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Effects of Chlordiazepoxide on Predator Odor-induced Reductions of

Playfulness in Juvenile Rats

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Abstract

The extent to which a non-sedative dose of chlordiazepoxide (CDP) is able to modify the behavioral responses toward a predator odor was assessed in juvenile rats. Play behavior was suppressed and defensive behaviors were enhanced in the presence of a collar previously worn by a cat, when tested 24 hours later in the same context as that where the exposure occurred, and when tested in a context different than that in which the exposure occurred for up to 3 hours after exposure. CDP had no effect on the ability of cat odor to suppress play when rats were tested in the presence of the odor or when tested 24 hours later in the same context where that exposure occurred. When rats were exposed to a worn cat collar in their home cage and tested in a different context CDP attenuated the ability of cat odor to reduce one measure of play (nape contacts) but not another measure (pins). Rats had an opportunity to hide during testing and CDP either decreased hiding or increased risk assessment from within the hide box in all of the testing scenarios. These data suggest that CDP can alter the defensive strategy used by juvenile rats that are confronted with a predatory threat and can also lead to an earlier return to pre-threat levels of playfulness when that threat becomes less immediate.

1. Introduction

Play is a highly motivated and robust behavior that is exhibited by the young of most mammalian species [5,15,21]. Play can take on many forms although one type of play that can be seen in a variety of mammals and that is readily observed in the behavioral repertoire of the laboratory rat is rough-and-tumble play wrestling [34,36,40,51]. In the young rat, this type of play follows a distinct and predictable ontogenetic pattern; beginning shortly after independent locomotion is attained, peaking at around 35 days of age, and then tapering off to low levels at around puberty [30,32]. Although play appears to be important for social and behavioral development [23,37,48,49], it is still easily disrupted by homeostatic challenges [2,4,44] and other non-specific stressors [38,41,50]. Given the apparent sensitivity of play to disruption by a variety of stressors, assessing this behavior following stress and/or threats could be particularly advantageous for developing animal models of psychiatric disorders as they may manifest during childhood and adolescence.

Anxiety is one of the most common psychiatric disorders among children, with approximately 13% of all children being diagnosed with some type of anxiety disorder [39]. Anxiety among children and adolescents can also lead to additional problems, such as difficulty in school, alcohol and drug abuse, and other psychiatric conditions such as depression [53]. Childhood and adolescence is also a vulnerable developmental period during which the foundation for developing an anxiety disorder during adulthood could be established [22]. While there has been considerable effort directed towards developing animal models of anxiety using adult rats, it has been only recently that specific attention has been given to the younger rat [20,26,46].

There has been an increased interest in recent years towards developing animal models of anxiety that take advantage of more ecologically valid stimuli for generating fear and anxiety. In particular, a number of laboratories have been using fear of predation, either by exposing animals to a live predator such as a cat or just the smell of a predator to further understand the neurobiological substrates of fear and anxiety [1,3,12,19]. However, relatively few studies have looked at the extent to which these types of stimuli may also be useful for understanding the neurobiological substrates of anxiety in the younger organism. Fear of predation develops fairly early in small prey species such as the rat, with young rats exhibiting a full range of defensive responses towards the odor of a predator as early as 18 days of age [24,52]. The smell of a predator can also have a robust effect on play behavior [31,42,43]. For example, we have shown that play is virtually abolished in the presence of a predator odor and continues to be suppressed for up to 7 days when the animals are returned to the context in which the odor was experienced. Play is also suppressed when animals are exposed to a predator odor in a context separate from that used for testing, although the suppression in this case is not as long-lasting [43]. These data suggest that the suppression of play during or following exposure to a predator odor may provide a sensitive and ecologically valid indicator variable for assessing anxiety in the young rat.

As a class, benzodiazepines are widely considered to be prototypical anxiolytics to which all other putative anxiolytics are compared yet the efficacy of benzodiazepines when tested in animals exposed to cat odor have not been as consistent as when tested in other animal models of anxiety. For example, chlordiazepoxide (CDP) has been reported to have minimal effects on how a rat responds to a cloth impregnated with cat odor when exposed in their home cage but had anxiolytic effects when the rats were tested shortly thereafter in either an elevated plus maze or in the social interaction test [55]. However, when given a clear opportunity to hide in the

presence of cat odor, the short-acting benzodiazepine midazolam has been shown to decrease hiding and increase approach towards the odor, consistent with an anxiolytic effect [17,27]. Similarly, the Blanchard lab has reported that a wide variety of benzodiazepines (diazepam, chlordiazepoxide, midazolam) can modulate responsiveness towards cat odor and suggested that benzodiazepines may be altering the defensive strategy towards the threat of predation such that some responses may be inhibited while others are disinhibited [6-8,14]. While benzodiazepines are not a common treatment option for childhood anxiety, assessing the effects of this class of anxiolytic in young rats will provide a benchmark to which other putative anxiolytics can be assessed. In order to determine whether benzodiazepines can also modulate fearfulness in juvenile rats, the effects of a non-sedative dose of chlordiazepoxide were assessed using two different models of cat odor-induced suppression of play.

2. Materials and Methods

2.1. Subjects and housing

Male Long-Evans rats were obtained from Harlan Sprague-Dawley at approximately 25 days of age. Animals were initially housed in groups of four in solid bottom cages (48 X 27 X 20 cm) and periodically handled for a few days in order to acclimate to the laboratory. Rats were then housed individually in solid bottom cages (27 X 21 X 14 cm) for the duration of testing. Food and water were always freely available. The colony room was maintained at 22° C with a 12/12 hr reversed light/dark cycle (lights off at 08:00), with all testing done during the dark phase of the light/dark cycle. All housing and testing was done in compliance with the NIH Guide for Care and Use of Laboratory Animals using a protocol approved by the Institutional Animal Care and Use Committee at Gettysburg College.

2.2. Apparatus

Play was assessed in a Plexiglas chamber (50 x 50 x 40 cm) that was painted black on all four sides. The floor of the chamber was covered with about 3 cm of pine shavings. A wooden hide box (20 x 26 x 50 cm) with a small (6 x 8 cm) opening was situated in one corner of the chamber. The room was darkened during testing and the outer chamber was illuminated by two 25W red light bulbs, whereas the hide box was not illuminated. Play bouts were recorded with a camera that was directly above the outer chamber and an infrared-sensitive camera that was placed inside the hide box. Video output from both cameras was directed through a quad multiplexer, encoded as digital files, and scored later using behavioral observation software (Observer XT: Noldus Information Technology) by an observer unaware of the treatment conditions.

The collars used in this study were Petwear Adjustable Safety Collars (Rose America Corporation, Wichita, KS). Worn collars were obtained from a domestic cat (spayed female that spent most of the time indoors) that had been wearing the collar for approximately 2 months. The collar was cut into 2.5 cm pieces and only those pieces of the collar that came in direct contact with the fur of the cat were used. The collars were stored in airtight containers at -10° C and warmed prior to testing by immersing the sealed container in hot (50° C) tap water for approximately 10 minutes. Care was taken to insure that the collars never got wet and the collars were always handled with gloved hands. Identical collars that were never worn by a cat were used for the control (unworn) condition.

2.3. Quantifying play, location, and risk assessment

Play was assessed by recording the number of contacts directed by each rat to the nape of the other rat (nape contacts) and the number of times each rat was pinned by the other rat. A

nape contact is scored if one rat brings its snout to within 1 cm of the nape of its partner; whereas a pin is defined as occurring if a rat is on its back with at least three paws in the air [34,40,45,51]. The number of nape contacts and pins for each pair of rats was treated as a single unit of data. The location of the rats was also recorded as the relative amount of time spent in the hide box by one rat, both rats, or neither rat and these data were used to provide an index of hiding. A measure of risk assessment was also measured by quantifying the amount of time at least one of the rats was engaged in a "head-out" posture. A head-out posture is a type of vigilant scanning of the environment from the relative safety of a confined space, such as a hide box, and has been suggested to be a sensitive measure of risk assessment [11,18,28]. An occurrence of a head-out posture was noted when at least one rat was inside the hide box, not moving, and had at least its entire head outside of the box and at least two hind paws within the box.

