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# Chronic stress alters behavior in the conditioned defensive burying test: role of the posterior paraventricular thalamus

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**Abstract**

In the present studies, we examined the effects of chronic restraint on behavior in the conditioned defensive burying paradigm, a well-validated test of anxiety. This test is based on the findings that rodents tend to cover or bury the source of a noxious or aversive stimulus. However, little is known about whether prior chronic stress exposure can alter this anxiety-related behavior. In the present study, we examined whether chronic restraint affects indices of behavior in the conditioned defensive burying paradigm. Furthermore, since the posterior division of the paraventricular thalamus (pPVTh) regulates neuroendocrine activity specifically in chronically stressed but not control rats, we hypothesized that the pPVTh may also regulate any chronic stress-induced changes in behavior observed in the defensive burying test. Chronically stressed rats (30-min restraint per day for seven consecutive days) exhibited decreased latency to bury compared to control rats regardless of the presence of lesions suggesting increased reactivity to the shock in these animals. Importantly, pPVTh-lesioned chronically stressed rats exhibited increased duration and height of burying compared to control rats with pPVTh lesions, whereas no differences existed between sham-lesioned control and chronically stressed rats. Since both burying height and duration of burying are considered indices of anxiety in the defensive burying test, the present results suggest that the intact pPVTh may be important in dampening behaviors related to anxiety in chronically stressed rats.


*Keywords:* Anxiety; Chronic restraint; Paraventricular thalamic nucleus; Amygdala; Corticosterone

## 1. Introduction

Chronic or repeated exposure to stress exerts powerful influences on activity within the hypothalamic–pituitary–adrenal (HPA) axis, and on memory, synaptic plasticity and behavior (Dallman et al., 2002; McEwen, 2002; Ottenweller et al., 1989). Chronic stress has different effects on subsequent responsivity of the HPA axis depending on whether the subsequent stress is novel or previously experienced (Dallman et al., 1992). Our recent findings pertaining to the neural circuitry that underlies chronic stress-induced HPA activity strongly suggest a critical role for the posterior paraventricular thalamus (pPVTh). Lesions of the pPVTh prevent habituation to repeated restraint (Bhatnagar et al., 2002) and augment the facilitated HPA responses seen in chronically stressed rats exposed to novel stress (Bhatnagar and Dallman, 1998). In both cases, pPVTh lesions did not alter HPA function in acutely stressed rats. These data suggest that, under normal conditions, the pPVTh inhibits HPA responses specifically in chronically stressed animals but has no functional effect in acutely stressed rats.

Given the importance of the pPVTh in regulation of chronic stress-induced HPA activity, we hypothesized that it would also play an important role in chronic stress-induced changes in physiology and behavior. Indeed, the pPVTh regulates circadian rhythms in body temperature in chronically stressed but not control rats (Bhatnagar and Dallman, 1999). In the present experiments, we asked whether the pPVTh regulates behavior in a test of anxiety differently in control vs. chronically stressed rats. The conditioned defensive burying paradigm is a well-validated test of anxiety (Pinel and Treit, 1978; Treit et al., 1981). Burying behavior is conditioned to an aversive stimulus, an electrified probe, and is readily observable in a single trial. Anxiolytic drugs such as diazepam and chlordiazepoxide decrease duration and height of burying without producing overt motor impair-

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ments (Pinel and Treit, 1983; Treit et al., 1981). Thus, increases in duration and height of buried bedding are thought to reflect increased anxiety. Defensive burying has been used to study animals of different genetic backgrounds (Pare, 1994) and following early environmental manipulations (Horvath et al., 1999; Felszeghy et al., 1993). However, little is known about whether chronic exposure to stress can alter behavior in this test even though chronic or repeated exposure to stress is closely associated with anxiety-related disorders (Korte, 2001; Haller, 2001; Tache et al., 2001).

