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5-hydroxytryptamine 2C receptors in the dorsal striatum mediate stress-induced interference with negatively-reinforced instrumental escape behavior

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Abstract

Uncontrollable stress can interfere with instrumental learning and induce anxiety in humans and rodents. While evidence supports a role for serotonin (5-HT) and serotonin 2C receptors (5-HT_{2C}R) in the behavioral consequences of uncontrollable stress, the specific sites of action are unknown. These experiments sought to delineate the role of 5-HT and 5-HT_{2C}R in the dorsal striatum (DS) and the lateral/basolateral amygdala (BLA) in the expression of stress-induced instrumental escape deficits and exaggerated fear, as these structures are critical to instrumental learning and fear behaviors. Using in vivo microdialysis, we first demonstrate that prior uncontrollable, but not controllable, stress sensitizes extracellular 5-HT in the dorsal striatum, a result that parallels prior work in the BLA. Additionally, rats were implanted with bilateral cannula in either the DS or the BLA and exposed to uncontrollable tail shock stress. One day later, rats were with injected 5-HT_{2C}R antagonist (SB242084) and fear and instrumental learning behaviors were assessed in a shuttle box. Separately, groups of non-stressed rats received an intra-DS or an intra-BLA injection of the 5-HT_{2C}R agonist (CP809101) and behavior was observed. Intra-DS injections of the 5-HT_{2C}R antagonist prior to fear/escape tests completely blocked the stress-induced interference with instrumental escape learning; a partial block was observed when injections were in the BLA. Antagonist administration in either region did not influence stress-induced fear behavior. In the absence of prior stress, intra-DS administration of the 5-HT_{2C}R agonist was sufficient to interfere with escape behavior without enhancing fear, while intra-BLA administration of the 5-HT_{2C}R agonist increased fear behavior but had no effect on escape learning. Results reveal a novel role of the 5-HT_{2C}R in the DS in the expression of instrumental escape deficits produced by uncontrollable stress and demonstrate that the involvement of 5-HT_{2C}R activation in stress-induced behaviors is regionally specific.

Keywords

Uncontrollable stress; serotonin; anxiety; amygdala; learned helplessness; instrumental learning

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1. Introduction

Despite the prevalence of stress-related psychiatric disorders such as depression and anxiety, available therapeutic options have limited success (Turner et al., 2008, Turner and Rosenthal, 2008, Markou et al., 2009) and little is known about how stress leads to the expression of these disorders. One factor contributing to the behavioral repercussions of exposure to a traumatic event is the nature of the experience, i.e. controllable stressors have less of an impact than uncontrollable stressors (Maier, 1976, Shapiro et al., 1996, Crombez et al., 2008). In fact, lack of perceived behavioral control over a stressful event may be critical to the development of stress-related disorders (Morgan et al., 2001). Exposure to an uncontrollable stressor not only alters behavior at the time of the stressful experience but also alters responding to subsequent aversive stimuli. Patients exhibiting symptoms of stress-related psychiatric disorders report difficulty engaging in goal-directed/instrumental behavior (Tull et al., 2007), have enhanced behavioral and neural fear responses (Rauch et al., 2000) and display exaggerated fear in response to a mild aversive stimuli (Jovanovic et al., 2009). Similarly, rats exposed to an uncontrollable stressor later have difficulty learning to escape from a simple instrumental shuttle box escape task (Maier, 1973, Jackson et al., 1980) and display reduced social exploration (Christianson et al., 2008) and exaggerated fear conditioning (Maier, 1990, Rau et al., 2005). Understanding the neurobiological mechanisms involved in the expression of uncontrollable stress-induced behavioral impairments is vital to the development of more effective therapeutic treatments of stress-related psychiatric disorders.

Serotonin (5-HT) has long been implicated in depression and anxiety (Graeff et al., 1996, Anderson and Mortimore, 1999, Blier and de Montigny, 1999, Ninan, 1999, Blier, 2001), and evidence supports a crucial role for 5-HT and the 5-HT_{2C} receptor (5-HT_{2C}R) in the development and expression of the behavioral consequences of uncontrollable stress. Uncontrollable, relative to controllable, stress intensely activates 5-HT neurons in the dorsal raphe nucleus (DRN) (Grahn et al., 1999, Greenwood et al., 2003a, Amat et al., 2005, Takase et al., 2005). As a result of this hyperactivation, the DRN is left in a 'sensitized' state so that exposure to a subsequent aversive stimulus elicits a larger 5-HT response, both within the DRN (Maswood et al., 1998, Amat et al., 2005) and at DRN projection sites (Amat et al., 1998a, b, Christianson et al., 2010). DRN sensitization is critical for the expression of stress-induced behaviors because manipulations that increase DRN activity (Maier et al., 1995a) or rapidly increase 5-HT (Greenwood and Fleshner, 2008, Greenwood et al., 2008) mimic the instrumental-escape deficit and exaggerated fear produced by uncontrollable stress. Additionally, inhibition of the DRN abolishes the behavioral consequences of prior exposure to uncontrollable stress. (Maier et al., 1995c, Christianson et al., 2010).

