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Research Report

Lesions of the habenula produce stress- and dopamine-dependent alterations in prepulse inhibition and locomotion

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ABSTRACT

The habenula complex modulates the activity of dopamine and serotonin systems in the brain. An important question remains whether there is a link between habenula dysfunction and monoamine-related disorders, such as schizophrenia. In this study, we describe an interaction between habenula lesions and stress that produces long-lasting effects on behavior. Mice received control lesions or bilateral electrolytic lesions of the habenula and were tested for fear-potentiated startle and freezing measures of conditioned fear. They were also tested for prepulse inhibition (PPI) and locomotor activity in the presence or absence of a dopaminergic agonist (apomorphine) or an atypical antipsychotic with mixed dopamine/serotonin antagonist properties (clozapine). There were no detectable effects of habenula lesions on fear conditioning and no effects on PPI in the absence of stress. However, following conditioned fear stress, habenula-lesioned animals showed decreased PPI which normalized with clozapine. Lesioned animals also showed diminished activity at baseline, with hyperlocomotion following apomorphine. These data support the hypothesis that the habenula may be normally involved in stress-dependent regulation of monoamine systems.

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1. Introduction

The habenula complex, a paired midline structure just medial to the thalamus, provides a neural pathway that mediates forebrain control over midbrain dopaminergic and serotonergic firing (Sutherland, 1982; Andres et al., 1999). The habenula receives convergent input from most limbic brain regions including the hypothalamus, central amygdala, substantia innominata, diagonal band of Broca, nucleus accumbens, septum, and prefrontal cortical regions (Sutherland, 1982; Ellison, 2002). In addition to receiving afferent input from

brainstem dopamine and serotonergic systems, it is a principle regulator of firing of the ventral tegmental area (VTA), dorsal raphe nucleus (DRN), and interpeduncular nucleus (IP) (Wang and Aghajanian, 1977; Reisine et al., 1982; Skagerberg et al., 1984; Nishikawa et al., 1986; Varga et al., 2003).

Given the anatomical and functional ability of the habenula to regulate dopaminergic and serotonergic transmission, it is plausible that habenula dysfunction may disrupt the regulation of behavior influenced by monoamine transmission. Past studies have indeed shown that lesions of the

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habenula lead to alterations in the normal regulation of noradrenergic, dopaminergic, and serotonergic transmission (Lisoprawski et al., 1980; Nishikawa et al., 1986; Murray et al., 1994; Murphy et al., 1996; Amat et al., 2001). However, the consequences of habenula damage on cognitive and motor behaviors have been mixed. Several laboratories have demonstrated lesion-induced impairment in a number of behaviors including avoidance learning (Thornton et al., 1994), water maze performance (Thornton and Davies, 1991; Lecourtier et al., 2004), and locomotive activity (Lee and Huang, 1988; Thornton et al., 1990; Murphy et al., 1996). On the other hand, other studies have found little influence of habenula damage on such behaviors (Thornton et al., 1994; Vale-Martinez et al., 1997).

At present, the variables which contribute to these differential outcomes are presently ill-defined; however, some evidence indicates that the functional consequences of habenula damage appear most apparent in lesioned animals which have been exposed to various types of stressors. For example, Thornton and Bradbury (1989) found that habenula lesions impair one-way avoidance learning only when physical effort or stress levels are increased. The addition of isolation and food deprivation stress also alters normal elevated plus-maze behavior in lesioned animals (Murphy et al., 1996). Furthermore, Maier and colleagues (Amat et al., 2001) have shown that an intact habenula is essential for subsequent long-term alterations in neural and behavioral responses after uncontrollable stress. Together, these results suggest that the habenula may play a more active and central role in the long-term modification of monoamine transmission and behavioral responses subsequent to aversive and stressful events. Electrophysiological and *c-fos* immunoreactivity studies in rodents have indeed demonstrated that the habenula is activated in response to various aversive stressors, including stimulation of the tail, restraint, novel environments, and footshock (Benabid and Jeaugey, 1989; Wirtshafter et al., 1994; Gao et al., 1996; Smith et al., 1997). We have previously reported that the habenula also showed increased expression of synaptic plasticity genes associated with long-term changes in neuronal activity in response to stress associated with fear conditioning (Ressler et al., 2002). Along with evidence indicating the habenula's role in regulating monoamine transmission, these results suggest that stress may induce long-term structural changes in the habenula which influence subsequent monoamine-dependent behaviors.

In the current study, we investigated the effects of habenula lesions on the acquisition and expression of conditioned fear and whether lesions altered behavioral measures of dopamine sensitivity and sensorimotor gating after stress associated with conditioned fear. Our results demonstrate that despite the correlational evidence of increased activity with conditioned fear, the habenula does not appear to be essential for the acquisition or expression of this form of learning. However, habenula lesions do lead to stress-dependent changes in behavioral measures of dopamine sensitivity as measured with apomorphine, a D1/D2 agonist. Sensorimotor gating deficits were also found that were reversible with clozapine, an atypical antipsychotic with mixed dopamine/serotonin antagonist properties. These data provide evidence for a role of the habenula complex in long-term stress-dependent

modulation of monoamine systems and associated behaviors. These data also suggest that abnormalities in this regulatory system produce changes in measures of sensorimotor gating and dopamine sensitivity in animals.

2. Results

2.1. Postsurgery PPI and fear conditioning

Two days after matching, animals received either discrete lesions restricted to the habenula complex or sham lesions (Fig. 1a). Of the 22 habenula-lesioned animals, 1 died after surgery and 3 had only unilateral habenula damage. Thus, 20 sham-lesioned and 18 habenula-lesioned animals were included in analysis. Damage to the habenula extended the rostrocaudal boundaries in most mice. In some mice, sparing was observed in the rostral ($n = 4$) or caudal ($n = 4$) pole of the habenula complex. In most cases, lesions included damage to the fasciculus retroflexus (FR). In some cases, damage extended past the boundaries of the habenula and included collateral damage to laterodorsal thalamus and dorsal areas of the mediodorsal thalamus. Collateral damage was also noted in the dorsal hippocampus in some mice; however, in these cases, damage was relatively small. Statistical comparisons between animals with and without minor hippocampal damage revealed no significant differences between groups; thus, mice with such damage were included in overall analyses. Fig. 1b shows histological reconstructions depicting the smallest and largest habenula lesions included in the analysis.