2.4. Experiment 1: Effects of CDP on play

In order to insure that the dose of CDP to be used in subsequent testing was not sedating the animals, the effects of CDP were initially assessed without any cat odor. Eight pairs of rats were acclimated to the same housing conditions and testing apparatus as those to be used in subsequent testing. Rats were given three days of acclimation prior to testing by placing them in the testing chamber as pairs for 5 minutes on each of these days. Pairings for this acclimation period were the same as those used for testing.

Rats were tested following either CDP (5 mg/kg) or vehicle (0.9% saline) over 2 days. This dose was selected on the basis of doses reported to be effective in similar models from the literature [9, 55] and on preliminary data from our lab suggesting that higher doses (e.g., 10 mg/kg) were more likely to yield sedative effects. Injections were given intraperitoneally (IP) 30

minutes before a 5 minute test session and were administered in a 1 ml/kg volume. Each pair of rats received each of the two treatment conditions in a counterbalanced order, with 48 hours separating each test.

2.5.Experiment 2: Effects of CDP when cat odor is presented in the testing context

A summary of the procedural timeline for this experiment and Experiment 3 can be seen in Figure 1. Beginning at approximately 25 days of age, rats were acclimated to the testing chamber by allowing them to play in pairs for 3 days. Rats were randomly allocated to a partner prior to the first acclimation day and these pairings remained the same throughout testing. On each of these days, the rats were placed in the chamber for 5 minutes. Rats were transported to and from the testing room in a covered cardboard box. These initial acclimation sessions were not recorded. On the fourth day of testing, the same pairs of rats were again placed in the testing chamber for 5 minutes but this session was recorded and used to provide baseline measures in order to insure that no group differences were present before treatment. Rats were then assigned to one of 4 treatment conditions based on the type of collar (unworn or worn) presented on the following day (conditioning day) and drug treatment (vehicle or CDP) on the conditioning day and on the subsequent day (test day). Both animals of each pair received the same treatment (collar and drug).

On the conditioning day, a single piece of collar (unworn or worn) was placed on the wall facing the opening of the hide box and kept in place by an alligator clip situated approximately 5 cm from the base of the chamber. Rats in the control group (n = 8 pairs) were exposed to an unworn collar on the conditioning day and received a vehicle injection 30 minutes before testing on both days. An additional group of rats (n = 8 pairs) was exposed to a worn cat collar on the conditioning day and received a vehicle injection on both days. This group allowed for a direct

assessment of the unconditioned and conditioned effects of cat odor on play. To test the effects of CDP on the unconditioned suppression of play, one group of rats (n = 8 pairs) was injected with 5 mg/kg of CDP and exposed to a worn cat collar on the conditioning day. To test the effects of CDP on the conditioned suppression of play, another group of rats (n = 8 pairs) were exposed to a worn cat collar on the conditioning day and injected with 5 mg/kg of CDP on the Test day. Rats from the latter two groups received a vehicle injection on those days when they did not receive CDP. All injections were given intraperitoneally (IP) 30 minutes before testing.

2.6. Experiment 3: Effects of CDP when cat odor is presented in the home cage

Beginning at approximately 29 days of age, rats were acclimated to the testing chamber by allowing them to play in pairs for 2 days. As in Experiment 2, rats were randomly allocated to a partner prior to the first acclimation day, these pairings remained the same throughout testing, and rats were transported to and from the testing room in a covered cardboard box. On each of these days, the rats were placed in the chamber for 5 minutes. These initial acclimation sessions were not recorded. On the third day of testing, the same pairs of rats were again placed in the testing chamber for 5 minutes but this session was recorded and used to provide baseline measures. On the next day rats were assigned to one of four conditions (see Figure 1). One group of rats (n = 12 pairs) was exposed to an unworn collar in their home cage while three groups of rats were exposed to a worn collar in their home cage 60 minutes before testing. Those rats exposed to an unworn collar received vehicle injections 30 minutes before exposure and 30 minutes after exposure. One group of rats (n = 12 pairs) that was exposed to pieces of a worn collar was also given injections of vehicle 30 minutes before and 30 minutes after exposure. This group assessed the effects of cat odor in this model. Of the remaining two groups of rats that were exposed to pieces of a worn collar, one group (n = 12 pairs) was injected with

vehicle before exposure and CDP (5 mg/kg) after exposure while the other group (n = 12 pairs) was injected with CDP before exposure and vehicle after exposure.

On the day of testing, rats were given their initial injection and returned to their home cage. Thirty minutes later, rats were taken in their home cages from the main holding area to a room adjacent to the one in which they were being tested for play. This room was dimly illuminated and the two cages were placed side by side. The lid for each cage, which contained both food and water, was replaced with an identical lid that had no food or water bottle. A single piece of collar, either unworn or worn depending on the group assignment, was suspended from one end of each lid using alligator clips, being careful not to allow the collar to touch the bedding. After a 5 minute exposure period the cage lids with the collars were replaced with the original cage lids and the rats were returned to the holding room. After 30 minutes the rats were again injected with either vehicle or CDP depending on group assignment, returned to their home cage, and tested 30 minutes afterwards. As in the previous experiments, tests lasted 5 minutes.

An additional group of rats was tested for the effects of CDP when injected prior to exposure and when a longer period lapsed between exposure and testing. One group of rats (n = 8 pairs) was injected with vehicle while another group of rats (n = 8 pairs) was injected with CDP 30 minutes before exposure to a worn cat collar in the home cage. Exposure was done as described above and all rats in this experiment were exposed to pieces of a worn cat collar. After exposure, rats were returned to the main holding room and then observed in the testing chamber as before for 5 minutes at 1, 3, and 6 hours after exposure.

3. Results

3.1. Experiment 1

CDP had no effect on either nape contacts or pins, $t(7) < 1.0$ for both measures. The mean ($\pm$ SEM) number of nape contacts following vehicle and CDP were 59.5 ± 3.6 and 64.6 ± 4.2, respectively. The mean ($\pm$ SEM) number of pins following vehicle and CDP were 28.0 ± 5.5 and 31.0 ± 4.5, respectively. CDP also did not affect the relative amount of time that 0, 1, or both rats were in the hide box at the same time during testing (data not shown). Therefore, any subsequent effects associated with CDP cannot be easily attributed to any sedative effect of the drug.

3.2. Experiment 2

The results for nape contacts and pins on the conditioning day and test day can be seen in Figure 2. The data for each measure was analyzed using a one-way Analysis of Variance (ANOVA) on each of the two days. For nape contacts there was a significant group effect on both the conditioning day, $F(3,28) = 15.31$, $p < .001$, and a marginal group effect on test day, $F(3, 28) = 2.75$, $p = .06$. Post-hoc analysis (Student Newman-Keuls, $p < .05$) of the data indicated that all 3 groups exposed to a worn cat collar had significantly fewer nape contacts than the control group on the conditioning day. For pins there was a significant effect on both the conditioning day, $F(3,28) = 6.72$, $p < .001$, and on the test day, $F(3,28) = 4.83$, $p < .01$. All 3 groups exposed to the worn cat collar had significantly fewer pins than the control group on both days. Those rats given CDP either on the conditioning day or the test day did not differ on either measure of play from those rats given vehicle and also exposed to the worn cat collar.

