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A Comparison of Different Forms of Methamphetamine on Locomotor Activity and Sign Tracking Performance in Rats

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A Comparison of Different Forms of Methamphetamine on Locomotor Activity and Sign Tracking Performance in Rats

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Experimental Psychology with a Concentration in Behavioral Neuroscience

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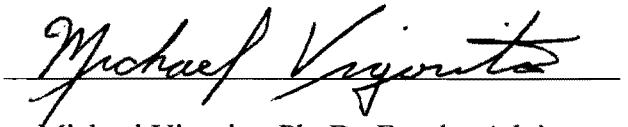

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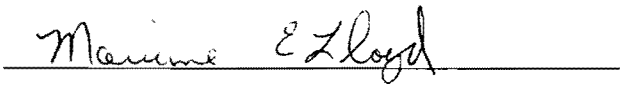

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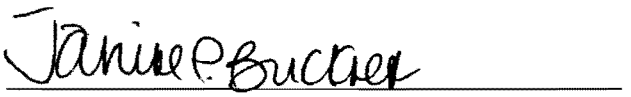

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Table of Contents

List of Figures

List of Tables

Abstract

Methamphetamine (METH) abuse is a major public health concern that needs to be better understood. Although METH obtained for research purposes adhere to a strict process of synthesis which tracks purity of a drug, METH is produced in clandestine laboratories with a variety of ingredients available in stores. Thus, the effect of impurities resulting from clandestine METH production on METH pharmacokinetics and drug use is poorly understood. Using rats as subjects, the present study examined the effects of d-METH (the psychoactive form of the drug) and d-METH adulterated with the levo form of the drug (l-METH) on two behaviors theorized to be associated with drug addiction, locomotor sensitization and sign tracking. Greater locomotor activity was observed in the groups treated with d-METH than in the l-METH alone and untreated controls, as well as behavioral sensitization to d-METH. Although l-METH has been reported to have some psychoactive properties the drug had no effect when given alone or when combined with the psychoactive form (i.e., dl-METH) (as is the case in clandestine-produced METH). Sign tracking, which reflects the impact of incentive salience on behavior, was also investigated. Incentives are previously neutral stimuli (or objects) associated with rewards (such as food or psychoactive drugs) that attain the same rewarding properties as the rewards themselves. As a reward-paired object gains incentive salience it gains the power to elicit compulsive approach behavior called sign tracking. The acquisition of incentive salience by reward-related stimuli (incentives) has been suggested to underlie the pathology of drug addiction (Robinson & Berridge, 1993). Incentives are believed to activate the same dopaminergic neural pathway involved in METH-induced locomotor activation. Thus we hypothesize that priming the dopaminergic pathway with a

sensitizing dose of d-METH should result in the faster acquisition of sign-tracking behavior compared to rats pre-treated with l-METH and untreated groups. The data were suggestive of a priming effect in the d-METH treated animals but not in the l-METH or dl-METH-treated animals.

A Comparison of Different Forms of Methamphetamine on Locomotor Activity and Sign Tracking Performance in Rats

Methamphetamine abuse is a major public health concern. According to the 2009 National Survey of Drug Use and Health (NSDUH), 502,000 individuals abused the drug within a month of taking the survey. This reflects an increase from the statistics reported in 2008, indicating past month methamphetamine abuse being 314,000. Treatment episodes for methamphetamine abuse have seen an increase within the past decade, as indicated by a report released by Substance Abuse and Mental Health Services Administration (SAMHSA) in February 2009. SAMHSA indicated that from 1997 to 2007, the number of admissions to treatment in which methamphetamine was the primary drug of abuse increased from 53,694 in 1997 to 137,154 in 2007 (SAMHSA, 2009). Amphetamine is the parent compound of a family of psychostimulants, which is available in two forms, l-amphetamine and d-amphetamine. The molecule of methamphetamine has a chiral center and exists as 2 enantiomers, d-methamphetamine (d-METH) which has been known to be the more active enantiomer and l-methamphetamine (l-METH), the less active enantiomer. D-METH has strong stimulant properties and has been widely associated with a higher abuse potential than l-METH (Fowler et. al., 2007). L-Meth is currently used in over the counter nasal decongestants such as Vick's Vapor Inhaler because it is a sympathomimetic vasoconstrictor. Other compounds in this family structure include methamphetamine, ephedrine, cathinone, "3,4 methylenedioxymethamphetamine" (MDMA), "3,4 methylenedioxyamphetamine" (MDA) and "3,4 methylenedioxy-N-ethylamphetamine" (MDE) (Meyer & Quenzer, 2005). Interestingly, amphetamine psychostimulants resemble the neurotransmitter,

dopamine (DA) in their chemical structure and this may account for its potent effects on the dopaminergic system. Amphetamines are referred to as sympathomimetic agents because they engage the actions of the neurotransmitters of the sympathetic nervous system (Julien et. al., 2010). Neurotransmitters in the sympathetic division of the autonomic nervous system include epinephrine, norepinephrine (NE) and DA. These neurotransmitters produce the signs and symptoms of the normal alerting response, but are engaged in excess under the influence of amphetamines. The main molecular targets of amphetamines include the dopamine (DAT), norepinephrine (NET), serotonin (SERT) and vesicular monoamine (VMAT) transporters. Unlike other psychomotorstimulants, amphetamine molecules do not block transporters, but use these transporters to enter the terminals and provoke neurotransmitter release from the vesicles into the cytoplasm (Meyer & Quenzer, 2005). Because of its molecular targets mentioned above, it is considered to be a catecholamine agonist and also has effects as a monoamine oxidase inhibitor (MAOI). Amphetamine's mechanism of action with NET, and SERT occur to a lesser extent when compared with its role with DAT (see Figure 1).

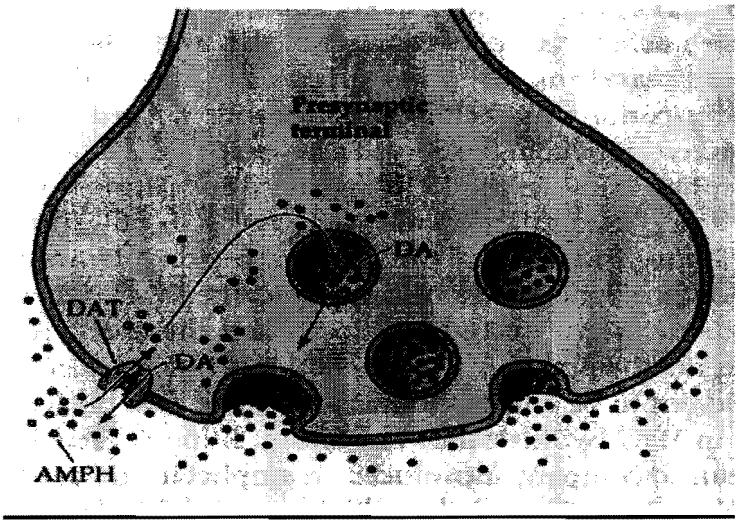

Figure 1 – *Mechanisms of amphetamine-stimulated DA release (Meyer & Quenzer, 2005)*

Methamphetamine is more potent in its effects of the central nervous system than amphetamine. In vitro studies have demonstrated that methamphetamine is twice as potent at releasing NE as DA, and its effect is much greater on noradenaline than serotonin (5-HT) release. Because of its long half life in the terminal plasma, lasting up to 10 hours, users experience a longer lasting high from a single dose of methamphetamine (Cruickshank & Dyer, 2009). This causes methamphetamine to be preferred by substance abusers over other stimulants such as cocaine which only have a half life ranging from .5 to 1.5 hours. The drug's high abuse potential and reinforcing properties have been attributed to, among other actions, the increased DA release onto the Nucleus Accumbens (NAc) (Cruickshank & Dyer, 2009). The mesolimbic DA system is referred to as the reward circuit in the brain which includes the pathway from the Ventral Tegmental Area (VTA) to the NAc. This pathway has been indicated as having a role in the highly reinforcing effects of most major drugs of abuse.

The clinical pharmacokinetics may differ in the methamphetamine typically used by individuals who abuse the drug. Drugs obtained for medical or research purposes adhere to a strict process of synthesis which track purity and impurity of a drug. This quality control in pharmaceutical industry has been demonstrated to be a reliable process producing desired effects of the drug. According to law enforcement, methamphetamine has been increasingly produced in clandestine laboratories using a variety of ingredients available in stores. The manufacturing of methamphetamine is called "cooking" (White House Drug Policy, 2009). Such laboratories which have been seized by law enforcement officials on a regular basis in the United States, do not adhere to quality

control guidelines in their synthesis of illicit methamphetamine. Therefore, street methamphetamine may produce unknown effects on the central nervous system. Based on anecdotal reports obtained from internet sources, it has been indicated that the levo form of methamphetamine (l-METH) has been used in the illicit synthesis of methamphetamine for recreational use (http://www.bluelight.ru/vb/archive/index.php/t-271041.html). It has been demonstrated that rushed synthesis of drugs can have deleterious effects on health. In the 1980s a clandestine laboratory rushing the synthesis of MPPP, a synthetic opioid, accidentally produced MPTP. This compound, targeting DA neurons resulted in permanent damages to the individuals who ingested it, producing Parkinson-inducing effects (Pinel, 2009).

