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Selective Dopamine D₃ Receptor Antagonism Significantly Attenuates Stress-Induced Immobility in a Rat Model of Post-Traumatic Stress Disorder

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Abstract

Post-traumatic stress disorder (PTSD) is a debilitating psychiatric syndrome that occurs in individuals exposed to extremely threatening or traumatic events. In both animals and humans, dopamine (DA) function appears to be dysregulated in brain areas involved in the conditioned fear response(s) that underlie PTSD. In this study, we determined the effect of the selective DA D₃ receptor antagonists YQA14A (6.25, 12.5 and 25 mg/kg i.p.) and SB-277011A (6 mg/kg i.p.) on tone-induced fear (assessed by measuring freeze time) in a modified version of the single-prolonged stress (SPS) model of PTSD in adult male Sprague-Dawley rats. Rats pretreated with vehicle and then subjected to restraint stress, forced swim and random foot shock (SPS) in the presence of a distinctive tone, displayed a significantly increased tone-induced contextual freeze time and fecal pellet mass following re-exposure to the tone. Rats pretreated with a single i.p. injection of 6.25 or 12.5 mg/kg of YQA14 or 6 mg/kg of SB-277011A showed significantly attenuated contextual freeze time in the presence of the tone when tested 14 days after exposure to SPS. Overall, our results indicate that selectively antagonizing dopamine D₃ receptors significantly decreases freezing time caused by an environment previously associated with stress. If our findings can be extrapolated to humans with PTSD, they suggest that dopamine D₃ receptors may play a role in the pathophysiology of PTSD, and may have therapeutic utility for the clinical management of PTSD.

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Keywords

Post-traumatic stress disorder; dopamine; D₃ receptor antagonists; YQA14; SB-277011A; single prolonged stress model

Introduction

Post-traumatic stress disorder (PTSD) is a complex psychiatric disorder, occurring in approximately 7-9% of the general population (Kessler et al., 1995), that typically results from an traumatic experience in vulnerable individuals (Breslau et al., 1998). The illness is characterized by a constellation of symptoms, including altered fear responses, hypervigilance, hyperarousal, re-experiencing and avoidance behavior, flashbacks and dysphoric nightmares (Association, 2013). The traumatic event that initiates the development of PTSD can vary widely (Yehuda, 2004). Studies over the last two decades indicate that PTSD symptoms may result from a dysregulation of limbic brain areas involved in mediating fear, stress and anxiety (Hendriksen et al., 2014; Izquierdo et al., 2016; Lopresto et al., 2016). Data obtained from functional and structural imaging studies in PTSD patients suggest that the medial prefrontal cortex, amygdala, sublenticular amygdala and hippocampus play a role in the formation of symptoms (Etkin and Wager, 2007; Liberzon and Sripada, 2008; Stark et al., 2015). Meta-analyses suggest that patients with PTSD have hypoactivation of the dorsal and rostral cortices and the ventromedial prefrontal cortex (Hayes et al., 2012; Stark et al., 2015). It has been postulated that the development and maintenance of PTSD could result, in part, from dysregulation of brain DA circuits (Felmingham et al., 2014; Jatzko et al., 2006; Lee et al., 2016; Sailer et al., 2008).

While numerous studies have shown that DA neurons are involved in appetitive behaviors, salience, motivation, prediction of reward error, addiction, and learning and memory (Anderson and Pierce, 2005; Berridge and Robinson, 1998; Di Chiara and Imperato, 1988; Flagel et al., 2011; Schultz et al., 2017; Tsai et al., 2009), additional data indicate that DA neurons may mediate fear and aversive behaviors (Brischoux et al., 2009; de la Mora et al., 2010; Kwon OB, 2015; Lammel et al., 2014; Matsumoto and Hikosaka, 2009). Thus, environmental stressors can significantly alter the physiology of DA neurons in the brain. Exposure of rats to aversive or stressful stimuli significantly increases extracellular DA levels within limbic brain areas, including the amygdala, medial prefrontal cortex and nucleus accumbens (Abercrombie et al., 1989; Anstrom et al., 2009; Holly et al., 2015; Imperato et al., 1992). DA has been reported to play a role stress-induced reinstatement to drug-seeking behavior (Logrip et al., 2012; Mantsch et al., 2016; McFarland et al., 2004; Sinha, 2008) and DA neurons are known to be involved in stress-induced fear conditioning (de Oliveira et al., 2006; Fadok et al., 2009; Pezze and Feldon, 2004; Yoshioka M., 1996). Furthermore, DA receptor antagonists attenuate fear-conditioned behaviors in rats (Brandao et al., 2015; Inoue et al., 2000; Ponnusamy et al., 2005; Swain et al., 2008).