Location of the rats over the course of the testing period is shown in Figure 3 as the relative amount of time (% total) the rats were in one of three possible states: both rats in the hide box, one rat in the hide box with the other rat in the main chamber, or no rats in the hide box. As with play, the data for each state was analyzed using a one-way ANOVA on each of the

two days. There were significant differences on the conditioning day for the state where both rats were in the hide box at the same time, $F(3,28) = 10.88$, $p < .001$, with both rats of a testing pair from all 3 groups exposed to the worn cat collar more likely to be in the hide box at the same time. In addition, the group that was given CDP prior to being exposed to a worn cat collar was less likely to be in this state than those two groups exposed to the worn collar but given vehicle. Significant differences continued on the test day, $F(3,28) = 3.47$, $p < .03$, with all 3 groups exposed to the worn collar differing significantly from the control group. On this day there were no significant differences among the 3 groups exposed to a worn cat collar. These data suggest that CDP decreased the relative amount of time both rats spent hiding in the presence of a worn cat collar.

For the state where one rat was in the hide box and one rat out in the main chamber, there were significant differences among the groups on the conditioning day, $F(3,28) = 3.26$, $p < .05$, but not on the test day, $F(3,28) = 0.33$. Post hoc analysis of the data from the conditioning day indicated that both groups exposed to a worn cat collar and given vehicle on this day differed significantly from the control group, with both groups less likely to be in this state. Those rats exposed to a worn collar and given CDP on the conditioning day differed significantly from the other 2 groups exposed to a worn cat collar and given vehicle but did not differ from the control group.

For the state where no rats were in the box (i.e., both rats in the main chamber) there were significant effects on both the conditioning day, $F(3,28) = 18.95$, $p < .01$, and on the test day, $F(3,28) = 7.69$, $p < .01$, with all 3 groups exposed to the worn cat collar less likely to be in this state than the control on both days. Furthermore, the groups that were exposed to the worn collar did not differ from one another on either day.

Risk assessment, as quantified by the amount of time (% total) engaged in a head-out posture can be seen in Figure 4. Analysis of these data on the conditioning day yielded no differences between the four groups, $F(3,28) = 1.45$. However, there were significant differences on the test day, $F(3,28) = 6.14$, $p < .01$, with rats exposed to a worn cat collar and administered CDP on either the conditioning day or the test day exhibiting significantly more risk assessment than the control group on the test day. Those rats exposed to the worn cat collar with vehicle did not differ significantly from either the control group or the two groups that received CDP.

3.3. Experiment 3

For the first part of this experiment rats were tested 1 hour after a 5-minute exposure to an unworn or worn cat collar in their home cage. Those rats exposed to a worn cat collar were injected with either vehicle or CDP 30 minutes before exposure or 30 minutes after exposure. The results for play behavior can be in Figure 5. Analysis of these data yielded significant differences for both nape contacts, $F(3,44) = 13.16$, $p < .001$, and pins, $F(3,44) = 9.35$, $p < .001$. All 3 groups exposed to a worn cat collar exhibited significantly fewer nape contacts and fewer pins than rats in the control group. None of the groups exposed to worn cat collar differed from each other.

The relative location of the rats and amount of time engaged in risk assessment behavior is shown in Table 1. The only significant effect was for the state where no rats were in the hide box, $F(3,44) = 6.37$, $p < .01$. Post-hoc analysis indicated that amount of time in this state was reduced in all 3 groups exposed to a worn cat collar.

In a separate group of rats, both vehicle-treated and CDP-treated rats were exposed to a worn cat collar and observed at 1, 3, and 6 hours after exposure to a worn cat collar. This experiment did not include a group exposed to an unworn collar so to confirm the effectiveness

of the worn collar the data from the baseline observations and the first set of observations (i.e., 1 hour after exposure) were submitted to a 2 x 2 ANOVA with repeated measures for time. As expected, there was a significant main effect of time for nape contacts, $F(1,14) = 4.74$, $p < .05$, and for pins, $F(1,14) = 16.29$, $p < .01$, with both measures of play decreasing 1 hour after exposure to the worn cat collar. There was no main effect of treatment nor was there a significant time x treatment interaction. A similar analysis was done with the location data and it was found that the likelihood that neither rat was in the box, $F(1,14) = 12.09$, $p < .01$, or one in the box, $F(1,14) = 18.52$, $p < .01$, was decreased after exposure to the cat collar. Conversely, it was more likely to find both rats in the box after exposure to the worn cat collar, $F(1,14) = 23.61$, $p < .01$. Just as with play, there was no main effect of group or a significant time x group interaction for any of these measures. Exposure to the worn cat collar had no initial effect on risk assessment.

Having confirmed that the worn cat collar was effective in reducing play and increasing hiding, the data from the three post-exposure time points were then submitted to a 2 x 3 ANOVA with repeated measures on time. The results for play are shown in Figure 6. For nape contacts, there was a significant main effect of time, $F(2,28) = 8.36$, $p < .001$, with nape contacts increasing over the course of the 3 observations. There was also a significant main effect of group, $F(1,14) = 6.46$, $p < .03$, with those rats given CDP exhibiting more nape contacts overall than the vehicle-treated rats. Although it looks as if the effect of CDP was maximized at 3 hours post-exposure, the treatment x time interaction was not significant, $F(2,28) = 1.70$. For pins, there was a significant main effect of time, $F(2,28) = 5.63$, $p < .01$, with pinning increasing over the course of the 3 observation periods. There was no significant main effect of treatment nor was there a significant treatment x time interaction.

The data for location and risk assessment, including baseline values, can be seen in Table 2. Analyzing the post-exposure data for location indicated significant main effects of time for the state where one rat is in the box, $F(2,28) = 5.73$, $p < .01$, and where both rats are in the box, $F(2,28) = 4.69$, $p < .02$. This pattern reflected a decrease in the time that both rats spent hiding together over the course of the 6 hour post-exposure period. There was no significant main effect of group nor was there a significant time x group interaction. An interesting pattern emerged for risk assessment and this can be seen in both Table 2 and Figure 7. Analysis of these data yielded a significant main effect of time, $F(2,28) = 6.47$, $p < .01$, which reflected a significant increase in time spent in risk assessment over the course of the 6 hour post-exposure period. There was also a significant time x group interaction, $F(2,28) = 3.88$, $p < .05$. Further analysis of this interaction indicated that levels of risk assessment remained steady in the vehicle-treated rats over the course of the 6 hours while risk assessment increased at 3 and 6 hours post-exposure in the CDP-treated rats when compared to 1 hour post-exposure.

4. Discussion

For a rat, the smell of a predator can have a profound effect on subsequent behavior with a cessation of non-defensive behaviors and an increase in defensive behaviors [1,12,19,54]. Fear towards the smell of a predator appears fairly early in development [24,52] and previous work from our laboratory has shown that the smell of a natural predator (cat) decreases play and increases defensive behaviors such as hiding and risk assessment [42,43]. The results from this study are consistent with these findings in that play behavior was suppressed in the presence of a collar previously worn by a cat, when tested 24 hours later in the same context as that where the rats had previously been exposed to the collar, and when tested in a context different than that in which the exposure occurred, for up to 3 hours after exposure.

The primary objective for this series of experiments was to determine whether a non-sedative dose of the prototypical benzodiazepine chlordiazepoxide was able to modulate the effects of predator odor exposure on defensive behaviors (hiding, risk assessment) and on a non-defensive behavior (play) in young rats. While many studies have examined the effects of benzodiazepines on defensive behaviors in response to predatory threat, less work has examined how benzodiazepines affect predatory threat-induced changes to non-defensive behaviors. When tested in the presence of a worn cat collar or when tested 24 hours later in the same context where that exposure occurred, CDP had no effect on the suppression of play (Experiment 2). However, when rats were exposed to a worn cat collar in their home cage and tested in a different context (Experiment 3) CDP attenuated the ability of cat odor to reduce nape contacts while having no effect on the reduction in pinning. These data show that animals treated with CDP and then exposed to the smell of a worn cat collar are more playful than those animals treated with vehicle, but only when tested in a different context and after a minimum amount of time has elapsed since the exposure. These data confirm those of Zangrossi and File [54] where CDP was shown to reverse the suppressant effect of cat odor on social interaction in adult rats and extend this finding by noting a similar effect in younger pre-pubertal rats engaged in a highly motivated and more specific type of social interaction.

