Anxiety Behavior Induced in Mice by Acute Stress

Jonathan Solomonow, Tulane University
Jeffrey G. Tasker, Tulane University

Abstract

The amygdala is known to be part of a limbic circuit critical for the integration of cognitive function, emotion, and memory. The basolateral nucleus of the amygdala (BLA) is implicated in fear memory formation and acts as an overall fear and anxiety response center in the brain. Stress activates the hypothalamic-pituitary-adrenal (HPA) neuroendocrine axis, ultimately causing the release of glucocorticoids from the cortex of the adrenal glands. Previous studies showed that a 30-minute restraint stress causes a glucocorticoid-induced suppression of inhibitory synaptic inputs to BLA neurons in rats, which should lead to an increase in BLA neuron excitability and result in an anxiety-like behavior. Here, we conducted behavioral experiments in mice to test for anxiety-like behavior induced by an acute stress using the elevated plus maze, open field test, and light-dark box test. We found that mice display more anxiogenic behavior following acute restraint stress.

Introduction

Stress plays a critical role in the regulation of emotional memories. The amygdala, particularly the basal lateral complex of the amygdala (BLA, including the basal, lateral and accessory basal nuclei), is the region containing the changing neural circuitry that lends itself to the consolidation, as well as the extinction, of fear memories. The BLA acts as a central relay center that processes sensory and cognitive inputs to influence memory consolidation in downstream pathways including the hippocampus and caudate putamen. This circuitry is implicated in anxiety and mood disorders such as post-traumatic stress disorder (PTSD), which is characterized by an inability to extinguish a conditioned fear response.[1]

Such responses are regulated by stress hormones, including glucocorticoids, which influence several areas of the brain involved in emotional processing, learning, and survival. Stress stimulation activates both a sympathetic response and an acute neuroendocrine response that together are responsible for triggering a large range of events. The sympathetic output activated by stressful stimuli stimulates the fight or flight response, which allows mammals to react quickly to life threatening events.[2] Sympathetic activation results in epinephrine and norepinephrine secretion into the bloodstream, which results in increased cardiac and respiratory rates, vasoconstriction, and increased skeletal muscle blood flow, along with decreased gastrointestinal activity, amongst other responses. The stress response therefore provides an emphasis on bodily responses critical for survival function.[2]

Stress exposure also activates the hypothalamic-pituitary-adrenal (HPA) neuroendocrine axis, which results in increased glucocorticoid secretion into the bloodstream. Stress promotes fear memory consolidation via rapid actions of glucocorticoids in the BLA.[3] Intra-BLA application of glucocorticoid agonists enhances the consolidation and extinction of fear memories, while GC antagonists directly inhibit such effects.[3]

The Tasker lab has shown that glucocorticoids cause a long-term depression of inhibitory synaptic inputs to BLA principal neurons.[4] This effect is dependent on the glucocorticoid activation of retrograde endocannabinoid signaling to presynaptic GABAergic neurons, which suppresses synaptic inhibition in a non-reversible manner. A prolonged decrease in synaptic inhibition should lead to long-term potentiation of excitatory inputs to the BLA neurons, which should increase BLA excitability. [4] Here, we hypothesized that stress results in an increase in BLA outputs that leads to an increase in anxiogenic

behavior. We conducted behavioral experiments on mice to test for the effect of acute restraint stress on performance on a series of behavioral tests for anxiogenesis, including the elevated plus maze, the open field test, and the light-dark box test, which are widely accepted as behavioral assays for anxiogenic behavior in rodents.

Methods

Animals

Twenty male C57BL/6 mice were used in accordance with a protocol approved by the Tulane University Institutional Animal Care and Use Committee. Rodents were acquired at 4-6 weeks of age, allowed to acclimate to the animal facility for 1-2 weeks, and tested at 5-9 weeks. Animals were housed individually at a constant temperature on a 12 hour light/dark cycle and were provided with *ad libitum* access to food and water.