In addition to the more well-studies D₁ and D₂ receptors, the far less studied D₃ DA receptor may play a role in mediating stress-induced behaviors. Swain et al. (Swain et al., 2008) reported that i.p. administration of the highly selective D₃ receptor antagonist, SB-277011A,

produces a significant dose-dependent attenuation of the expression of conditioned fear (induced by scrambled foot shocks) in adult male Wistar rats. Place aversion precipitated by the stress of naloxone-induced morphine withdrawal is attenuated by SB-277011A in a dose-dependent manner (Rice et al., 2012). Stress-induced reinstatement of cocaine-seeking behavior in male Long-Evans rats is dose-dependently blocked by pre-stress administration of SB-277011A (3, 6, and 12 mg/kg i.p) (Xi et al., 2004). Importantly, bilateral microinjection of SB-277011A into the nucleus accumbens (NAc), but not into the dorsal striatum, significantly attenuates the stress-induced reinstatement of cocaine-seeking behavior (Xi et al., 2004), implicating a specific locus of action in the brain. In addition, *in vitro* studies indicate that dopamine D₃ receptor mRNA is present in limbic brain regions (i.e., amygdala, mesencephalic DA neuron cell bodies) that modulate responses to stressful internal and environmental stimuli linked to anxiety and fear (Heidbreder et al., 2005; Sokoloff and Le Foll, 2017). In humans, D₃ receptor mRNA expression closely resembles that of seen in the rodent brain (Landwehrmeyer et al., 1993; Suzuki et al., 1998).

There is an ongoing debate regarding the characteristics that constitute a valid animal model for PTSD. One such model of PTSD is the single prolonged stress (SPS) model, originally proposed by Liberzon et al. (Liberzon et al., 1997), a model possessing both face and construct validity (Yamamoto et al., 2009). In addition, specific neuroendocrinological abnormalities associated with PTSD are present in the SPS model, as well as dysregulation of GABAergic and glutamatergic pathways and heightened amygdalar activity (Schoner et al., 2017; Yamamoto et al., 2009). Thus, rats exposed to SPS exhibit enhanced startle response, hyperarousal, increase in freeze time and impaired fear extinction (Knox et al., 2012; Schoner et al., 2017; Wu et al., 2016; Yamamoto et al., 2009). With respect to the predictive validity of the SPS model, it has been reported that the anxiety-like behaviors shown by rats after undergoing SPS exposure are attenuated by chronic administration of the selective serotonin reuptake inhibitors (SSRIs) fluoxetine (Shafia et al., 2017) or paroxetine (Takahashi et al., 2006; Wang et al., 2008). This finding is consistent with clinical data suggesting that SSRIs have efficacy in the chronic treatment of PTSD (Kamo et al., 2016; Roberts, 2006; van der Kolk et al., 2007; Xu et al., 2011), although complete remission of symptoms occurs in only 20-30% of PTSD patients (Berger et al., 2009). Finally, it should be noted that the SPS model meets the criteria listed for PTSD in the Diagnostic and Statistical Manual on Mental Disorders V (Borghans and Homberg, 2015).

Given the localization of DA D₃ receptors in brain areas that are postulated to modulate responses to aversive stimuli and that antagonism of DA D₃ receptors mitigates stress-induced behaviors, we postulated that selective DA D₃ receptor antagonists might have efficacy in the SPS model of PTSD. We therefore used a modified SPS model, wherein we exposed animals to forced swim, restraint and random, inescapable footshocks in the presence of a distinctive tone and then retested the animals two weeks later in the presence of only the distinctive tone. The animals' freeze time to the tone was measured and taken as an index of re-elicited fear. The effects of acute i.p. administration of the highly selective DA D₃ receptor antagonists SB-277011A (Reavill et al., 2000) and YQA14 (Song et al., 2014; Song et al., 2012) in rats subjected to this modified SPS animal model of PTSD were assessed.