As with all benzodiazepines, CDP is well known to have sedative properties. Consequently, the dose used in the present series of experiments (5 mg/kg) was chosen on the basis of values provided in the literature as being effective in other rodent models of anxiety involving cat odor [e.g., 9, 55] and on preliminary data collected in our laboratory. In several preliminary experiments, including the results reported in Experiment 1, we found no evidence for any sedative effects associated with 5 mg/kg in our model but some indication that a higher

dose (e.g., 10 mg/kg) could result in slight sedation, as evidenced by decreases in play at this dose. Therefore, it is likely that the dose used in these experiments was optimal for maximizing the anxiolytic potential of this particular compound in this model.

It is interesting that the effect of CDP on play was limited to nape contacts. This suggests that pinning may be particularly sensitive to disruption by predatory threat, more so than nape contacts, and less likely to be modulated by benzodiazepines. Since an animal on its back (the posture being assumed when pinned) may be particularly vulnerable to attack by a predator there may be a prolonged reluctance to engage in this posture if there is still any perceived threat of predation. This also highlights the importance of using multiple measures to assess anxiety-induced changes in ongoing behaviors and how anxiolytics may modulate these changes.

It is well established that the threat of predation increases defensive behaviors and these were quantified in the present study by looking at hiding and one measure of risk assessment. Since pairs of rats were tested in these experiments, hiding was quantified along a discrete continuum from maximum hiding, when both rats were in the hide box, to minimum hiding, when neither rat was in the hide box. Under control conditions (i.e., unworn collar and vehicle), rats in all of the experiments spent a disproportionate amount of time (approximately 75%) together in the hide box even though play was at expected levels and occurred almost exclusively in the hide box. In our earlier work where hiding was measured as in this study, it was found that both rats were in the hide box at the same time about 40% of the time under control conditions and that 75% of pinning occurring in the hide box [43]. Both of these studies highlight the extent to which young rats will opt to play in a smaller yet more protected space when give the opportunity, even when there is no immediate threat.

When given on the day of exposure and tested in the presence of a worn cat collar, CDP decreased the relative amount of time both rats were in the hide box at the same time while increasing the amount of time when one was in the box and the other was out of the hide box. This suggests that CDP changed the behavior of the pair of rats under these conditions in a way that may be facilitating a joint defensive strategy; one rat taking a more active approach towards assessing the risk with the other remaining in the relative safety of the hide box. These results are consistent with the observations of Dielenberg and colleagues [17] showing that the short-acting benzodiazepine midazolam decreased hiding and increased approach time towards a worn cat collar when an option to hide was available.

This particular strategy of decreased hiding with CDP was not apparent on the following day when rats were tested in the same context as that in which the odor was experienced. However, there was more risk assessment among CDP-treated rats in the form of increased head-out scanning from the safety of the hide box. Interestingly, this was observed among those rats that received CDP on either that day or the day before. CDP does not seem to be simply attenuating the effect of cat odor on risk assessment. Rather, CDP appeared to be potentiating a modest and non-significant increase in risk assessment seen in vehicle-treated rats that were exposed to the cat odor. CDP also increased risk assessment in Experiment 3 when tested 3 and 6 hours after exposure to a worn cat collar and this seemed to coincide with changes in nape contacts. However, it cannot be readily determined from these data whether the increase in risk assessment among CDP-treated rats preceded the increase in nape contacts, which might be expected if play returns once the perceived threat is reduced. Therefore it's not clear if the animals are actually using the information being gathered by scanning the outer chamber from the safety of the hide box in a way that would lead to an earlier return to play behavior.

An important aspect of our experimental design was that rats were tested in same-treatment pairs and, in the case of Experiment 2, exposed to cat odor in pairs as well. Accordingly, we need to interpret our results with this in mind. Since individual rats were not identified it is not clear if any of the pharmacological effects observed were more predominant in one rat of the pair over the other. This could be a particularly relevant issue for interpreting the location data from Experiment 2. For example, it is not clear if one animal of the pair was more likely than the other to be outside of the hide box or whether both animals of the pair were equally likely to be out in the main chamber in the presence of cat odor. In their exhaustive work with the visible burrow system, the Blanchard lab has shown that the dominant male in a mixed-sex colony is the first to leave the burrow after a cat has been placed in the open area [10]. Dominant rats have also been reported to be more likely to take risks and show less anxiety-like behavior in the elevated plus maze [16]. Although it is not clear if the rats in our experiments had ample time to develop a stable dominance/subordinate relationship there is evidence for dominance to be established in pairs of rats given limited opportunities to play [25,33,35]. Therefore, a closer examination of possible individual differences in the effectiveness of benzodiazepines in these models may be an interesting line of future inquiry.

How an animal responds to a predatory threat and how benzodiazepines can modulate that response seems to depend upon the salience of the threat, options that are available to the animal, and baseline levels of the behaviors that are being measured [7-10,12]. Based on our results (see Table 3), an overall pattern begins to emerge that is consistent with this perspective. For example, CDP affected either hiding or risk assessment in each of the three testing scenarios although how this effect actually played out was dependent on the scenario. When the threat was particularly salient (worn collar present during testing) the main effect of CDP was to reduce

hiding and facilitate approach by one animal of the pair towards the putative threat. When the threat became somewhat less salient, less immediate, and perhaps more diffuse (testing in either the same context without the collar or in a different context but reasonably close in time to that exposure), then CDP didn't affect hiding but increased risk assessment from within the hide box. On the other hand, when rats were tested in a different context than that in which the exposure occurred and after a minimum amount of time had elapsed since that exposure (e.g., > 1 hour) CDP continued to facilitate an increase in risk assessment and also led to more play in the form of increased nape contacts. So the effect of CDP in our model is to enhance behaviors that may serve to assess risk when the threat is most salient and then as the threat becomes less immediate we continue to see higher levels of risk assessment but also see the beginnings of an earlier return to a normal non-defensive behavior for rats of that age (e.g., play). This is consistent with previous ideas on how benzodiazepines modulate anxiety produced by a predatory threat [8,13,29] and suggest comparable effectiveness in the younger pre-pubertal rat.

Along with higher incidences of anxiety disorders being noted in recent years among children and adolescents [47] is an increased likelihood of treating younger patients with anxiolytics commonly used in the adult population. For example, one recent study reports that the use of anxiolytics in child/adolescent populations has seen a 2.9 fold increase during the 10 year period from 1987-1996 [56]. Since much of what we know about the effects of anxiolytics on fear and anxiety derives from studies using adult rats and/or mice, a need for more research using younger animals seems clearly warranted. The present study helps provide a benchmark from which the effectiveness of other putative anxiolytics in the younger rat can be assessed. Although there are methodological differences between the current study and previous studies with adult rats, the overall pattern of how CDP affected defensive behaviors in pre-pubertal rats

is not inconsistent with what has been reported in adults. Our finding that CDP also facilitates an earlier return to pre-threat levels for at least one component of play in young rats suggests that incorporating play into studies of fear and anxiety could be a particularly sensitive index for determining the efficacy of other putative anxiolytics in the pre-pubertal rat.

Acknowledgements

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Figure Legends

Figure 1. Procedural timelines for Experiment 2 and for both studies from Experiment 3. Time above each arrow indicates elapsed time between the events on either end of that arrow.