One week following lesions, animals were given a post-surgery PPI session to examine whether lesions produced changes in PPI. As seen in Fig. 2a, repeated-measures ANOVA revealed no significant change in PPI after surgery, $F(1,36) = 2.58$, $P > 0.05$, and no difference in PPI levels between sham and habenula animals, $F(1,36) = 0.01$, $P > 0.05$. Likewise, we found no session \times lesion interaction, $F(1,36) = 1.26$, $P > 0.05$, indicating that habenula and sham lesions did not produce differential effects on PPI.

Following the PPI sessions, mice were given a pretraining fear test followed by a fear training session which consisted of 5 pairings of a tone conditioned stimulus (CS) with a footshock (US) on each of three consecutive days. One day later, mice were tested for behavioral levels of fear in the absence of shock. A repeated-measures ANOVA revealed that over the course of training, both habenula- and sham-lesioned animals showed significant increases in freezing, $F(1,36) = 184.60$, $P < 0.01$ (Fig. 2b). No group differences were detected in the rate of acquisition or overall freezing level, $F_s < 1.64$, $P_s > 0.05$ indicating that both groups showed similar acquisition rates of conditioned fear to the tone. There were also no differences between groups in motor reactivity to the US on each day, suggesting similar pain sensitivity to the footshock, $t_s(36) < 1.10$, $P_s > 0.05$ (Fig. 2c). During testing in the absence of shock, sham and habenula animals showed comparable levels of conditioned fear as measured by percent fear-potentiated startle, $t(36) = 0.77$, $P > 0.05$ (Fig. 2d) and freezing, $t(36) = 0.35$, $P > 0.05$ (Fig. 2e); thus, both groups equally retained the ability to express fear.

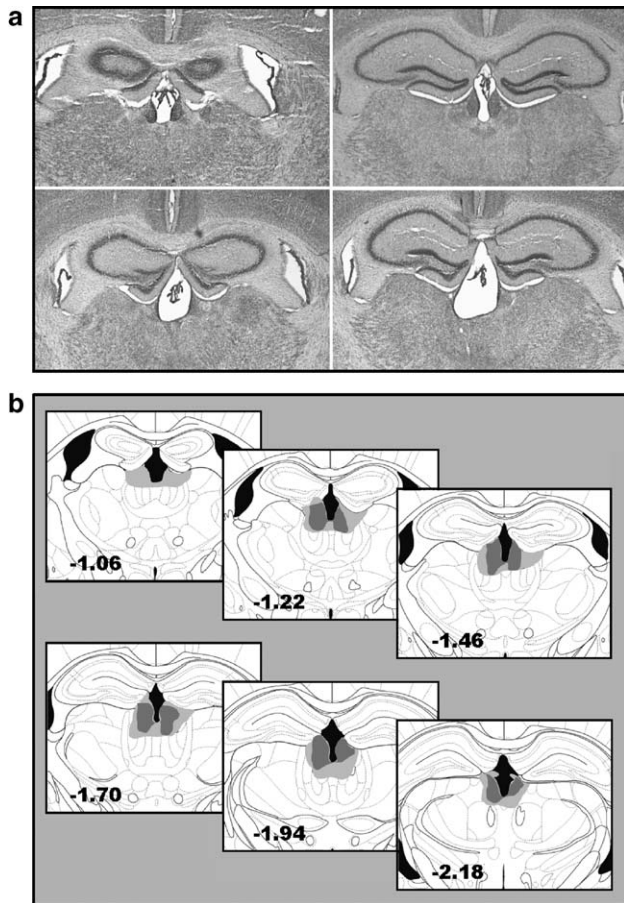


Fig. 1 – (a) Representative photomicrographs of sham-lesioned animals (top row) and habenula-lesioned animals (bottom row). Arrows demonstrate location of habenula in control and lesioned animals. (b) Serial histological reconstruction of electrolytic lesions of the habenula in mice included in study. Reconstructions have been transcribed onto modified plates 40, 41, 43, 45, 47, and 49 as adapted from Paxinos and Franklin (2001) and illustrate the largest (light grey) and smallest (dark grey) lesions included in statistical analyses.

2.2. Postconditioned fear PPI

Because previous studies have demonstrated that long-term and short-term stress paradigms produce subsequent sensitization of various behaviors known to be dependent on monoamine systems, animals were again placed back into the testing chambers and given a PPI test 3 days after conditioned fear stress (2 weeks after surgery).

The results of this poststress PPI session revealed a relative PPI increase in the sham controls compared to habenula-lesioned animals (Fig. 3a) which was evident across several different prepulse intensities (Fig. 3b). A repeated-measures ANOVA comparing pre- to poststress PPI measures revealed a significant lesion \times session interaction $F(1,36) = 7.18, P < 0.02$. Subsequent analyses indicated that after conditioned fear stress, habenula animals showed less PPI than shams, $t(36) < 4.05, P < 0.01$. Conditioned fear stress increased PPI levels in sham ($P < 0.01$) but not habenula animals ($P > 0.05$).

Contextual fear, as expressed in the FPS paradigm, is associated with increased baseline startle. Thus, to examine whether differences between habenula and sham animals in PPI were influenced by differences in the expression of contextual fear, we analyzed pre- and poststress mean startle responses in the absence of the prepulse. This analysis revealed no significant main effects or interaction, $F_s(1,36) < 0.2, P_s > 0.05$ (data not shown), thus differences in PPI could not be attributed to differences in startle responses.

