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Anxiety-related behavioral inhibition in rats: A model to examine mechanisms underlying the risk to develop stress-related psychopathology

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Abstract

Behavioral inhibition (BI) is an adaptive defensive response to threat; however, children that display extreme BI as a stable trait are at risk for development of anxiety disorders and depression. The present study validates a rodent model of BI based on an ethologically relevant predator exposure paradigm. We demonstrate that individual differences in rat BI are stable and trait-like from adolescence into adulthood. Using *in situ* hybridization to quantify expression of the immediate early genes *homer1a* and *fos* as measures of neuronal activation, we show that individual differences in BI are correlated with the activation of various stress-responsive brain regions that include the paraventricular nucleus of the hypothalamus and CA3 region of the hippocampus. Further supporting the concept that threat-induced BI in rodents reflect levels of anxiety, we also demonstrate that BI is decreased by administration of the anxiolytic, diazepam, Finally, we developed criteria for identifying extreme BI animals that are stable in their expression of high levels of BI and also demonstrate that high BI (HBI) individuals exhibit maladaptive appetitive responses following stress exposure. These findings support the use of predator threat as a stimulus and HBI rats as a model to study mechanisms underlying extreme and stable BI in humans.

Keywords

Anxiety; homer; psychological stress; hypothalamus; adolescence

Introduction

Behavioral inhibition (BI) is an adaptive defensive response to threat that has been evolutionarily conserved across species to promote survival (Porges 2003). Individual differences in BI are evident early in life and are moderately stable throughout development (Fox *et al*. 2005). When children exhibit extreme BI, characterized by prolonged and excessively inhibited responses to strangers and novelty, this trait-like tendency is viewed as

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a temperamental disposition and is a considerable risk factor for the development of anxiety and stress-related disorders (Caspi *et al.* 1995; Caspi & Silva 1995). Importantly, it is children with both high and stable levels of BI that are at greatest risk to develop psychopathology, which tends to be social anxiety disorder (Biederman *et al.* 2001). Because other disorders are comorbid with social anxiety disorder (Hettema *et al.* 2005; Stein *et al.* 2001; Zimmermann *et al.* 2003), it is likely that extreme and stable BI is a broad risk factor for the later development of other anxiety and depressive-related psychopathologies (Gladstone *et al.* 2005; Goldsmith & Lemery 2000; Hirshfeld *et al.* 1992).

We have developed a rat model of BI that is elicited by ferret exposure, a naturalistic and ethologically relevant predatorial threat. Our laboratory and others previously demonstrated that exposure to predator stimuli elicit conditioned and unconditioned anxiety- and defensive-like behaviors such as BI, freezing and withdrawal in rats (Campeau *et al.* 2008; Masini *et al.* 2006; McGregor *et al.* 2002; Nanda *et al.* 2008). In our model, we define BI as combination of threat-induced freezing and hypervigilant behaviors. Eliciting BI by exposure to potentially threatening situations is consistent with methods used in human and non-human primate models of BI (Kagan *et al.* 1988; Kalin & Shelton 2003). In addition, predatorial threat is an important aspect of the model because rodents tend not habituate to predator exposure (Blanchard *et al.* 1998), which allows for the performance of longitudinal studies with repeated assessments.

Because BI has trait-like characteristics associated with human and non-human primate anxiety (Fox *et al.* 2005; Kalin & Shelton 2003), we characterized the stability of BI from adolescence to adulthood in rats and determined the ability of the anxiolytic, diazepam, to modulate BI. Given the importance of the hypothalamic-pituitary-adrenal (HPA) axis (Blanchard *et al.* 1998; File *et al.* 1993; Masini *et al.* 2005; Morrow *et al.* 2000; Perrot-Sinal *et al.* 1999) and other stress-responsive regions such as the amygdala and hippocampus in mediating the physiological and behavioral response to predator exposure (Campeau *et al.* 2008; Dielenberg *et al.* 2001; Masini *et al.* 2005; Roseboom *et al.* 2007), we determined the extent to which activity of these regions is associated with individual differences in BI. We assessed regional activity by quantifying *in situ* hybridization signals for the immediate early genes *homer1a* and *fos*. Finally, we assessed the extent to which rats with extreme BI are vulnerable to the influences of stress on appetitive behaviors, as reductions in appetitive behaviors are common maladaptive characteristics of stress-related psychopathology (Lo Sauro *et al.* 2008; O'Brien & Vincent 2003).

Methods

Animals

Male and female Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 225–250 g were housed individually in clear, plastic cages in a temperature and light-controlled vivarium (24 °C, lights on from 0700–1900 h). Except where indicated, food and water were available *ad libitum*. All rats were handled by the experimenters for at least five days prior to testing to minimize handling- and novelty-related stress. Six male ferrets (Marshall Farms, North Rose, NY) were used in these studies and were housed in pairs; food and water were available *ad libitum*. All facilities are AAALAC approved; the protocols were in accordance with the guidelines of the National Institutes of Health and the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison.

Drug treatment

Diazepam (DZP) was obtained from Sigma-Aldrich (St. Louis, MO) and was dissolved at 1 mg/ml in 40% propylene glycol and 10% ethanol in sterile water, which also served as the vehicle (VEH). To minimize the stress of the injection procedure, beginning three days prior to testing rats received daily intraperitoneal (IP) injections of 0.2 mL of 0.9% saline. On the test day, rats received either VEH or 1 mg/kg DZP given in a volume of 1 mL/kg given IP 30 min prior to behavioral testing. This dose has been shown in numerous publications to produce anxiolytic-like effects in a variety of rodent models (Duzzioni *et al.* 2008; Jochum *et al.* 2007; Nicolas & Prinssen 2006; Wilson *et al.* 2004).

Ferret predator paradigm

Rats were placed into empty standard rat housing cages (length 18” by width 10” by height 8”) without bedding, and cage lids were flipped upside down and clipped into place to allow for an unobstructed side view of the rats. The cage containing the rat was transferred into the ferret colony room and placed directly in front of the six ferrets, approximately 30 cm from the ferret cages, such that the rat could still hear, see and smell the ferrets with no direct physical contact. All ferret exposures were videotaped for 15 min and rat behavior was assessed by a trained reviewer. The behaviors that were scored are described in the “behavioral testing” section below.