The present study examines the effects of the different forms of methamphetamine on two behaviors, sensitization of locomotor activity and sign tracking. Locomotor activity is defined as movement and physical activity demonstrated by the animal. Locomotor sensitization is the increased locomotor activity after repeated exposure to the same dose of psychomotor stimulant, such as methamphetamines. Comparisons of both amphetamine and d-methamphetamine have produced locomotor sensitization using open field measurements (Hall et. al., 2008). The present study endeavors to determine if differences in locomotor sensitization effects emerge in comparison among the three methamphetamine treatment groups. It has been reported in human research that at high doses, l-METH intoxication is similar to that of d-METH but the pharmacodynamic effects (heart rate, blood pressure, intoxication ratings, drug liking) are shorter lived and thus, not as desired as d-METH (Mendelson et.al., 2006). In a study conducted by Volkow et. al. (1997), DA increase must occur quickly in order for

a drug to be perceived as reinforcing, as seen with many drugs of abuse, especially d-METH. Additionally, Mendelson et. al. (2006) reported that d-METH and d,l-METH had similar effects in humans and may have similar abuse potential. Microdialysis experiments examining l-METH have indicated that a much higher dose (12mg/kg and 18 mg/kg) is needed in order to induce stereotyped behaviors (Kuczenski et.al., 1995). The Kuczenski et. al. experiments also showed that a much higher dose of l-METH (12 mg/kg and 18 mg/kg) is needed in order to increase extracellular DA sooner and to higher peak levels, and even at these doses, DA levels are still not as high and did not peak as rapidly as those seen with d-METH.

We hypothesize that differences in locomotor sensitization will emerge among groups, with more robust locomotor activity appearing in the groups treated d-METH and little or no locomotor sensitization in the groups treated with l-METH and saline. But if l-Meth has some psychoactive properties as suggested by some human and animal studies, the l-Meth group may fall between the d-Meth and saline groups. Hall et. al., used 0.5 and 1.0 mg/kg doses for amphetamine and d-METH. The present study will examine a 1.5mg/kg dose of d-METH and l- METH, but a 3mg/kg of racemic METH (i.e., d,l-METH mixture) , so that this treatment group is receiving the same amount of d- (1.5mg/kg) and l- (1.5mg/kg) METH per injection as the other treatment groups. If l – METH is an ineffective form of the drug then the 1.5mg/kg d-METH and the 3.0mg/kg racemic METH should show equivalent effects on locomotor behavior. However, if l-METH interacts with the d-METH, the effect of the 3.0mg/kg racemic METH will show differential effect on locomotor behavior than the 1.5mg/kg d-METH. Previous research has indicated that head shaking may be a better measure of METH –induced motor

sensitization than general locomotor behavior (Kass, et.al., 2010). In fact the emergence of head shaking when higher doses are used may interfere with the measurement of locomotor behavior in the form of general activity. Thus, a locomotor sensitization effect may not be readily observed. Although we expect METH to increase locomotor behavior compared to controls, the sensitization to METH over days may not be observed in locomotor activity.

The present experiment will also examine whether prior METH exposure of the three different treatments has an effect on behavior in a Pavlovian sign-tracking procedure. A Pavlovian conditioning procedure involves presentation of a conditioned stimulus (CS) which is followed with a presentation of an unconditioned stimulus (US), such as food. In the sign tracking procedure when subjects approach, contact or interact with the CS, they are displaying sign tracking behavior, but when subjects approach the location of the US during the CS presentation they are displaying goal tracking behavior (Brown & Jenkins, 1968). The emergence of sign tracking behavior shows that stimuli associated with rewards (incentives) such as food or drugs of abuse can attain the same rewarding properties as the rewards themselves. The acquisition of incentive salience by reward related cues has been suggested to underlie the pathology of drug addiction because the incentive properties of drug-related or drug predictive cues may impact drug use related behavior (Robinson & Berridge, 1993). The increased incentive salience or "incentive sensitization" may impact drug addiction by increasing the behavior directed at obtaining drugs and drug related cues (Robinson & Berridge, 2003). The sign tracking procedure is an effective procedure for evaluating the acquisition of incentive salience brought on by reward-predictive cues (Brown & Jenkins, 1968; Tomie et. al., 2001). It

has also been demonstrated that administration of psychostimulant drugs such as cocaine or amphetamines may cause long-lasting enhancements of behavior toward reward-predictive cues (Norquist et. al., 2007).

If sign tracking is a case of incentive sensitization, then it may be expected that rats treated with methamphetamine prior to sign track training should show faster acquisition of sign tracking compared with controls. Methamphetamine may prime the neural pathway responsible for behavioral sensitization so that when reward-predictive cues are presented, they should respond to it sooner and more strongly. In a study conducted recently by Flagel, et al., (2010), selectively bred rats for novelty seeking behavior were compared with controls in a signtracking procedure with measurements of dopamine levels analyzed during presentation of the CS. The selectively bred signtracking rats demonstrated higher release of DA onto the NAc than goal seeking rats. This indicates that DA plays an important role in reward learning, more specifically with the association of incentive salience to reward related cues. Since methamphetamine has a potent mechanism of action with DA, the present study may produce faster acquistion of signtracking in the rats treated with d-METH when compared with controls and l-METH. Some previous research using the sign tracking paradigm, failed to yield sign tracking behavior in rats after prior exposure to d-amphetamine (Simon et. al., 2009; Mendez et. al. 2009). Conversely, the researchers found that more goal tracking behavior emerged as a result of prior d-amphetamine exposure. Using a different dependent measure of sign tracking, latency to approach the CS, Fitzwater & Spear (2011) observed shorter latencies in adult Sprague Dawley rats previously treated with d-amphetamine. However, they did not observe an increase in the amount of interaction with the CS.

The previous experiments did not expose the animals to the sign tracking procedure prior to drug administration. In the sign tracking procedure, rats first learn to associate the sound of the moving object with food, therefore they goal track before they begin to sign track . Once they begin to sign track, most rats spend less time goal tracking and more time sign tacking as seen in our pattern of data from a previous study (Michaels, Casachahua, & Vigorito , 2010). It may be possible that METH pretreatment enhances attention towards any signaled reward. Since goal tracking usually occurs first, methamphetamine pretreatment may enhance goal tracking and interfere with the establishment of sign tracking. It has been demonstrated that stimulants such as cocaine enhance responding to all CS's including goal tracking (Taylor & Jentsch, 2001). If the animals are given some initial training in the sign tracking procedure before methamphetamine treatment, then once methamphetamine is administered, the drug may have the opportunity to enhance sign tracking behavior, since they are sign tracking and goal tracking rather than exclusively goal tracking.

In the present study, rats received some initial training in the sign tracking procedure before drug administration and locomotor sensitization measurements, which has not been done in the previously noted experiments. The sign tracking procedure implemented in these experiments took place two months after drug administration (Mendez et. al., 2009) or seven days after final drug injection (Simon et. al., 2009). The present study resumes sign tracking training the day after final injection. The CS used in the previous experiments was either a lever (Mendez et. al., 2009; Fitzwater & Spear, 2011) or light (Simon et. al., 2009). The CS in the present study uses presentation of a sipper from a bottle containing water, similar to that used in previous experiments

examining ethanol consumption in the sign tracking procedure (Tomie et. al., 2001). We hypothesize that more robust sign tracking behaviors will emerge in the d-METH and d,l-METH treatment groups compared with l-METH and controls, given the differences from previous research in procedure, which are noted above.

Experiment 1

Method

Subjects. Sixteen male Long Evans Rats, approximately 6 to 8 weeks old were obtained from Harlan. They were kept on a 12:12 light/dark cycle, with experiments performed during the light cycle. Rats were double housed in translucent standard home cages for 4 weeks prior to the start of the present study and weighed daily prior to and during the study. They remained double housed in the standard cages for the duration of the experiments and received food (Purina lab pellets) and water *ad libitum.*

Apparatus

Open Fields. Locomotor activity was measured in two square open field structures consisting of Plexiglas walls that were 90 cm square and 90 cm high. The field was illuminated with a 60 W fluorescent light. A digital camcorder was mounted above the two open field structures and a video tracking system (Any-Maze) was used to record rats' activities during the daily test sessions.