Figure 2. Mean (± SEM) number of nape contacts and pins when tested on the Conditioning day in the presence of either an unworn or worn cat collar and on the Test day in the absence of any collar. * $p < .05$ compared to the group exposed to an unworn collar and given vehicle on both days.

Figure 3. Mean (± SEM) amount of time spent in each of the three possible states for location of the two rats on either the Conditioning day or Test day.   * $p < .05$ compared to the group exposed to an unworn collar and given vehicle on both days. # $p < .05$ compared to the group exposed to a worn collar and given vehicle on both days.

Figure 4. Mean (± SEM) amount of time at least one rat of the testing pair was engaged in risk assessment (head out posture) on either the Conditioning day or Test day. * $p < .05$ compared to group exposed to an unworn collar and given vehicle on both days.

Figure 5. Mean (± SEM) number of nape contacts and pins when tested 1 hour after exposure to either an unworn collar or worn collar. Rats received an injection of either vehicle or CDP 30 minutes before exposure and 30 minutes after exposure. * $p < .05$ compared to the group exposed to an unworn collar and given vehicle at both time points.
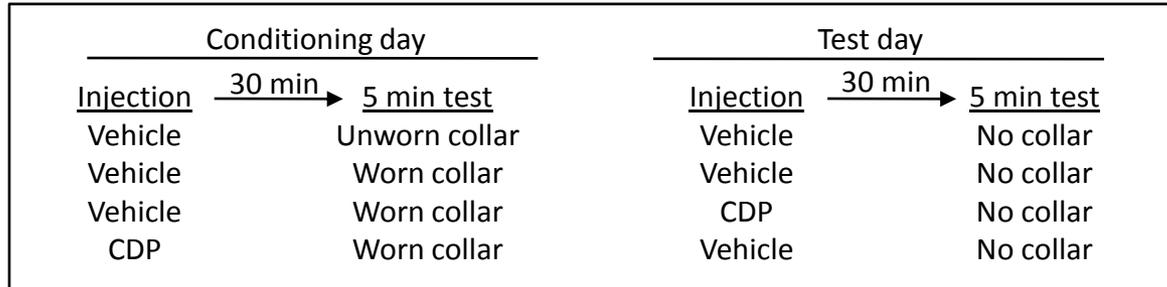
Figure 6. Mean (± SEM) number of nape contacts and pins when tested 1, 3, and 6 hours after exposure to a worn collar. Rats received an injection of either vehicle or CDP 30 minutes before exposure. Horizontal dashed line represents the mean value for both groups on the baseline day.

Figure 7. Mean (± SEM) amount of time at least one rat of the testing pair was engaged in risk assessment (head out posture) at 1, 3, and 6 hours after exposure to a worn collar. Rats received

an injection of either vehicle or CDP 30 minutes before exposure. Horizontal dashed line

represents the mean value for both groups on the baseline day.

Figure 1

A. Experimental groups and procedural timeline for Experiment 2

Conditioning day			Test day		
Injection	30 min →	5 min test	Injection	30 min →	5 min test
Vehicle		Unworn collar	Vehicle		No collar
Vehicle		Worn collar	Vehicle		No collar
Vehicle		Worn collar	CDP		No collar
CDP		Worn collar	Vehicle		No collar

B. Experimental groups and procedural timeline for the two studies in Experiment 3

Injection 1	30 min →	Home cage exposure	30 min →	Injection 2	30 min →	5 min test
Vehicle		Unworn collar		Vehicle		No collar
Vehicle		Worn collar		Vehicle		No collar
Vehicle		Worn collar		CDP		No collar
CDP		Worn collar		Vehicle		No collar