Experimental design

In Experiment 1a (n = 15), we examined the extent to which predator threat elicits BI in adolescent male rats. The behavior of adolescent male rats (42 or 43 days old) was first observed at baseline, followed immediately by another baseline measurement (n = 7) or 15 min of ferret exposure (n = 8). Experiment 1b sought to elucidate gender differences in BI response, as well as stability of behavioral responding from adolescence to adulthood. To achieve this, male (n = 19) and female (n = 18) adolescent rats (42 or 43 days old) were exposed to the ferret predator paradigm and re-exposed 28 days later (70 or 71 days old). This experiment was repeated in male rats (n = 24) that were exposed to ferrets twice in adolescence (at 36 and 38 days of age) and twice in adulthood (at 85 and 87 days of age) during the dark phase (testing between 1100–1500 h, lights off from 0900–1700 h). To further establish rodent BI as a model of anxiety-like behavior, in Experiment 2 we employed the use of an anxiolytic drug to pharmacologically validate the model. Rats (n = 8/group) received either vehicle or 1 mg/kg diazepam IP, as described above, and 30 min later were exposed to the predator paradigm. The aim of Experiment 3 (n = 54) was to examine how individual differences in brain reactivity are related to individual differences in BI. Based on our previous studies indicating that *homer1a* and *fos* expression are increased in response to ferret exposure in our paradigm (Nanda *et al.* 2008; Roseboom *et al.* 2007), we use *in situ* hybridization to quantify *homer1a* and *fos* mRNA levels and then determined the relationship to the expression of BI. Forty-five rats were exposed to the ferret predator paradigm and nine rats remained in their home cage serving as controls. All rats were killed by decapitation 1 h following cessation of ferret exposure and the brains were rapidly removed and placed in isopentane at −20°C to −30°C. The whole brains were stored at −80°C until they were sliced into 20 μm coronal sections for *in situ* hybridization analysis. Finally, we examined the extent to which extremely inhibited animals exhibit other maladaptive behaviors after the cessation of threat exposure. In Experiment 4, sixty adult rats were initially screened, and extreme and stable groups of low BI (LBI) and high BI (HBI) were identified as those animals that consistently ranked in the bottom or top quartile, respectively, in two tests separated by 48 hours. The eight HBI and seven LBI rats were subjected to the feeding test as described in the next section.

Behavioral testing

Definitions of the behaviors that were scored were previously described in detail (Nanda *et al.* 2008). Briefly, the durations of the following behaviors were scored: rearing (raising both front paws off the floor of the test cage), grooming (licking and rubbing with paws of any accessible area of skin and fur), locomotion and BI. Locomotion is defined as the duration of movement of all four feet. BI is defined as the sum of freezing (this behavior is scored if a period of at least 3 seconds passes, during which there is an absence of all movement except those required for respiration) and hypervigilance, which is characterized by an absence of locomotion with accompanying minor head movements associated with sensory acquisition (sniffing and vibrissa movement).

The feeding test was performed essentially as previously described (Jochman *et al.* 2005). Rats were habituated for three consecutive days to nightly food deprivation (from 1600 to 0900 h). During this time, rats were also habituated to the feeding test environment, which consisted of placing the rat in a novel empty housing cage containing wire mesh flooring in a room separate from where the ferret exposure occurred. On the first test day, rats were entered into a 30 min baseline feeding test. On the following day, all rats were given a 15 min direct ferret exposure immediately prior to the feeding test. Direct ferret exposure consisted of exposing the rat to a ferret by placing the rat in a protective wire cage (length 7.75” by width 6” by height 5.5”) within the ferret homecage as previously described (Roseboom *et al.* 2007). During the feeding test, rats were rated for the amount of time spent eating, and food was weighed before and after testing to determine the amount of food eaten.

In situ hybridization histochemistry

The single-stranded *homer1a in situ* hybridization probe was obtained by PCR amplification of rat whole brain cDNA and corresponded to bases 5231–5699 of GenBank accession number U92079.1. The *fos* probe corresponded to bases 1045–1868 of GenBank accession number NM_022197.1. The respective PCR products were subcloned into pCRII-Topo (Invitrogen, Carlsbad, CA, USA). Each plasmid was linearized with the restriction enzyme *SpeI* (Promega, Madison, WI, USA) and the antisense [α-^{35}S] uracyl tri-phosphate (UTP)-labeled riboprobe was generated by *in vitro* transcription using the T7 Riboprobe System (Promega) as previously described (Herringa *et al.* 2004). Coronal sections (20 μM) were fixed in 4% paraformaldehyde and subsequently processed for *in situ* hybridization as previously described (Hsu *et al.* 2001).

Quantification of *in situ* hybridization was as previously described with the specific parameters detailed below (Nanda *et al.* 2008). Probed sections were exposed to phosphor screens for 4 days and scanned with a Typhoon phosphorimager (GE Healthcare, Piscataway, NJ, USA) at 50 μM resolution. ImageQuant 5.0 software (GE Healthcare) was used to analyze positively hybridized probe signals. The experimenter quantifying the signals was blind to the experimental condition associated with each image. Four sections per animal were analyzed for the paraventricular nucleus of the hypothalamus (PVN), 14 for the basolateral amygdala (BLA), 15 for the medial amygdala (MeA) and 20 each for the dentate gyrus (DG), CA1 and CA3 regions of the hippocampus. Signal intensity was assessed using regions of interest (ROI) with fixed dimensions along the rostral-caudal extent of the PVN (1.2 by 1.55 mm ellipse), BLA (0.9 by 1.35 mm ellipse), MeA (1.3 by 1.9 mm ellipse), DG (0.4 by 0.4 mm square), CA1 (0.45 by 0.55 mm rectangle) and CA3 (0.45 by 0.55 mm rectangle) in each hemisphere. Background signal was calculated by using an identically shaped ROI placed over an adjacent region that showed low levels of hybridization. A background ROI was placed over the thalamus for the BLA and MeA quantification, and a ROI placed over the internal capsule was used for the PVN, DG, CA1 and CA3 quantification. The sections that were used for signal quantification corresponded