Sign Tracking Chambers. Rats were trained in four (23 x 18 x 23.5 cm) standard conditioning chambers that are equipped with a lever, two stimulus lights, a retractable bottle and sucrose pellet dispenser and food tray. The lever and stimulus lights were not used in this experiment. The approaches to the bottle presentation, (i.e., the conditioned

stimulus (CS)) were defined as licks on the sipper which were counted with lickometers, and the approaches to the unconditioned stimulus (US) (i.e., head pokes into the food tray) were recorded with infrared sensors attached to the two clear sides of the food tray. All equipment was controlled by programs written in Med-PC IV software (Med Associates Inc.).

The four chambers were similar in construction but with some minor differences. Within chambers 1 and 2, the food trays were approximately 4.3 cm x 4.3 cm, and were located in the middle of the same metal wall 2.5 cm above the grid floor. Within chambers 3 and 4, the food trays were approximately 5 cm x 5 cm, and were located in the left (2.5 cm away from plastic wall) of the same metal wall 1 cm above the grid floor. A retractable bottle mechanism from Med Associates extended the bottle to an opening in the plastic wall of the chamber approximately 2.5 cms above the grid floor. The bottle nozzles were inserted so they were flush with the inside chamber wall so that the rats would not touch the nozzles with their paws while licking. The Med Associates lickometer wires were connected to the bottle nozzle and to the chamber grid floor.

Drugs

Methamphetamine, both d-METH and l-METH were obtained from Sigma-Aldrich Chemical Corporation. Three different mixtures of methamphetamine were used in the present study. The first was d-METH, the second was l-METH and the third was racemic METH, a 50/50 combination of d and l-METH. Each of the three types of methamphetamine was dissolved in physiological saline on the days of injections. The methamphetamine compounds were administered intraperitoneally (IP).

Procedure

Adaptation. Prior to the first phase of sign tracking training, rats were adapted to the chambers where they received sucrose pellets used as the US in the sign tracking task to decrease neophobia and adapt to the chambers. This lasted a period of four days with trials of 15 minutes for each of the four groups.

Pretraining. During the pretraining phase of the present study, rats were moved one squad at a time (n = 4/squad) from their home cages to one of four Sign Tracking chambers. Each 30 minute session consisted of 30 trials with 60 seconds between trials. During a trial the bottle CS was presented for 10 seconds and immediately followed by the delivery of the US, a single sucrose pellet. Lick on the bottle sipper tube and head pokes in the food tray were recorded as the primary dependent variables in the experiment. The pretraining phase lasted 3 days to assess sign tracking and goal tracking behavior preferences. The purpose of this phase is to ensure that the rats were displaying goal tracking and sign tracking behavior before the drug exposure phase of the experiment.

Drug Treatment/Open Field Testing. The second phase of the study continued offline from the sign tracking procedure. The rats were divided into four groups, each group receiving a different drug treatment, with the groups matched on the amount of head poking and sign tracking behavior established in the pretraining phase. Drug treatment and open field testing occurred on five consecutive days with animals tested two at a time. Each day immediately after an injection, the rats were placed in one of the two open field structures for 30 minutes to measure locomotor behavior. The AnyMaze software divided the floor area into 16 equal-sized squares so that locomotion can be

measured in terms of number of line crossings. . The dependent variables were the total distance traveled and number of line crossings per session. Group d-Meth ($n = 4$) received 1.5mg/kg IP injections of d-METH. Group d.l-Meth ($n = 4$) received 3.0mg/kg IP injections of racemic methamphetamine (d,l-METH). Group l-METH ($n = 4$) received 1.5mg/kg IP injections of l-METH. Group four ($n = 6$) received saline (1ml/kg).

On the fifth day after open field measurements, blood samples were collected from each rat. Blood was collected from the lateral tail vein into EDTA filled Microvette CB300 pipettes. The data are not reported in this thesis.

Sign-track training. Sign tracking training continued during the third phase of the study. Each group of rats was placed in the sign tracking chambers for 30 minute trials, as they were in pretraining. The sign tracking procedure lasted for 10 days to assess sign tracking or goal seeking differences between and within groups after prior meth-amphetamine exposure.

Data Analysis. The open field and sign tracking data were analyzed using separate mixed design ANOVAs with drug treatment (4) as the between-subjects factor and days (5 or 7) as the within-subjects factor.

Results

Open Field. In examining the total distance traveled of the four treatment groups, a main effect of drug emerged, $F(3, 12) = 13.98, p < .001, \eta\rho^2 = .78$. Post-hoc analysis (Tukey *HSD*) confirmed that rats treated with d-METH traveled significantly greater distance than l-METH ($p = .001$) and saline ($p = .002$) but not dl-METH ($p > .05$). In addition, rats treated with dl-METH traveled significantly greater distance than l-

METH (p = .007) and saline (p = .008). The effect of days, F (4, 48) = p < .001, $\eta\rho^2$ = .46, was due to significantly greater distance traveled on the first day compared with all other days (p <.001). A significant drug x days interaction, F (12, 48) = 2.15, p = .03, $\eta\rho^2$ = .35, indicates that there was a greater decline in distance traveled in rats treated with d-METH and dl-METH than in the two groups that were not treated with d-Meth (see *Figure 2*). To confirm the nature of this interaction, separate 2 x 5 ANOVAs were conducted on the two groups treated with d-Meth and the two groups not treated with d-Meth. The groups that were not treated with the psychoactive form of METH (groups l-Meth and Saline) showed no significant change in distance traveled over days, F (4, 24) = 1.54, p > .05. However, the distance traveled in groups treated with the psychoactive form of the drug (d-Meth and dl-Meth) significantly declined by day 2, F (4,24) = 20.54, p < .001). But, as can be seen in Figure 2, the distance traveled did not differ on Days 2 to 5 (*ps* > .05).

In examining number of line crossings *(see Figure 3)*, a significant main effect of drug emerged, F (3, 12) = 13.49, p < .001, $\eta\rho^2$ = .77. Post-hoc analysis (Tukey *HSD*) confirmed that rats treated with d-METH crossed significantly greater amount of lines than l-METH (p = .002) and saline (p = .003) but not dl-METH (p > .05). Additionally, rats treated with dl-METH crossed significantly greater amount of lines than l-METH (p = .004) and saline (p = .006). In contrast to the distance traveled measure a significant drug x days interaction did not emerge, F (12, 48) = 1.76, p = .08, $\eta\rho^2$ = .31 However, a significant main effect of days was observed, F (4, 48) = 3.276, p = .02, $\eta\rho^2$ = .21. Pairwise comparisons indicated that the main effect of days in line crossings was due to a higher amount on day1 compared with day 4 (p = .01) and day 5 (p = .01).

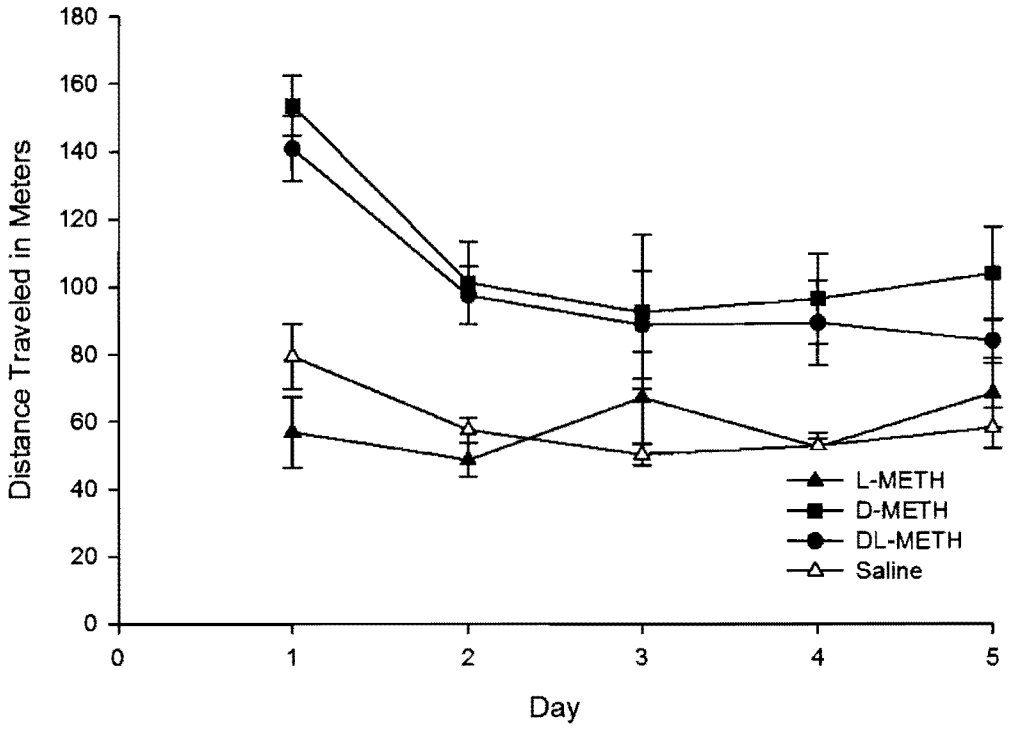

Figure 2. Total distance traveled in the four treatment groups across the five days of IP injections.