The 5-HT_{2C}R has recently emerged as a relevant target in the treatment of neuropsychiatric disorders (Millan, 2005). Consistent with data implicating 5-HT_{2C}R activity in stress-related behaviors in humans (Murphy et al., 1989, Gatch, 2003, Millan et al., 2005, Olie and Kasper, 2007, Pjrek et al., 2007, Van Veen et al., 2007, Stein et al., 2008) and rodents (Bagdy et al., 2001, Burghardt et al., 2007, Heisler et al., 2007, Greenwood et al., 2008), we have observed that activation of the 5-HT_{2C}R with systemic administration of the 5-HT_{2C}R agonist CP809101 is sufficient to interfere with instrumental-escape learning and increase shock-elicited freezing (Strong et al., 2009). Moreover, systemic blockade of the 5-HT_{2C}R removes the deficit in escape learning (Strong et al., 2009) and social avoidance (Christianson et al., 2010) produced by prior uncontrollable stress. These data suggest that activation of 5-HT_{2C}R in specific projection sites of the DRN contribute to the expression of the behavioral consequences of uncontrollable stress. Indeed, Christianson et al. (2010) recently demonstrated that 5-HT_{2C}R activation in the basolateral amygdala (BLA) is

necessary and sufficient for the social avoidance produced by uncontrollable stress. The specific brain regions in which 5-HT_{2C}R activation contributes to the instrumental escape deficit and exaggerated fear produced by uncontrollable stress, however, remain elusive.

The dorsal striatum (DS), a critical region for instrumental learning (Balleine et al., 2009), and the BLA, a key structure in fear and anxiety behaviors (LeDoux, 2003, Shekhar et al., 2005), stand out as likely candidates for being the proximal mediators of the stress-induced instrumental-escape deficit and exaggerated fear. The DRN projects to both the DS (Yin et al., 2005, Corbit and Janak, 2010) and the BLA (Kim and Jung, 2006), where 5-HT can interfere with instrumental behaviors (Mitchell et al., 2007, Tanaka et al., 2009) and enhance fear (Campbell and Merchant, 2003), respectively. Twenty four hours after uncontrollable, but not equal controllable, stress, 2 brief foot shocks elicit a large increase in extracellular 5-HT in the BLA (Amat et al., 1998a). The current studies investigate whether a similar sensitized 5-HT release occurs in the striatum. Additionally, we utilized site-specific administration of selective 5-HT_{2C}R antagonist and agonist compounds to investigate the involvement of 5-HT_{2C}R activation in discrete brain regions, the DS and the BLA, in the deficit in instrumental escape learning and exaggerated fear produced by uncontrollable stress.

2. Experimental Procedures

2.1 Animals

Adult, male Fischer 344 rats (250–300g; N=161) were used in all behavioral experiments based on prior work optimizing stress-induced behaviors in this strain (Greenwood et al., 2003a, Greenwood et al., 2007, Strong et al., 2009). Similarly, adult, male Sprague-Dawley rats weighing 250–300g (N=21) were used in microdialysis experiments based on our prior work optimizing the procedure in this strain (Amat et al., 1998a, b, Amat et al., 2005, Christianson et al., 2010). In accordance with our prior work on the role of 5-HT_{2C}R in stress induced behaviors (Greenwood et al., 2008, Strong et al., 2009), all rats were individually housed in Nalgene Plexiglas cages (45 × 25.2 × 14.7 cm) at a temperature of 22° C. Lights were maintained on a 12:12 hour light/dark cycle. Animals had *ad libitum* access to food and water. All experimental protocols were approved by the University of Colorado Animal Care and Use Committee.

2.2 Stress Protocols

2.2.1 Controllable/Yoked-Uncontrollable Stress—The controllable/yoked-uncontrollable stress procedure was used in the microdialysis experiment and followed protocols known to produce differential effects of controllable vs. uncontrollable stress on brain and behavior (Amat et al., 1998a, Christianson et al., 2008, Christianson et al., 2010). Briefly, copper electrodes with electrode paste were wrapped around the tail and shocks were administered by a Precision Regulated Animal Shocker (Coulbourn Instruments, Allentown, PA). Rats were restrained in 14 × 11 × 17 cm (length by width by height) acrylic boxes with a wheel 7 cm wide and 9.5 cm in diameter located on the wall opposite the tail (Maier, 1990). Tail shocks were presented on a variable interval-60 second schedule (VI-60). Turning the wheel at the front of the chamber terminated each tail shock after a ¼ turn of the wheel. If the response was performed within 5 seconds of shock onset the response requirement doubled for the next trial until a maximum of 4 full wheel-turns was reached. If the response was made after 5 seconds but before 20 seconds the requirement was reduced by half and if no response was made by 30 seconds the shock terminated and the requirement was reset to ¼ turn. This procedure was used to ensure that the rat learned the correct response rather than reflexively turning the wheel. In order to maintain escape behavior the shock intensity was 1.0 mA for the first 33 trials, 1.3 mA for the following 33

trials and 1.6 mA for the remaining 34 trials (Amat et al., 2005). A second rat, in the uncontrollable stress condition, was yoked to each controllably stressed rat, but had no control over shock termination and received exactly equal duration tail shock. Animals in the home cage control group remained in their cages. Rats were returned to their home cages following the termination of shock. The entire stress procedure lasted 2 hours and occurred from 0800–1000, 24 hours prior to microdialysis.

2.2.2 Uncontrollable Stress—In the intra-DS and intra-BLA microinjection experiments, only uncontrollable stress was administered. This uncontrollable stress procedure produces exaggerated shock-elicited freezing and interference with shuttle escape behavior that is indistinguishable from that produced by the yoked stress treatment. Briefly, 100, 5 second tail shocks were presented on a VI-60 schedule while the rats were restrained in acrylic tubes (23.4 cm length $\times$ 7 cm diameter). Shock intensity began at 1.0mA and was increased to 1.5mA after 1 hour. The entire shock session lasted for 1 hour and 50 minutes. Five second shocks were used because the controllable stress treatment maintains shocks at an average duration of 5 seconds (data not shown). Stress was performed between 0700 and 0900. Rats were returned to their home cages after stress.