2.3. Clozapine pretreatment and PPI

To test whether a dysregulation of the dopaminergic and/or serotonergic systems might be contributing to PPI differences, habenula and sham-lesioned animals were tested for PPI in the presence of clozapine (6 mg/kg) or vehicle using a random crossover experimental design with a 3 day drug washout period between tests. A repeated-measures ANOVA analysis revealed a significant lesion \times pretreatment interaction, $F(1,36) = 7.30, P < 0.01$, indicating that clozapine produced a differential effect on lesioned groups (Fig. 3c). As expected, habenula-lesioned animals displayed significantly less PPI than sham animals when pretreated with vehicle, $t(36) = 2.27, P < 0.03$. In the sham-lesioned animals, there was no PPI difference between clozapine and vehicle pretreatment, $t(19) = 0.33, P > 0.05$. In contrast, clozapine pretreatment resulted in a significant PPI increase in habenula-lesioned animals, $t(17) = 3.70, P < 0.01$. To rule out any effect of order of treatment in this crossover design, we examined order of treatment (vehicle first vs. clozapine first) as a variable. We found no sequence effect or sequence interactions, $F_s(1,36) < 1.1, P_s > 0.05$, suggesting no carryover effects of treatment order. Thus, these data demonstrate that the relative PPI deficit displayed by habenula-lesioned animals that had previously been stressed was eliminated with clozapine pretreatment but was again present in the absence of clozapine.

2.4. Systemic injections of apomorphine and locomotor activity

Given the putative role of the habenula in dopaminergic control, and the role of dopamine in modulating PPI, we explored group differences in locomotive activity in an open-field before and after acute administration of the dopamine agonist apomorphine. Habenula and sham-lesioned animals were first given a 5-min novel open-field test session in the absence of drug. This test revealed that habenula animals displayed less activity than shams, $t(24) = 3.87, P < 0.01$. Two days later, both groups were given systemic injections of apomorphine (2 mg/kg, ip) 30 min before a second open-field test session. The administration of apomorphine produced an increased locomotor response in both habenula and sham groups, $F(1,24) = 184.60, P < 0.01$. However, the magnitude of increase was significantly larger in the habenula group, as reflected by the significant session \times lesion interaction, $F(1,24) = 10.97, P < 0.01$, and a direct comparison of the mean activity change between test sessions, $t(24) = 3.31, P < 0.01$ (Fig. 3d). These results indicated that previously stressed habenula-lesioned animals displayed less locomotor activity in the absence of drug and were more sensitive to the activity

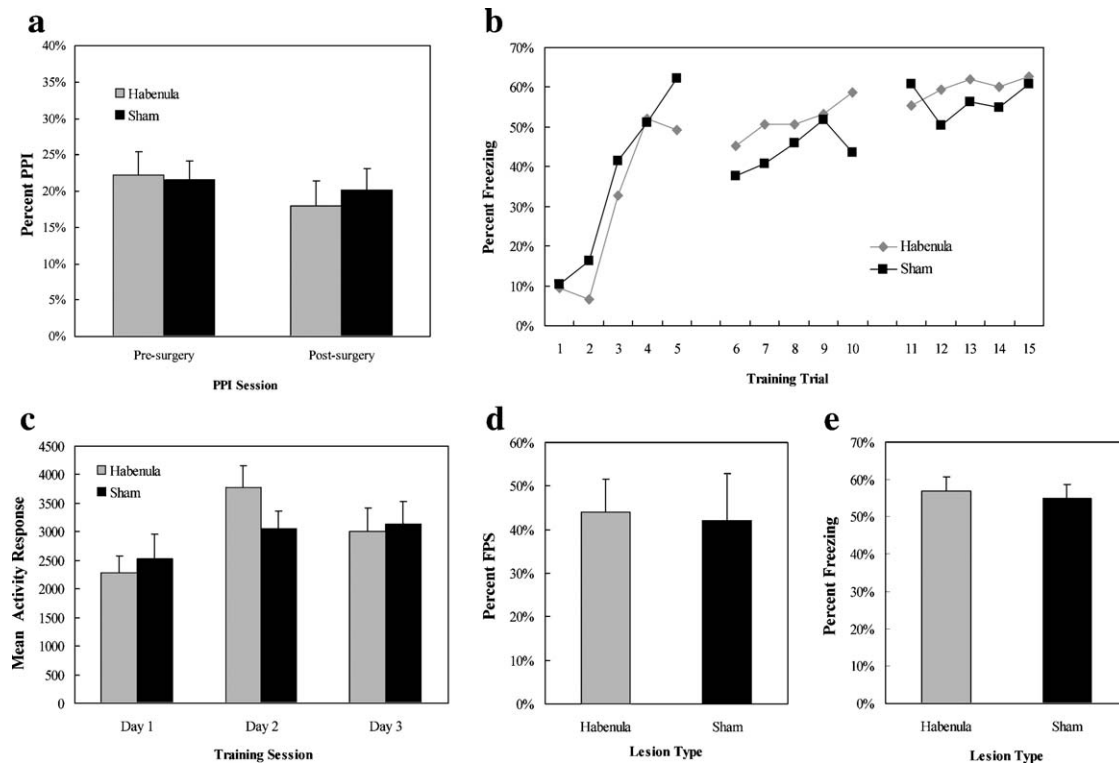


Fig. 2-(a) Pre- and postsurgery PPI sessions designed to examine the effects of habenula damage on PPI. Mean percent PPI scores represent the average inhibition computed over 4 prepulse intensities (2, 4, 8, or 10 dB above a 63 dB white noise background). Postsurgery test was performed 1 week after surgery. (b) Freezing response to the presence of the tone conditioned stimulus (CS) during acquisition of conditioned fear. Training consisted of 5 tone + shock trials on each of 3 consecutive days (15 total trials). (c) Mean reactivity score in response to the 0.4 mA footshock used as the unconditioned stimulus (US) during fear conditioning. (d-e) Retention of conditioned fear as measured by (d) fear-potentiated startle and (e) freezing. The retention test was conducted 24 h after last training session. Bars represent means + SEM.

enhancing effects of apomorphine when compared to sham animals.