Experimental Protocols

For the 5 days prior to experimentation, the mice were transported to the handling room and handled for two minutes each day. On the day of testing, animals were transported to the handling room (adjacent to testing area) and were habituated to it for at least 45 min prior to testing. In the testing room, experimental subjects were then subjected to restraint stress for 30 min in a flexible plastic cone (Decapicone) with an open nose hole and that was tied at the tail to secure the mouse. The control animals were placed in a new housing cage with no food or water for 30 min prior to testing. After each test run, the testing chambers were cleaned with 70% ethanol solution to remove olfactory cues. Animals were tested during their light cycle. The same animals were used for multiple tests, but were alternated between the experimental and control groups for each test. There was ≥ 1 week interval between the different tests.

Elevated Plus Maze

The elevated plus maze is made of a black plexiglas platform containing four perpendicular arms (each 30 cm x 5 cm) extending from a central open square (5 cm^2). Two opposing arms are enclosed with 15-cm high walls ("closed arms"), and the remaining 2 opposing arms are without walls ("open arms"). The maze is elevated on legs 38 cm above the ground. A floor fan and a white noise maker were turned on during testing to cancel out surrounding ambient noise. Mice were introduced into the central open area facing a closed arm, and were allowed to move around freely in the maze for 5 min. A camera mounted above the maze was used to record the movement of the animals on video tape. Video tapes were analyzed following the tests for anxiety behavior for the percent time spent in the open arms, closed arms and center region of the elevated plus maze.[5,6]

Open Field Test

The open field test was conducted in an open-aired black Plexiglas cube with dimensions of 40 cm x 40 cm x 30 cm. At the beginning of the test, each animal was introduced into the same left back corner of the arena, and was allowed to explore the arena freely for 5 min. A camera mounted above the apparatus was used to video record the movement of the animal. Video tape was analyzed following the test for anxiety behavior. The floor of the field was subdivided into 16 equal sized squares (4 center, 12 peripheral). The amount of time the mouse spent in the center squares vs. the peripheral squares was calculated and used to measure anxiogenic behavior.[5]

Light-Dark Box Test

The light-dark box test was performed in the same arena as the open field test, but included an opaque black box insert that covered half the area, dividing it into two compartments, one light and one dark, with a small opening between the two. A single bare light bulb was hung above the open compartment. The mice were tested for 10 min in the light-dark box test. A camera mounted above the apparatus was used to record the movement of the animals. Video tape was analyzed following the tests, quantifying the time spent in the light vs. dark compartments to monitor anxiogenesis and the number of times the animal crossed between the two compartments to monitor locomotor activity.[5]

Results

Anxiety-like behavior was measured in the elevated plus maze based on the ratio of time spent in the closed arms of the maze to the time spent in the open arms in the acutely stressed mice compared to the controls (Fig. 1). Mice display a natural curiosity to explore new territory, but an innate fear of open