2.3 In vivo microdialysis and quantification of striatal 5-HT

A uni-lateral dialysis cannula guide was implanted into the striatum (tip of guide targeted at +0.5 A/P, $\pm$ 3.0 M/L, and -4.6 D/V) (Atallah et al., 2007, Corbit and Janak, 2010) under inhaled isoflourane anesthesia (3% in O₂). A stylet was placed in the cannula to maintain patency. After the recovery period, rats were assigned to controllable stress, yoked-uncontrollable stress or home cage treatment. Six hours after stress, rats were placed into microdialysis bowls and microdialysis probes (CMA 12, MW cut-off 20kD, 3mm) were inserted into the guide. Artificial cerebrospinal fluid (aCSF) was perfused through the probes using a CMA infusion pump at a flow rate of 0.2 μ l/minute. Rats were left alone in the dialysis room with food and water for at least 15 hours on a light cycle that matched the vivarium. After 15 hours, the flow rate increased to 1 μ l/minute for a 90 minute equilibration period, at which time 8 samples were then collected at 30 minute intervals. Sample collection began 24 hours after the beginning of stress. Immediately prior to the onset of the 4th baseline sampling period rats were gently moved onto a grid floor surrounded by Plexiglass walls located adjacent to the microdialysis bowl. The grid floor is identical to the grid floor of the shuttle box used in behavioral testing experiments. No change in extracellular 5-HT was observed during the 30 minute sampling period after the rats were moved from the dialysis bowls onto the grid floor (sample 4). At the onset of the 5th sampling period each rat received 2, 5 second foot shocks separated by 1 minute. This foot shock procedure is identical to the beginning of the fear/instrumental learning test. The rats remained on the grid floor for the remainder of the experiment as they would normally do during the shuttle box behavior test. At the beginning of the 5th sample each rat received 2, 5 second foot shocks (0.8mA) separated by 1 minute. This procedure is identical to the beginning of the fear/instrumental learning test described below. Here we sought to determine whether as a result of a sensitized DRN, rats in the IS group would exhibit greater extracellular 5-HT in the DS in response to foot shock than ES or HC rats.

2.3.1 5-HT Quantification—Dialysates were immediately placed in an -80°C freezer until analysis. Samples were analyzed with high-pressure liquid chromatography (HPLC) using methods previously described (Amat et al., 2005).

2.4 Surgery

Under ketamine (0.75 mg/kg i.p.) and medetomidine (0.5 mg/kg i.p.) anesthesia, bilateral cannula (26 gauge, Plastics One, Roanoke, VA) were implanted as previously described

(Greenwood et al., 2007). Cannula were aimed at either the DS: +0.5 A/P, $\pm$ 3.0 M/L, and -4.6 D/V from Bregma (Atallah et al., 2007) or the BLA: -3.0 A/P, $\pm$ 4.8 M/L, -6.2 D/V from Bregma (Christianson et al., 2010), based on the atlas by Paxinos and Watson (Paxinos, 1998). Atipamezole (0.5 mg/kg i.p.) was administered following surgery to reverse the effects of medetomidine. All rats were inoculated with .25 mL/kg (subcutaneous) penicillin (Combi-Pen, Agribios, St. Joseph, Missouri) immediately following surgery and allowed 7–10 days of recovery before any other manipulation occurred. Following experiment completion brains were sliced at 40 μ m and stained with Cresyl Violet for cannula placement verification.

2.5 Drug Microinjections

Rats were subjected to either uncontrollable stress or home cage conditions 24 hours prior to drug microinjections. On the day of behavioral testing, a microinjector extending 0.5mm (intra-DS) or 1.0mm (intra-BLA) beyond the tip of the guide cannula was inserted. Drug doses and injection volumes were based on prior work examining the effects of these compounds on stress-induced behaviors (Strong et al., 2009, Christianson et al., 2010). The selective 5-HT_{2C}R antagonist SB242084 (Tocris Bioscience, Ellisville, Missouri) was dissolved in 0.9 % sterile and sonicated to achieve a concentration of 50mM. SB242084 was administered intra-DS at a volume of 1.0 μ L/side and intra-BLA at a volume of 0.5 μ L/side (Strong et al., 2009, Christianson et al., 2010). The selective 5-HT_{2C}R agonist CP-809101 (Tocris Bioscience) was dissolved in 0.9 % sterile saline and gently warmed at 45 ° C for 10–15 minutes in a water bath to achieve a concentration of 6mM and was administered intra-DS at a volume of 1.0 μ L/side or intra-BLA at a volume of 0.5 μ L/side (Strong et al., 2009, Christianson et al., 2010). Microinjections were made 15 minutes prior to behavioral testing. In the experiment involving pretreatment of the antagonist in the DS, SB242084 was administered 5 minutes prior to CP809101. In these experiments, pretreatment with SB242084 in the DS blocked the effects of the agonist, CP809101 (Fig. 3D/3E); therefore, a SB242084 pretreated group was not included in the intra-BLA agonist study. Additionally, prior work by our lab has shown that the behavioral effects of these compounds also are selective when injected systemically (Strong et al., 2009).

2.6 Behavioral Testing

Fear and instrumental learning were assessed sequentially in shuttle boxes (50.8cm $\times$ 25.4cm $\times$ 30.48 cm, Coulbourn Instruments, Whitehall, PA) using procedures previously described (Maier, 1990, Greenwood et al., 2003a, Takase et al., 2005, Greenwood et al., 2008, Strong et al., 2009). At the beginning of each session, rats were placed into shuttle boxes and allowed to explore for 5 minutes. During this 5 minute period (pre-shock period), fear behavior was assessed using a sampling procedure in which each rat was scored every 10 seconds as either freezing, defined as an absence of all movement except for that required for respiration, or not freezing. Rats then received two 0.8 mA foot shocks delivered through both sides of the grid floor. Foot shocks were terminated when the rat fully crossed over to the opposite side of the shuttle box (fixed ratio 1, FR-1). The latencies to cross were recorded (FR-1 latencies). Following the second FR-1 trial, shock-elicited freezing was observed for 20 minutes. Shock-elicited freezing is a measure of fear conditioned to cues present in the shuttle box (Fanselow and Lester, 1988).