2.5. Control lesions and tests

To examine whether group differences in PPI were the consequence of subcortical damage in general, the timing of lesions with respect to testing, or the number of PPI sessions, we conducted an experiment on a separate group of animals that differed from the original design in two ways. First, animals were given 2 weeks with an additional intervening PPI session, rather than 1 week, between surgery and conditioned fear stress. Second, a third group of control-lesion animals was included. This control group received lesions of the lateral geniculate body which resulted in thalamic damage that was similar in size to habenula damage.

When we tested these animals for PPI before and 2 weeks after surgery, a repeated-measures ANOVA revealed no significant main effects or interaction, $F_s < 0.80$, $P_s > 0.05$. This indicated that behavioral differences could not be detected within a 2-week postsurgery time window in the absence of stress. Between weeks 2 and 3, animals received fear conditioning and testing followed by a poststress PPI test (Fig. 4c). As seen in our initial experiment (Fig. 4b), after stress, habenula-lesioned animals displayed a relative PPI deficit

when compared to control- and sham-lesioned animals (Figs. 4a and 4c) as reflected by a significant lesion by session interaction, $F(2,22) = 3.60$, $P < 0.05$. Subsequent t test indicated that habenula animals displayed lower PPI than control- and sham-lesioned animal, $t_s > 2.6$, $P_s < 0.03$. No differences were detected between sham and control animals, $P > 0.5$.

3. Discussion

Our findings indicate that lesions of the habenula do not result in immediate behavioral deficits. Groups of animals matched with comparable PPI levels before surgery demonstrated similar PPI performance either 1 or 2 weeks after surgery, regardless of lesion type. Likewise, no differences were seen in reactivity to shock or the acquisition and retention of conditioned fear. Following conditioned fear, habenula-lesioned animals displayed significantly less PPI than control-lesioned animals. Compared to sham animals, habenula-lesioned animals also displayed lower baseline and greater apomorphine-induced locomotor activity after fear conditioning.

Given past studies showing that lesions of the habenula result in alterations in the normal regulation of monoamine transmission (Lisoprawski et al., 1980; Nishikawa et al., 1986;

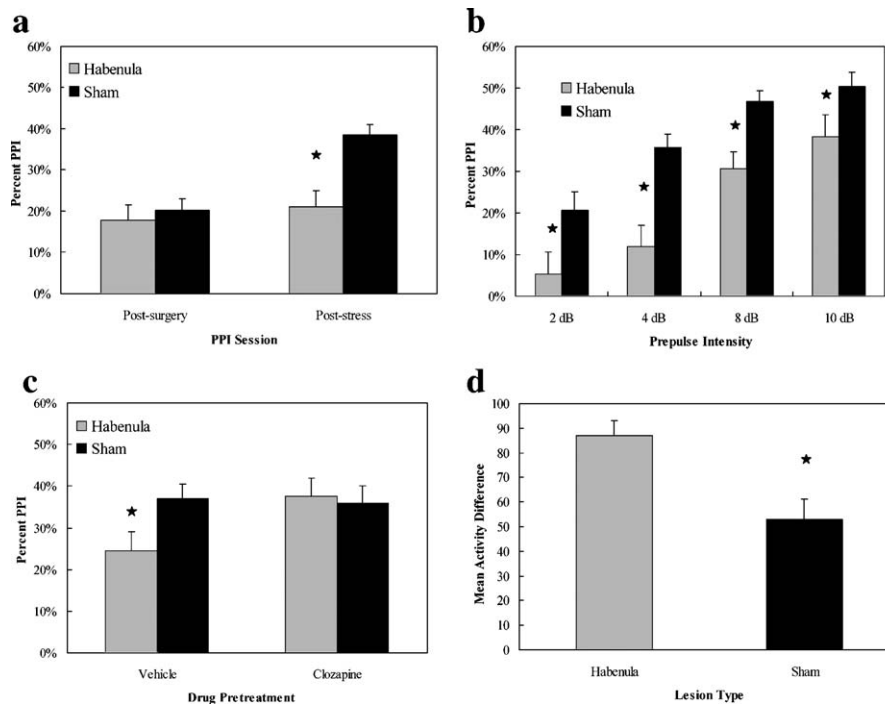


Fig. 3 – (a) Pre- and poststress PPI sessions designed to examine the effects of habenula damage and conditioned fear stress on PPI. Mean percent PPI scores represent the average inhibition computed over 4 prepulse intensities (2, 4, 8, or 10 dB). Poststress session was conducted 1 week after prestress session (2 week after surgery) during which time animals were given fear conditioning. (b) Mean percent PPI scores at each of the prepulse intensities. (c) Effects of clozapine on PPI in habenula- and sham-lesioned mice. Bars depict the mean percent PPI computed over 4 prepulse intensities. Mice were given i.p. injections of either vehicle or clozapine (6 mg/kg) 30 min before behavioral testing using a random crossover experimental design. (d) The effects of apomorphine on motor activity in habenula- and sham-lesioned animals. Bars represent mean increases in motor activity in response to apomorphine. The mean was computed by subtracting the number of compartment entries on session 1 (no drug, shams: $M = 34.5$, $MSE = 3.7$; habenula: $M = 17.5$, $MSE = 2.3$) from number of compartment entries on session 2 (apomorphine, shams: $M = 87.3$, $MSE = 6.7$; habenula: $M = 104.5$, $MSE = 7.5$). Apomorphine (2 mg/kg) was administered 30 min before session 2. Bars represent means + SEM (* P s < 0.05).

Murray et al., 1994; Murphy et al., 1996; Amat et al., 2001), the failure to find differences in the acquisition and retention of cued fear following classical conditioning suggests that any monoamine changes did not significantly influence these behaviors. While the impact of monoamine alterations on conditioned fear is far from clear, past studies investigating the influence of DA on cued conditioning suggest that decreases (Davis et al., 1993; Borowski and Kokkinidis, 1996; Munro and Kokkinidis, 1997), but not increases in DA mesolimbic activity may produce impairments. Concerning alterations in serotonergic activity, results obtained using the fear-potentiated startle paradigm have shown that systemic injections of buspirone, a partial 5-HT_{1A} receptor agonist, block fear-potentiated startle (Kehne et al., 1988; Mansbach and Geyer, 1988; Risbrough et al., 2003). However, lesions of the raphe nuclei failed to block disrupt fear-potentiated startle or the effect of buspirone on potentiated startle (Davis et al., 1988), suggesting the modulation of DRN activity does not effect fear as measured by potentiated startle.