Given the important role of the pPVTh in regulating chronic stress-induced changes in HPA activity and the lack of information about the effects of chronic stress on behavior in the defensive burying paradigm, we examined behavior in the defensive burying test of sham- and pPVTh-lesioned animals that were exposed to chronic restraint (for seven consecutive days) or to no restraint at all. Our previous work in chronically stressed animals has measured indices of neuroendocrine function and physiology on Day 8 after 7 days of stress (Bhatnagar and Dallman, 1998; Bhatnagar et al., 2000, 2002). To allow us to make comparisons between the effects of chronic stress on neuroendocrine function, physiology and behavior, we wanted to measure behavior in the defensive burying paradigm on Day 8 after 7 days of chronic stress. However, testing in the burying apparatus requires habituation to the apparatus for 3–4 days immediately before testing so that exposure to environmental novelty does not interfere with latency to contact the probe or burying behavior (Treit et al., 1981). Use of such a habituation procedure would mean that chronically stressed rats would also be habituated during the last 4 days of chronic stress, whereas control rats would only be habituated to the apparatus. To determine whether we could avoid this potential confounding situation of pairing restraint with habituation to the apparatus in chronically stressed rats, we first determined whether habituation to the apparatus could be administered for 4 days before the 7 days when chronic stress would normally take place. In Experiment 1, we found that habituating naïve (nonstressed) rats 7 days before testing did not alter behavior compared to rats habituated for the 4 days immediately before the testing day. Therefore, in Experiment 2, sham- and pPVTh-lesioned rats were exposed to 4 days of habituation. For the next 7 days, control rats were undisturbed whereas chronically stressed rats were restrained for 30 min each day. Both control and chronically stressed rats were then tested in the defensive burying test on Day 8.

## 2. Methods

### 2.1. Animals

All experiments used young adult male Sprague–Dawley rats supplied by Harlan Sprague Dawley (Indianapolis, IN). Body weights ranged from 200 to 225 g upon arrival at the animal housing facilities at the Department of Psychology, University of Michigan. Rats were individually housed in hanging metal cages, and were allowed ad libitum access to rat chow and water. They were maintained on a 12-h light/dark schedule (lights on at 07:00 h), and all experiments took place during the trough of the diurnal rhythm. Animals were briefly handled the day before experiments were conducted. All experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan.

### 2.2. Defensive burying apparatus

A rectangular Plexiglas burying apparatus (28.6 cm wide × 38.1 cm long and 38.1 cm high) was used. An electrified probe consisting of wires wrapped around a wooden dowel was positioned in the apparatus such that it protruded 10.2 cm into the chamber and was 7.6 cm from the bottom of the chamber. Contact with the probe produced a shock of 1 mA. Bedding in the chamber was approximately 5.1 cm in depth. The probe was not present during the 4-day habituation period. A background white noise generator was used for habituation and testing.

### 2.3. Behavioral testing

On testing day, rats were tested individually for 30 min with the electrified probe in place. The height of bedding behind the probe was measured before testing and at the end of each 30-min test period and was expressed as the height of bedding above the 5.1 cm of bedding already placed in the chamber. Fresh bedding was placed in the apparatus before testing of each animal. Behavior during the 30-min test period was video recorded and later scored by two independent observers. Behaviors measured were: latency to first contact the probe and be shocked, latency to initiate burying from the beginning of the test and from first contact, total number of shocks received, height of buried bedding and duration of burying behavior over the entire test period.

### 2.4. Experiment 1

In this experiment, rats were divided into two groups (seven rats per group). In one group, rats were individually habituated to the defensive burying apparatus for 30 min/day for the 4 days preceding the day of testing. The other group of rats was habituated to the apparatus for 30 min/day for 4 days 1 week before the day of testing. Thus, habituation was done on the 4 days before testing or 1 week before testing. Habituation and testing were conducted in the homeroom as described above.

### 2.5. Experiment 2

Two separate experiments were conducted and the data were pooled for final analysis in Experiment 2. All rats

underwent stereotaxic surgery (see below). Before surgery, half the rats were assigned to the sham-lesioned group and the other half were assigned to the pPVTh-lesioned group. Following 4 days of recovery from surgery, all animals were habituated to the defensive burying apparatus for 30 min/day for 4 days in the homeroom. Based on the results of Experiment 1, all rats were habituated on the four consecutive days 1 week before testing. Half of each of the sham- and pPVTh-lesioned groups were undisturbed during this 1-week period (control rats). The other half were exposed to repeated restraint (chronically stressed rats). These chronically restrained rats were taken from their home cage placed in a ventilated Plexiglas restraint tube (length 12.6 cm, internal diameter 5.7 cm) for 30 min/day (generally between 10:00 h and 10:30 h) for each of seven consecutive days. All animals were tested on Day 8. There were a total of four groups in this study: sham-lesioned control rats (Control–Sham), sham-lesioned chronically restrained rats (Chronic–Sham), pPVTh-lesioned control rats (Control–Lesion) and pPVTh-lesioned chronically restrained rats (Chronic–Lesion). There were a total of 41 rats used in the two experiments that provide data for Experiment 2. We excluded rats that did not exhibit any burying behavior after receiving shock (5/41) and also excluded rats with missed lesions. Therefore, the final *n*'s per group were: Control–Sham, 6–7; Chronic–Sham, 6–9; Control–Lesion, 6–9; Chronic–Lesion, 6, depending on the measure. The variations in *n*'s for each measure result from missing data points and/or technical problems with the videotaping.