to the following anterior/posterior co-ordinates relative to Bregma–PVN (−1.5 to −2.0 mm), BLA and MeA (−1.9 to −3.6 mm) and the DG, CA1 and CA3 (−1.7 to −4.1 mm). For the BLA, MeA, DG, CA1 and CA3 regions, the highest average signal volume (signal intensity multiplied by area of ROI) from consecutive sections was determined for each hemisphere in each region using a rolling average. For the BLA and MeA, the rolling averages consisted of three sections, while the DG, CA1 and CA3 rolling averages consisted of five sections. In each rat, the highest average signal volume from the left and right hemispheres were averaged together. For PVN, because the signals for the left and right hemispheres were in close proximity, a single ellipse was drawn that contained signal from both hemispheres. In addition, because there were only 4 sections per animal that contained a PVN signal, the highest signal volume obtained from a single section was used instead of a rolling average.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.01 software (GraphPad Software, San Diego, CA, USA). Because some BI data sets were not normally distributed and various transformations failed to normalize the distribution, analyses of BI, with the exception of the effects of gender on BI, were performed using non-parametric methods. Comparisons between two groups were made using Mann-Whitney *U*-tests and comparisons between multiple groups were made using Kruskal-Wallis *H*-tests followed by Dunn's post tests for multiple comparisons. Correlations were made using Spearman's rank correlation. For analysis of the impact of gender on BI it was possible to normalize the distribution by log transforming the data, and a two-factor ANOVA was performed followed by Bonferroni post tests. In addition, Pearson correlations were performed including co-varying for gender. The feeding data in experiment 4 was normally distributed and was analyzed parametrically using a two-way ANOVA followed by comparing each treatment group individually using Bonferroni post tests.

Results

Experiment 1a: ferret exposure induces behavioral inhibition in adolescent rats

To examine the extent to which animals exhibit BI early in development, we assessed the effects of ferret exposure on adolescent male rats (42 or 43 days old). The study design was such that all rats (n = 15) received a baseline assessment of BI in the absence of the ferret. Baseline testing consisted of placing the rat in an empty rat housing cage and transporting the animal to a separate housing suite. This exposure was followed immediately by another 15 min exposure in the baseline environment (control group; n = 7) or a 15 min ferret exposure (ferret exposed group; n = 8). The control rats were transported in the facility for approximately the length of time that was required to move a rat to the ferret colony room before being placed back in the baseline room for a second exposure. A Kruskal-Wallis *H*-test analysis of BI over the 15 min sessions revealed significant differences between the control and ferret exposed groups ($\chi^2 = 18.39$, df = 3, $p < 0.001$; Figure 1). Dunn's post hoc analyses revealed that the ferret exposed group exhibited significantly more BI than the control group during the second test session ($p < 0.001$). These results show that similar to adult rats, ferret exposure reliably elicits BI in adolescent rats.

Experiment 1b: behavioral inhibition in rats is stable from adolescence to adulthood and within adulthood

Because BI in children is considered trait-like, and stable extreme BI is a considerable risk factor for the later development of psychopathology, we examined the extent to which individual differences in ferret-induced BI in adolescent rats is predictive of adulthood BI. Thirty-seven adolescent rats (male: n = 19; female: n = 18) 42 or 43 days old were exposed to ferrets for 15 min and were re-exposed 28 days after initial exposure, at ages 70 or 71. A

two-way repeated measures ANOVA was performed with gender as the between subjects factor and age (adolescent vs. adult) as the within subjects factor. Significant main effects of both gender ($F_{(1,35)} = 13.68$, $p < 0.001$) and age ($F_{(1,35)} = 58.09$, $p < 0.0001$) were observed as was a significant gender by age interaction ($F_{(1,35)} = 12.98$, $p < 0.001$). The main effects demonstrated that males exhibited more BI than females, and that more BI occurred during adolescence. Post hoc analysis revealed that males exhibited significantly more BI than females in adulthood ($t = 5.076$, $df = 36$, $p < 0.001$; Figure 2A). A correlational analysis revealed that the levels of BI displayed in adolescent male and female rats predicted adult levels ($r = 0.475$, $n = 37$, $p < 0.01$; Figure 2B); a result that remained significant when co-varying for gender ($r = 0.43$, $n = 37$, $p < 0.01$). Similar stability in individual differences from adolescence to adulthood was found in a subsequent study involving 24 male rats assessed during the dark phase of their light dark cycle in which BI was measured twice in adolescence (at 36 and 38 days of age) and twice in adulthood (at 85 and 87 days of age). We found that BI was stable between the two measures taken in adolescence ($r = 0.538$, $n = 24$, $p < 0.01$) and between the two measures taken in adulthood ($r = 0.723$, $n = 24$, $p < 0.001$). Analysis of the average BI scores from adolescence and adulthood also revealed a significant correlation ($r = 0.559$, $n = 24$, $p < 0.01$).

Experiment 2: diazepam reduces threat-induced BI

To further validate predator threat-induced BI as a model of anxiety-like behavior, we examined the ability of the anxiolytic diazepam (DZP) to decrease the expression of BI. Adult male rats (n = 8/group) were treated with either vehicle (VEH) or DZP (1 mg/kg) 30 min prior to ferret exposure. Male rats were used because they were found to engage in significantly more BI than female rats in Experiment 1b. As predicted, DZP-treated rats engaged in significantly less BI than VEH-treated rats ($U = 13$, $df = 16$, $p < 0.05$; Figure 3A). The effects of DZP appeared to be selective to BI as no significant differences were observed in the duration of grooming ($U = 29$, $df = 16$, $p = 0.792$; Figure 3B), rearing ($U = 20$, $df = 16$, $p = 0.235$; Figure 3C) or locomotion ($U = 24$, $df = 16$, $p = 0.442$; Figure 3D).