Following conditioned fear, habenula-lesioned animals displayed significantly less PPI than control-lesioned animals. These results demonstrate that fear conditioning differential-

ly modified PPI in lesioned groups. The second study indicates that these effects were not the consequence of general subcortical damage, the timing of lesions with respect to testing, or the number of PPI sessions. In that experiment, thalamus-control lesioned animals did not differ from sham animals. Furthermore, there were no differences when conditioned fear stress was delayed 2 weeks rather than 1 week after surgery. As seen in our initial experiment, only after stress did habenula-lesioned animals displayed a relative PPI deficit when compared to control- and sham-lesioned animals (Figs. 4b, c). Thus, it appears that the stress associated with conditioned fear somehow ‘unmasks’ differences that were not otherwise obvious in animals with lesions confined to the habenula complex.

In both PPI experiments, the differences between sham and habenula-lesioned groups were driven largely by a stress-induced increase in PPI. The differential change in PPI may be explained in a number of ways. First, while previous studies have shown that long-term or developmental stressors like social isolation and maternal deprivation decrease PPI (see, Weiss and Feldon, 2001), evidence also indicates that some stressors can increase PPI. For example, Grillon and Davis (1997) have reported that the anticipation

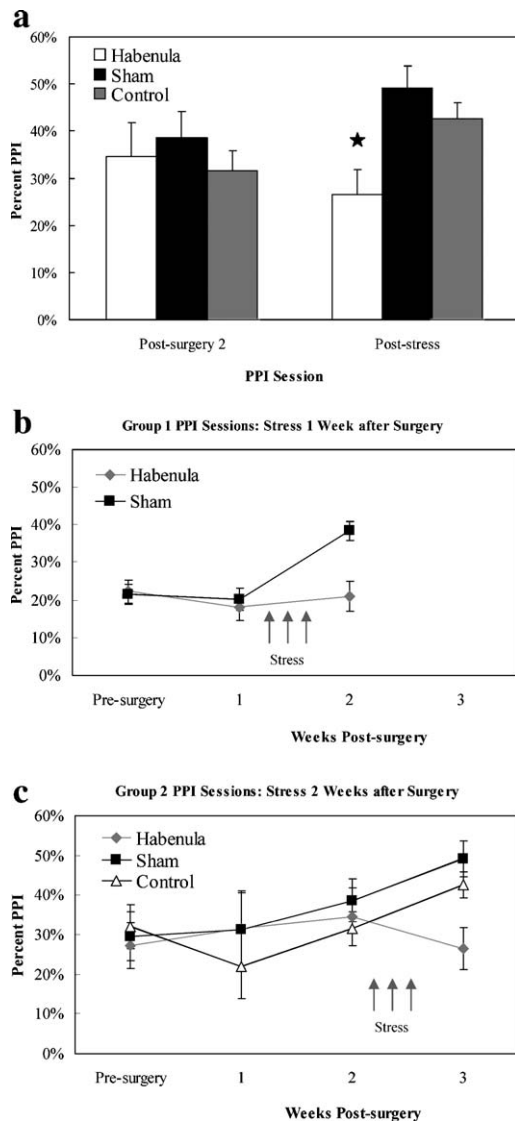


Fig. 4—(a) Mean percent PPI scores pre- and poststress for habenula, geniculate (control), and sham-lesioned animals. Poststress session was conducted 1 week after prestress session (2 week after surgery) during which time animals were given fear conditioning. Mean percent PPI scores represent the average inhibition averaged computed over 4 prepulse intensities (2, 4, 8, or 10 dB). **(b–c)** Comparison of mean percent PPI scores for Groups 1 and 2. Animals in Group 1 received stress between weeks 1 and 2 after lesion surgery. Group 2 animals that received stress between weeks 2 and 3 postsurgery (* $P < 0.05$).

of shock can increase PPI in humans. In rats, 5 days of repeated mild footshock can also potentiate PPI (Pijlman et al., 2003). The increases in PPI seen in these and the current study are likely due to some stress-induced plasticity, and possibly due to a learned expectancy of the footshock. Although some studies have shown that environmental context plays an important role in the process of sensitization in some paradigms (Le Moal, 1995), we found no evidence of a difference in either cue- or context-mediated fear conditioning. This suggests that in both groups the

animals were aware of the tone-shock association, and thus may anticipate the footshock in the train/testing context. However, habenula lesions may have decreased the ability of this potential anticipation to enhance PPI. It is possible that changes in arousal mechanisms or attentional states may prevent the PPI enhancement effect, but not affect fear potentiated startle or freezing in these animals. Interestingly, these data suggest that there may be a dissociation between learned fear at the level of amygdala output pathways mediating startle and freezing and a potential separate pathway mediating a learned fear effect on PPI that depends upon the habenula. Future studies are warranted to examine the effects of other types of nonassociative stress on PPI.