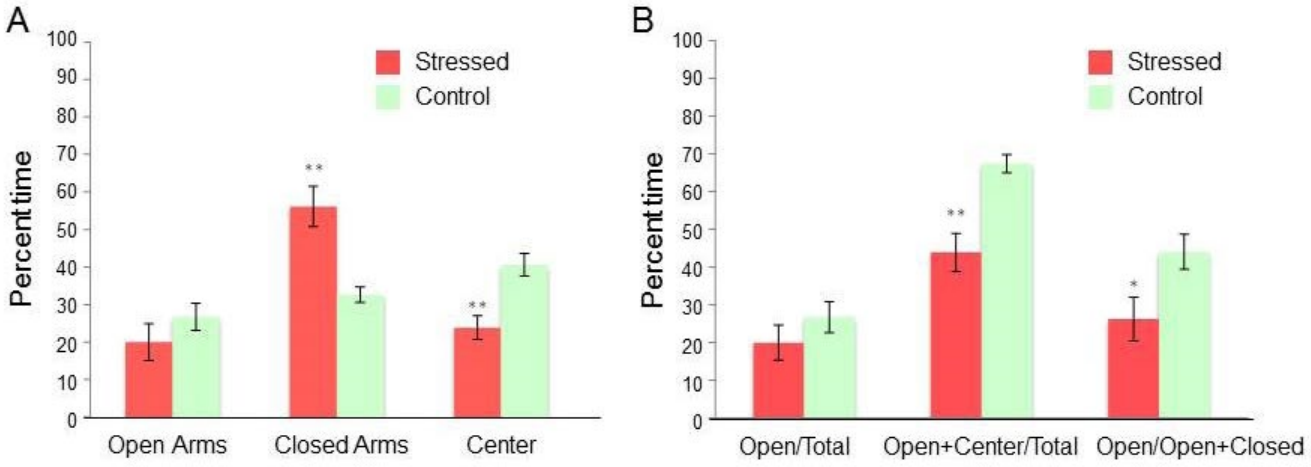

Fig. 1. Stressed mice show increased anxiety-like behavior in the elevated plus maze. A. Mice that underwent restraint stress spent more time in the closed arms and less time in the center, but not the open arm. **B.** Percent time spent in open arms, with or without center time, over total time. In all ratios, stressed mice showed a lower percentage time spent in the open arms, suggesting an increase in anxiety-like behavior. *, $P < 0.05$; **, $P < 0.01$.

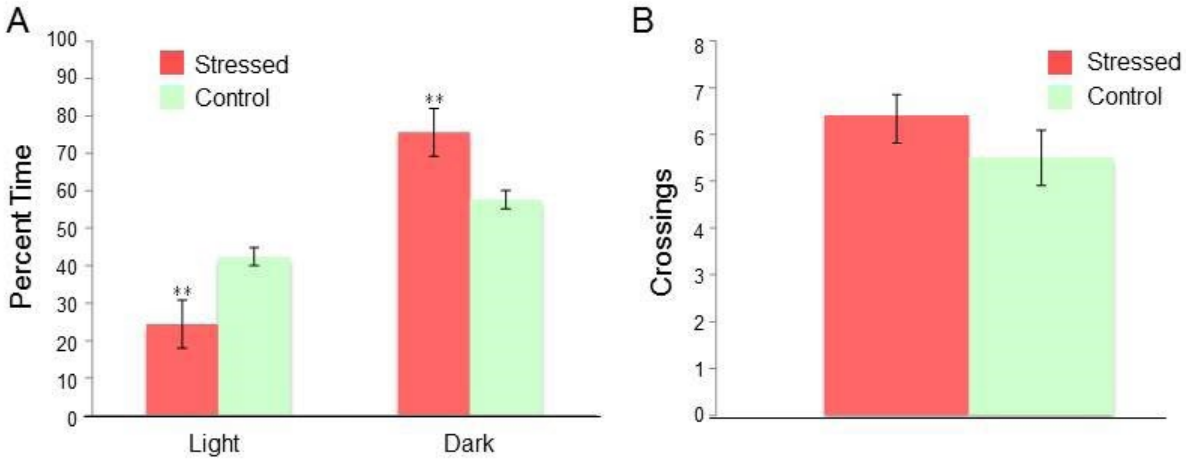

Fig. 2. Stressed mice show increased anxiety-like behavior in the light-dark box test. A. The mice in the stressed group (red bars) spent less time in the lighted compartment and more time in the dark compartment than the control mice (green bars) in the light-dark box. **B.** There was no difference in the overall locomotor activity, as shown by numbers of crossings from the light to the dark compartments, between the stressed mice (red bar) and the control mice (green bar). **, $P < 0.01$.

spaces. Therefore, less time spent in the open arms and/or more time spent in the closed arms indicate higher levels of anxiety-like behavior. Location of the mouse in the open or closed arms was defined by the mouse having all four of its paws cross over into the arm. This criterion was used for placement of the mice in all three tests. A two-way analysis of variance test revealed a statistically significant difference between the acute stress and control groups for time spent in the closed arms (P=.001) and time spent in the center region ($P = 0.001$). The average time spent in the open arms did not reach statistical significance ($P = 0.15$). However, when the time spent in the open arms was combined with the time spent in the center and set over the total time, a statistically significant difference was seen between the stressed and control groups ($P = 0.001$). When the time spent in the center was excluded, and the time spent in the open arms was set over the time spent in the open arms + time spent in the closed arms, the stressed group was also statistically different from the control group ($P = 0.02$).