METHODS

Animals

Sixty-five male Sprague-Dawley rats (200 grams at the start of the SPS exposure, Taconic Farms, Germantown, NY) were used. The animals were individually housed and maintained on a 12-h light/dark cycle, with access to food and water *ad libitum*. All experimental procedures took place during the dark cycle. The experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Furman University, and were in accord with the Guide for the Care and Use of Laboratory Animals (National Research Council).

Modified Single Prolonged Stress (SPS)

We used a modified version of the SPS model, where the third part involved exposing animals to random, inescapable footshocks in the presence of a distinctive tone. The animals were exposed to the SPS procedure in 3 stages:

- 1) **Forced swim:** Each rat was individually placed in a plastic bucket (300 mm diameter, 440 mm height), filled with approximately 4 gallons of water (24°C) and forced to swim for 20 min. The animal was allowed 15 min to recuperate before proceeding to the restraint stage.
- 2) **Restraint stress:** Rats were restrained for 2 h inside a clear polyethylene tube (Braintree, Scientific), with only the tail protruding. A sliding gate was adjusted according to the size of the rat to ensure complete immobilization. Holes along the sides of the tube and a hole in the end of the tube allowed the rats to breathe freely.
- 3) **Inescapable foot shock:** After immobilization, each animal was placed individually in an operant chamber (285 mm width x 215 mm depth x 21 mm height) and subjected to 1.5-s, 0.5 mA foot shock delivered through a stainless steel grid floor by a shock generator-scrambler (Operant Conditioning Console model 81335: Lafayette Instrument Company, Lafayette, IN, USA), for 20 min. Foot shocks were delivered with intervals of 20 s. Following inescapable shock, each rat was left undisturbed in its home cage for 14 days.

Control for novelty

Since a tone by itself could conceivably induce freezing anxiety or immobility, the animals to freeze or become immobile, the SPS animals were individually placed in a transparent polyethylene chamber (960 mm width x 400 mm height x 240 mm depth) and exposed to a 2900 Hz tone at 85 decibels for 5 minutes. Freeze time and fecal pellet mass were determined and used as measures of anxiety.

Determination of the effect of the SPS on freeze time and fecal pellet mass

Animals were placed in a polyurethane container for 5 min. The tone was played once for 20 s and freeze times and fecal pellet mass were recorded. The next day, the same animals were exposed to SPS. Immediately following exposure to SPS, the animals were returned to their

home cages and undisturbed for 14 days. On day 14, the animals were re-exposed to the tone once for 20 s and for the next 5 min, freeze times and pellet mass were measured.

Effect of DA D₃ receptor antagonism on SPS-induced anxiety

We then determined the effect of the DA D₃ receptor antagonist YQA14 on freeze time. Animals were randomly assigned to five groups (SPS Control, Sham Control, 6.25, 12.5 or 25 mg/kg of YQA14). YQA14 dose was dissolved in 25% 2-hydroxypropyl- β -cyclodextrin (Sigma-Aldrich, St Louis, MO, USA). The doses of YQA-14 were chosen based on previous data indicating that these doses significantly inhibited DA D₃ receptor-mediated behavioral responses in rats (Song et al., 2014; Song et al., 2012). The animals in the control and drug groups were exposed to SPS and returned to their home cages and left undisturbed for 14 days. The animals in the Sham Control group did not undergo SPS. On day 14, animals in all five groups were administered 1 ml/kg i.p. of the appropriate treatment 30 min prior to being exposed to the distinctive tone, once, for 20 sec. Subsequently, over the next five minutes, freeze times were recorded.

We similarly determined the effect of another highly selective DA D₃ receptor antagonist, SB-277011A, on freeze time. Animals were randomly assigned to two groups (SPS Control or 6 mg/kg SB-277011A). The 6 mg/kg dose of SB-277011A was chosen based on previous data showing that this dose significantly decreased foot shock-induced reinstatement of cocaine-seeking behavior (Xi et al., 2004). SB-277011A was dissolved in 25% (w/v) of 2-hydroxypropyl- β -cyclodextrin (vehicle) and animals in the SPS Control group received only the vehicle. Both groups were administered 1 ml of drug solution per kg weight at time of injection. The experiment was conducted exactly as outlined in above for YQA-14.

