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D-578, an orally active triple monoamine reuptake inhibitor, displays antidepressant and anti-PTSD effects in rats.

Aloke K. Dutta,*1 Soumava Santra1, Arman Harutyunyan[2], Banibrata Das,1 Michael Lisieski,[2] Liping Xu1, Tamara Antonio3, Maarten E.A. Reith3, Shane A. Perrine2

1. Wayne State University, Department of Pharmaceutical Sciences, Detroit, MI 48202

2. Department of Psychiatry and Behavioral Neurosciences, School of Medicine, Wayne State University

3. New York University, Department of Psychiatry, New York, N.Y. 10016.

Corresponding author:

Aloke K. Dutta, Ph.D., Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy & Health Sciences, Wayne State University, Detroit, MI, 48202.

Tel: 1-313-577-1064, Fax: 1-313-577-2033, e-mail: adutta@wayne.edu

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Abstract

Significant unmet needs exist for development of better pharmacotherapeutic agents for major depressive disorder (MDD) and post-traumatic stress disorder (PTSD) as the current drugs are inadequate. Our goal in this study is to investigate behavioral pharmacological characterization of a novel triple reuptake inhibitor (TRI) D-578 which exhibits nanomolar potency at all three monoamine transporters (Ki; 16.2. 16.2, 3.23 nM, and 29.6, 20.6, 6.10 nM for the rat brain and cloned human dopamine, serotonin and norepinephrine transporters, respectively) and exhibited little to no affinity for other off-target CNS receptors. In a rat forced swim test, compound D-578 upon oral administration displayed high efficacy and not stimulating in locomotor behavior. The effects of D-578 and paroxetine were next evaluated in a rat model for traumatic stress exposure - the single prolonged stress (SPS) model - which has been shown to have construct, predictive, and behavioral validity in modeling aspects of PTSD. Our results show that SPS had no effect on the acquisition of conditioned fear, but impaired extinction learning and extinction retention of fear behavior compared to sham treatment. D-578, but not paroxetine, attenuated the extinction and extinction-retention deficit induced by SPS. These findings suggest that D-578 has greater efficacy in normalizing traumatic stress-induced extinction-retention learning in a model for PTSD compared to paroxetine. Overall these results suggest that D-578, in addition to producing a robust and efficacious antidepressant effect, may attenuate maladaptive retention of fearful memories and support further testing of this agent for the pharmacotherapy of depression and PTSD.

Key Words: Major depressive disorder; Post-traumatic stress disorder; Triple reuptake inhibitors; Pharmacotherapy; selective serotonin reuptake inhibitor; Pharmacotherapy

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1. Introduction

Both major depressive disorder (MDD) and post-traumatic stress disorder (PTSD) are debilitating illnesses affecting greater than 15-20% of the population in the United States (Kessler et al., 2005). It is believed that 20% of all individuals suffer from a MDD at least once in their lifetime. Depression in some cases may lead to life threatening acts and suicide (Pompili, 2019). PTSD is a chronic and debilitating illness which is caused by exposure to traumatic events (Benjet et al., 2016). It has been estimated by epidemiologic studies that PTSD exists in ~8% of the US population, and in up to 30% of certain at-risk groups (Breslau et al., 1991; Kessler et al., 1995).

Over the years different classes of antidepressants have been developed. However, a significant unmet need persists for improved therapy, as large numbers of people with clinically-diagnosed depression, an estimated 15-30%, are still refractory to the current existing therapies. Moreover, a number of people suffer from relapse after treatment with current therapies (Rush et al., 2006). It is evident from the preclinical and clinical studies that dopamine plays an important role in the pathophysiology and treatment of depression (Dunlop and Nemeroff, 2007). However, one of the missing components in the current pharmacotherapy of depression is a dopaminergic activity in spite of existing evidence pointing to the presence of a strong dopaminergic component in MDD (Dunlop and Nemeroff, 2007).

Similar to depression, there is a great unmet need in the treatment of PTSD. The two FDA-approved pharmacotherapeutics currently used for PTSD are the selective serotonin reuptake inhibitors (SSRIs) paroxetine and sertraline (Bandelow et al., 2012). However, these agents are limited by their efficacy with low effect size (ES = 0.23, 95% CI,

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0.12–0.33) and do not work for a significant number (30%) of people and produce side effects (Hoskins et al., 2015; Ipser and Stein, 2012; Marks et al., 2008b; Thomas and Stein, 2017). Besides serotonergic neurotransmission, noradrenergic and dopaminergic pathways have been implicated in PTSD symptomatology. Various studies indicated that PTSD patients suffer from reward deficits with social anhedonia (Nawijn et al., 2015). It is conceivable that augmentation of dopaminergic activity could address these untreated symptoms related to anhedonia associated reward functioning in the PTSD population.