Experiment 3: Threat-induced hippocampal and PVN activation predicts individual differences in BI

To determine if activation of stress responsive brain regions is predictive of individual differences in BI and anxious temperament, brains from adult male rats sacrificed 1 hr post-ferret exposure (n = 45) were compared to those from rats that remained in their home cage (n = 9) through *in situ* hybridization analysis of the immediate early genes *homer1a* and *fos*. *Homer1a* mRNA expression was assessed in the amygdala (basolateral amygdala (BLA) and medial amygdala (MeA)), hippocampus (dentate gyrus (DG), CA1 and CA3 regions) and the PVN. Since no *homer1a* signal was detected in the central amygdala (CeA), this region was not examined. Consistent with data from our previous amygdala microarray studies, predator stress significantly induced *homer1a* expression in the BLA ($U = 32$, $df = 54$, $p < 0.0001$) and MeA ($U = 10$, $df = 54$, $p < 0.0001$). In addition, significant increases in *homer1a* mRNA occurred in the DG ($U = 32$, $df = 54$, $p < 0.0001$), CA1 ($U = 82$, $df = 54$, $p < 0.01$), CA3 ($U = 57$, $df = 54$, $p < 0.001$) and PVN ($U = 23$, $df = 54$, $p < 0.0001$; Figure 4B). Furthermore, individual differences in *homer1a* mRNA expression in the CA3 and PVN were significantly correlated with BI ($r = 0.368$, $n = 45$ in the CA3, Figure 5A; $r = 0.353$, $n = 45$ in the PVN, $p < 0.05$ two-tailed, Figure 5B). No significant correlations between *homer1a* expression and BI were observed in the DG ($r = 0.053$, $n = 45$, $p = 0.727$), BLA ($r = 0.217$, $n = 45$, $p = 0.168$) or MeA ($r = 0.184$, $n = 45$, $p = 0.227$); a trend for a significant correlation was found in the CA1 ($r = 0.288$, $n = 45$, $p = 0.055$). We also assessed the relationship between BI and *fos* mRNA expression since *fos* is another immediate early gene that has a different expression pattern. Significant predator stress-induced increases in *fos* mRNA expression were observed in the BLA ($U = 2$, $df = 54$, $p < 0.0001$), MeA ($U = 0$,

df = 54, $p < 0.0001$), PVN (U = 3, df = 54, $p < 0.0001$), CA1 (U = 0, df = 54, $p < 0.0001$) and CA3 (U = 0, df = 54, $p < 0.0001$). No significant correlations between *fos* expression and BI were observed in the BLA ($r = 0.219$, $n = 45$, $p = 0.149$), MeA ($r = 0.216$, $n = 45$, $p = 0.155$), PVN ($r = 0.223$, $n = 45$, $p = 0.142$) and CA1 ($r = 0.072$, $n = 45$, $p = 0.639$), although a trend for a positive correlation existed for the CA3 region ($r = 0.259$, $n = 45$, $p = 0.086$). Similar to *homer1a*, the lack of a distinct *fos* mRNA signal within the CeA prevented quantification of this region.

Experiment 4: Developing criteria to select pathologically extreme rats and demonstrating that they display disrupted appetitive responses

To further develop the BI phenotype as a model of psychopathology, we sought to characterize differences between rats that stably display extremely high or low levels of BI. Since the selection of temperamentally extreme groups of animals requires screening large numbers of individuals, we examined the stability of BI across two ferret exposures separated by 48 hrs in 255 adult male rats. Consistent with earlier findings, BI was stable across the two exposures ($r = 0.586$, $n = 255$, $p < 0.001$; Figure 6A). To identify the animals that displayed extreme and stable levels of BI, the BI durations from each of the 2 tests were ranked by quartiles. Rats in the bottom quartile on both tests were considered to have low and stable levels of BI (LBI), rats consistently in the middle quartiles were considered to have mid-range stable levels of BI (MBI) and rats consistently in the top quartile were considered to have extremely high and stable BI (HBI) (LBI, n = 34; MBI, n = 23; HBI, n = 39). When assessing the stability of the selected LBI, MBI, and HBI groups as expected a strong correlation was found between the first and second tests ($r = 0.902$, $n = 96$, $p < 0.001$; Figure 6B).

To determine if animals with extreme levels of threat-induced BI have altered appetitive behaviors as an indicator of additional maladaptive responding, HBI and LBI rats were identified and tested in a feeding paradigm. Rats were selected as being extreme and stable using the quartile method described above. Of the sixty rats that were screened, 8 met criteria for LBI (13% of population) and 7 for HBI (12% of population). As a baseline, HBI and LBI rats were food deprived for 17 hrs and were tested for feeding behavior over a 30 min period when they had access to chow in a non-threatening environment (Baseline). The rats were again food deprived and on the next day were exposed to 15 min of direct ferret stress as described in the methods section which was immediately followed by the feeding test occurring in the same non-threatening environment in which the baseline feeding test was performed. A two-way repeated measures ANOVA with temperament (HBI vs. LBI) as the between subjects factor and testing session (baseline vs. post-stress) as the within subjects factor revealed a significant difference in total feeding time between baseline and post-stress testing ($F_{(1,13)} = 27.00$, $p < 0.001$) and between LBI and HBI temperaments ($F_{(1,13)} = 13.20$, $p < 0.01$; Figure 7A). Importantly, a significant stress by temperament interaction was also observed ($F_{(1,13)} = 25.78$, $p < 0.001$). There were no significant differences in feeding between HBI and LBI animals in the baseline condition, but when tested after threat exposure; there was a significant reduction in feeding in the HBI compared to LBI animals. Compared to LBI animals, the HBI animals spent 60% less time feeding after predator exposure ($t = 5.803$, df = 14, $p < 0.001$). Similar results were obtained when analyzing the total amount of food eaten, which revealed significant main effects of stress ($F_{(1,13)} = 16.26$, $p < 0.01$) and temperament ($F_{(1,13)} = 4.726$, $p < 0.05$; Figure 7B) as well as a significant stress by temperament interaction ($F_{(1,13)} = 27.49$, $p < 0.001$). HBI animals ate 56% less food post-stress compared to LBI animals ($t = 4.552$, df = 14, $p < 0.001$). These findings demonstrate that prior stress exposure differentially affects HBI and LBI rats such that after the offset of the stressor, the HBI rats display a deficit in adaptive appetitive behaviors.

