cue while in the apparatus. Each mouse was given an IP injection of saline both before entering the apparatus and one hour after in the home cage. Preference test 2 was also the same procedure, however the “Mini Moon Lite” was not turned on during the test, and therefore the only illumination in the apparatus was due to the CS in the nose poke hole.

**Results**

**Pretest**
A one-way ANOVA was run on the activity data, with the single factor of group. A two-way ANOVA was run on the left time data, with the factors of group and conditioning subgroup. A repeated measures ANOVA was run on the nose poke data, with the within subjects factor of side (light, dark) and the between subjects factor of group. A p-value less than 0.05 was considered significant.

**Activity** The average activity per trial for the pretest is shown in figure 1. The pretest showed that the activity for the paired and unpaired groups was similar.

**Left Time** Shown in figure 2 is the mean time spent on the left side of the box per trial. The group of mice receiving the cue on the right side in the unpaired group spent slightly more time on the right side of the box [F(1,45)=5.2, p=0.02], most likely due to a sampling error.
A repeated measures ANOVA was run for the conditioning data. The within subjects factors were trial and trial type. The between subjects factor was group for the activity and percent time data. The between subjects factors for the left time data were group and conditioning subgroup. A repeated measures ANOVA was also run on the nose poke data. The within subjects factors were side (light, dark) and trial type. The between subjects factor was group. A p-value less than 0.05 was considered significant.

**Activity** - Figure 4 shows the average activity measured in counts per minute for each of the six conditioning trials. The activity for the paired CS+ group remained above that of the paired CS- and both of the unpaired groups throughout all of the conditioning trials [F(1,40)=119.4, p=0.000]. This shows that the animals were activated by the ethanol. Over time, the activity of each group decreased slightly, most likely due to habituation.

**Left Time** - The amount of time spent on the left side of the box is shown in figure 5. There is no distinction between the groups and conditions.

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**Nose Pokes** - Figure 3 shows the average number of nose pokes made per trial by the paired and unpaired groups in the light and dark nose poke holes. Both groups nose poked slightly more in the hole with the light cue compared to the dark hole [F(1,39)=5.1, p=0.000]. The unpaired group also poked significantly more in the light hole than the paired group [F(1,39)=5.1, p=0.030].

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**Conditioning trials**
Percent Time- Figure 6 shows the percent of time spent on the light side of the box. There was no significant difference between groups with respect to percent of time spent on the light side.

Nose Pokes- Figures 7 and 8 illustrate the average number of nose pokes per trial on conditioning trials 1 and 6, respectively. Both graphs show that there were more nose pokes on CS+ day compared to CS- days (for trial 1 [F(1,22)=4.4, p=0.047], for trial 6 [F(1,43)=13.1, p=0.001]). However, neither graph shows a significant difference between groups.

Preference tests
The statistics run on the preference tests were the same as were run on the pretest.

Test 1
Activity- Figure 9 shows the activity measurement for test 1. This shows a significant difference in groups with
respect to activity measured \[F(1,46)=6.7, \ p=0.013\]. The paired group has a higher activity count even though both groups received saline.

Left Time - The measure of left time is shown in figure 10. There was no significant difference between groups.

Nose Pokes - The average number of nose pokes during test 1 is shown in figure 11. The paired group poked significantly more in the CS+ hole compared to the CS- hole \[F(1,42)=5.0, \ p=0.031\].

Test 2

Activity - Figure 12 shows the activity for test 2. There was no significant difference between groups.

Left Time - The amount of time per minute spent on the left side of the box is shown in figure 13. There was no significant difference between groups.
Nose Pokes- Figure 14 shows the average number of nose pokes on the light and dark sides per group. There were significantly more nose pokes on the light side compared to the dark side \[F(1,44)=5.2, p=0.028\]. Also, the paired group made significantly more pokes on the dark side than the unpaired group \[F(1,44)=5.9, p=0.019\].

Discussion
Although the Swiss mice did not show a preference for the light cue during the conditioning trials, they did learn the association and were able to exhibit sign-tracking during preference test 1.

When comparing the nose poke counts for conditioning trials 1 and 6 (see figures 7 and 8 respectively) we can see that there was no sign-tracking observed in the presence of ethanol. The ratio of light pokes to dark pokes is not changed significantly in either group. There is a decrease in the overall amount of nose pokes from conditioning trial 1 to conditioning trial 6, which is most likely due to habituation.

The preference for the light cue can be seen when comparing figures 3 and 11, which show the nose poke counts for the pretest and preference test 1, respectively. During the pretest, both groups poked more in the light cue holes. However during preference test 1, the paired group showed a clear preference for the nose poke hole with the light cue. The fact that the paired group shows a preference for the light cue after conditioning can be indicative of sign-tracking. The act of nose poking was not necessary to obtain the drug, and was not rewarded. Another interpretation of these results could be that the increase in nose pokes by the paired group on the light side is a reflection of their increased activity.

It is interesting that the amount of time spent on the light side of the box was not a good indication of sign-tracking in this experiment. In the previous experiment by Cunningham & Patel (2007), the NZB/B1NJ mice showed sign-tracking over the conditioning trials by increasing the amount of time spent on the star side of the floor. A reason that the animals in our experiment did not spend more time on either side of the box could be that the mice were too activated by the ethanol during conditioning. In Cunningham & Patel, the DBA/2J mice had a much
higher mean activity during the conditioning trials with ethanol, compared to the NZB/B1NJ mice. The DBA/2J mice did not show an increase in time spent on the star side during conditioning trials, however they did show a preference for the star side during the saline test. This shows that although the DBA/2J mice did not show sign-tracking, they did learn the association of the star cue and the ethanol. One could argue that the difference in activity was the reason that the DBA/2J mice could not show sign-tracking during the conditioning trials. The activity of the DBA/2J mice was much higher than the activity of the NZB/B1NJ mice.

This activity difference could apply to our experiment and explain why sign-tracking was not observed during the conditioning trials. The Swiss mice were very active during the ethanol trials. However, even during the saline preference tests in our experiment the mice did not spend a greater amount of time on the light side of the box. In Cunningham & Patel (2007), the DBA/2J mice did show a preference during the saline test. The fact that the Swiss mice did not spend more time on the light side of the box could mean that the mice did not learn the association well enough. Perhaps Swiss mice need more conditioning trials in order to make a stronger association and be able to show a preference for the cue during the conditioning and the preference sessions.

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