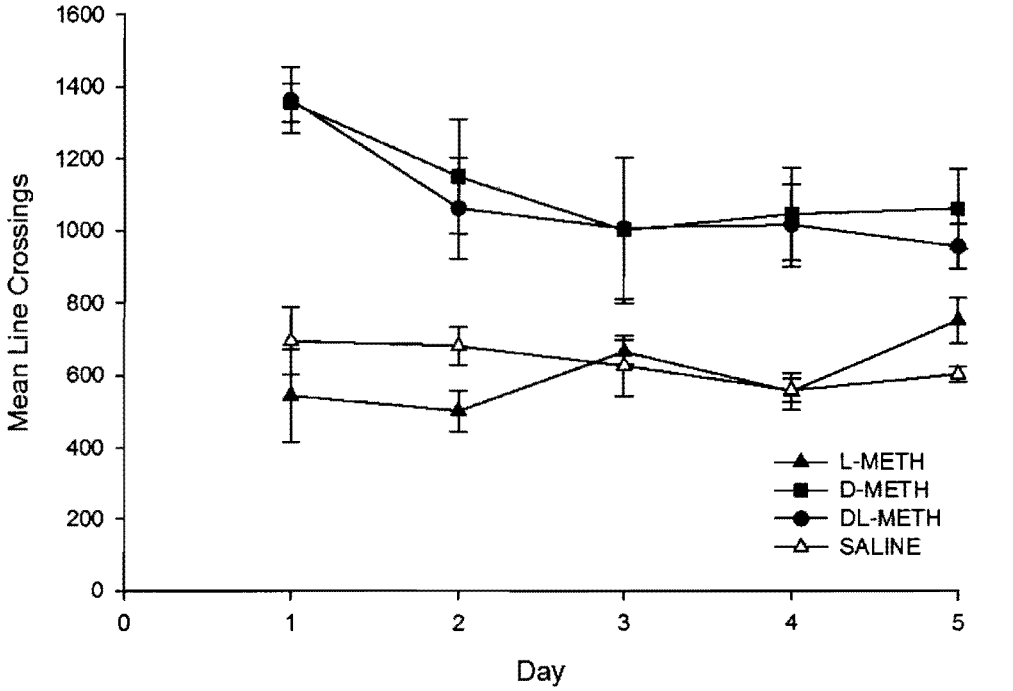

Figure 3. Number of line crossings in the four treatment groups across the five days of IP injections.

Sign Tracking. Daily session licks on the bottle sipper were collected prior to (3 days) and after drug treatment (7 days). Groups were assigned to the drug conditions after the third day of pre-training. Based on the lick rates on the third day of pre-training the rats were divided into 4 equal groups and each group was assigned to one of the 4 drug conditions. The data from the first 3 days were then analyzed with a Groups (4) x Days (3) mixed ANOVA to confirm that there were no initial group differences before drug treatment was initiated. The session licks for the four groups on the first three days of sign tracking before the drug was introduced are shown in Figure 4 (left panel). An ANOVA on the data found that there was not a significant main effect of drug, F (3,12) = 0.52, p = .753, nor a drug x days interaction, F(6, 24) = 0.567, p = .753). Most rats did show some sign tracking (i.e., licking of the bottle sipper) during the first 3 days. Allowing for the emergence of some sign-tracking behavior before the introduction of drug treatment was the goal of pre-training. The lick rates overall appeared to increase slightly over the first three days of pre training however significance did not emerge, F (2, 24) = 2.56, p = .098.

In order to evaluate the effect of drug treatment on the continued acquisition of sign tracking, ANOVAs were conducted using the daily lick totals from seven days of sign tracking after drug treatment (see *Figure 4*, right panel). When examining all four treatment groups (4 x 7 ANOVA), only a significant main effect of days emerged, F (6, 72) = 6.65, p = .001 (see *Figure 4*). Because of the small number of subjects in each group additional ANOVAS were done for exploratory purposes. As indicated by the open field data, both forms of d-METH had more psychomotor activating properties than l-Meth and saline. Thus, when combining the 2 groups that received d-METH either in

racemic mixture or with the d-METH alone (d-Meth; n = 8) and combining the l-METH and saline groups (No d-Meth; n = 8), a 2 x 7 ANOVA yielded a significant main effect of treatment, F (6, 84) = 7.163, p = .001, with higher lick rates observed in the d-METH treatment group. The interaction between treatment and days was not significant, F (1, 14) = 3.169, p = .09. This re-plotted data is shown in *Figure 5*).

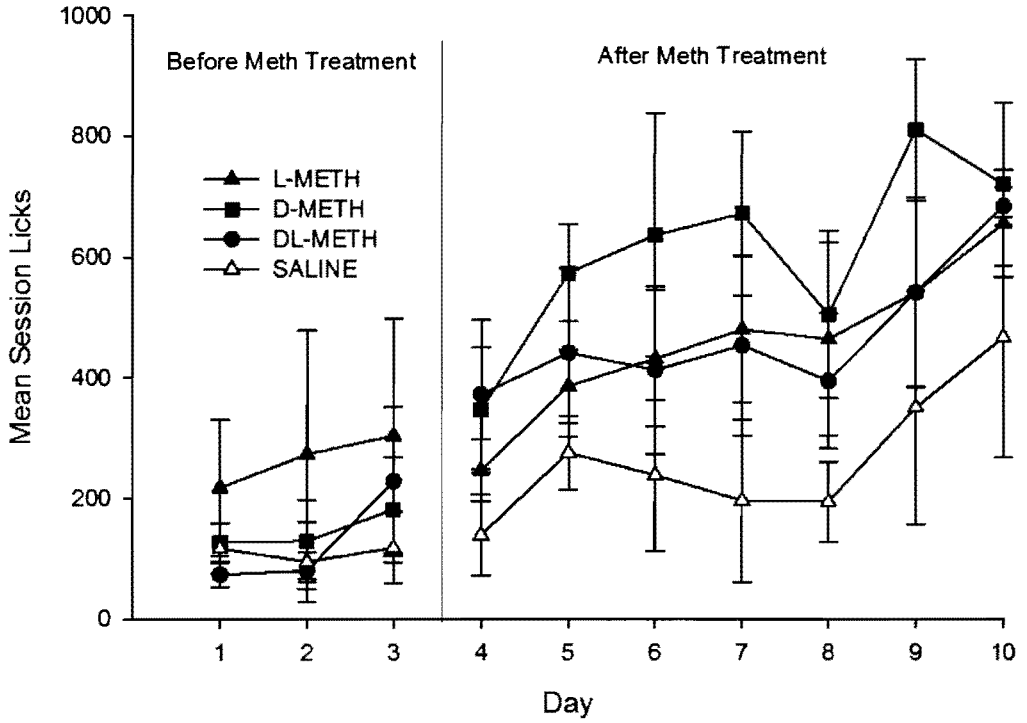

Figure 4. Data above show total licks of the d-METH (n = 4), d,l-METH (n = 4), l-METH (n= 4) and saline (n = 4) treatment groups prior to drug administration (left panel) and after drug administration (right panel).

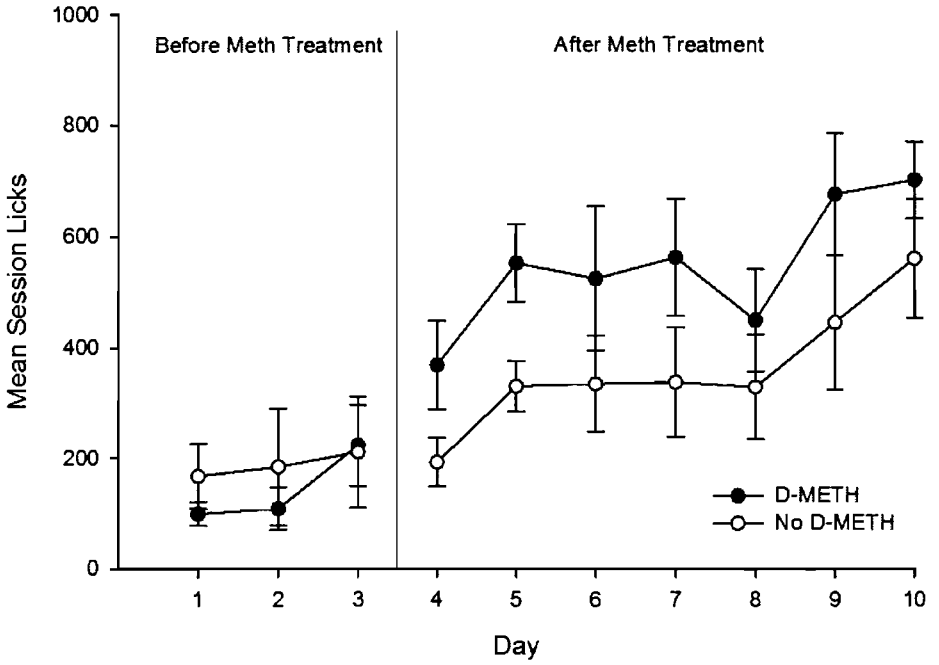

Figure 5. Data above show total licks of the d-METH (n = 8) compared with no d-Meth (n = 8) treatment groups prior to drug administration and after drug administration.