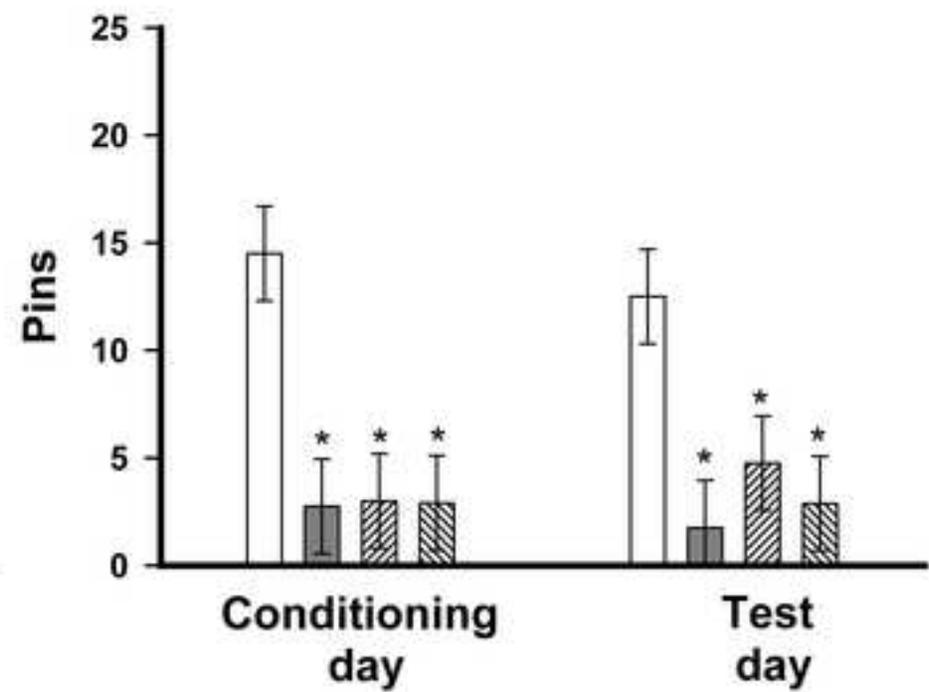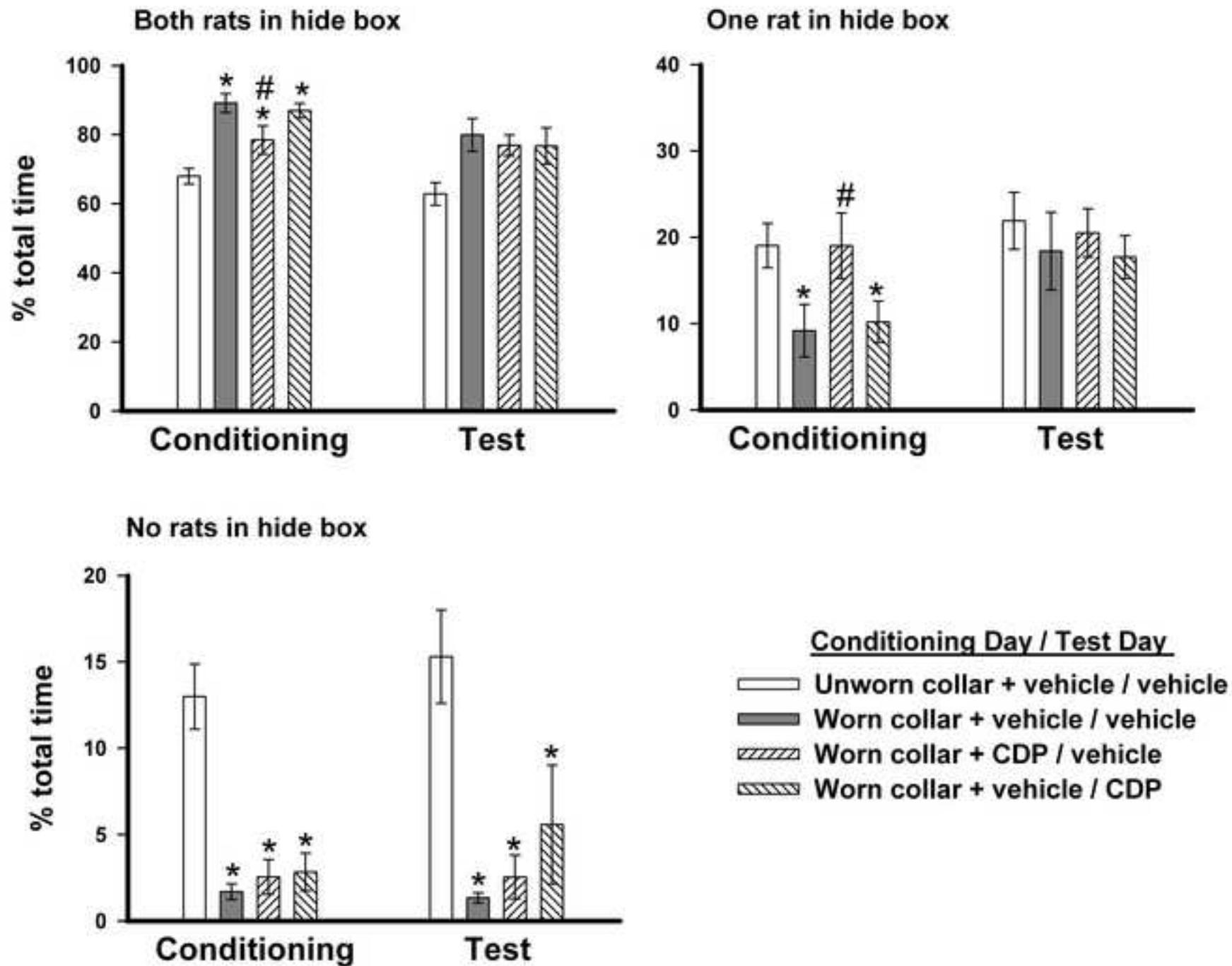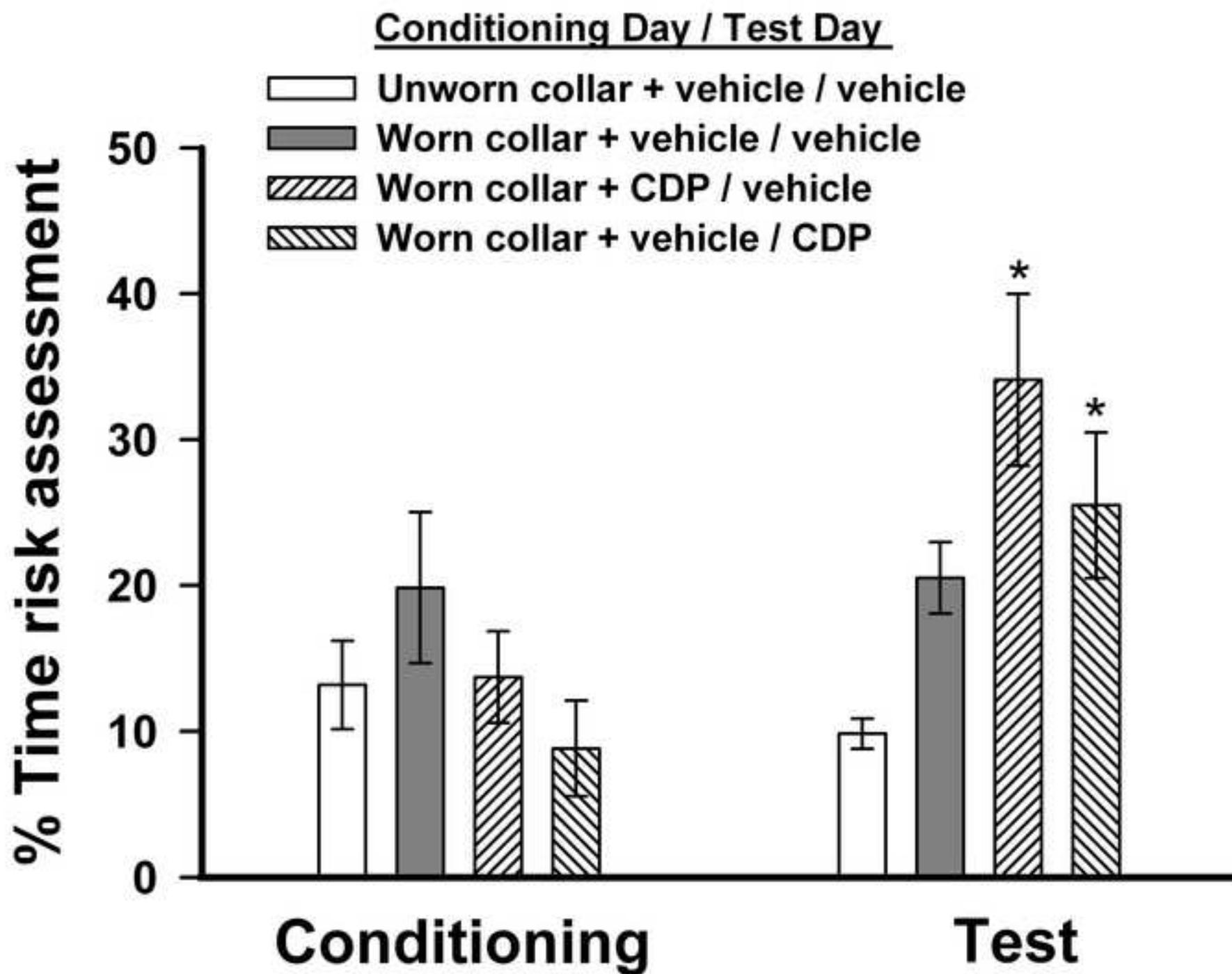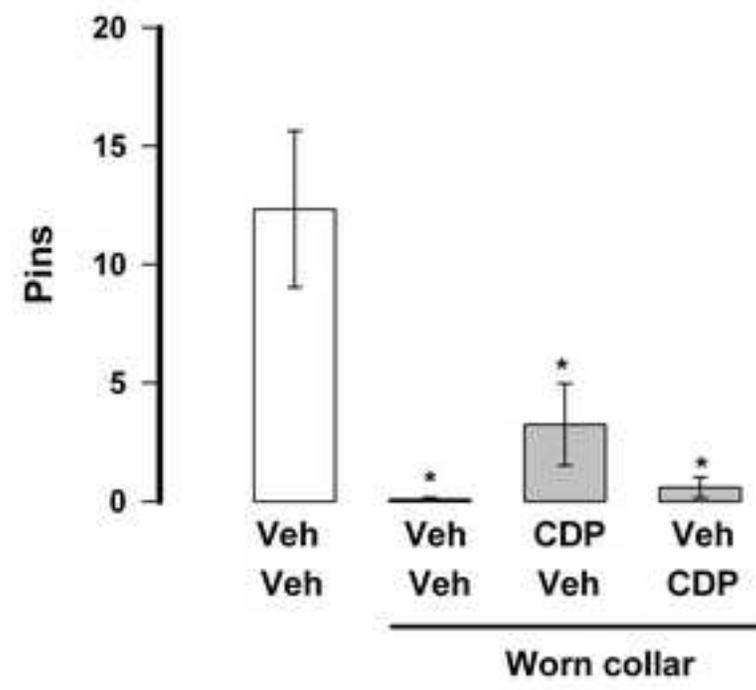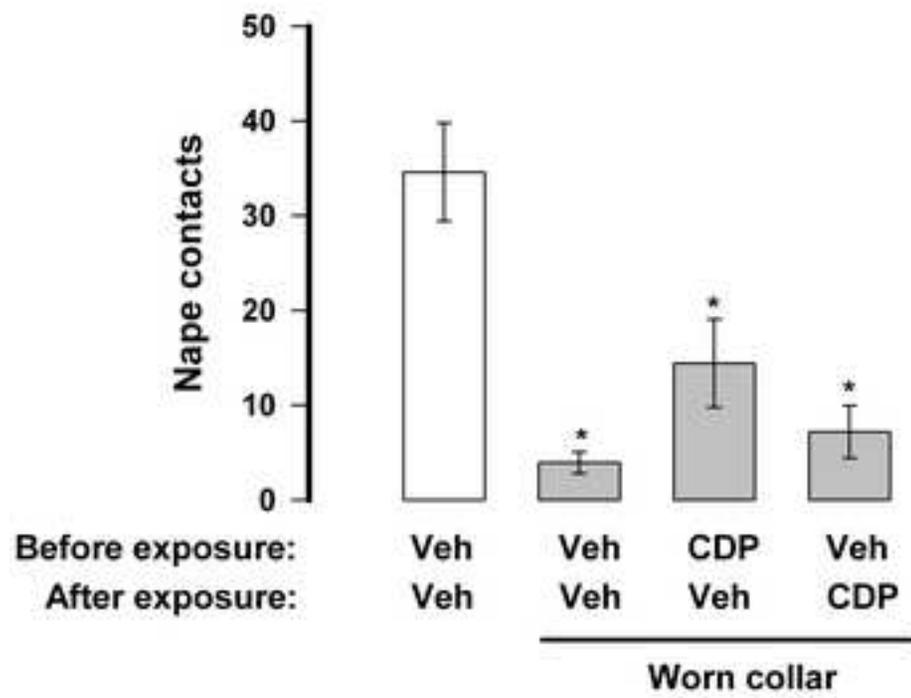| Injection	30 min →	Home cage Exposure	1 hr →	Test	2 hrs →	Test	3 hrs →	Test
Vehicle		Worn collar		No collar		No collar		No collar
CDP		Worn collar		No collar		No collar		No collar