Discussion

Understanding the molecular mechanisms underlying anxiety-related psychopathology through the use of an ethologically relevant rodent model is crucial for identifying novel drug targets and developing new pharmacological treatments. Our previous rodent work (Nanda *et al.* 2008; Roseboom *et al.* 2007) used ferret exposure, an ethologically relevant threat, to study stress-induced behavioral responses and changes in gene expression. In the present studies, we extend this work by establishing a rodent model of BI temperament analogous to human and non-human primate BI. Importantly, we emphasize the selection of stable and extreme animals as at-risk individuals, consistent with what is observed in human children (Biederman *et al.* 2001).

Because BI in humans is assessed in childhood, we aimed to establish a developmental model to allow for the identification of individuals that will be susceptible to the deleterious effects of stress. Our findings demonstrate that, as in monkeys and humans, rat BI is present early in life and is trait-like. Specifically, we found that BI is stable when repeatedly tested in adolescence and in adulthood; and that the level of BI expressed by an individual in adolescence is predictive of the level of BI in adulthood. To further establish that rodent BI reflects anxiety, we demonstrate that diazepam selectively decreases BI without affecting other behaviors such as rearing, grooming and locomotion. This finding is consistent with other studies using benzodiazepines to validate rodent models of anxiety (Duzzioni *et al.* 2008; Jochum *et al.* 2007; Nicolas & Prinssen 2006; Wilson *et al.* 2004). Additionally, it is noteworthy that we observed an effect of diazepam on BI levels in an unselected population. Given that previous studies show no effect of diazepam in tests of anxiety-like responding in non-anxious animals (do-Rego *et al.* 2006), it is likely that we would find an even greater effect of diazepam in a population that is enriched with HBI rats.

Human and non-human primate studies have demonstrated involvement of a network of brain systems in mediating anxiety, fear and associated psychopathology (Charney 2003; Kalin *et al.* 2004; Kalin *et al.* 2005; McEwen 2007). We assessed the activity of brain regions using the expression of the immediate early genes *homer1a* and *fos*. These genes were chosen as they are highly-inducible in response to increases in neuronal activity, with each gene showing somewhat different patterns of activation. Additionally, we have shown that expression of both these genes are induced by our predator stress paradigm (Nanda *et al.* 2008; Roseboom *et al.* 2007). We chose to measure mRNA expression by *in situ* hybridization over immunodetection of protein as induction of mRNA transcription is more proximal to increases in neuronal activity, and therefore may more accurately reflect alterations in cellular activity. In this study, we found similarities between brain regions associated with BI in rats with regions identified in our non-human primate imaging studies (Fox *et al.* 2008). Using *homer1a* mRNA expression, we determined individual differences in hippocampal CA3 region activity were predictive of individual differences in BI. Given the importance of the hippocampus in contextual fear conditioning and in memory consolidation and retrieval, it is logical that hippocampal activity would play an important role in regulating the expression of BI (Davidson *et al.* 2000; Kalin & Shelton 2003; McEwen 2007). Similar to CA3, there was a correlation between CA1 region *homer1a* expression and BI that approached statistical significance. As both the CA3 and CA1 are activated during acute stress and functionally respond to stress in similar ways (Joels 2009; McEwen 1999), it is not surprising that they exhibit similar activation relative to BI.

We also found a positive correlation between PVN activity and BI. Involvement of the PVN in BI is expected, as BI is associated with increased HPA activity that has been described as a component of anxious temperament in humans (Schmidt *et al.* 1997). Additionally, rodent studies have also suggested a relationship between HPA axis activity with trait-like anxiety

(Landgraf *et al.* 1999; Veenema *et al.* 2007). Furthermore, the PVN receives direct and indirect inputs from the hippocampus and amygdala and has a central role in orchestrating the physiological response to stress (Herman *et al.* 2005; Jankord & Herman 2008).

The lack of a significant correlation between BLA and/or MeA *homer1a* expression with BI was unexpected given the role of the amygdala in human and non-human primate BI (Fox *et al.* 2008; Schwartz *et al.* 2003). Moreover, numerous studies using predator odor as a stimulus have shown that both BLA and MeA play roles in the unconditioned response to predator threat (Blanchard *et al.* 2005; Li *et al.* 2004; Muller & Fendt 2006; Takahashi *et al.* 2005). Lack of a positive finding should be interpreted with caution since only one time point was examined in this study, and other time points may have revealed a significant relation between amygdala activation and BI expression.

We also examined the expression of the immediate early gene *fos* as an additional marker of neuronal activation (Herrera & Robertson 1996). Similar to previous studies examining the effects of predator or predator odor on *fos* expression in rats (Campeau *et al.* 2008; Day *et al.* 2004; Dielenberg *et al.* 2001; Masini *et al.* 2005), we observed significant stress-induced increases in *fos* expression. However, we did not see significant correlations between BI and *fos* expression. This lack of finding supports the use of more than one immediate early gene in these types of studies, since different patterns of gene expression often occur in different neuronal populations.