### 2.6. Ibotenic acid lesions of the pPVTh

Rats were anesthetized with a mixture of ketamine, xylazine and acepromazine (77:1.5:1.5 mg/ml given intraperitoneally at 0.1 ml/100 g body weight) and placed in a stereotaxic apparatus with the skull flat (the tooth bar at − 3.3 mm). For pPVTh lesions, the following coordinates were used (from bregma): AP, − 2.8; ML, 0.0 mm; DV, − 6.2 mm (from the surface of the skull; as in Bhatnagar and Dallman, 1999). A Hamilton microsyringe containing 10 ng/250 nl of ibotenic acid (Sigma, St. Louis, MO) was lowered to the pPVTh, and the drug injected over a 1-min period, with the needle remaining in place for another 5 min before removal. In sham-lesioned animals, the needle was lowered into place and 250 nl of vehicle (0.1 M PBS/0.9% saline) was injected as described above. All animals were allowed 4 days of recovery from surgery before habituation to the testing apparatus.

### 2.7. Confirmation of pPVTh lesions (Fig. 1)

At the end of Experiment 2, brains were collected, postfixed in 4% formalin followed by 30% sucrose and sliced at 30 μm on a sliding microtome. One series of sections was stained with cresyl violet and an adjacent series stained immunocytochemically for glial fibrillary acidic protein (GFAP) to assess the damage produced by the ibotenic acid lesion. Free-floating sections to be reacted with GFAP antibody were incubated with 10% normal horse serum in 0.1 M PBS solution for 20 min at 4 °C. Sections

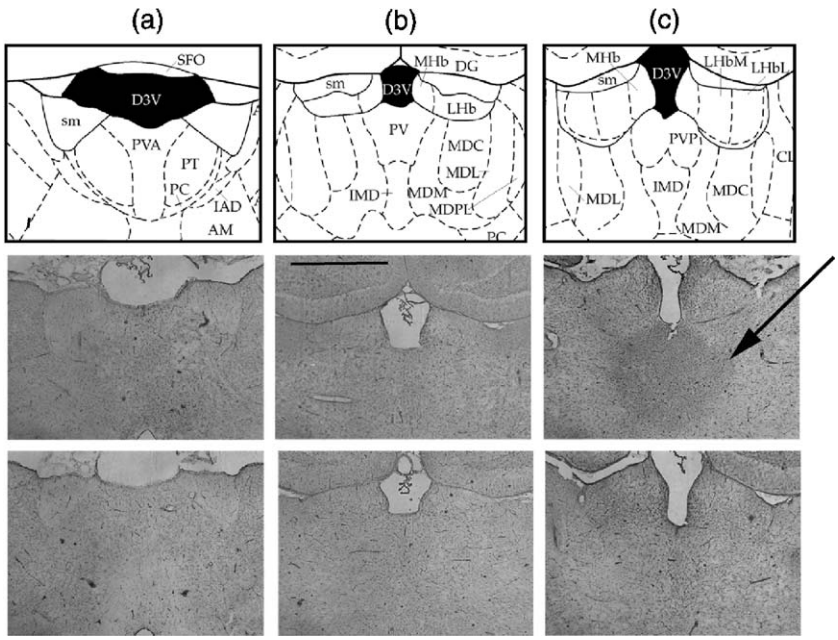


Fig. 1. Representative lesions of the pPVTh. Images from the Paxinos and Watson atlas of the anterior (a, referred to as PVA), medial (b, referred to as PV) and posterior (c, referred to as PVP) PVTh are shown in the top row. In the second and third rows, sections of the anterior, medial and posterior PVTh stained for glial fibrillary acidic protein are shown. In the second row, a lesion limited to the posterior division of the PVTh is shown, the outer border of which is indicated by an arrow. In the third row, a representative sham-lesioned rat with no damage to any of the divisions of the PVTh is shown.