Statistical Analysis

The data were analyzed using Student's t test and using one-way analysis of variance, followed by the Newman-Keuls inter-group analyses. The *a priori* significance level was $p < 0.05$.

Drugs

YQA14 was synthesized at the Beijing Institute of Pharmacology and Toxicology. SB-277011A was synthesized at MegaPharma Pharmaceuticals kft, Budapest Hungary. 2-Hydroxypropyl- β -cyclodextrin was purchased from Tocris-Cookson Chemicals (St. Louis, MO). All drug doses are expressed as the salt weight.

RESULTS

Validation of the SPS model

There was a significant increase (approximately 6-fold, Student's paired t test, $t = 3.68$, $p < 0.01$) in freeze time when rats were exposed to a 2900 Hz tone in a neutral area 14 days after SPS exposure, compared to the sham controls (Figure 1). In addition, there was a significant increase in fecal pellet mass in the SPS rats (approximately 5-fold, Student's paired t test, $t = 5.32$, $p < 0.0001$) compared to the sham controls (Figure 2). Thus, the modified SPS

procedure produced a robust and significant increase in the primary anxiety measure – freeze time.

Effect of the dopamine D₃ receptor antagonist YQA-14 on freeze time

One-way ANOVA indicates a significant treatment effect on the freeze time ($F(4,33) = 14.04$, $p < 0.001$, Figure 3). *Post hoc* analyses indicated that the freeze time in the SPS control rats (treated with vehicle on the test day) was significantly greater than that of sham controls (Newman-Keuls value = 7.56, $p < 0.0001$). *Post hoc* analyses indicated that freeze time was significantly decreased by 6.25 and 12.5 mg/kg i.p. of YQA14 (Newman-Keuls values of 7.08, $p < 0.0001$ and 8.56, $p < 0.0001$, respectively) given 30 minutes before exposing animals to the tone on the test day, compared to the SPS control group. The 6.25 and 12.5 mg/kg doses of YQA14 produced significantly greater decreases (Newman – Keuls values 4.87, $p < 0.01$ and 6.40, $p < 0.001$, respectively) in freeze time than 25 mg/kg of YQA14. It should be noted that freeze time was completely blocked by 12.5 mg/kg of YQA14. In contrast, there was no significant difference in freeze time between the SPS control group and the 25 mg/kg i.p. dose of YQA14 (Figure 3).

Effect of the DA D₃ receptor antagonist SB-277011A on freeze time

In order to validate our findings with YQA14, we assessed the effect of the highly selective DA D₃ receptor antagonist SB-277011A on tone-induced freeze time. Statistical analysis indicated that 6 mg/kg i.p. of SB-277011A significantly decreased freeze time compared to animals in the SPS control group (Student's unpaired t test, $t = 5.81$, $p < 0.0001$, Figure 4).

Discussion

One finding of this study is that all rats exposed to our modified SPS procedure expressed a significantly greater degree of conditioned fear (i.e. increased freeze or immobility time) when re-exposed to the tone that was uniquely paired with the concluding portion of the SPS procedure. The increased freeze time is believed to result from enhanced contextual and cued fear conditioning and impaired fear extinction and retention of extinction memories (Knox et al., 2012; Schoner et al., 2017; Yamamoto et al., 2009), which also occur in patients with PTSD (Bowers and Ressler, 2015). Our results are consistent with previous reports indicating that in the SPS model, animals have an increase in freezing time and display behaviors indicative of anxiety and stress (Borghans and Homberg, 2015; Yamamoto et al., 2009). Furthermore, our results indicate that our modifications of the SPS procedure replacing exposure to ether by foot shock stress, produced a robust and significant conditioned fear response. We saw a significant increase in contextual freezing in the SPS rats 14 days after exposure to the stressors. In addition, preliminary data suggest that the increase in freeze time in the SPS rats is present for up to 8 weeks (data not shown).