Discussion

We expected that after some initial training in the sign-tracking procedure rats treated with d-METH would demonstrate faster acquisition of sign tracking behavior than rats treated with saline or the non-psychoactive form of methamphetamine. This prediction was based on the assumption that d-Meth treatment would sensitize, and therefore prime, the same neural pathway that mediates sign-tracking behavior. Although not statistically significant, the effect of d-METH treatment on sign tracking was in the predicted direction.

Although both forms of d-METH produced greater locomotor behavior than l-Meth and Saline, locomotor sensitization was not observed in the open field data. In fact

there was a slight decline by day 2 and this decline was significant in the distance traveled analysis. The decline in locomotor behavior is not unexpected because such declines result from increased stereotypy competing with locomotor behavior (Flagel & Robinson 2007; Kass, Liu, Vigorito, Chang & Chang 2010). For example, an increase in rearing activity and head shaking, which are indicators of behavioral sensitization, is not detectable by the AnyMaze program being used in the study and therefore sensitization may be indicated by a decline in locomotor behavior with repeated drug treatment. However; in this experiment the decline occurred on the second day with no further declines on subsequent days. The initial drop was most likely due to habituation to the novel context of the open field apparatus rather than due to the emergence of stereotypy that interferes with locomotor behavior. It is desirable to demonstrate a sensitization effect since the hypothesis is based on the assumption that it is the sensitization process that is involved in the Meth effects and the emergence of sign tracking. It may be that the 30 minute session length of the open field test was not of sufficient duration to reveal significant declines in locomotor behavior with increasing drug exposure. Thus in Experiment 2 the duration of the open field test session was increased to 60 minutes.

It was surprising to see that l-METH may have reduced the effects of d-METH on sign tracking. We expected no effect of the addition of l-METH to d-METH, because each treatment group was receiving the same dose of d-METH and the literature suggests that that l-METH has limited psychoactive properties. We considered that there may have been a slightly greater effect of the racemic METH compared to d-METH alone, but not a weaker effect. If this is a real effect it may be an important finding.

Experiment 2

The purpose of Experiment 2 was to extend the findings of Experiment 1. Because the data were trending in the predicted direction, the same sign tracking procedure was used for Experiment 2, except that the number of animals per group was increased. Another procedural change was the duration of the open field test, which was increased from 30 minutes to 60 minutes. This increase in the session length was introduced to look for evidence of sensitization. It may be that the 30 min session in Experiment 1 was too brief for stereotypy to emerge at sufficient levels to be reflected in the locomotor behavior data. In order to address the drop in locomotor behavior from day 1 to day 2, we adapted the rats to the open field apparatus the day before drug treatment began so that the open field was no longer novel on the first day of drug treatment.

Because one of the main research questions surrounds the impurity of street drugs impacting behavior and Experiment 1 suggested that l-METH may have influenced the effect of d-METH, we modified the design analyses in the experiment. We are interested in examining whether or not d-METH (pure) when mixed with a "dirty" solvent (l-METH) versus a clean solvent (saline) will yield a difference in locomotor activity, behavioral sensitization and sign tracking. In order to investigate the data specific to this research question, we conducted an analysis looking at the interaction of drug treatment (d-METH or no drug) with the solvent (dirty, [i.e. with l-METH] or clean [i.e., saline]) as outlined in Table 1. This analysis results in the same four groups tested in Experiment 1, but allow for an analysis of a possible interaction between d-METH and l-METH. When analyzing the open field data the addition of days as a factor results in a Drug (2) x

Solvent (2) x Days (5) mixed ANOVA; for the sign tracking data after drug treatment the design is a Drug (2) x Solvent (2) x Days (7) mixed ANOVA.

Table 1.
Drug Treatment (2) x Solvent (2) Between-Groups Factorial design

		Drug Treatment	
		d-METH	No Drug
Solvent	Dirty (with l-METH)	n = 7	n = 8
	Clean (Saline)	n = 8	n = 7

By running these analyses, it allows the examination of a possible interaction between d-METH with the solvent and it provides a baseline for future experiments investigating whether additional adulterants may have a significant impact on the d-METH effects on behavior. Moreover if an interaction is not observed this design also has the advantage of increasing *n* for each of the drug conditions from 7 to 14 (i.e., the main effects of drug and solvent.

Method

Subjects. Thirty male Long Evans Rats, approximately six to eight weeks old were obtained from Harlan. They were kept on a 12:12 light/dark cycle, with experiments performed during the light cycle. Rats were double housed in translucent standard home cages for 4 weeks prior to the start of the present study and weighed daily prior to and during the study. They remained double housed in the standard cages for the duration of the experiments and received food (Purina lab pellets) and water *ad libitum.*

Apparatus. The same two open fields and the same four sign-tracking chambers from Experiment 1 were used in this experiment.

Drugs. Three different mixtures of METH were used as in Experiment 1: d-METH, l-METH and dl-METH (racemic METH) The drugs were dissolved in physiological saline on the days of injections and administered ip.

Procedure

Adaptation to Open Field. All rats were adapted to the open field apparatus for 20 minutes the day prior to the start of sign track training.

Adaptation to Sign Tracking Chamber. All rats were adapted to the chambers during 4 daily 15 minute sessions where they received sucrose pellets used as the US in the sign tracking task.

Pretraining. During the pretraining phase of the present study, rats were tested in the Sign Tracking chambers in squads of four. Each 30 minute session consisted of 30 trials with 60 seconds between trials. Licks on the bottle and head pokes were recorded as described in Experiment 1.

Drug Treatment/Open Field Testing. The second phase of the study continued offline from the sign tracking procedure. The rats were divided into four groups, each group receiving a different drug treatment, with the groups matched on the amount of head poking and sign tracking behavior established in the pretraining phase. Initial analyses of the pretraining data indicated no significant mean differences among the groups prior to treatment. Drug treatment and open field testing occurred on five consecutive days with animals tested as described in Experiment 1 except that the session length was 60 minutes rather than 30 minutes. The dependent variables were the total distance traveled and number of line crossings per session. Group d-Meth ($n = 7$)

received 1.5mg/kg IP injections of d-METH. Group d.l-Meth (n = 8) received 3.0mg/kg IP injections of racemic METH (d,l-METH). Group l-METH (n = 8) received 1.5mg/kg IP injections of l-METH. Group four (n = 7) received saline (1ml/kg).

On the fifth day after open field measurements, blood samples were collected from each rat. Blood was collected from the lateral tail vein into EDTA filled Microvette CB300 pipettes

Sign-track training. Sign tracking training continued during the third phase of the study. Each group of rats was placed in the sign tracking chambers for 30 minute trials, as they were in pretraining. The sign tracking procedure lasted for 10 days to assess sign tracking or goal seeking differences among groups after prior METH exposure.

Data Analysis.

Open Field. The open field data were analyzed using mixed design ANOVAs with drug treatment (D or No D-METH) (2) x solvent (Dirty or Clean) (2) x days (5). Drug treatment and solvent were the between-subjects factors. Days was the within-subjects factor.

Sign Track Training. The sign tracking and head poking data were analyzed using separate mixed design ANOVA drug treatment (D or No D-METH) (2) x Solvent (Dirty or Clean) (2) x days (10). Drug treatment and solvent were the between-subjects factors and days was the within-subjects factors.

Results

Open Field. The drug (2) x solvent (2) x days (5) ANOVA on the total distance traveled revealed a significant main effect of drug, $F(1, 26) = 42.48$, $p < .001$, $\eta\rho^2 = .62$.

Pairwise comparisons indicated that the effect of drug was due to significantly greater distance traveled in rats treated with both forms of d-METH (i.e., d-Meth and racemic dl-Meth) ($M = 219.65 \pm 13.44$) when compared with rats treated with No d-METH (95.74 ± 13.44) ($p < .001$). There was also a significant drug x days interaction, $F(4, 104) = 3.30$, $p = .01$, $\eta\rho^2 = .11$. Post-hoc analysis on the distance traveled revealed that the interaction emerged as a result of significantly lower amount demonstrated by the rats treated with d-METH on day 5 compared with all other days of treatment ($ps < .04$). As can be seen in Figure 6 this interaction was due to a significant decline in distance traveled across the five days in rats treated with d-METH but no decline in the rats that were not treated with d-METH. The decline in the d-METH-treated rats likely reflects a sensitization effect because the decline occurred after several days of drug treatment when rats were likely engaged in increasing stereoypies that interfered with locomotor behavior. There was not a significant three way drug x solvent x days interaction, nor a drug x solvent interaction, indicating that the solvent (saline or l-Meth) did not affect the impact of repeated d-Meth treatment on distanced traveled.