Figure 2
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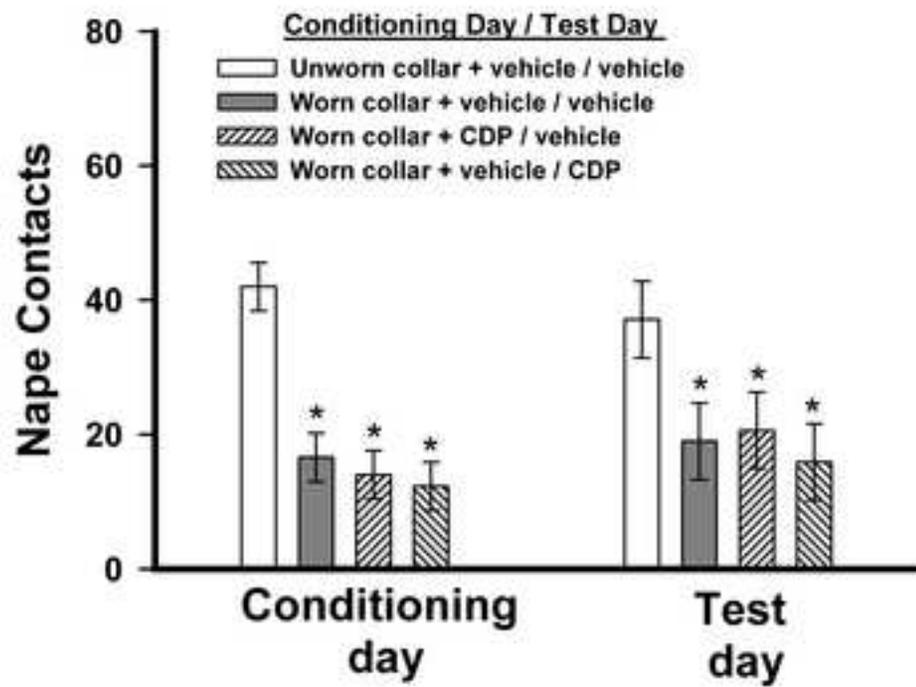
Figure 3
Click here to download high resolution image

Both rats in hide box

One rat in hide box

No rats in hide box

Conditioning Day / Test Day
- Unworn collar + vehicle / vehicle
- Worn collar + vehicle / vehicle
- Worn collar + CDP / vehicle
- Worn collar + vehicle / CDP

Figure 4

Conditioning Day / Test Day
- ☐ Unworn collar + vehicle / vehicle
- ■ Worn collar + vehicle / vehicle
- ▨ Worn collar + CDP / vehicle
- ▨ Worn collar + vehicle / CDP

% Time risk assessment vs. Conditioning / Test

Figure 5
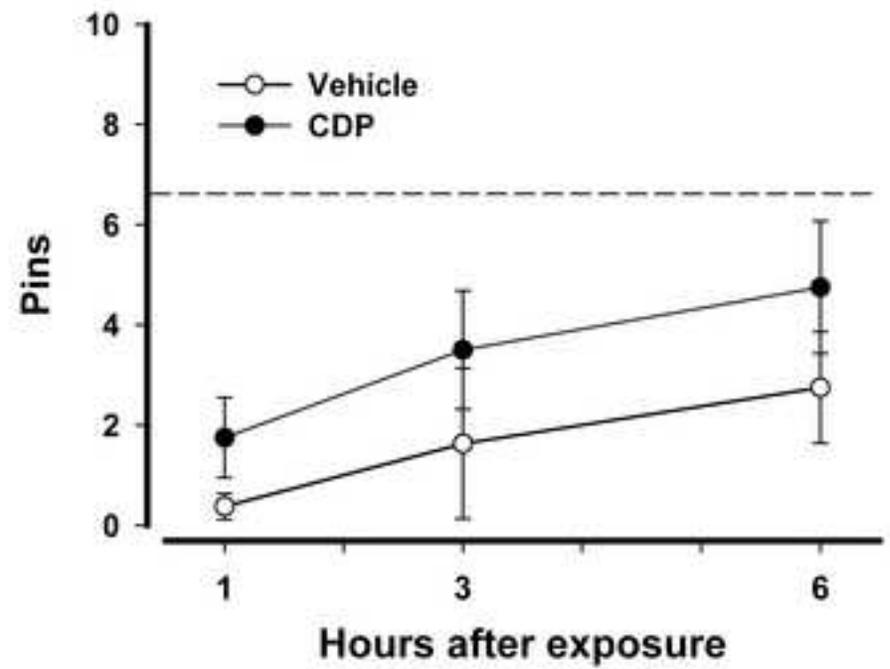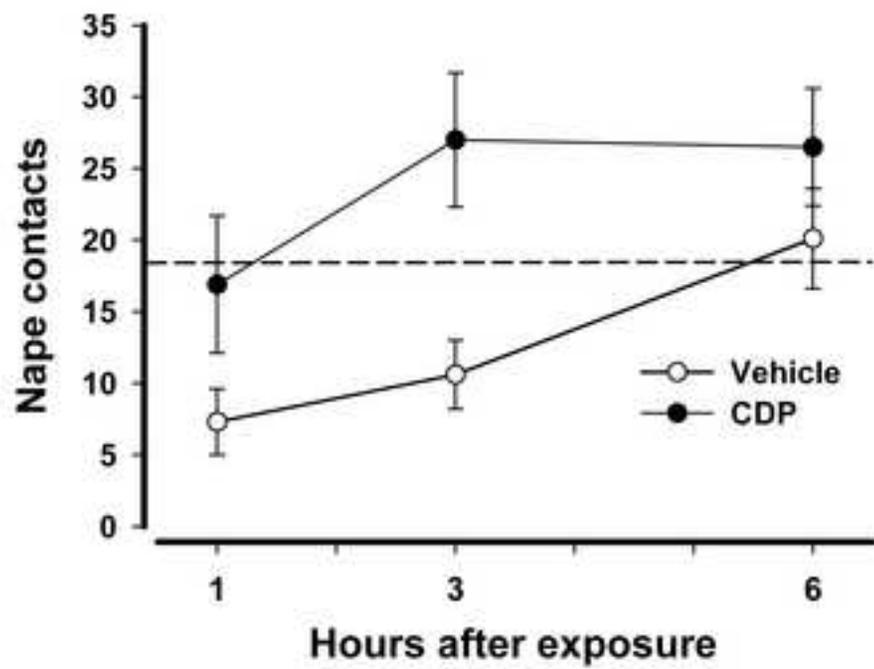**Click here to download high resolution image**