A second finding of the present work is that a single i.p. injection of 6.25 or 12.5 mg/kg of YQA14, given 30 mins before animals were re-exposed to the tone, significantly decreased the freeze time. In fact, the tone-induced freeze time was completely inhibited by 12.5 mg/kg i.p. of YQA14. To our knowledge, this study is the first to report that i.p. administration of 2 structurally distinct and highly selective DA D₃ receptor antagonists,

SB-277011A and YQA-14, were efficacious in our novel rat model of SPS and PTSD. *In vitro* data indicates that YQA-14 is highly selective for DA D₃ receptors versus D₁ ($> 10^5$), D₂ (5×10^5), D₄ ($> 10^5$) and D₅ receptors ($> 10^5$) (Song et al., 2012). Furthermore, YQA-14 does not produce D₂ receptor antagonist-like behaviors (e.g. inhibition of locomotor activity, catalepsy, a left shift in electrical brain stimulation reward rate frequency curves (Song et al., 2014). One could posit that the YQA14-induced inhibition of freeze time was due to elicitation of stimulatory motoric effects. This is unlikely, as repeated i.p. administration of 12.5 or 25 mg/kg of YQA14 does not significantly affect basal locomotor activity in rats (Song et al., 2014). One could argue that YQA14 produces appetitive or rewarding effects, thereby decreasing the aversive response to the tone on the test day, thus possibly decreasing the magnitude of the freeze time. However, i.p. administration of either 12.5 or 25 mg/kg of YQA14 does not produce appetitive effects in either naïve rats or in rats previously trained to self-administer cocaine and does not significantly alter brain stimulation reward thresholds. The decrease in the contextually enhanced freeze time could have resulted from YQA14 impairing memory and thus decreasing the association between the tone and the SPS exposure. However, the systemic administration of selective DA D₃ receptor antagonists has been shown to enhance social memory and attenuate scopolamine-induced memory deficits (Joyce and Millan, 2005; Laszy et al., 2005; Nakajima et al., 2013). Finally, it is also unlikely that the YQA14-treated rats exhibited lower freeze times due to an inability to perceive the associated tone as a result of drug-induced sensory deficits, as YQA14 (6.25, 12.5 and 25 mg/kg i.p.) does not block contextual (light and tone)-induced self-administration of cocaine in rats (Song et al., 2014). We do not know why the 25 mg/kg dose of YQA14 did not significantly decrease SPS-induced freeze time. We believe that this is unlikely to have been due to interaction with D₂ receptors as this dose of YQA14 does not produce dopamine D₂ receptor-mediated behaviors (i.e. changes in locomotor activity and appetitive drug seeking behaviors) (Song et al., 2014). It is possible that at 25 mg/kg, YQA14 interacts with other receptors mitigate its DA D₃ receptor antagonist efficacy, although this dose of YQA14 does attenuate contextual cue-triggered drug-seeking mediated by DA D₃ receptor antagonism (Song et al., 2014).

Acute i.p. administration of 6 mg/kg of SB-277011A, a highly selective DA D₃ receptor antagonist, prior to exposure to the cue tone on the test day, significantly decreased freeze time in rats. SB-277011A's systemic efficacy in rats appears not to be due to DA D₂ receptor antagonism because, unlike DA D₂ receptor antagonists, SB-277011A does not produce: 1) catalepsy (up to 78.8 mg/kg, (Reavill et al., 2000) 2) alterations in basal locomotor activity (up to 41.3 mg/kg, (Reavill et al., 2000); 3) significant elevation of serum prolactin levels (up to 93.3 mg/kg, (Reavill et al., 2000); 4) inhibition of amphetamine-induced locomotor activity (Reavill et al., 2000); 5) antagonism (up to 93.3 mg/kg) of quinelolane-induced decrease in striatal DA levels (Reavill et al., 2000) (which is due to activation of dopamine D₂ receptors); 6) rightward shifts in electrical brain-stimulation reward rate frequency function (up to 12 mg/kg i.p., (Vorel et al., 2002); 7) conditioned place aversion up to 24 mg/kg, (Rice et al., 2012) or 8) increased cocaine intake in rats under a fixed ratio 1 or 2 schedule of reinforcement (up to 24 mg/kg, (Xi, 2005). Furthermore, *in vitro* data indicate that SB-277011A has 100-fold selectivity for the dopamine D₃ receptor, as well as 66 other receptors, ion channels and enzymes (Reavill et al., 2000). As with