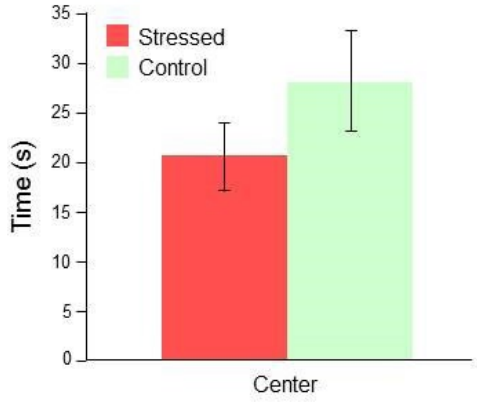

Fig. 3. Open field test of anxiety-like behavior. The mice in the stressed group (red bar) spent less average time in the center of the open field than the control mice (green bar), although this did not reach statistical significance.

In the light-dark box test, the stressed mice spent more time on average in the dark compartment and less time in the light compartment compared to the control mice (P = 0.01) (Fig. 2A). There was no significant difference in the number of crossings from one compartment to the other between the two groups (P = 0.13) (Fig 2B), which indicates no difference in locomotor activity.

In the open field test of anxiety, the stressed mice spent more time in the center of the open field on average than the control mice, however, this difference did not reach statistical significance (P = 0.12) (Fig. 3), indicating a trend toward a difference in anxiety-like behavior in this test.

Discussion

This experiment was designed to explore the behavioral outcome of the long-term depression of synaptic inhibition caused by stress-induced endocannabinoid suppression of GABA release onto BLA neurons, shown in a preliminary study by Di and Tasker using a brain-slice electrophysiological approach.[7] We studied the anxiogenic response in mice to a 30-min restraint test. A 30-min acute stress protocol was chosen based on studies showing that 30 min is the time required for peak glucocorticoid levels in the bloodstream.[4] Previous studies testing stress exposure for intervals between 15 min and one hour found that a 30-min stressor has the strongest effect on anxiogenic behavior.[8] Since the BLA is known to be central to the regulation of emotion and fear, stress-induced suppression of GABA-mediated inhibition of the BLA neurons would be expected to lead to increased anxiogenic behavior. We tested this using three widely used behavioral tests of anxiety-like behavior, the elevated plus maze, open field, and light-dark box test. We found that stressed mice displayed a significant increase in anxiety-like behavior compared to control mice in the elevated plus maze and the light-dark box, and a trend toward increased anxiety-like behavior in the open field test.

Following exposure to the 30-min restraint stress, mice spent relatively less time in the open arms and more time in the closed arms of the elevated plus maze. Although significance was not reached in the open arm times, the group differences did reach significance when the open-arm times relative to the open- plus closed-arm times or the open-arm plus center times relative to total time were considered. Acute stress exposure also caused a clear increase in the time mice spent in the dark compartment and decrease in the time spent in the light compartment of the light-dark box, but no change in the total crossings from one compartment to the other. This suggests that the acute stress caused an increase in anxiety-like behavior without affecting locomotor behavior.

In the open field test, the stressed mice showed a trend toward less time in the center of the open field than the controls, but this difference did not reach statistical significance. This is not unexpected, as the open-field test requires a greater separation between the behaviors of control and experimental subjects to achieve significance, and the increase in anxiety elicited by a single acute stress might not be robust enough to attain this difference.[5] An increase in the number of subjects tested or an increase in the severity of the acute stress might make this difference between the two groups reach statistical significance.

There are several factors and variables that come into play with these behavioral tests that could cause either no effect or even results that are opposite of those expected. One of the most important factors when testing for anxiety-like behaviors is to ensure a

low level of baseline stress prior to experimentation, both in the days leading up to the experiment and on the day of experimentation. An animal that is stressed will present a higher basal level of stress activation, which would make it difficult to distinguish the effect of the restraint stress compared to an already elevated baseline stress level in the control animals. We handled the mice for five days leading up to the experiment to allow them to acclimate to the experimenter's olfactory cues and handling. Even the transport of animals can cause anxiety due to the motion and noise. Handling of the animals during the experiment, such as moving it from the home cage to the temporary holding cage, must be conducted carefully to minimize stress.

The lack of significant data in the open field test may have been the result of re-stressing animals that had already been stressed. In each cohort of 20 animals, 10 were used as controls and 10 as experimentals first in the elevated plus maze. The second experiment was the light-dark box test, which was conducted two weeks later; the control and stressed groups were switched, such that the animals that were stressed during the elevated plus maze test were used as controls for the light-dark box test, and the controls for the first test were stressed for the second test. For the elevated plus maze and light-dark box tests, therefore, each animal experienced the restraint stress only once. In the third experiment, the open field test, on the other hand, the animals that had been stressed during the elevated plus maze underwent the restraint stress a second time, albeit 4 weeks later. Being subjected to the restraint stress a second time could have caused those animals to become habituated to the restraint and, therefore, mount a blunted stress response, which could have resulted in a decreased glucocorticoid effect in the BLA and reduced anxiogenic behavior.