The drug (2) x solvent (2) x days (5) ANOVA analysis of the number of line crossings revealed a significant main effect of drug, $F(1, 26) = 45.15$, $p < .001$, $\eta\rho^2 = .64$. Pairwise comparisons indicated that the effect of drug was due to a significantly greater amount of line crossings in the rats treated with both forms of d-METH ($M = 1720.63 \pm 99.56$) than rats treated with No d-METH ($M = 774.55 \pm 99.56$) ($p < .001$). As with the distance traveled data, there was also a significant drug x days interaction, $F(4, 104) = 5.94$, $p < .001$, $\eta\rho^2 = .19$. Post-hoc analysis indicated that the interaction emerged as a result of the significant decline in line crossings demonstrated by the rats treated with

d-METH on days 3, 4 and 5 compared with days 1 and 2 (*ps* < .03). Thus, a sensitization effect may be inferred from the decline in traveling across the chamber floor as indicated by the line crossings measure, demonstrated by rats treated with d-METH (see *Figure 7*) The analyses did not reveal a significant 3-way interaction, nor did a significant 2-way interaction with solvent confirming that the type of solvent did not affect the impact of d-METH on the sensitization of stereotyped behavior.

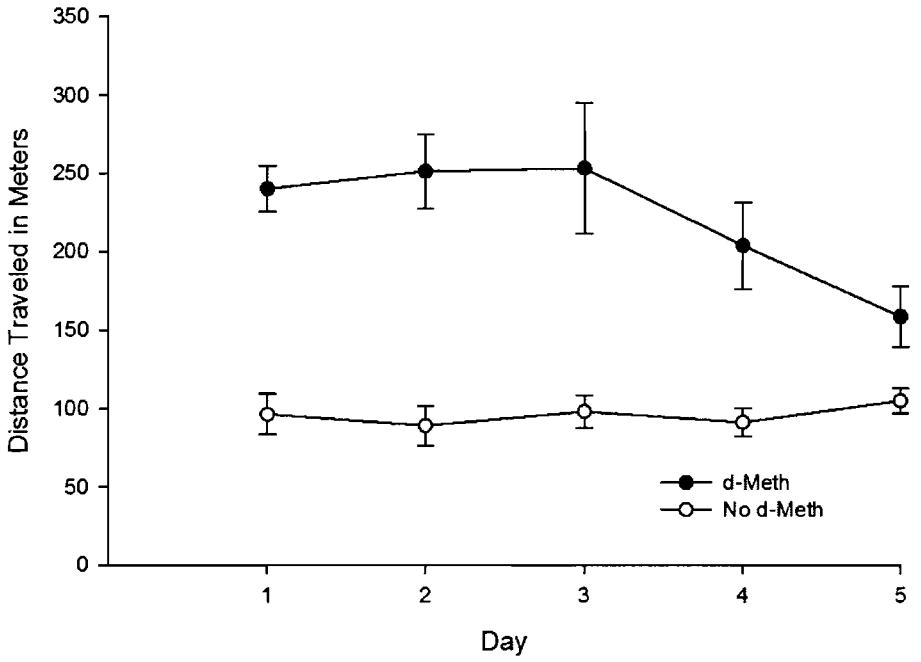

Figure 6. Total distance traveled in the groups separated by treatment of d- or no d-METH across the five days of IP injections.

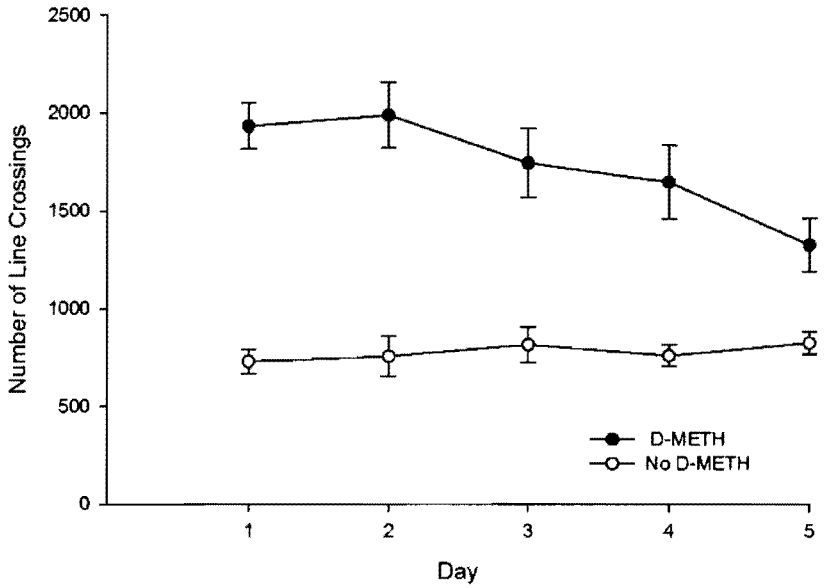

Figure 7. Total number of line crossings in the groups separated by treatment of d- or no d-METH across the five days of IP injections.

Sign Tracking. Daily session licks on the bottle sipper were collected prior to (3 days) and after drug treatment (10 days). Groups were assigned to the drug conditions after the third day of pre-training. Based on the lick rates on the third day of pre-training the rats were divided into 4 groups and each group was assigned to one of the 4 drug conditions. The data from the first 3 days (*Figure 8*, left side) were then analyzed with a Groups (4) x Days (3) mixed ANOVA to confirm that there were no initial group differences before drug treatment was initiated, $F(6, 52) = 1.229, p = .31$. A drug (2) x solvent (2) x days (10) mixed ANOVA revealed a main effect of days, indicating that all rats increased session lick rates across the ten days after treatment, $F(9, 234) = 9.81, p < .001$, $\eta\rho^2 = .28$ (see *Figure 8, right side*). The main effect of drug $F(1, 26) = .833, p > .05$ and solvent, $F(1, 26) = 1.09, p > .05$, failed to be significant When examining the means across all 10 days in Figure 8 it can be observed that the acquisition of sign tracking was steepest in the d-Meth group compared to the other groups. Interestingly,

the d-Meth rats were slightly lower in mean lick rates than the No d-METH groups in the first few days after treatment, but this seemed to reverse during days 7 through 10. This suggests that d-Meth enhanced the acquisition of sign tracking. However, the drug x solvent x days interaction was not significant ,F (9, 234) = 1.32, p > .05. When the drug x days interaction is plotted ignoring the solvent (*Figure 9*) the d-METH-treated rats again show a steeper learning curve, but again the drug x days interaction failed to be significant, F (9, 234) = 1.39, p > .05 .

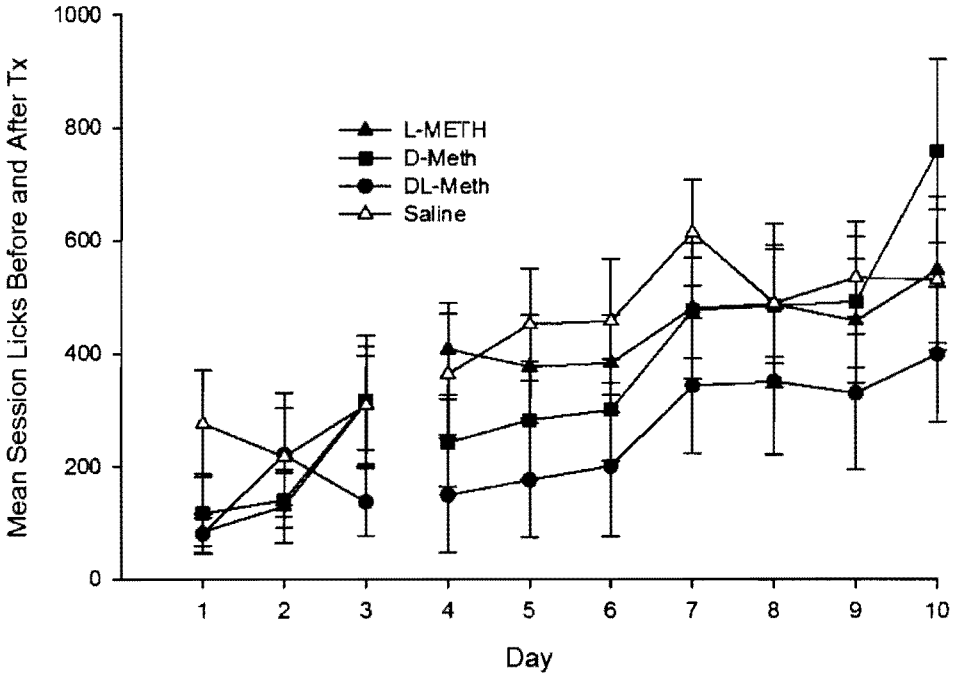

Figure 8. Before and after METH treatment mean lick rates for the four groups.