YQA-14, SB-277011A's decrease in freeze time is not due to: 1) memory impairment, as SB-277011A enhances memory (Joyce and Millan, 2005; Nakajima et al., 2013); 2) increased locomotor activity (Reavill et al., 2000); 3) sensory deficits, as SB-277011A does not block contextual (light and tone) cue-induced intake of 0.75 mg/kg of cocaine in rats under a fixed ratio 1 schedule of reinforcement (Xi, 2005); 4) appetitive or rewarding efficacy, as SB-277011A self-administration is not maintained in naïve rats or in rats with a history of cocaine self-administration (Xi, 2005).

Antagonism of DA D₃ receptors by SB-277011A significantly decreases: 1) expression of conditioned fear (Swain et al., 2008); 2) foot shock-induced reinstatement of i.v. cocaine self-administration (Xi et al., 2004) and 3) conditioned place aversion produced by naloxone-precipitated withdrawal in morphine-treated rats (Rice et al., 2012). These data, although obtained from non-PTSD models, are similar to our finding in the SPS model as they suggest that antagonism of DA D₃ receptors attenuates conditioned stress/aversive stimuli. Interestingly, both YQA-14 and SB-277011A significantly decreased contextual freeze time after a single administration. This is in contrast to fluoxetine, paroxetine or D-cycloserine, which require repeated administration to attenuate enhanced contextual freezing in SPS rats (Takahashi et al., 2006; Wang et al., 2008; Yamamoto et al., 2009).

In conclusion, the results the present study indicate that acute i.p. administration of SB-277011A or YQA14 prior to re-exposure to stress-associated contextual environmental cues, significantly attenuates the freeze time of rats exposed to single prolonged stress. The effects of SB-277011A and YQA14 in the SPS model is most likely due to antagonism of DA D₃ receptors in the brain. It is important that the efficacy of selective D₃ receptor antagonists be tested in other animal models of PTSD, including those that involve traumatic brain injury. Furthermore, it remains to be determined if the efficacy of these drugs in the SPS model is maintained after chronic administration. It is also critical to determine if selective D₃ receptor antagonists significantly attenuate the increased fast negative feedback of the hypothalamic-pituitary-adrenal, increased acoustic startle response and failure to retain extinction memory.

Finally, it is well established that a high degree of comorbidity between PTSD and drug addiction exists. Indeed, patients diagnosed with PTSD are significantly more likely to abuse drugs and become addicts (Kessler et al., 1995; Pietrzak et al., 2011; Seal et al., 2011). There are considerable data indicating that highly selective DA D₃ receptor antagonists are efficacious in decreasing the appetitive and reinforcing actions of cocaine, methamphetamine, nicotine, heroin and ethanol in rats (Heidbreder et al., 2005; Heidbreder and Newman, 2010; Sokoloff and Le Foll, 2017). Thus, if the present results can be translated to humans, they suggest that highly selective DA D₃ receptor antagonists may be efficacious in attenuating PTSD symptoms, while simultaneously decreasing the likelihood of drug abuse and addiction.

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Timeline

Figure 1:
Timeline for PTSD Experiments

Figure 2:

SPS group freeze times pre- and post-SPS. There was a significant difference between pre- and post-SPS freeze time (**Student's paired t test; $p < 0.01$).

Effects of SPS on Amount of Fecal Pellets

Figure 3:

SPS group fecal pellet mass pre- and post-SPS. There was a significant difference between pre- and post-SPS testing fecal pellet mass within the SPS group (Student's paired t test; $p < 0.0001$).

Effects of YQA 14 on Freeze Time Post SPS Exposure

Figure 4:

Effects of YQA-14 on post - SPS freeze times. There was a significant difference in freeze time between test groups post-SPS (One-way ANOVA; $p < 0.001$). YQA-14 at 6.25 or 12.5 mg/kg significantly attenuated post-SPS freeze time. **** $p < 0.0001$, compared to SPS control group.

Effects of SB-277011A on Freeze Time Post SPS Exposure

Figure 5:

Effect of SB277011A on post-SPS freeze time. There was a significant difference in freeze time between test groups post-SPS (Student's t test, $p < 0.0001$).