The post shock freezing period was followed by 25 fixed-ratio 2 (FR-2) escape trials. During FR-2 trials, rats were required to cross through the shuttle box door twice in order to terminate the foot shock (0.8 mA). An escape latency of 30 seconds was assigned if a correct escape response did not occur within 30 seconds, at which time the shock was terminated. Shocks occurred with an average inter-trial interval of 60 seconds and a single

test session lasted approximately 1 hour. All behavioral tests occurred between 0900 and 1200 by an experimenter blind to treatment condition of the animals.

2.7 Statistical Analysis

Data were analyzed with analysis of variance (ANOVA) with stress and drug conditions treated as between-subjects factors and dialysis sample, fear or escape trial blocks as repeated measures. Extracellular 5-HT was converted to percentage of baseline by dividing each sample by the average of the first 4 samples X 100. Pre-shock freezing scores were averaged into 1 pre-shock score and shock-elicited freezing into 10, 2 min blocks. The two FR-1 trials were averaged into a single FR-1 latency score. FR-2 escape latencies were averaged into 5 blocks of 5 trials each. Average post-shock freezing and average FR-2 escape latencies were analyzed with ANOVAs that included an additional group of off-site controls when appropriate. Significant main effects and interactions were followed by Fisher's protected least significant difference (PLSD) post hoc analysis. Results were considered significant when $p < 0.05$.

3. Results

3.1 Prior stressor controllability modulates extracellular 5-HT in the striatum in response to a subsequent stress challenge

Rats were exposed to no stress, controllable stress or uncontrollable stress treatment and 24 hours later striatal extracellular 5-HT concentrations were determined by microdialysis and HPLC. Microdialysis probe placement is shown in Figure 1A. All rats in the controllable stress group learned to escape the tail shock and quickly reached the maximum response criterion and maintained escape behavior as observed previously (Amat et al., 2005). Twenty four hours later, 2 brief foot shocks were delivered after 4 baseline samples and dialysis continued for 4 samples after shock. Only rats with dialysis probes within the striatum were included; the resulting sample sizes were Controllable Stress, $n = 8$; Uncontrollable Stress, $n = 7$; No Stress, $n = 6$. Extracellular 5-HT was converted to percentage of baseline by dividing each sample by the average of the first 4 samples X 100 (Fig. 1B). As in prior studies (Christianson et al., 2010) stress did not influence basal 5-HT levels (data not shown). A large, but transient increase in extracellular 5-HT was found in response to foot shock in rats treated with prior uncontrollable stress, but not with controllable stress or home cage control groups. A repeated measures ANOVA revealed a significant effect of Stress, ($F(2, 18) = 4.183$; $p = 0.032$), Sample, ($F(7, 126) = 4.834$, $p < 0.001$), and the Stress by Sample interaction, ($F(14, 126) = 2.118$; $p = 0.015$). During the foot shock sample, there was significantly greater extracellular 5-HT in the DS of rats treated with prior uncontrollable stress compared to rats treated with prior controllable stress or home cage treatments (Sample P1; $p < 0.01$). Groups did not differ from one another during any other sample time. Within group comparisons between baseline and post-shock samples revealed a significant elevation in 5-HT from baseline only in the uncontrollable stress group; the extracellular 5-HT during the shock sample was significantly greater than the baseline samples (Sample P1; $p < 0.05$). No other between-groups or within-groups comparisons reached significance; controllable stress and home cage control groups did not differ from each other or their baselines at any time.

3.2 Expression of the stress-induced escape deficit, but not exaggerated fear, is dependent upon 5-HT_{2C}R activation in the DS

The 5-HT_{2C}R antagonist SB242084 was injected into the DS and shock-elicited freezing (Fig. 2B/2C) and shuttle box escape (Fig. 2D/2E) behaviors were observed 15 minutes later. After exclusion of rats with misplaced cannula ($n=10$), group sizes were No Stress/Saline, $n=7$; No Stress/SB242084, $n=7$; Uncontrollable Stress/Saline, $n=7$; Uncontrollable Stress/

SB242084, $n=15$. Off-site controls were not included in this analysis because only one animal from the Uncontrollable Stress/SB242084 group had misplaced cannula. Cannula placements are shown in Figure 2A. Pre-shock freezing was minimal and did not differ between groups (Fig. 2B, pre-shock). Uncontrollable stress produced exaggerated shock-elicited freezing, regardless of intra-DS treatment with SB242084 (Fig. 2B). A significant main effect of uncontrollable stress on post-shock freezing ($F(1, 32) = 37.068$; $p < .0001$) was found, but neither the main effect of drug nor the interaction between stress and drug reached significance. Average post-shock freezing scores are shown in Figure 2C.

While no differences in FR-1 escape latency were observed across all groups and treatments (Fig. 2D, FR-1), uncontrollable stress did interfere with FR-2 escape learning behavior and this effect of uncontrollable stress was blocked by intra-DS administration of the 5-HT_{2C}R antagonist SB242084 (Fig. 2D). Reliable main effects of stress ($F(1, 32) = 29.310$; $p < .0001$), drug ($F(1, 32) = 10.066$; $p = .0033$), and time ($F(4, 128) = 3.638$; $p = .0077$) were found. Two-way interactions between drug and stress ($F(1, 32) = 9.907$; $p = .0036$), time and drug ($F(4, 128) = 5.532$; $p = .0004$), time and stress ($F(4, 128) = 2.614$; $p = .0383$), and the three-way interaction between time, drug and stress ($F(4, 128) = 5.322$; $p = .0005$) were all significant. Post hoc analysis revealed that during the first block of FR-2 trials, both stressed groups had significantly slower escape latencies compared to non-stressed groups. The stressed-saline group differed from all other groups for the remainder of the trials. No other group differences were significant. Average FR-2 latency scores are displayed in Figure 2E.