Figure 6

Figure 7
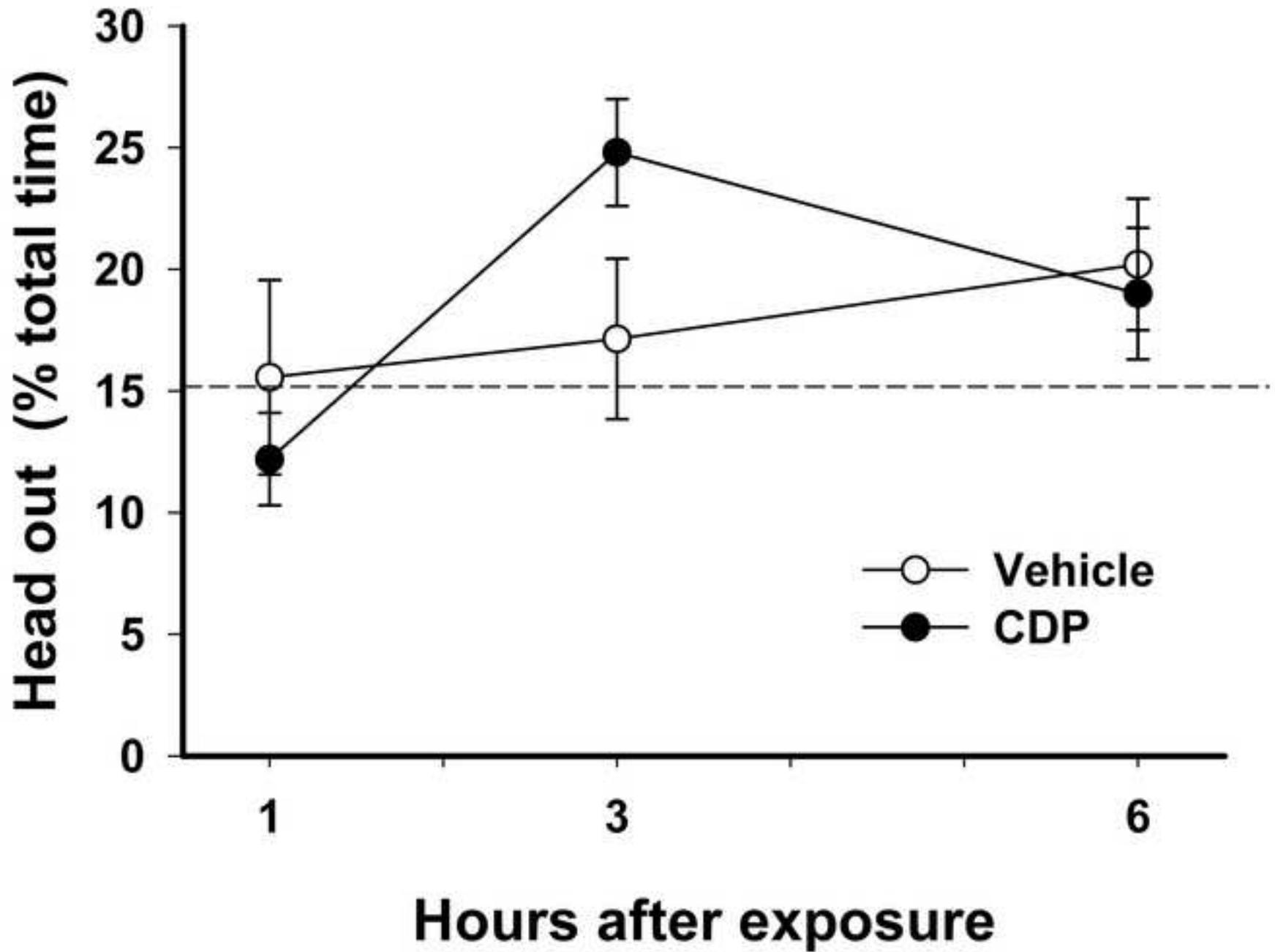**Click here to download high resolution image**

Table 1

Table 1. Mean (± SEM) percentage of total time in each of the three states for relative location within the testing chamber and percent of time (mean ± SEM) engaged in a form of risk assessment behavior (head out posture) when rats are exposed to either an unworn or worn collar in the home cage and tested 1 hour later.

	Unworn collar	Worn collar		
	Vehicle-vehicle	Vehicle-Vehicle	Vehicle-CDP	CDP-Vehicle
None in box	6.92 ± 2.12	1.24 ± 0.34*	1.38 ± 0.58*	1.09 ± 0.31*
One in box	16.94 ± 5.29	8.71 ± 2.69	10.33 ± 3.39	10.42 ± 3.37
Both in box	76.05 ± 5.56	90.02 ± 2.52	88.29 ± 3.43	88.49 ± 3.34
Head out posture	15.31 ± 2.73	13.68 ± 3.32	19.06 ± 4.78	19.95 ± 1.87

CDP = chlordiazepoxide (5 mg/kg)

*p < .05 when compared to rats exposed to unworn collar and given vehicle before and after exposure

Table 2

Table 2. Mean (± SEM) percentage of total time in each of the three states for relative location within the testing chamber and percent of time (mean ± SEM) engaged in a form of risk assessment behavior (head out posture) on the day before testing (Baseline) and at three different time points after exposure to a worn cat collar.

		Time after exposure on test day		
	Baseline	**1 hour**	**3 hours**	**6 hours**
None in box				
Vehicle	8.75 ± 2.98	1.95 ± 1.21	3.08 ± 1.47	2.59 ± 1.26
CDP	5.96 ± 1.83	0.91 ± 0.07	3.60 ± 2.09	4.37 ± 2.46
One in box				
Vehicle	15.58 ± 4.45	1.45 ± 0.37	10.58 ± 3.90	6.36 ± 3.59
CDP	17.61 ± 2.63	5.48 ± 2.94	13.40 ± 3.44	8.98 ± 2.08
Both in box				
Vehicle	75.53 ± 6.58	96.28 ± 1.39	85.88 ± 4.10	91.01 ± 4.87
CDP	75.85 ± 3.47	93.58 ± 2.96	82.92 ± 5.25	86.46 ± 2.94
Head out posture				
Vehicle	16.32 ± 1.56	15.56 ± 4.04	17.14 ± 3.26	20.29 ± 2.68
CDP	14.97 ± 1.86	12.22 ± 1.85	24.75 ± 2.16	19.02 ± 2.66

Table 3

Table 3. Summary of the effects of chlordiazepoxide in three different scenarios on the various measures used to assess the effects of predator odor-induced fear in juvenile rats.

	Behavioral measure			
Testing scenario	**Hiding**	**Head out posture**	**Nape contacts**	**Pins**
Collar + Context	↓	0	0	0
Context only	0	↑	0	0
Different context	0	↑	↑	0

Note:
 0 = no effect of CDP compared to vehicle-treated rats
 ↓ = CDP decreased behavioral measure when compared to vehicle-treated rats
 ↑ = CDP increased behavioral measure when compared to vehicle-treated rats