While BI expressed in the presence of a predator is adaptive, excessive BI or altered behavior occurring in the absence of a threatening context is likely a sign of maladaptive emotional responding (Rotenberg & Boucsein 1993). In addition to characterizing the behavioral consequences and neural systems associated with rodent BI, we used our model to identify pathological at-risk individuals. To examine maladaptive pathological responding in relation to our model, we developed criteria to select animals that stably expressed extreme HBI or extreme LBI. To be categorized into the extreme groups, individuals had to stably display levels of BI that were in the upper or lower quartile when tested on two separate occasions. Using these criteria, we found a 12% prevalence of HBI in rats, which is similar to the prevalence of extreme BI reported in human children (Kagan *et al.* 1988). We then demonstrated that HBI, but not LBI rats, fail to engage in appetitive behaviors when tested in a non-threatening environment following stress exposure. This finding is consistent with human stress-related psychopathology in which disruptions of appetitive behavior is common (Lo Sauro *et al.* 2008; O'Brien & Vincent 2003). It is of interest that data from our laboratory and others suggest that an overactive corticotropin releasing factor system may mediate stress-induced decreases in appetitive drives (Bakshi *et al.* 2002; Gosnell *et al.* 1983; Jochman *et al.* 2005; Krahn *et al.* 1986; Negri *et al.* 1985) and may be important in the pathophysiology of anxiety and depressive disorders (Koob & Heinrichs 1999; Vale *et al.* 1981). Given the importance of mesolimbic dopamine neurotransmission in regulating appetitive behaviors (Bello & Hajnal 2010; Berridge 2009), it is also possible that alterations in this system may underlie the differential effects of stress on feeding behavior observed in HBI and LBI rodents.

Previous studies establishing rodent models of psychopathology have used rats or mice that are selectively bred for anxiety-associated traits (Liebsch *et al.* 1998; Touma *et al.* 2008). Similar to high-anxiety-bred (HAB) and low-anxiety-bred (LAB) rodent models (Liebsch *et al.* 1998; Muigg *et al.* 2009), our data suggest HBI animals have impaired coping mechanisms and greater brain reactivity to stressful or novel situations. Other data from our laboratory demonstrates that animals with high levels of BI tend to display increased anxiety-related responses in the elevated plus maze test (unpublished data), and this is consistent with HAB rats (Liebsch *et al.* 1998). Although selective breeding studies provide

insights into the pathophysiology of the anxious temperament (Knapman *et al.* 2009; Muigg *et al.* 2009; Touma *et al.* 2008), this approach differs significantly from our studies which focus on unselected and unmanipulated populations to identify animals that display extreme and stable BI. Furthermore, in relation to rodent anxiety, few studies have examined the issue of stability of BI expression over time (Cavigelli *et al.* 2007).

In summary, the present studies establish that rodent BI has many similarities to human and non-human primate BI. In the rodent model, BI is evident early in life, is trait-like and responds to anxiolytic treatment. Furthermore, individuals with extreme and stable high BI demonstrate other maladaptive responses. In addition, these studies implicate the PVN and hippocampus in modulating the expression of BI. Taken together, these studies form a solid foundation for further work aimed at identifying the molecular mechanisms that underlie the relationship between BI and stress-induced psychopathology. Moreover, the rodent model can identify novel systems that may serve as both markers for identifying at-risk individuals and as targets for new pharmacological treatments aimed at either preventing or ameliorating stress-induced psychopathology.

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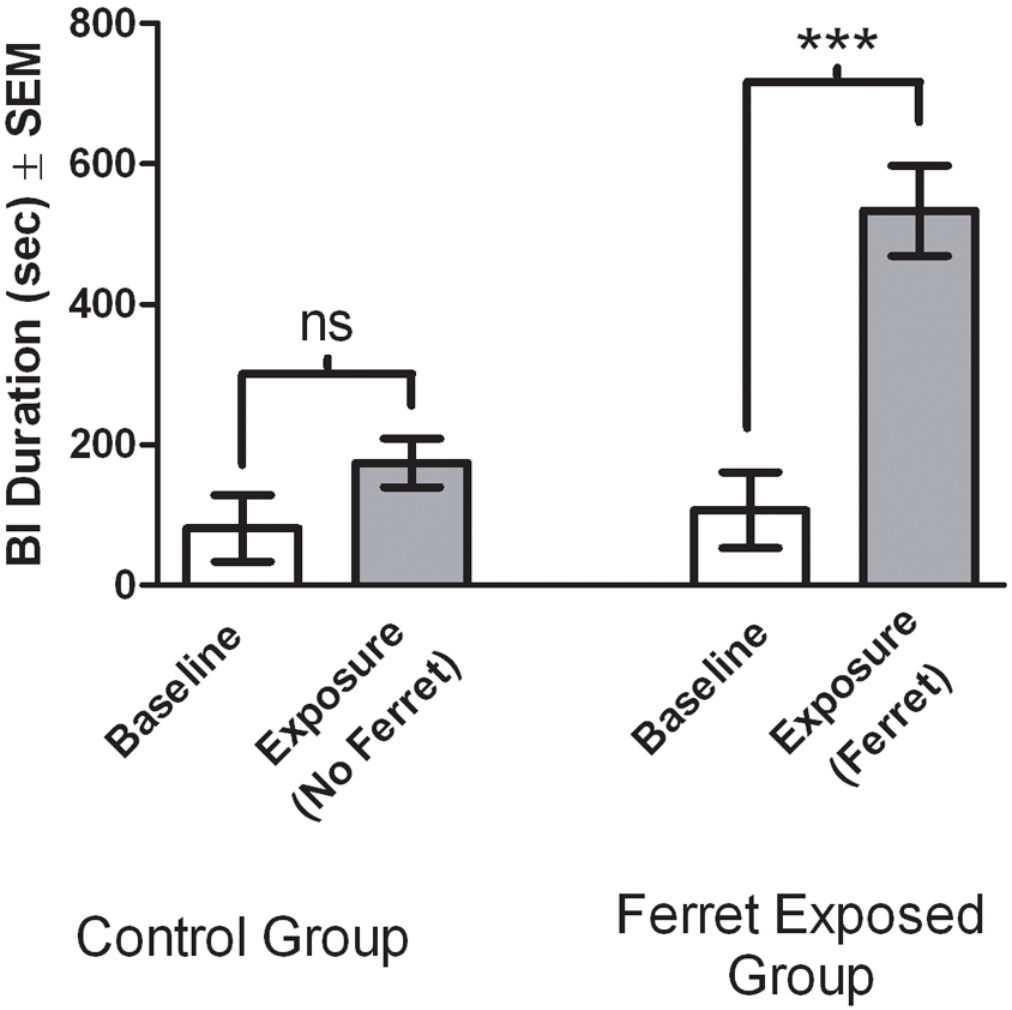

Figure 1. Ferret exposure induces behavioral inhibition in adolescent rats

The control rats were exposed to a baseline session followed by exposure to the same environment as baseline, and the experimental group was exposed to baseline followed immediately by exposure to the ferret. In the Ferret Exposed Group, rats exhibited significantly more BI in the presence of the ferret compared to the baseline condition. Bars represent the mean ± SEM. ***, $p < 0.001$.