3.3 5-HT_{2C}R activation in the DS is sufficient to interfere with escape learning without enhancing fear

The 5-HT_{2C}R agonist CP809101 was administered 15 minutes prior to behavioral testing, 5 minutes after injection of the 5-HT_{2C}R antagonist SB242084. Cannula placements are shown in Figure 3A. After exclusion of rats with misplaced cannula ($n=4$), group sizes were Saline, $n=8$; CP809101, $n=8$; SB242084 + CP809101, $n=8$. Rats with misplaced cannula that received CP809101 ($n=2$) were included as off-site controls (Fig. 3C/3E). The effect of the 5-HT_{2C}R agonist on freezing is shown in Figure 3B. Average post-shock freezing scores are shown in Figure 3C. Pre-shock freezing was minimal and did not differ between groups (Fig. 3B, pre-shock). There was a reliable main effect of time on freezing behavior ($F(9, 189) = 15.656$; $p < .0001$), but neither the main effect of drug treatment nor the interaction between time and drug were significant. Freezing behavior of rats that received injections of CP809101 through misplaced cannula (off-site control) was indistinguishable from that of all other groups.

No differences in FR-1 escape latency were observed across all groups and treatments (Fig. 3D, FR-1). Activation of the 5-HT_{2C}R in the DS prior to behavioral testing was sufficient to interfere with escape learning, and this effect was blocked by pre-treatment with the 5-HT_{2C}R antagonist SB242084 (Fig. 3D). Significant main effects of drug treatment ($F(2, 21) = 4.464$; $p = .0242$) and time ($F(4, 84) = 9.587$; $p < .0001$) on escape behavior were found, but the interaction between time and drug was not significant. Average FR-2 latencies are displayed in Figure 3E. Off-site control rats behaved similarly to saline injected rats.

3.4 5-HT_{2C}R activation in the BLA is not necessary for stress-induced exaggerated freezing but contributes to the expression of the escape deficit

The 5-HT_{2C}R antagonist SB242084 was injected in the BLA 15 minutes prior to assessment of shock-elicited freezing (Fig. 4B/4C) and escape behavior (Fig. 4D/4E). After exclusion of rats with misplaced cannula ($n=9$), group sizes were No Stress/Saline $n=13$; No Stress/SB242084, $n=10$; Uncontrollable Stress/Saline, $n=15$; Uncontrollable Stress/SB242084,

n=10. Rats with misplaced cannula that received uncontrollable stress and SB242084 (n=5) were included as off-site injection controls. Cannula placements are shown in Figure 4A. No differences in pre-shock freezing were observed across all groups and treatments (Fig. 4B, preshock). A reliable main effect of stressor exposure on freezing behavior ($F(1, 44) = 13.688$; $p = .0006$) was found; however, no other main effect or interaction was significant. Average post-shock freezing scores are shown in Figure 4C. Freezing behavior of rats that were exposed to uncontrollable stress and injected with SB242084 through misplaced cannula (off-site control) was indistinguishable from that of all other stress groups.

While 5-HT_{2C}R blockade had no effect on conditioned fear, SB242084 reduced the stress-induced interference with escape behavior. No differences in FR-1 escape latencies were observed across all groups and treatments (Fig. 4D, FR-1), but significant main effects of drug ($F(1, 44) = 4.940$; $p = .0314$), stress ($F(1, 44) = 49.280$; $p < .0001$) and time ($F(4, 44) = 29.967$; $p < .0001$) were found. Significant two-way interactions were observed between time and drug ($F(4, 176) = 3.199$; $p = .0145$) and time and stress ($F(4, 176) = 13.969$; $p < .0001$). The three-way interaction between time, drug and stress ($F(4, 176) = 4.564$; $p = .0016$) was also significant. Post hoc analysis showed that uncontrollable stress increased escape latency during all trial blocks regardless of treatment with saline or SB242084. 5-HT_{2C}R blockade in the BLA reduced stress-induced interference with escape learning during the last 3 trial blocks, i.e. the stressed drug group was different from both the stressed saline group and both non-stressed groups. Average FR-2 latencies are displayed in Figure 4E. Off-site control rats behaved similarly to uncontrollable stressed, saline injected rats.

3.5 5-HT_{2C}R activation in the BLA is sufficient to enhance fear but not interfere with escape behavior

The 5-HT_{2C}R agonist CP809101 was administered 15 minutes prior to behavioral testing. Cannula placements are shown in Figure 5A. After exclusion of rats with misplaced cannula (n=16), group sizes were Saline, n=11 and CP809101, n=11. Rats with misplaced cannula that received CP809101 (n=12) were included as off-site injection controls (Fig. 5C/5D). The effect of the 5-HT_{2C}R agonist on freezing is shown in Figure 5B. Average post-shock freezing scores are shown in Figure 5C. Pre-shock freezing was minimal and did not differ between groups (Fig. 4B, pre-shock). Main effects of drug treatment ($F(1, 20) = 8.913$; $p = .0073$) and time ($F(9, 20) = 11.253$; $p < .0001$) on freezing behavior were significant but the interaction between time and drug was not significant. Freezing behavior of rats that received injections of CP809101 through misplaced cannula (off-site control) was indistinguishable from that of saline injected rats.