Table 1
Latency to first contact the electrified probe, the latency to initiate burying and the height of burying are shown for rats that were habituated to the testing apparatus for the 4 days immediately before testing day or for rats that were habituated 1 week before testing day

| Habituation group | Latency to contact (s) | Latency to bury (s) | Height (cm) |
|---|---|---|---|
| Four days immediately before testing | 11.1 ± 2.8 | 207 ± 107 | 2 ± 0.9 |
| Four days one week before testing | 8.7 ± 1.6 | 244 ± 60 | 1.4 ± 0.7 |

There were seven rats per group at the beginning of the experiment.

were then incubated overnight at 4 °C at with a monoclonal mouse anti-GFAP (1:1500; Boehringer Mannheim, Indianapolis, IN) in 0.1 M PBS cocktail containing 1% normal horse serum (Vector, Burlingame, CA), 0.3% Triton X-100 (Sigma) and 0.25% BSA (Sigma). Sections were subsequently incubated with a biotinylated anti-mouse IgG (rat adsorbed) in horse serum (Vector), diluted 1:200 in the above PBS cocktail for 2 h at room temperature, washed and then incubated with an avidin–biotin–peroxidase complex (Vector) for 2 h. The chromagen was diaminobenzidine in 0.3% $H_2O_2$. All brains were examined in a blind fashion. Only animals exhibiting damage of, at least, approximately two-thirds of the posterior division of the PVTh by visualization of GFAP staining were included in the study. The posterior division of the PVTh was defined as extending from −2.56 to −3.3 mm from bregma (as in Bhatnagar and Dallman, 1999). All animals exhibiting damage limited to the anterior and medial subdivisions were excluded, although animals exhibiting some damage to the medial subdivision were included if, at least, two-thirds of the posterior subdivision was also lesioned. Although it is possible that some ibotenic acid leaked into the ventricles, we did not see any damage, as assessed by increased GFAP staining above background, in any regions adjacent to the PVTh in those animals that were included in the study. Our rate of successfully lesioning the pPVTh using the criteria described above was 65%. Fig. 1 shows representative sham and pPVTh lesions.

## 3. Statistics

A one-way analysis of variance (ANOVA) was used to analyze data in Experiment 1. A two-way ANOVA [Stress (Control or Chronic) × Lesion (Sham or pPVTh Lesion)] was used to analyze data collected in Experiment 2 and significant effects were followed by Fisher's post hoc tests. The significance level was set at $P \leq .05$ for all analyses.

## 4. Results

### 4.1. Experiment 1

We examined behavior of rats habituated to the testing apparatus for 4 days before the day of testing or 7 days before the day of testing. Four out of seven rats exhibited measurable burying behavior in each group. We found that these two groups did not exhibit any differences in height of buried bedding, latency to first contact the probe and latency to bury the probe (Table 1). Based on these results, in Experiment 2, we habituated both control and chronically stressed rats for 4 days 1 week before testing on Day 8. Chronically stressed rats were exposed to 30-min restraint per day during this week whereas the control rats were undisturbed during this week in Experiment 2.

### 4.2. Experiment 2

There were no significant effects in the latency to first contact the probe and be shocked (Fig. 2a) and no significant difference in the number of times rats received shocks from contacting the probe (data not shown).

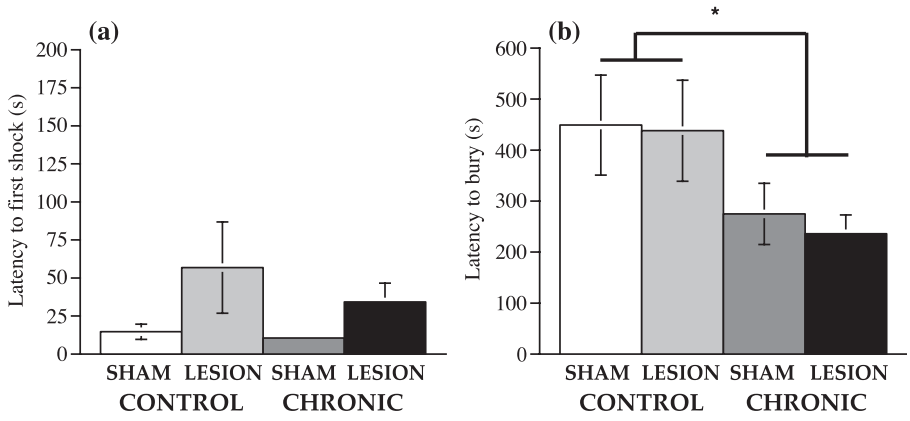


Fig. 2. Behaviors in the defensive burying paradigm are shown for control and chronically stressed (30-min restraint per day for 7 days) rats that were sham-lesioned or had lesions of the pPVTh. The latency to first contact the electrified probe and be shocked is shown in a, and the latency to exhibit measurable burying behavior following the shock is shown in b. Chronically stressed rats, regardless of lesion, exhibit decreased latency to initiate burying compared to both groups of control rats. * denotes significant difference between control and chronically stressed rats ($P < .05$).