Given the broad antagonism at both dopamine (D4 > D2 > D1) and serotonin (5HT2A) receptors by clozapine, we cannot determine from these experiments which receptor populations may be most affected by this manipulation. Since both mixed dopamine/serotonin antagonists (clozapine) and relatively specific D2 antagonists (haloperidol) as well as 5-HT1A agonists have been shown to reliably increase PPI in mice (Dulawa et al., 1997, 2000; Dulawa and Geyer, 2000; Olivier et al., 2001), it is possible that the increase in PPI observed in sham-operated animals represents changes in dopaminergic and/or serotonergic tone. Habenula lesions have been shown to result in increased DA activity (Lisoprawski et al., 1980; Nishikawa et al., 1986). In this case, the relative PPI deficit could reflect an overactivity of the subcortical dopaminergic system, consistent with a sizeable literature on the effects of dopamine dysregulation on PPI (Braff et al., 2001; Geyer et al., 2001). It has also been well established that central dopaminergic and serotonergic pathways originating from the VTA and DRN are activated by a variety of physical and psychological stressors, including conditioned fear (Inoue et al., 1994; Beck and Fibiger, 1995; Yoshioka et al., 1995, 1996; Morrow et al., 1999). Furthermore, evidence shows that prior exposure to acute or chronic stressors can result in behavioral and pharmacological sensitization effects caused by changes dopaminergic (Pani et al., 2000; de Jong et al., 2005) and serotonergic tone (Adell et al., 1988; Chung et al., 2000; Matuszewich and Yamamoto, 2003). In support of this view, Watkins and Maier have shown that the habenula is essential for the normal sensitization of DRN neurons after uncontrollable stress (Maier et al., 1995; Maswood et al., 1998; Grahn et al., 1999; Amat et al., 2001). The stress-induced alteration in the habenulo-DRN pathway results in increase excitation of DRN 5-HT neurons and changes in subsequent stress behaviors. Lesions of the habenula disrupt normal stress-induced alterations in DRN activity and behavior. In the context of the current study, stress associated with fear-conditioning may have induced sensitization of serotonergic systems responsible for PPI increases in control-lesioned animals. In habenula-lesioned animals, the absence of normal excitatory input to the DRN may prevent sensitization or shock-expectancy that leads to normally increased PPI.

In addition to changes in PPI, we found that habenula lesions followed by conditioned fear stress produced hypolocomotion at baseline and hyperlocomotion in response to apomorphine. Past studies have shown that habenula lesions

produce no change (Thornton et al., 1994; Vale-Martinez et al., 1997) or an increase in baseline locomotive activity measurements (Lee and Huang, 1988; Thornton et al., 1990; Murphy et al., 1996). These differential outcomes are likely due to the extent of lesion damage. Thornton et al. (1994) have shown that lesions confined to the habenula do not result in hyperactivity; whereas damage that includes the FR produces persistent elevations in locomotor activity. In the current study, we found a lower baseline activity measurement for habenula animals in the open-field session given before apomorphine treatment. It is unclear from our experimental design whether this difference reflects dopaminergic alterations secondary to the habenula lesion. However, increased immobility has been reported in habenula-lesioned animals which have previously been exposed to various types of stressors (Murphy et al., 1996), suggesting that conditioning may have induced subsequent hypolocomotor behavior. Given that low doses of dopamine agonists reliably induce hypolocomotion (Storey et al., 1995), the decrease in baseline activity could paradoxically reflect an increased basal level of DA activity. The hypolocomotor effects of DA agonists have been attributed to stimulation of D2 autoreceptors or D3 receptors which are more sensitive than their postsynaptic counterparts (Kim et al., 2001). Alternatively, since serotonergic hypoactivity is also known to reduce exploratory behavior as a whole (Lipska et al., 1992), we cannot rule out the contribution of lesion-induced changes in serotonergic tone in habenula animals.

In the current study, all animals showed increased motor activity in response to systemic injections of apomorphine, a mixed D1/D2 dopamine receptor agonist. This is in accordance with past results showing that similar doses of apomorphine induce increased locomotor behavior in rodents (Presti et al., 2004). Although both groups showed apomorphine-induced motor responses, habenula-lesioned mice demonstrated significantly larger increases in locomotion, suggesting an enhanced sensitivity to apomorphine. It has previously been reported that the threshold for apomorphine induced stereotyped behaviors is lower in animals with bilateral excitotoxic habenula lesions (Carvey et al., 1987). These results are consistent with hyperlocomotor responses to dopamine agonists resulting from increased sensitivity of the subcortical dopaminergic system (Flores et al., 1996; Wan and Corbett, 1997).

Future studies will need to address whether the effects of conditioned stress can be generalized to overall effects of stress and arousal in lesioned animals. Additionally, it will be important to more directly examine the physiological changes within the dopamine, serotonin, GABA, and glutamate systems as well as to examine the effects of these manipulations with drugs that have more specific (e.g., D1 vs. D2 vs. D4) mechanisms of action. Finally, replicating these data with chemical lesions will be important to rule out the effects of lesioning on fibers of passage.

In summary, these findings suggest that in the presence of stress, as demonstrated here with conditioned fear stress, the habenula may normally contribute to the regulation of sensorimotor gating mechanisms and certain monoamine-dependent behaviors. These results are consistent with the hypothesis that habenula dysregulation may

be involved in disorders of monoamine dysfunction, such as schizophrenia.

4. Experimental procedures

4.1. Animals and experimental design

Male C57BL/6J mice, 4 to 9 weeks of age and weighing 20–30 g, were obtained from Jackson Labs (Bar Harbor, ME) and used as subjects. Mice were kept in groups of five to six in plastic cages (30 × 20 × 16 cm) on corn dust bedding. They were housed at 24 °C with a 12/12-h light/dark cycle with ad libitum access to food and water. All experiments were conducted on mice between 5 and 10 weeks of age. The experiments were approved by our Institutional Protocol Approval Committee and were in accordance with Yerkes Primate Research Center Regulations.

Two groups of animals were used for behavioral experiments. Initial experiments were conducted with 42 lesioned mice –20 with sham lesions and 22 with bilateral habenula lesions. Animals in this first group received a presurgery PPI session followed by lesion surgery. One week after surgery, animals received a postsurgery PPI session followed 1 day later by conditioned fear training and testing (conditioned fear stress). After conditioned fear stress animals received a third PPI test (poststress PPI session), which revealed differences between lesioned groups. To examine the effects of clozapine on the group differences, all animals were tested for PPI in the presence of clozapine (6 mg/kg) or vehicle with a random crossover experimental design using a within-groups comparison for drug effect. As with other studies which have employed this design, a 3 day drug washout period was given between tests (e.g., Johansson et al., 1995; Heldt et al., 2004). Of the initial 42 mice, approximately one half were subsequently given open-field testing after an additional 1 week drug washout period (habenula lesioned, $n = 13$; sham lesioned, $n = 13$). All animals were sacrificed for histological evaluation 3–5 days after behavioral testing.