No differences in FR-1 escape latency were observed across all groups and treatments (Fig. 5D, FR-1). Activation of the 5-HT_{2C}R in the BLA prior to behavioral testing had no effect on escape behavior (Fig. 5D). A significant main effect of time ($F(4, 20) = 3.430$; $p = .0122$) on escape behavior was found, but neither the main effect of drug nor the interaction between time and drug were significant. Average FR-2 latencies are displayed in Figure 5E. Off-site control rats behaved similarly to saline injected rats.

4. Discussion

The ability to select the correct behavioral strategy in order to escape danger is vital to an organism's survival and can utilize instrumental learning mediated by the DS. These studies are the first to demonstrate that stress can interfere with instrumental escape behavior through a 5-HT_{2C}R-mediated mechanism in the DS. The present data indicate that 1) extracellular 5HT in the striatum during a mild stress challenge is sensitized by prior uncontrollable, but not controllable, stress, 2) 5-HT_{2C}R activation in the DS is necessary and

sufficient for the expression of stress-induced deficits in instrumental learning, and 3) although 5-HT_{2C}R activation in the BLA is not necessary for enhanced post-shock freezing produced by uncontrollable stress, 5-HT_{2C}R activation in the BLA is sufficient to enhance fear and contributes to the stress-induced interference with instrumental learning.

The current results are exciting because they are the first to demonstrate that extracellular 5-HT in the dorsal striatum in response to a mild stress challenge is sensitive to prior stressor controllability. Uncontrollable stress led to the expression of exaggerated shock-elicited freezing and poor escape performance. These behaviors are thought to be mediated by hyper-activation and sensitization of 5-HT neurons in the DRN and subsequent exaggerated 5-HT release in DRN projection sites (Maier et al., 1995c, Amat et al., 1998a, b, Maswood et al., 1998, Grahm et al., 1999, Christianson et al., 2010). The DRN projection sites known to respond to a sensitized DRN now include the DS, where 2 foot shocks elicited an increase in extracellular 5-HT 24 hours after uncontrollable, but not controllable, stress. The increase in extracellular 5-HT in the striatum could be the result of a sensitized DRN based on work demonstrating that 1) the majority of 5-HT input to the DS originates in the DRN (Imai et al., 1986, Waselus et al., 2006), and 2) electrical stimulation of the DRN (McQuade and Sharp, 1997) and other stressors such as forced swimming (Kirby et al., 1995) both increase extracellular 5-HT in the striatum. Although the use of two different rat strains in the dialysis and behavioral experiments (Sprague Dawley and Fischer 344, respectively) could negatively influence the ability to compare the two data sets, both Sprague Dawley and F344 rats display similar neurochemical (Grahm et al., 1999, Greenwood et al., 2003b, Greenwood et al., 2005, McDevitt et al., 2009) and behavioral (Greenwood et al., 2003a, Amat et al., 2005, Greenwood et al., 2005) responses to uncontrollable stress. We are therefore confident that the current observations apply equally to both strains.

5-HT_{2C}R activation in the DS is both necessary and sufficient for the deficit in instrumental escape behavior produced by uncontrollable stress. The escape deficits produced by the 5-HT_{2C}R agonist CP809101 were likely mediated by activation of DS 5-HT_{2C}R because the deficit was not observed in rats with misplaced cannula and was totally blocked by pre-treatment with intra-DS SB242084. The identified role of DS 5-HT_{2C}R in stress-induced interference with shuttle box escape is consistent with prior work indicating that stress interferes with instrumental tasks involving the DS in both humans (Schwabe and Wolf, 2011) and rodents (Dias-Ferreira et al., 2009) and that 5-HT_{2C}R in the DS are involved in stress-related behaviors. 5-HT_{2C}R knockout mice, for example, display a disruption in striatal function (Abdallah et al., 2009) and an anxiolytic phenotype in response to noxious stimuli (Heisler et al., 2007). Additionally, alterations of escape behavior produced by 5-HT_{2C}R manipulations in the DS occurred in the absence of changes in fear, supporting prior work (Maier, 1990, Maier and Watkins, 2005, Greenwood and Fleshner, 2008, Greenwood et al., 2010) indicating that the instrumental escape deficit produced by uncontrollable stress is separable from exaggerated fear and each occur via different mechanisms.

The current data extend our understanding of the repercussions of uncontrollable stress and indicate that the stress-induced shuttle box escape deficit is likely a consequence of interference with instrumental processes rather than simply a motor impairment. Although a motor deficit can certainly interfere with escape performance under specific laboratory conditions (Greenwood et al., 2008, Greenwood et al., 2010), the data suggest that the escape deficit measured using the current experimental protocol represent a stress-induced interference with instrumental learning processes. Importantly, the escape deficits produced by uncontrollable stress or intra-DS 5-HT_{2C}R agonist both develop over trials. This observation is consistent with the hypothesis of Jackson et al. (1980) that escape learning is predominantly an associative process and prior uncontrollable stress reduces sensitivity to

correct escape contingencies as the result of subsequent associative interference (Jackson et al., 1980, Jackson and Minor, 1988).

Although 5-HT_{2C}Rs in the DS are clearly involved in the escape deficit produced by uncontrollable stress, the mechanism remains unknown. Dopamine (DA) in the striatum supports the association of an action with a particular outcome (Lex and Hauber, 2010) suggesting that 5-HT_{2C}R activation in the DS could interfere with instrumental process by interfering with DA activity in the DS. 5-HT_{2C}R activation can decrease DA neurotransmission in the DS (Alex et al., 2005). Additionally, activation of 5-HT_{2C}R in the striatum can increase the activity of inhibitory interneurons (Blomeley and Bracci, 2005, Blomeley and Bracci, 2009), and systemic blockade of 5-HT_{2C}R activation enhances neuronal activity in the striatum (De Deurwaerdere et al., 2010). It is possible that sensitized extracellular 5-HT in the striatum during exposure to aversive stimuli 24 hours after uncontrollable stress could interfere with instrumental escape learning through a DA-dependent mechanism and/or a net inhibitory effect on striatal output as a consequence of 5-HT_{2C}R activation.