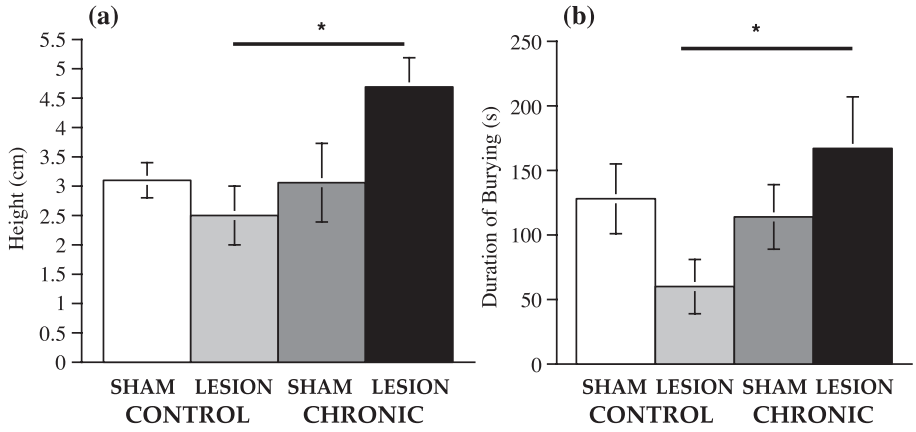


Fig. 3. Burying behavior in the defensive burying paradigm is shown for control and chronically stressed (30-min restraint per day for 7 days) rats that were sham-lesioned or had lesions of the pPVTh. The height of buried bedding is shown in a and the total duration of burying behavior during the test session is shown in b. Groups connected by horizontal lines are significantly different ($P<0.05$) from each other.

There was a significant main effect of stress [$F(1,22)=5.8$, $P=.02$] in the latency to bury from the beginning of the test session (Fig. 2b). Similarly, there was a significant main effect of stress in terms of latency to exhibit burying behavior after first contact with the probe [$F(1,22)=5.4$, $P=.03$; data not shown]. In both cases, chronically stressed rats exhibited lower latencies to begin burying than did control rats, regardless of lesions.

There was a significant Stress × Lesion interaction in burying height [$F(1,28)=3.9$; $P<.05$]. Post hoc tests showed that chronically stressed animals with pPVTh lesions buried significantly more than control animals with pPVTh lesions (Fig. 3a). No such differences were observed between sham-lesioned control and chronically stressed rats. Similarly, there was a significant Stress × Lesion interaction in duration of burying behavior [$F(1,24)=4.3$; $P<.04$]. Post hoc tests indicated that chronically stressed rats with pPVTh lesions exhibited burying behavior for a longer duration than control rats with pPVTh lesions (Fig. 3b). No other significant effects were found.

In summary, chronically stressed animals, either sham- or pPVTh-lesioned, exhibited decreased latency to begin burying either from the beginning of testing or from the onset of shock compared to both groups of control rats. Furthermore, chronically stressed rats with pPVTh-lesioned exhibited increased height and duration of burying compared to similarly lesioned control rats.

## 5. Discussion

We examined whether chronic exposure to restraint stress alters behavior in the defensive burying test, a validated model for testing anxiety-related behaviors. Chronic restraint decreased the latency to bury compared to control rats regardless of whether either group of rats was lesioned or not. pPVTh lesions resulted in increased burying height and duration of burying in chronically stressed rats compared to controls with such lesions. Since duration of burying and burying height are considered primary indices of anxiety in the defensive burying test, pPVTh lesions produced increases in anxiety in chronically stressed compared to control rats. Therefore, these data suggest that the intact pPVTh is important in dampening anxiety behaviors related to chronic stress exposure.

