5-Hydroxytryptamine 2C Receptors in the Basolateral Amygdala Are Involved in the Expression of Anxiety After Uncontrollable Traumatic Stress

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Background: Exposure to uncontrollable stressors often increases anxiety-like behavior in both humans and rodents. In rat, this effect depends on stress-induced activity within the dorsal raphe nucleus (DRN). However, the role of serotonin in DRN projection regions is largely unknown. The goals of this study were to 1) assess the effect of uncontrollable stress on extracellular serotonin in the basolateral amygdala during the anxiety test, 2) determine whether DRN activity during a poststress anxiety test is involved in anxiety-like behavior, and 3) determine the role of the serotonin 2C receptor (5-HT_{2C}) in uncontrollable stress-induced anxiety.

Method: Rats were exposed to tail shocks that were either controllable or uncontrollable. On the following day, anxiety-like behavior was assessed in a Juvenile Social Exploration (JSE) test. Basolateral amygdala (BLA) extracellular serotonin concentrations were assessed during JSE by in vivo microdialysis 24 hours after uncontrollable stress, controllable stress, or no stress. In separate experiments, drugs were administered before the JSE test to inhibit the DRN or to block 5-HT_{2C} receptors.

Results: Exposure to uncontrollable shock reduced later social exploration. Prior uncontrollable stress potentiated serotonin efflux in the BLA during social exploration, but controllable stress did not. Intra-DRN 8-OH-DPAT and systemic and intra-BLA 5-HT_{2C} receptor antagonist SB 242,084 prevented the expression of potentiated anxiety in uncontrollably stressed rats. Intra-BLA injection of the 5-HT_{2C} agonist CP 809,101 mimicked the effect of stress.

Conclusions: These results suggest that the anxiety-like behavior observed after uncontrollable stress is mediated by exaggerated 5-HT acting at BLA 5-HT_{2C} receptors.

Key Words: 5-HT_{2C}, learned helplessness, PTSD, rat, serotonin, social exploration

The pathobiologies of stress-induced anxiety disorders, such as acute stress disorder (ASD) and posttraumatic stress disorder (PTSD), are poorly understood, yet PTSD has an estimated lifetime prevalence of 7.8% (1), and treatment of PTSD is inadequate (2). ASD and PTSD symptoms include avoidant, anxiety-like behaviors that present just after stressor exposure or develop with time, respectively (3). Stress victims who display ASD symptoms are more likely to develop chronic PTSD (4), and early intervention can reduce the occurrence of chronic PTSD (5). Although much has been reported about the therapeutic mechanisms of PTSD pharmacotherapy, relatively little is known about the biological basis of anxiety *expression* after trauma.

The sequelae of a stressor depend on both environmental and genetic factors. In terms of environmental factors, controllable stressors tend to have less measurable impact than those that are not controllable (6), and a lack of behavioral control over stress may be critical to the development of PTSD (7,8). In one well-characterized paradigm, exposure to inescapable tail shock (IS) induces a number of behavioral consequences that do not follow exposure to exactly equal escapable tail shocks (ES). These outcomes include shuttle escape failure, enhanced fear conditioning, and reduced social exploration, among others, collectively described as "learned helplessness effects" (for a review, see Maier and Watkins [6]).

Exaggerated anxiety-like behavior is one of the most striking consequences of exposure to IS, relative to ES, as well as to acute trauma in humans. Rats exposed to IS later show greater fear conditioning (9), postshock freezing (10), neophobia (11), and reductions in social interaction (12,13) than do rats exposed to equal ES. The development of anxiety-like behaviors after IS depends on an intense activation of serotonergic neurons in the dorsal raphe nucleus (DRN; for a review, see Maier and Watkins [6]) during stress that is thought to "sensitize" DRN-serotonin (5-HT) neurons to respond to later stressors in exaggerated fashion (14–16). Acute activation of 5-HT neuronal systems is thought to be a critical component of anxiety expression because acute administration of selective serotonin reuptake inhibitors (SSRIs) evokes anxiety-like behaviors in both humans and rodents (17–20) and mimics the effects of IS (21). We have reported that IS, but not ES, reduces exploration of both adult (13) and juvenile (12) conspecifics, a behavior that is commonly thought to "assay" anxiety-like behavior (22). Furthermore, the effect of IS was dependent on DRN activation at the time of stress (12).

Only a subset of neurons in the caudal DRN respond differentially to IS and ES (23), and precisely this subset projects to the basolateral amygdala (BLA) (24), a key structure in the mediation of anxiety. Environmental information about threatening stimuli is relayed to the BLA, a structure with glutamatergic projections to a wide range of limbic structures involved in the mediation of overt fear and anxiety behaviors (25,26). Importantly, BLA output is modulated by 5-HT (25). 5-HT_{2C} receptor agonists produce anxiogenic effects (18) and lead to activation in BLA projection regions (27), whereas 5-HT_{2C} receptor antagonists are anxiolytic

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(28). Recently, Strong *et al.* (29) demonstrated that a systemic 5-HT_{2C} antagonist blocked, and 5-HT_{2C} agonists mimicked, the effects of IS on later freezing and shuttle escape behavior.