It is of interest to note that distinct sub-regions of the DS mediate different aspects of instrumental behavior (Balleine et al., 2009). Goal-directed learning is initiated in the medial DS (Yin et al., 2005, Corbit and Janak, 2010), whereas habit learning is thought to be stored in the lateral DS (Yin et al., 2006). The fact that escape learning in the current paradigm occurs rapidly in non-stressed rats supports a role for the medial DS in this early stage of learning. The lateral DS, however, has been recently implicated in the acquisition of simple procedural learning (Yin, 2010), and could thus play a role in the acquisition of the shuttle box escape contingency. The cannula in the current studies were aimed at the border between the medial and lateral DS, but no differences in behavior were observed after comparing effects of drug injections between rats with cannula placements towards the extremes of either the medial or lateral DS. While the involvement of 5-HT_{2C}R in the DS in the behavioral consequences of uncontrollable stress remains an important and novel observation, the role of 5-HT_{2C}R in the discrete sub regions of the striatum warrants further study.

The current experiments also investigate the involvement of 5-HT_{2C}R activation in the BLA in stress-induced behaviors. Extracellular 5-HT in the BLA is sensitized 24 hours following exposure to uncontrollable, relative to controllable, stress (Amat et al., 1998a, Christianson et al., 2010). The BLA is involved in fear behavior (LeDoux, 2003), is critical for shock-elicited freezing (Kim et al., 1993), and has been implicated in stress-related psychiatric disorders such as anxiety (Graeff et al., 1996, Walker et al., 2003) and PTSD (Brunetti et al., 2010). In rodents, 5-HT_{2C}R activation in the BLA can elicit anxiety-like behaviors in the open-field test (Campbell and Merchant, 2003), potentiated auditory fear conditioning (Burghardt et al., 2007), and reduced social exploration (Christianson et al., 2010). 5-HT_{2C}R activation is also necessary for ethanol-withdrawal-induced anxiety (Overstreet et al., 2006). Here we demonstrate that 5-HT_{2C}R activation in the BLA is sufficient to increase fear behavior (e.g. freezing) during shock-elicited fear conditioning, an effect similar to systemic administration of the 5-HT_{2C}R agonist (Strong et al., 2009). Despite these observations, 5-HT_{2C}R blockade in the BLA failed to prevent the expression of stress-induced exaggerated fear. This is consistent with the previous report that systemic administration of SB242084 also had no effect on this behavior (Strong et al., 2009). Importantly, the current data do not preclude a role for 5-HT in stress-induced exaggerated fear behavior as 5-HT is clearly necessary (Maier et al., 1993, Maier et al., 1995b, Maier et al., 1995c) and sufficient (Greenwood et al., 2008) for this stress-induced behavior.

5-HT_{2C}Rs in the BLA seem to be critical for the expression of some anxiety-like behaviors produced by uncontrollable stress but not others. Whereas, 5-HT_{2C}R activation in the BLA is necessary for the 5-HT-mediated potentiation of short-duration (3 min test), unconditioned, anxiety-like, behaviors such as stress-induced reductions in social exploration (Christianson et al., 2010), the current results suggest that BLA 5-HT_{2C}R may not be necessary for 5-HT-mediated sustained fear behaviors, such as the shock-elicited exaggerated freezing that is measured in the shuttle box test over a longer period (20 min). Indeed, while short-duration fear is mediated by the amygdala, the expression of sustained fear involves the bed nucleus of the stria terminalis (BNST) (Walker et al., 2003) and lesions of the BNST can prevent stress-induced exaggerated freezing (Hammack et al., 2004). It is possible, therefore, that while 5-HT_{2C}R activation in the BLA is sufficient to increase fear-like behavior per se, stress-induced exaggerated freezing may also require 5-HT activity in another region such as the BNST. Finally, a 5-HT receptor in the BLA other than the 5-HT_{2C}R may be responsible for exaggerated freezing following uncontrollable stress. The 5-HT_{2A} receptor is one candidate, as systemic 5-HT_{2A} blockade prevents stress-induced potentiation of acoustic startle (Jiang et al., 2009).

Surprisingly, while intra-BLA blockade of the 5-HT_{2C}R had no effect on stress-induced exaggerated freezing behavior, the antagonist did partially reduce the escape deficit produced by prior uncontrollable stress. These data could reflect a contribution of BLA 5-HT_{2C}R to the expression of the instrumental escape deficit. The DS receives inputs from the BLA (Kelley et al., 1982, Baldwin et al., 2000), and the BLA can play a role in acquisition of instrumental learning (Balleine et al., 2003, Wang et al., 2005, Lazaro-Munoz et al., 2010) and other forms of striatal-dependent learning (Graham et al., 2009). The BLA has been reported to drive striatal plasticity during the acquisition of instrumental learning (Popescu et al., 2007, Popescu et al., 2009); therefore, it is possible that exaggerated release of 5-HT in the BLA during escape testing (Amat et al., 1998a) could interfere with the BLA contribution to the processes supporting striatal-dependent instrumental learning through a mechanism involving the 5-HT_{2C}R. Additionally, activation of central amygdala (CeA)-dependent process can constrain the expression of instrumental behavior in the Sidman Avoidance task (Lazaro-Munoz et al., 2010). It is therefore possible that the partial 5-HT_{2C}R antagonist-mediated reduction in the stress-induced escape deficit was the result of unintended activation of 5-HT_{2C}R in CeA.