Both burying height and duration of burying behavior are important indices of anxiety in the defensive burying test. Known anxiolytic drugs such as diazepam and chlordiazepoxide decrease burying height and duration of burying (Treit et al., 1981, 1986; Pesold and Treit, 1992). However, latency to bury measures are not always consistently altered with burying behavior in response to some anxiolytic drugs, leading to the suggestion that the latency to bury measure is inversely related to an animal's reactivity to the anxiety-provoking situation of the presence of an aversive object (Craft et al., 1988; Lopez-Rubalcava et al., 1996, 1999). Therefore, in our study, the decreased latency exhibited by chronically stressed animals suggests increased reactivity. Some evidence suggests that binding of corticosterone to central mineralocorticoid and/or glucocorticoid receptors is important in regulating latency behavior in the conditioned defensive burying test. Animals with injections of mineralocorticoid receptor antagonists into the hippocampus exhibit increased latency to bury (Bitran et al., 1998). Additionally, Korte et al. (1996) have shown that animals treated with intracerebroventricular administration of combined mineralocorticoid and glucocorticoid receptor antagonists exhibit increased immobility, which could increase latency to bury, but no change in burying behavior was observed. These animals were exposed to the electrified prod on Day 1 (as in the present study) but behavior on the next day in the presence of the nonelectrified prod was examined. Although it is not unclear how this learned behavior on the second day is associated with behaviors measured on the first day, the

work of Bitran (1998) and Korte (1996) and colleagues suggests an important role for corticosteroid receptors in mediating some behaviors in the defensive burying test. Applying these results to our study, it is possible that binding of daily stress-induced release of corticosterone to its receptors increases reactivity and latency to initiate burying in chronically stressed animals. One way to test this possibility is to chronically stress adrenalectomized rats that are replaced with various doses of corticosterone and examine resultant effects on behavior in the defensive burying test.

Burying height and duration were elevated in chronically stressed animals compared to control but only if these groups had lesions of the pPVTh. That is, pPVTh lesions resulted in increased anxiety in chronically stressed vs. control rats. These data suggest that the intact pPVTh is important in dampening anxiety-related behaviors in chronically stressed compared to control animals and is consistent with our previous studies in which the intact pPVTh is important in dampening HPA responses to subsequent stress in chronically stressed animals. The neuroanatomical pathways by which the pPVTh may mediate behaviors in the defensive burying test are not known. The efferent projections of the pPVTh are very limited (Moga et al., 1995). One major set of projections is to the amygdala, including the central, basolateral and basomedial nuclei (Moga et al., 1995). The amygdala plays an important role in regulating anxiety-related behaviors (Walker and Davis, 2002; Davidson, 2001). In the defensive burying test, lesions of the central amygdala increase latency to bury and produce a nonsignificant decrease in burying behavior (Kopchia et al., 1992) though injections of the anxiolytic drug midazolam into either the central or basolateral amygdala had no effect in this test (Pesold and Treit, 1995). Roozendaal et al. (1993) have examined the effects norepinephrine injected into the central amygdala on retention of conditioning on the second day in the presence of the nonelectrified probe. Animals bred for high avoidance behavior exhibit increased burying behavior following norepinephrine injection into the central amygdala. Application of these data on retention on the day after exposure to the electrified prod to our results is limited since our testing protocol was a 1-day protocol that did not measure retention. Nonetheless, these data suggest that one way that the pPVTh could regulate amygdala function in the defensive burying test is to alter its sensitivity to norepinephrine differentially in chronically stressed vs. control rats.

Alternatively, some evidence suggests that corticotropin-releasing factor (CRF) actions alter anxiety-related behaviors in the defensive burying paradigm. Blockade of central CRF by intracerebroventricular injections of CRF antagonists decrease burying time and height but increase latency to bury (Korte et al., 1994; Basso et al., 1999; Heinrichs et al., 2002). These effects are the opposite of what we found in chronically stressed animals with pPVTh lesions and suggest that CRF may be a mediator of pPVTh regulation of anxiety-related behaviors. Both the role of the amygdala and of CRF in pPVTh regulation of anxiety-related behaviors in chronically stressed animals need to be specifically examined. The results of such experiments would be important for understanding the pathways by which chronic stress alters behavior in tests of anxiety.

Together, the present results indicate that chronically stressed animals are more reactive in some anxiety provoking situations, as evidenced by the latency to initiate burying data. Furthermore, the intact pPVTh regulates anxiety-related behavior in the conditioned defensive burying test such that it serves to dampen these behaviors in chronically stressed compared to control animals. The use of pPVTh lesions in this study revealed an important role for this region in dampening anxiety-related behavior in chronically stressed animals. These data suggest that observations of anxiety-related behaviors exhibited by intact animals manipulated in other ways, for example, following exposure to more severe forms of chronic stress in adulthood or perinatal manipulations, may reflect alterations within pPVTh-related neuroanatomical circuitry. In this regard, pPVTh projections to amygdaloid subnuclei may be particularly important.

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