A second group of 26 mice was used to examine the effects of control lesions and the temporal aspects of behavioral findings from the initial experiments above (habenula lesioned, $n = 7$; sham lesioned, $n = 10$; control lesioned, $n = 8$). As with the first group of animals, this second group received a presurgery PPI session followed by lesion surgery. However, in contrast to the first group, this group received two postsurgery PPI sessions prior to stress. These sessions were conducted 1 and 2 weeks after surgery. Conditioned fear stress started 1 day after the second postsurgery PPI session and a poststress PPI session was given 3 weeks after surgery (for group comparisons, see Figs. 4b–c).

4.2. Drugs

Clozapine is a mixed dopamine/serotonin antagonist with highest affinity for the D4 receptor, with antagonist properties at the D2/D1 and 5HT(2A) receptors as well. Clozapine was used in these studies because of its clinical role as an atypical antipsychotic and its efficacy in reversing PPI in dopaminergic models of schizophrenia. Apomorphine is a nonselective dopamine agonist with actions at the D1/D2 receptors. Apomorphine was used in these studies because of its demonstrated efficacy in both clinical and preclinical studies of exacerbating dopaminergic symptoms in models of schizophrenia. Clozapine and apomorphine were obtained from RBI/Sigma (Sigma Chemical, St. Louis, MO). Both clozapine (6 mg/kg) and apomorphine (2 mg/kg) were dissolved in a vehicle solution consisting of saline with 10% DMSO. Mice were injected intraperitoneally (i.p.) with 200 μ l of drug or vehicle 30 min before behavioral testing. The drug concentrations were chosen on the basis of previous studies of doses required to obtain

behavioral effects in mice and rats (e.g., Zhang et al., 1998; Dirks et al., 2003; Heldt et al., 2004).

4.3. Startle apparatus

Startle reflexes were measured in eight identical startle response systems (SR-LAB, SDI, San Diego, CA). Each system consisted of a nonrestrictive Plexiglas cylinder, 5.5 cm in diameter and 13 cm long, mounted on a Plexiglas platform which was located in a ventilated, sound-attenuated chamber. The floor of each cylinder consisted of a cradle-shaped grid which contained seven 3.0 mm diameter stainless steel bars spaced 1 cm apart through which shock could be delivered. Cylinder movements were detected by a piezoelectric accelerometer mounted under each platform and were digitized and stored by an interfacing computer assembly. Movements were sampled each millisecond (ms). Startle amplitude was defined as the peak accelerometer voltage that occurred during the first 100 ms after the onset of the startle stimulus. Reactivity to shock was defined as the peak accelerometer voltage that occurred during the first 100 ms after the onset of the footshock. Activity measurements used to detect immobility were sampled during a 5-s "window" starting 10 s before the footshock (training) or startle stimulus (testing). Response sensitivities were calibrated (SR-LAB Startle Calibration System) to be nearly identical in each of the two startle chambers. Startle, prepulse and background stimuli were presented through a high-frequency speaker located 15 cm above the chambers. Sound intensities were measured by an audiometer (Radio Shack, #33-2055).

The tone conditioned stimulus (CS) was a 70-dB SPL, 12-kHz tone generated by a Tektronix function generator audio oscillator (Model CFG253, Beaverton, OR) and was delivered through a high frequency speaker (Motorola, Model 948) located 13 cm above each cylinder. The tone CS was 30 s in duration. The unconditioned stimulus (US) was scrambled shock generated by programmable constant current shock generators (SDI, San Diego, CA) located outside the isolation chambers. Footshock intensity was 0.4 mA. Shock levels were verified by using a 1 k Ω resistor across the bars of the shock grids and measuring the voltage drop between the bars to calculate the constant current. The chamber ventilation fans produced a 55 dB white-noise background which was present during acclimation, pre-exposure, fear conditioning, and testing sessions. Stimuli presentation and data acquisition were controlled by an IBM PC-compatible computer using SR-Lab software.

4.4. Prepulse inhibition procedure

For 3 consecutive days, each mouse was placed in the cylinder for approximately 15 min during which time no stimuli were presented. The following day, mice were placed in the cylinder and after 5 min were given 10 startle stimuli at each of four different startle stimulus intensities (90, 100, 110, 120 dB) with an interstimulus interval (ISI) of 30 s. The purpose of this acclimation procedure was to familiarize the mice to handling, the startle apparatus, and the startle stimulus.

One day after acclimation, mice were given a presurgery test session for prepulse inhibition (PPI) for the purpose of assigning mice into groups with similar mean startle and percent PPI scores prior to surgery. Each PPI test session (pre- and postsurgery) consisted of five different trial types. Startle stimuli (115 dB, 50 ms) were presented alone or were preceded by noise prepulses (20 ms) of 2, 4, 8, or 10 dB above a 63 dB white noise background (i.e., 65, 67, 71, or 73dB) with a fixed interval (100 ms) between onsets of the prepulse and startle stimuli. Each session began with a 5-min acclimation period followed by the five different trial types presented in random order nine times for a total of 45 trials. Intertrial intervals ranged from 20 to 40 s. The effect of clozapine treatment on poststress PPI was evaluated using a random crossover experimental design. On session 1, approximately one-half of the habenula- and sham-lesioned mice were given

clozapine pretreatment 30 min before testing; the remaining mice were given vehicle pretreatment. Session 2 was conducted following a 4-day drug washout period. On this session, drug pretreatment for each mouse was reversed.