5. Conclusion

These experiments provide a more complete picture of the role of 5-HT_{2C}R in discrete brain regions, the DS and the BLA, in specific behavioral consequences of uncontrollable stress. The data reveal a novel role for the 5-HT_{2C}R in the DS in the expression of instrumental escape deficits produced by uncontrollable stress. The DS is implicated as an important DRN projection site mediating the effects of acute increases in 5-HT on learning deficits associated with uncontrollable stress and stress-related psychiatric disorders. 5-HT_{2C}R in the DS could, more generally, contribute to the expression of habit behavior produced by prior stressor exposure (Schwabe and Wolf, 2011) and interference with goal-directed behavior present in patients exhibiting symptoms of post-traumatic stress (Tull et al., 2007). Finally, the results identify the DS as a brain region at which drugs targeting the 5-HT_{2C}R may increase therapeutic efficacy, perhaps through modulation of DA-mediated learning mechanisms.

Highlights

- Prior uncontrollable stress sensitizes extracellular 5HT in the striatum
- 5-HT_{2C}R in DS are necessary for stress-induced instrumental learning deficits

- 5-HT_{2C}R activation in DS interferes with instrumental escape learning
- 5-HT_{2C}R activation in BLA enhances conditioned fear
- 5-HT_{2C}R in BLA contributes to stress-induced instrumental learning deficits

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Figure 1.

Prior stressor controllability modulates extracellular 5-HT in the striatum in response to a subsequent stress challenge. **(A)** Graphic reconstruction of 3-mm dialysis probe placements within the striatum. **(B)** Mean extracellular 5-HT in the striatum before and after 2 footshocks. Rats received prior controllable stress, uncontrollable stress or homecage control treatment. 5-HT is expressed as a percentage of the average of four baseline samples (B1 – B4). The 2, 5 sec, footshocks were delivered immediately after the 4th baseline sample (B4) and dialysis continued for 4 samples after shock (P1 – P4). Data represent group means $\pm$ SEM. * $p < 0.05$ relative to all other groups. Illustrations were adapted from Paxinos and Watson and are listed in relationship to bregma in mm.

Figure 2.

Expression of stress-induced escape deficits, but not exaggerated fear, is dependent upon 5-HT_{2C} receptor activation in the DS. Rats received uncontrollable stress or home cage treatment 24 hrs prior to behavioral testing. Fifteen minutes before behavioral testing, rats received intra-DS microinjections of either the 5-HT_{2C} receptor antagonist SB-242084 or saline. **(A)** Cannula tip placement within DS. **(B)** Freezing behavior presented in two minute blocks (pre-shock scores are not different and therefore overlap). **(C)** The mean percent shock elicited freezing for the entire 20-min observation period. **(D)** Shuttle box escape latencies for one block of 2 FR-1 trials (FR-1) and five blocks of 5 FR-2 trials (FR-2). The No Stress groups overlap. **(E)** The mean escape latency for all 25 FR-2 escape trials. Data

represent group means $\pm$ SEM. * $p < 0.05$ relative to no-stress groups; ϕ $p < 0.05$ from all other groups.

Figure 3.

5-HT_{2C} receptor activation in the DS is sufficient to interfere with shuttle box escape learning. Freezing behavior and shuttle box escape latencies were sequentially measured. Fifteen minutes prior to behavioral testing rats received intra-DS microinjections of either the 5-HT_{2C} receptor agonist CP-809101 or saline. In the antagonist+agonist group, intra-DS pretreatment with SB-242084 occurred 5 minutes prior to intra-DS administration of CP-809101. (A) Cannula tip placement within DS; off-site placements are denoted with an x. (B) Mean freezing behavior presented in two minute blocks (pre-shock scores are not different and therefore overlap). (C) The mean percent shock elicited freezing for the entire 20-min observation period. (D) Shuttle box escape latencies for one block of 2 FR-1 trials

(FR-1) and five blocks of 5 FR-2 trials (FR-2). **(E)** The mean escape latency for all 25 FR-2 escape trials. Data represent group means $\pm$ SEM. * $p < 0.05$ relative to all other groups.

Figure 4.

5-HT_{2C} receptors in the BLA contribute to the stress-induced interference with shuttle box escape, but not exaggerated freezing. Rats received uncontrollable stress or home cage treatment 24 hrs prior to behavioral testing. Fifteen minutes before behavioral testing, rats received intra-BLA microinjections of either the 5-HT_{2C} receptor antagonist SB-242084 or saline. (A) Cannula tip placement within BLA; off-site placements are denoted with an x. (B) Mean freezing behavior presented in two minute blocks (pre-shock scores are not different and therefore overlap). (C) The mean percent shock elicited freezing for the entire 20-min observation period. (D) Shuttle box escape latencies for one block of 2 FR-1 trials (FR-1)

and five blocks of 5 FR-2 trials (FR-2). (**E**) The mean escape latency for all 25 FR-2 escape trials.

Data represent group means $\pm$ SEM. * $p < 0.05$ relative to no-stress groups; θ $p < 0.05$ from stress/saline group.

Figure 5. 5-HT_{2C} receptor activation in the BLA is sufficient to increase fear behavior but had no effect on shuttle box escape behavior. Fifteen minutes prior to behavioral testing rats received intra-BLA microinjections of the selective 5-HT_{2C} receptor agonist CP-809101 or saline. (A) Cannula placement within the BLA; off-site placements are denoted with an x. (B) Mean freezing behavior presented in two minute blocks (pre-shock scores are not different and therefore overlap). (C) The mean percent shock elicited freezing for the entire 20-min observation period. (D) Shuttle box escape latencies for one block of 2 FR-1 trials (FR-1) and five blocks of 5 FR-2 trials (FR-2). (E) The mean escape latency for all 25 FR-2

escape trials. Data represent group means $\pm$ SEM. * $p < 0.05$ relative to saline or off-site control group.