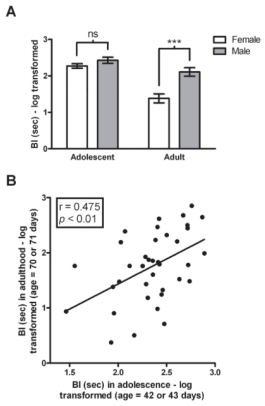

Figure 2. Behavioral inhibition is dimorphic and stable across development

BI was measured in male (n = 19) and female (n = 18) adolescent rats (age 42 or 43 days) and again in the same rats in adulthood (age 70 or 71 days). (A) Male rats engaged in more BI during adulthood than female rats. Bars represent the mean ± SEM. (B) BI exhibited in adolescent male and female rats is trait-like and maintained across development into adulthood.

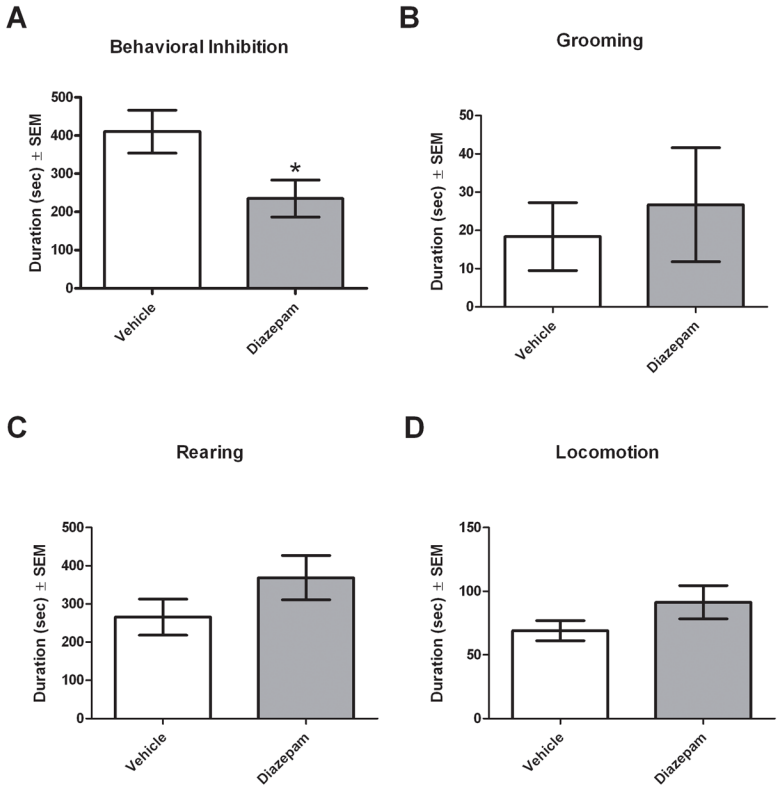

Figure 3. Diazepam administration decreases behavioral inhibition

Rats were treated with either vehicle or diazepam (1 mg/kg) 30 min prior to ferret exposure. Diazepam-treated animals exhibited significantly less BI than vehicle-treated animals (A). No significant differences in grooming (B), rearing (C) or locomotion (D) between vehicle and diazepam treated groups were observed. Bars represent the mean ± SEM for 8 independent determinations in the vehicle and diazepam treated groups. *, $p < 0.05$ compared with vehicle.

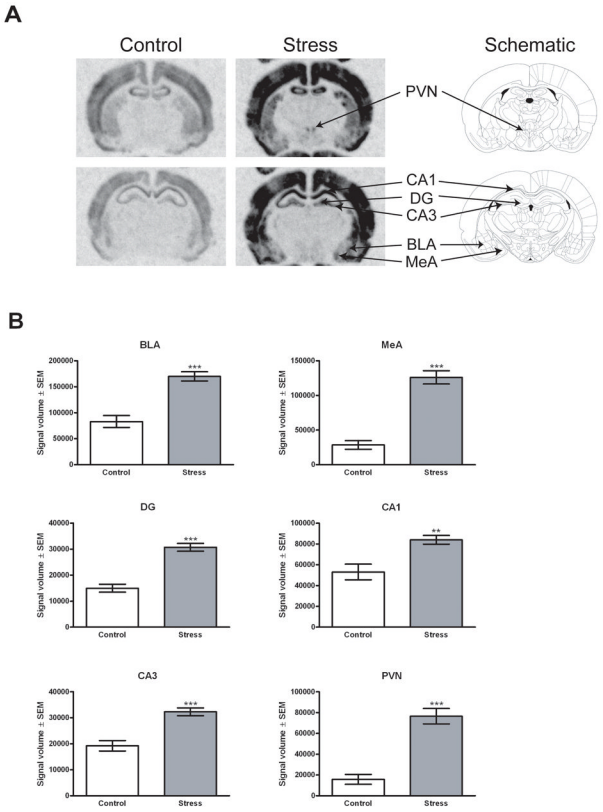

Figure 4. Ferret exposure increases *homer1a* mRNA levels within the BLA, MeA, DG, CA1, CA3 and PVN

Forty-five rats were exposed to the ferret and sacrificed 1 h following cessation of exposure. Nine rats served as controls and remained in their home cages until sacrificed. (A) *In situ* hybridization was used to detect *homer1a* mRNA in control and stressed rats and the location of the signals for the basolateral amygdala (BLA), medial amygdala (MeA), dentate gyrus (DG), CA1 region of the hippocampus (CA1), CA3 region of the hippocampus (CA3) and paraventricular nucleus of the hypothalamus (PVN) are indicated on the phosphor images from a representative ferret exposed rat and schematic. Schematic images representing Bregma −1.80 mm and Bregma −3.14 mm are adapted from a published atlas (Paxinos & Watson 1998). (B) Ferret exposure significantly increased *homer1a* expression in the BLA, MeA, DG, CA1, CA3 and PVN. Bars represent the mean quantified signal volume (signal intensity multiplied by ROI area) ± SEM for each group (9 independent determinations for control, 45 independent determinations for stress). **, $p < 0.01$ compared to control. ***, $p < 0.001$ compared to control.