4.5. Fear-conditioning procedure

Prior to fear training, mice were given a pretraining test to examine baseline levels of startle in the presence of the tone (Heldt et al., 2000; Jones et al., 2005). This was done to ensure that mice did not display significant unconditioned excitatory effects to the tone before training which could be interpreted as associative fear-potentiated startle. During testing, each mouse was placed in the cylinder and 5 min later presented with 4 startle stimuli at each of three different startle stimulus intensities (95, 105, 115 dB). These initial 12 startle-alone trials served to minimize the contribution of very high startle responses that often occur with the first few startle stimuli. After these initial trials, mice were presented with 4 additional startle-alone trials and 4 tone + startle trials at each of the three startle stimulus intensities. On tone + startle test trials, the startle stimulus was presented 29.750 s after the onset of the tone (the point at which shock would have been administered in training; see below). All trials were presented in a pseudo-random order with the constraint that each trial type occurred only once in each consecutive six trial block. The intertrial interval (defined as the interval between startle stimuli) was 1 min.

One day after the pretraining test, each mouse was placed in the cylinder and 5 min later presented with the 1st of 5 training trials at an average intertrial interval of 3.5 min (range: 2.5-4.5 min). Each training trial consisted of a 30-s tone co-terminating with a 0.250-s, 0.4-mA footshock. Training was carried out over three sessions for a total of 15 training trials. Fear-potentiated startle was assessed 24 h after the last training session (a total of 15 training trials). This test session was similar to the pretraining test, with the exception that after the initial startle-alone trials, mice were presented with 10 additional startle-alone trials and 10 tone + startle trials, at each of the three startle stimulus intensities.

4.6. Open-field apparatus and procedure

The open field consisted of a circular arena (60 cm diameter) made of black Plexiglass with a wall 20 cm high. The central and periphery floor of the open field was delineated by a 6 mm circular white line located 10 cm from the outside wall. The black floor was additionally divided into 9 equal-area compartments by 6 mm white lines. All four paws had to cross a dividing floor line to be considered an entry into the central, periphery, and/or a compartment of the open field. All testing was conducted under standard room lighting where behavior was continuously videotaped by a video camera placed over the structure and then encoded using a continuous sampling method. General motor activity was quantified by the total number of compartment entries.

Before testing, mice were acclimated to a normally illuminated test room for 5 min. Then mice were placed in the periphery of the arena at the start of the 5-min test period. At the end of the test, the animal was returned to its home cage. The effects of apomorphine (2 mg/kg) treatment were assessed on session 2.

4.7. Surgery and histology

Stereotaxic surgery was performed 2 days after PPI matching. Mice were anesthetized with Ketamine and Metomidine, i.p. Small holes were drilled in the skull above the lesion site, and a Kopf Model NE-300 electrode insulated to within 0.5 mm of the tip was lowered to the following coordinates from bregma: anteroposterior = -1.5, mediolateral = \pm 0.3, dorsoventral = -3.2. Lesions were

made by passing a 20 μ A anodal current for 75 s. For the sham-operated mice, the electrode was lowered to the same coordinates but no current was passed. For lateral geniculate bilateral control lesions, coordinates from bregma were: anteroposterior = -2.3 , mediolateral = ± 2.0 , dorsoventral = -3.5 . After surgery, the incision was closed with cyanoacrylate glue. Mice were given postsurgical i.p. injections of Antisedan (4.0 mg/kg; Metomidine reversing agent) and placed on a heated pad until fully recovered from anesthesia. All mice were returned to home cages for 7 days of recovery before testing; and body weight, eating, and drinking were monitored daily.

Following behavioral testing, lesioned mice were perfused intracardially with 0.9% saline followed by 4% paraformaldehyde. The brains were stored in a solution of 20% sucrose in paraformaldehyde at 4 °C for at least 6 h and stored at -80 °C. Coronal sections (15 μ m) of brains were cut on a cryostat (Leica; Nussloch, Germany) at -20 °C and mounted on gelatin-coated slides. Slides from each mouse were stained with cresyl violet and lesion locations were referenced from bregma.

4.8. Data reduction and statistical analysis

4.8.1. Prepulse inhibition

Mean startle amplitudes for the startle-alone test trials and each of the 5 prepulse + startle test trials were calculated for each mouse by averaging the startle amplitude of each trial type. Each mean prepulse + startle amplitude score was converted to a percent PPI. The percent PPI was obtained as follows; Percent PPI = [(mean startle-alone amplitude) – (mean prepulse + startle amplitude)] / (mean startle-alone amplitude) \times 100.

4.8.2. Fear-potentiated startle

Mean startle amplitudes for the startle-alone test trials and tone + startle test trials were calculated for each mouse by averaging the startle amplitude of each trial type across the three different startle intensities. Percentage of fear-potentiated startle was computed for each mouse by dividing the difference between these two trial types (light + startle stimulus minus startle stimulus alone) by the mean startle amplitude on startle-stimulus-alone trials: percent fear-potentiated startle = (difference/startle-alone) \times 100.

4.8.3. Freezing

Detailed descriptions of methods used to measure conditioned freezing have been previously reported (Jones et al., 2005). Briefly, activity measurements during the presentation of the CS were first converted to the average voltage output for each second of the 5-s activity window. Based on the voltage output, each mouse was given an immobility score of 0 or 1 (0 = moving, 1 = immobile) for each 1 s of the 5-s activity window. A mean percent immobility score was computed by averaging the 5 immobility scores and multiplying by 100. The percent immobility score was used as an index of freezing, and in previous work has demonstrated a high correlation with observational ratings of freezing ($r_s > 0.89$).

4.8.4. Analyses

To examine the effects of lesion type on behavioral measures, most results were analyzed by means of repeated measures ANOVAs with Lesion Type as a between-subject factor and Session (Pre, Post) as within-subject factors. Subsequent analyses were done with lower-order ANOVAs, pairwise *t* tests, and independent samples *t* tests (LSD) where appropriate. For ease of presentation, the effect of prepulse intensity on PPI can be assumed to be statistically significant in all startle analyses. To more closely examine differences between lesion groups, sometimes the overall analysis was followed by subsequent *t* test (LSD) at each of the prepulse intensities. One-way ANOVAs were used to evaluate reactivity to shock, and retention of fear-potentiated startle and freezing. A random crossover design was used to

evaluate PPI in the presence of clozapine or vehicle. This analysis included a Sequence (A–B, B–A) between-subject factor to identify any treatment order carryover effects.

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