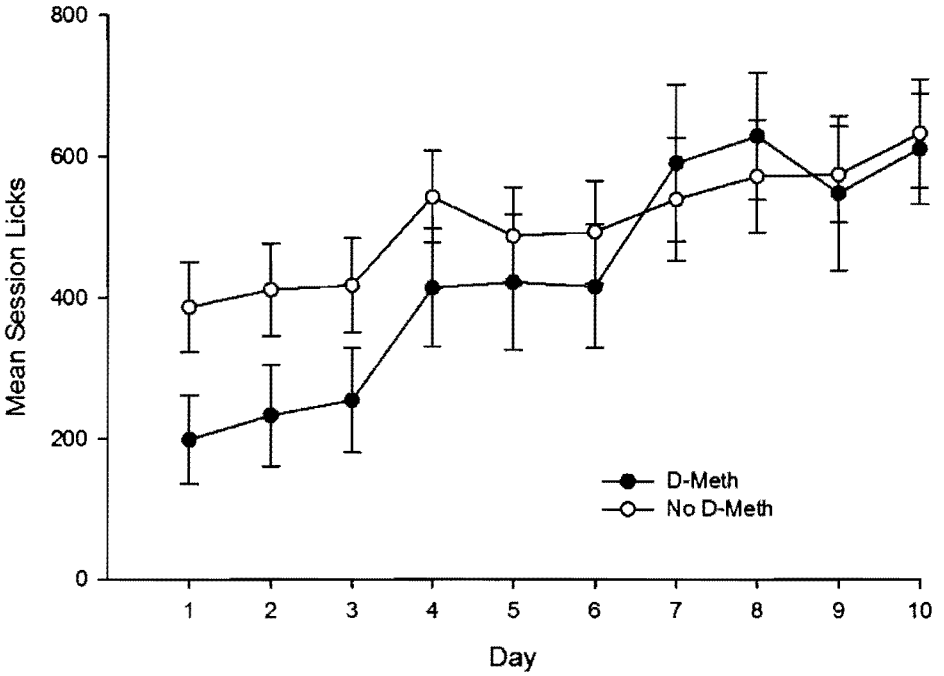

Figure 9. Data show total licks of rats treated with some form of d-METH (Drug) (n = 15) or no d-METH (No Drug) (n = 15) groups after drug administration.

To further evaluate the sign tracking data the post-treatment licks were corrected for baseline licking during initial training phase by calculating the ratio:

$$\text{Ratio} = \frac{\text{Licks during the session}}{\text{Licks during the last Before training session (B3)}}$$

A score of 1 indicates no change compared to the initial training baseline while increasing values indicate increases above baseline. A drug (2) x solvent (2) x days (10) analysis on these ratio data as conducted above yielded no additional significant effects, thus an additional factor was added to analyze the ratio data. Several studies indicate that there are considerable individual differences among rats with respect to their tendency to engage in sign tracking behavior. Although some rats quickly develop sign tracking behavior when exposed to the sign-tracking procedure many rats do not display

goal tracking behavior instead (Flagel & Robinson, 2007). Rats that show sign tracking early can be described as high responders, whereas those rats that develop sign tracking later or not at all can be described as low responders. To assess whether d-METH treatment affected high responders or low responders differently the rats in each of the four groups were split in half, based on the licks during baseline, to create low and high responders. A significant main effect of days, F (9, 198) = 5.63, $p < .001$, and a significant days x type of responder interaction, F (9, 198) = 3.74, $p < .001$, indicated that there was a greater increase (relative to baseline) in sign tracking over days in the low responders than the high responders. When looking at the ratio scores of the high sign tracking responders compared with low responders, it is suggestive that there may have been a ceiling effect in the high responders (see *Figure 10*). However, an ANOVA on the high responder ratios alone confirmed that the ratios did increase significantly over days, F (9, 108) = 2.85, $p < .01$. Although, Figure 9 suggests the predicted greater rate of increase in the d-METH treated groups, the drug x days interaction was not significant, F (9, 108) = 1.17, $p > .05$. A potential ceiling effect was not an issue with the low responders, but yet again a significant drug effect failed to emerge, F (9, 90) = 0.65, $p > .05$.

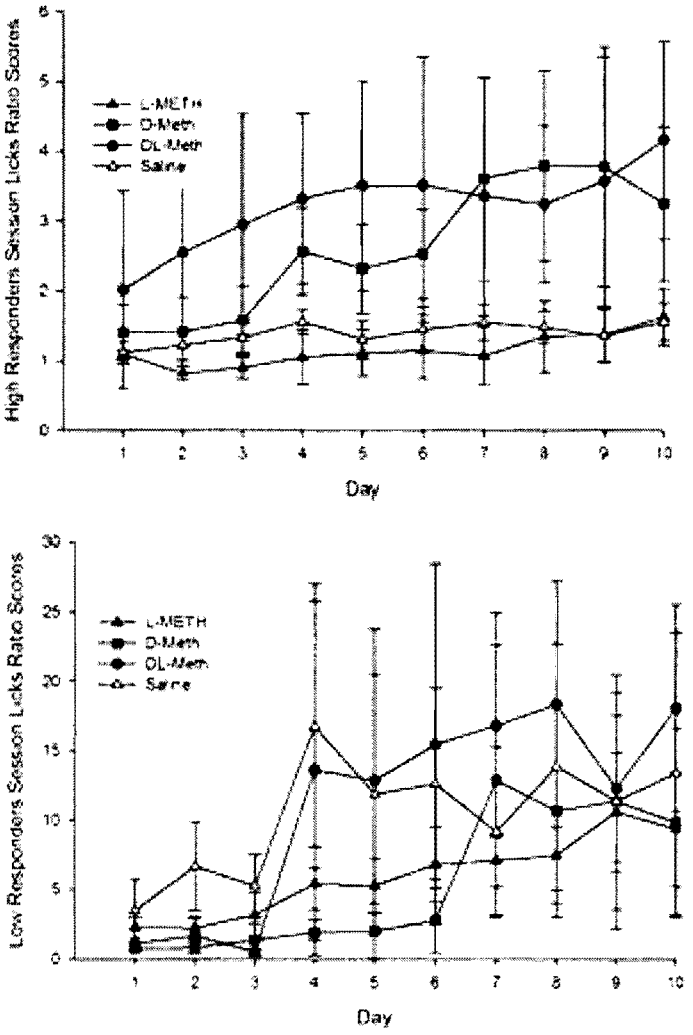

Figure 10. Ratio scores comparing high sign-tracking responders (top) with low responders (bottom). The graphs are on different scales in order to show the treatment group separation in the top graph.

Because the mixed ANOVA was not picking up the growth in responding across time points in the d-METH group, we conducted an analysis that would pull out this information accurately. It was clear from the plotted mean lick rates across the 10 days that d-METH rats had the most growth from day 1 to day 10 after treatment. A multilevel linear model analysis (MLM) was better equipped to verify what we were seeing in the graph (see *Figure 8*). The other groups seemed to stay level with some increase across the 10 days but

not to the magnitude seen in the d-METH rats. MLM takes into account both group level and individual level differences in performance on lick rates across the 10 days after treatment. Unfortunately, ANOVA was focusing directly on mean change only and not individual change for a group, which is something MLM handles. Additionally, ANOVA was not telling us about linear change over time. In choosing a model to run MLM, there are other alternatives to model the actual covariance structure. We chose an unstructured covariance type which estimates the variance and covariance parameters directly from the data rather than making assumptions about what they are. Our fixed effect was treatment (4) and our random effect was time (10). In MLM, each subject has its own value of each parameter and at the other levels, it considered the fixed (treatment – does not vary across subjects) and random effects (varies across subjects). In addition, MLM looked at the correlation between intercept and slope, thus, we were able to look at whether a treatment group has a larger mean intercept and slope (and variances there of) than the saline group. In terms of the treatment x days interaction, here, we were looking at the linear trend of treatment x days, which is more focused than the ANOVA interaction. In estimates of the covariance structure, an interclass correlation (ICC) of .48 was calculated, which indicated that 48% of the variablilty in the data was due to differences between subjects. Results indicated that days was significant, $F(1, 30) = 26.71, p < .001$. When looking at the treatment x days interaction, a significant effect emerged and the effect reflected the differences in slopes of the d-METH group relative to the control group across the time points, $b = 47.74$, $t(30) = 2.36$, $p = .02$. The other treatment groups did not reflect significantly different slopes relative to the treatment group across time points, $b = 24.25$, $t(30) = 1.16$, $p > .05$ (d,l-METH) and $b = 21.36$, $t(30) = 1.06$, $p > .05$ (l-METH). These results are also presented in Table 2.