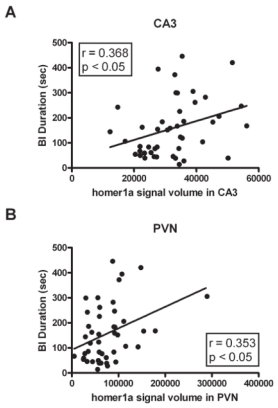

Figure 5. *homer1a* mRNA levels in the CA3 region of the hippocampus and the PVN correlate with levels of behavioral inhibition

In situ hybridization was used to assess the level of *homer1a* mRNA in rat brains (n = 45) obtained 1 h following ferret exposure. Regression analyses show that *homer1a* expression in the CA3 (A) and PVN (B) are significantly correlated with BI.

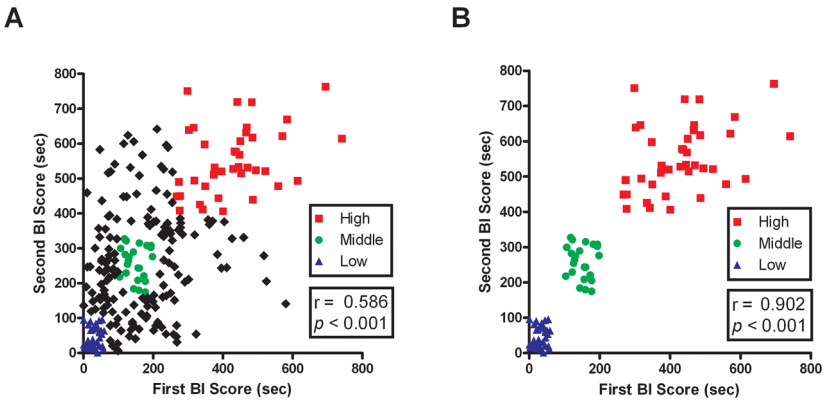

Figure 6. The expression of behavioral inhibition is stable trait-like characteristic and rats that express extreme and stable levels of BI can be identified

(A) Scatter-plot shows the relationship between the level of BI obtained from two exposures separated by 48 hr in a population of rats (n = 255). The duration of BI from the first exposure is significantly correlated to the duration of BI from the second exposure. Rats in the bottom quartile from both tests were labeled LBI (triangles), rats consistently in the middle quartile were labeled MBI (circles) and rats consistently in the top quartile were labeled HBI (squares). (B) Scatter-plot represents only animals in the LBI, MBI and HBI groups. As expected, the correlation of BI between first exposure and second exposure markedly increases when animals are selected for stability.

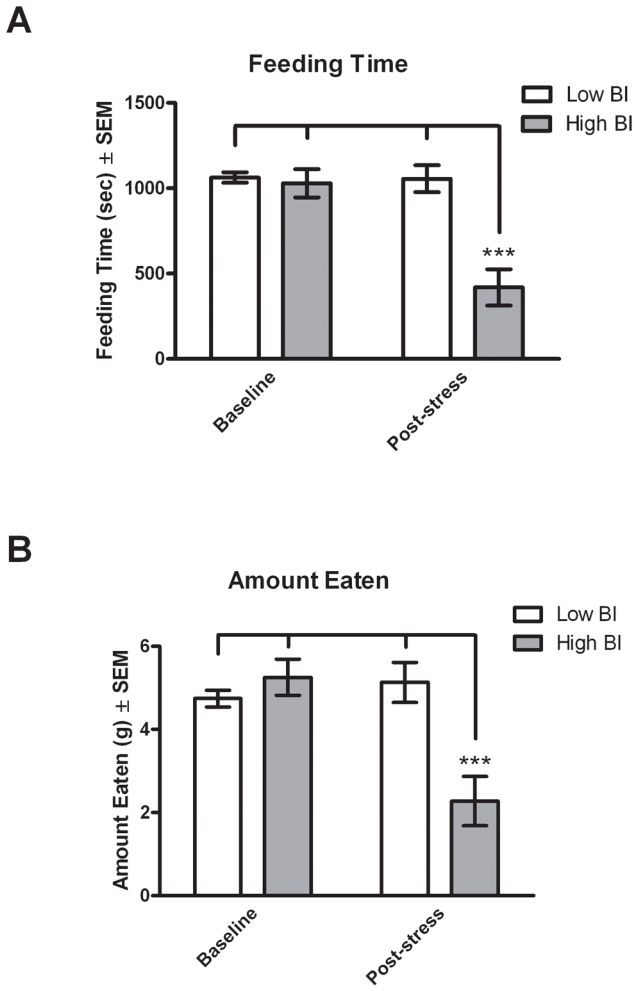

Figure 7. Ferret exposure induces feeding reductions in HBI rats

Food deprived extreme and stable (HBI and LBI) rats were tested for feeding behavior in a non-threatening environment immediately following ferret exposure. At baseline, no difference was observed between HBI and LBI in both feeding time (A) and amount of food eaten (B). However, following ferret stress, HBI rats spent 60% less time feeding (A) and ate 56% less food than LBI rats (B). Bars represent mean ± SEM. ***, $p < 0.001$ compared to LBI post-stress.