A previous study by Paylor and colleagues showed that in some tests the behavior of previously tested mice differed from the behavior of naïve mice, while for other tests there was no difference. One of the tests sensitive to testing order was the open field test.[8] As discussed above, this sensitivity to testing order may provide an explanation for the lack of significant difference between the control and stressed mice seen in our open field test.

Another factor that must be considered is the timing of testing of the animals in the light-dark cycle. All the animals were tested during the light phase of the light-dark cycle, which, because they are nocturnal animals, is the less active phase of the cycle. Furthermore, the mice were tested in a fully lit room, which may have put additional external stress on the animals and increased the baseline stress level of the controls. In future studies, we could consider either testing during the dark phase of the cycle or in a dimly lit room.

Finally, previous studies have shown that restraint stress may have an effect on locomotor activity in the first few minutes of each test due to confusion or shock.[10] A study by Morilak et al. discussed the possibility that testing directly following restraint may have an effect on the anxiety response.[10] While locomotor activity was accounted for and did not change significantly in our experiments, it may be beneficial in future studies to introduce a 5-min delay following the restraint stress and prior to introducing them into the anxiety testing apparatus, which would allow the animal time to adjust to being out of the restraint tube and be more active. Different lengths of time between the restraint stress and anxiety testing could be looked at in an attempt to find a balance between reducing the effect of the shock of the stress and sampling during the peak stress response.[8]

Overall, we conclude from this study that there is compelling evidence in support of our hypothesis that an acute restraint stress causes anxiogenic behavior in mice. Future experiments will include inhibiting the production of glucocorticoids or blocking glucocorticoid receptors to test for the glucocorticoid dependence of the stress-induced anxiogenic effect.

References

1. Mitra R, Adamec R, Sapolsky R. Resilience against predator stress and dendritic morphology of amygdala neurons. *Behav Brain Res*. 2009; 205(2):535-43.

2. Goldstein, David S. Stress-induced Activation of the Sympathetic Nervous System. *Baillière's Clinical Endocrinology and Metabolism* 1987; 1(2): 253-78.

3. Atsak P, Hauer D, Campolongo P, Schelling G, Fornari RV, Roozendaal B. Endocannabinoid Signaling within the Basolateral Amygdala Integrates Multiple Stress Hormone Effects on Memory Consolidation. *Neuropsychopharmacology* 21 January 2015; doi: 10.1038/npp.2014.334.

4. Di S, Malcher-Lopes R., Halmos KC, Tasker JG. Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: A fast feedback mechanism. *J Neurosci.* 2003; 23: 4850-4857.

5. Bailey KR., Crawley JN. Anxiety-Related Behaviors in Mice. In: Buccafusco JJ, ed. *Methods of Behavioral Analysis in Neuroscience, 2nd Edition*. Chapter 5. Boca Raton (FL): CRC Press; 2009.

6. Walf AA, Frye CA. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc.* 2007; 2(2):322-8.

7. Di S, Tasker JG. Nongenomic glucocorticoid inhibition of GABA synaptic transmission via endocannabinoid release in the rat basolateral amygdala. *Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011*.

8. MacNeil G, Sela Y, McIntosh J, Zacharko RM. Anxiogenic behavior in the light-dark paradigm following intraventricular administration of cholecystokinin-8S, restraint stress, or uncontrollable footshock in the CD-1 mouse. *Pharmacol Biochem Behav*. 1997; 58(3):737-46.

9. McIlwain KL, Merriweather MY, Yuva-Paylor LA, Paylor R. The use of behavioral test batteries: effects of training history. *Physiol Behav*. 2001; 73(5):705-1.

10. Morilak DA, Cecchi M, Khoshbouei H. Interactions of norepinephrine and galanin in the central amygdala and lateral bed nucleus of the stria terminalis modulate the behavioral response to acute stress. *Life Sci.* 2003; 73(6):715-26.