Table 2.
MLM parameter information

	b	*SE*b	95% *CI*
d-METH x days	47.75	20.24	6.42, 89.07
dl-METH x days	24.25	20.9	-18.44, 66.93
l-METH x days	21.36	20.24	-19.97, 62.68

Discussion

The results of these experiments revealed that rats treated with some form of d-METH demonstrated higher locomotor activity in open field than rats treated with l-METH and saline. Although there is some literature suggesting that l-METH has psychoactive properties similar to d-METH (Kuczenski, et al,1995; Mendelson et al, 2005) there was no evidence of this in the present experiment using locomotor behavior in an open field as the measure of psychoactive effects. Methamphetamine administration acutely increases locomotor behavior, but with repeated presentation of the drug behavioral sensitization is typically observed. Although there was no indication of a sensitization effect in Experiment 1 indirect evidence of a sensitization effect was obtained in Experiment 2. By doubling the duration of the open field test and eliminating a novelty effect by adapting the rats to the open field prior to drug administration, a decrease in locomotion was observed by days 4 and 5 in the d-METH treated rats, but not the control rats. The observed decrease in locomotion after several days of treatment with d-METH is likely due to the constraints of the AnyMaze software not detecting repetitive rearing behaviors and stereotyped head bobbing in rats treated with d- and d,l-METH. Thus, the emergence of these stereotyped behaviors would result in less traveling across the chamber floor, therefore decreasing total distance traveled and total

amount of line crossings when compared with rats treated with l-METH and saline. The significant decrease in both measures towards the end of drug treatment was observed in Experiment 2. Assessment of head shaking (Kass et.al, 2010), repetitive head bobbing and rearing (Doremus-Fitzwater & Spear, 2011) may be a better measure of METH-induced sensitization than general locomotor activity because after repeated exposure to the same dose of psychostimulant, these behaviors emerge, but stereotyped behavior is much more difficult to measure. However, previous research comparing amphetamine and d-METH revealed a sensitization effect across days when examining locomotor activity alone (Hall, et.al., 2008). The sensitization of locomotor behavior itself may have emerged because of use of a lower dose (.5 and 1.0 mg/kg) administered than in the present study (1.5 mg/kg). Higher doses (2.5 mg/kg and above) typically produce stereotyped behaviors more quickly and more robustly. The dose for the present study was chosen to induce some sensitization that could be measured automatically with AnyMaze, albeit indirectly.

Despite using a dose of d-METH that induced behavioral sensitization, the sign tracking data did not yield a significant effect of drug in enhancing the acquisition of sign tracking using the ANOVA for statistical analysis. Previous research examining acute prior exposure to amphetamine and cocaine has been reported to augment responding of goal tracking behavior (Holden & Peoples, 2010; Simon, Mendez & Setlow, 2009; Taylor & Jentsch, 2001). Previous failed attempts to see a priming of sign tracking with psychostimulants may have been due to the effects of the drugs in enhancing any response to signals for food. Because goal tracking emerges before sign tracking, the enhancement of goal tracking may have interfered with a priming effect on sign tracking.

Therefore, the present study examined the effects of d-METH on sign tracking performance after sign tracking had already begun to emerge. If rats were engaging in both goal tracking and sign tracking behavior at the time that drug treatment was initiated, then upon the return to sign tracking the drug treatment should preferentially prime sign tracking since sign tracking and behavioral sensitization are hypothesized to be mediated by a common neural pathway. Although the results suggested a priming effect (i.e., a greater rate of the acquisition of sign tracking) the effect did not achieve statistical significance. It is possible that a higher dose of d-METH may be needed in order to prime sign tracking behavior. As mentioned above the present experiment used a moderate dose (1.5 mg/kg), enough to detect a sensitization effect, but not a dose as high as typically used in behavioral sensitization experiments (e.g., 2.5 mg/kg).

The present experiments may also have been influenced by the substantial individual differences in sign tracking known to exist within a rat strain. In addition to affecting the ANOVA tests, our handling of these individual differences early in training may have impacted on the results. For example, there seemed to be higher lick rates at the start of the post-treatment phase demonstrated by rats assigned to the No d-METH groups prior to treatment (although not significantly different). This slight group difference may have been due to assigning the treatment groups with a slight bias against our hypothesis (rats with slightly greater lick rats assigned to the control conditions). If an effect of d-METH would emerge in sign tracking, we were demanding that it be strong enough to override the slight differences during pre-training. There may be a priming effect of d-METH on sign tracking, our assignment strategy may have weakened our ability to detect it in the Treatment (2) x days (10) mixed ANOVA. However, when

conducting an MLM analysis, we separated out all four treatment groups and were able to verify the growth in responding to the sign tracking procedure demonstrated in the d-METH group relative to the saline group in the ten days following treatment. Our significant treatment x days interaction reflected the differences in slopes seen in the d-METH group compared to the saline group. Thus, we were able to demonstrate that rats treated with d-METH had a greater linear trend in lick rates across the ten days when compared with saline treated rats. As can be seen in Figure 7, we determined that the significant growth in responding across the ten days was much greater in d-METH rats than the saline group. Interestingly, the d,l-METH group did not significantly differ from the saline group across the ten days, and Figure 7 suggests that pretreatment of l-METH may have attenuated sign tracking performance in this treatment group (d,l-METH). Because this group received the same dose of d-METH as the d-METH group, we predicted that their performance would be similar to rats in the d-METH group; however, this was not the case.

Additionally, in order evaluate if the effects of d-METH on the acquisition of sign tracking was influenced by individual differences in the propensity to sign track (Flagel & Robinson, 2007) the rats were divided into high responders (i.e., rats with highest lick rates during initial training) and low responders, but no effect of this factor was observed. It may have been more effective to compare the top third (high responders) with the lower third (lower responders) to increase the difference between groups, but there were not sufficient animals to take this approach.

If the priming of sign tracking with psychostimulants is a phenomenon that is difficult to detect with typical analyses, it may be helpful to use other measures of sign

tracking besides physical interactions (e.g., licks) with the object CS. In a recent study (Doremus-Fitzwater & Spear, 2011) the successful priming of sign tracking in adolescent and female rats with amphetamine pre-treatment was observed when the latency to approach the CS was used. Anecdotal observations in the present experiment suggested that some of the rats treated with both forms of d-METH were not physically interacting with the bottle CS, but were at the location of the CS prior to, during and shortly after CS presentation. Thus latency to approach the CS is an additional variable to be considered in future sign tracking experiments (Doremus-Fitzwater & Spear, 2011),

DA has been indicated to play an important role with the association of incentive salience toward reward related cues. In a microdialysis experiment conducted by Flagel, et al., (2010), selectively bred rats for high novelty seeking behavior were compared with controls in a sign tracking procedure with measurements of DA levels analyzed during CS presentation. The selectively bred novelty seeking rats (which were also high sign trackers) demonstrated higher release of DA onto the NAc than the low novelty seeking rats (which were also low goal trackers) . Because d-METH has a potent mechanism of action with DA, we hypothesized that it may produce faster and more robust acquisition of sign tracking when compared with controls and l-METH. It is clear that more research is needed in order to examine the effects of both a high dose l-METH group and a higher dose l-METH within the d,l-METH group to see if differences in locomotor behavior may emerge compared to our findings in the present experiment. If a higher dose of l-METH produces both stereotyped behaviors in animals (Kuczenski et.al., 1995) and psychoactive effects in humans (Mendelson et.al., 2006) , then it may be that more locomotor behavior may emerge in rats treated with the higher l-METH dose.

Additionally, higher doses of l-METH and d-METH may augment sign tracking behavior in order to sensitize them to a greater degree before signtracking. In Experiment 1 there was some indication that addition of l-METH to d-METH (i.e., the racemic METH) reduced the effects of D-METH on locomotor behavior, but effect was not confirmed in Experiment 2. Because l-METH in higher doses produces higher DA release and increase in extracellular concentrations (Kuczenski et.al., 1995), high doses of l-METH may also increase in behavior toward reward predictive cues. The pattern of data in the present experiments suggests that both d-METH and d, l-METH produce higher locomotor activity than rats treated with l-METH and saline.

Because METH induced sensitization may produce the incentive salience for a reward related cue, the impact of these cues on drug addiction and context- or cue-induced relapse should continue to be a pertinent focus for drug abuse research. Overall, drugs that are typically used by abusers, often obtain their supply from clandestine laboratories, which are not known to have the same quality control procedures in place as drugs obtained for research. In the present study, we endeavored to examine the impact of mixing different forms of METH in order to evaluate whether this would yield differences when compared with the pure form of the drug typically used in research. In future study, it may be prudent to look at actual street METH as a comparison, using the same procedures described here.

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