If IS leads to an anxiety-like state that is dependent on DRN sensitization, it is possible that 5-HT released in the BLA, which is increased in response to anxiogenic stimuli after IS (30), plays a critical role in the generation of IS-potentiated anxiety-like behavior. The idea here is that after IS, when the rat is presented with a stimulus such as a juvenile conspecific, 5-HT levels in the BLA rise and activate 5-HT_{2C} receptors, which may in turn enhance BLA output and increase anxiety-like behaviors. Using the Juvenile Social Exploration (JSE) test, the current set of experiments aimed to determine whether 1) prior IS exaggerates the release of 5-HT in the BLA produced by a juvenile social interaction, 2) DRN 5-HT activation is required to produce IS-augmented anxiety, 3) BLA 5-HT_{2C} receptors are critical to the anxiety-like effect of IS, and 4) 5-HT_{2C} agonism is sufficient to mimic IS effects on JSE (Supplement 1).

Methods and Materials

Rats

Adult (60–70 days old and weighing 275–350 g at the time of testing) and juvenile (28–32 days old and weighing 90–100 g at the time of testing) male Sprague-Dawley (Harlan, Indianapolis, Indiana) rats were used in all experiments. Rats were housed with free access to food and water in groups of two for microinjection experiments, in groups of four when drugs were administered intraperitoneally (IP), and in single cages for the microdialysis experiment. The vivarium maintained a 12-hour light-dark cycle; all experimental procedures were conducted within the first 6 hours of the light phase. All procedures were conducted in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the University of Colorado Institutional Animal Care and Use Committee.

Stress Induction Procedures

In experiments involving ES, 100 electric tail shocks were administered as previously described (14) (Supplement 1). In brief, rats were restrained, and shock was delivered through the tail on a variable interval 60-sec schedule. Turning a wheel inside the restraining box terminated the shock. Shock terminated automatically if no response was made within 30 sec. Rats in the yoked-IS shock group received exactly equal shock but were not permitted control—turning the wheel had no consequence. Animals in the homecage control (HC) group remained in the vivarium. When only IS was involved, 100 5-sec shocks were delivered on a variable-interval 60-sec schedule.

Juvenile Social Exploration Tests

JSE testing was conducted as described previously (31). In a separate testing room, each experimental subject was allocated a single plastic tub cage with shaved wood bedding and a wire lid. To begin, the test rats were placed into the test cage, and after 45 min, a 28 (± 2)-day-old juvenile was introduced to the cage for 3 min and an observer, blind to treatment, timed exploratory behaviors (sniffing, pinning, and allogrooming) initiated by the adult (31). To observe JSE during in vivo microdialysis the observers were not always blind to treatment and scored two tests simultaneously. In all other experiments, the observers were naive to treatment conditions and scored only one rat at a time. Juveniles were used for multiple tests but were never used for the same adult rat. Testing order was counterbalanced for stress and drug treatments.

Table 1. Type and Location Coordinates of Microdialysis and Microinjection Cannulae

Target	Cannula Type	AP	LM	DV
BLA	CMA 12 (Carnegie medicine, Sweden)	−3.0	+4.8	−6.2
DRN	26 g, 15.5-mm length (Plastics One, Roanoke, VA)	−8.1	0	−5.1
BLA	26 g, 7.5-mm length (Plastics One)	−3.0	±4.8	−6.2
CeA	26 g, 7.5-mm length (Plastics One)	−2.0	±4.0	−6.2

Coordinates are in millimeters and were measured from bregma and dura.

AP, anterior-posterior; BLA, basolateral amygdala; CeA, central nucleus of the amygdala; DRN, dorsal raphe nucleus; DV, dorsal-ventral; LM, lateral-medial.

Surgical Procedures

All surgery was performed under inhaled isoflurane anesthesia (3% in O_2). Microdialysis and microinjection guide cannulae were implanted and fixed in place with stainless steel screws and acrylic cement as previously described (14). Exact coordinates and cannulae specifications are listed in Table 1. A stylet was placed in the cannulae and each rat was inoculated with .25 mL/kg (subcutaneous) penicillin (Combi-Pen, Agrilabs, St. Joseph, Missouri). At the end of the experiment, brains were collected, sliced at 40 μm, and stained with cresyl violet for cannulae verification. Subjects were only included if tissue damage from the cannulae tips fell within the target nucleus.

In Vivo Microdialysis and Quantification of 5-HT

Microdialysis probes (CMA 12, MW cutoff 20 kDa, 2 mm) were inserted into the guide and Ringer's solution (145 mmol/L NaCl, 2.7 mmol/L KCl, 1.2 mmol/L CaCl) was perfused through the probes at a flow rate of .2 μL/min. After 12 hours, the flow rate increased to 1.5 μL/min. After a 90-min equilibration period, eight samples were collected at 15-min intervals. Dialysates were immediately placed in an −80°C freezer until analysis. Samples were analyzed with high-pressure liquid chromatography using standard methods (14).

Drugs

The selective 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT, Sigma, St. Louis, Missouri) was dissolved in .9% saline. The selective, brain-penetrant 5-HT_{2C} antagonist 6-chloro-2,3-dihydro-5-methyl-N-[6-[(2-methyl-3-pyridinyl) oxy]-3-pyridinyl]-1H-indole-1-carboxyamide dihydrochloride (SB 242,084, Tocris, Ellisvile, Missouri) was dissolved in .9% saline.

Experimental Procedures

Overview. Except where noted, all experiments were conducted in this fashion: Rats were implanted with cannulae and allowed 7 to 10 days to recover. JSE baseline tests were given, and 24 hours later, stress was administered. Because the focus of the current experiments was on the expression of stress-induced anxiety, pharmacologic manipulations were made before a post-stress JSE test given 24 hours after stress.

In Vivo Microdialysis and Quantification of BLA 5-HT. Rats were assigned to either ES or yoked IS stress or HC treatment (n = 7/group). After stress, microdialysis began as described earlier. Rats were left alone in the dialysis room with food and water for at least 12 hours on a light cycle that matched the