The concept behind the development of triple uptake inhibitors (TRIs) that target the serotonin, norepinephrine, and dopamine transporters (SERT, NET, and DAT) concurrently, has gained support based on the evidence from preclinical and clinical studies for the treatment of depression. It has also been predicted that agents potentiating all three monoaminergic systems might selectively benefit PTSD patients with the dysphoric/ anhedonic phenotype (Friedman and Bernardy, 2017).

In our work towards the development of TRIs, we embarked on designing and synthesizing novel pyran based asymmetric compounds (Dutta et al., 2014; Santra et al., 2012; Zhang et al., 2006; Zhang et al., 2005). Our structure-activity relationship (SAR) studies led to the identification of an earlier lead compound D-473 which showed high efficacy in a rat model of depression (Dutta et al., 2014). In this manuscript, we report development of an orally active TRI, D-578 and its pharmacological characterization in a rat forced-swimming test, locomotor activity assay and conditioned fear behavior in a rodent model of PTSD.

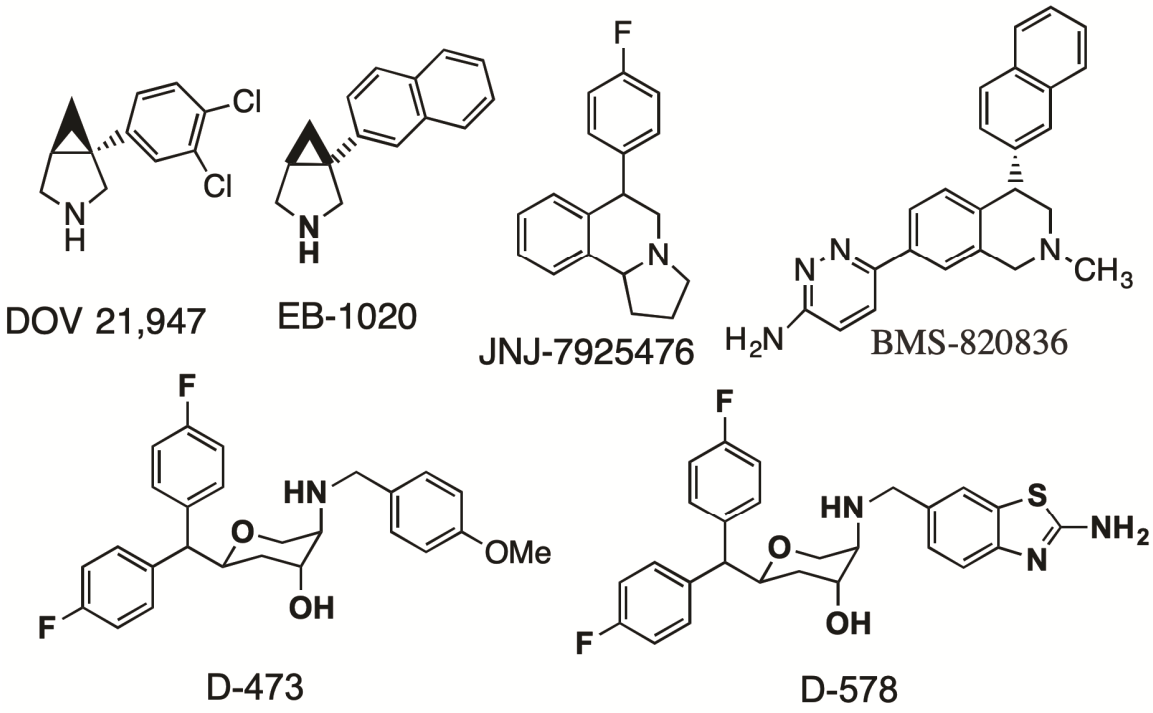


Figure 1

Fig. 1: Molecular structures of triple reuptake inhibitors.

2. Materials and Methods

2.1 Reagents and drugs

Synthesis of D-578 (2*S*,4*R*,5*R*)-5-(((2-aminobenzo[*d*]thiazol-6-yl)methyl)amino)-2-(bis(4-fluorophenyl)methyl)tetrahydro-2*H*-pyran-4-ol are shown in Scheme 1 & 2 and is described below. This compound was synthesized by asymmetric synthetic pathway as developed by us (Santra et al., 2012; Zhang et al., 2005). [Ring 2,5,6-^{3}H]dopamine (45.0 Ci/mmol) and [1,2-^{3}H]serotonin (27.9 Ci/mmol) were obtained from Perkin-Elmer (Boston, MA, U.S.A). Imipramine, desipramine, fluoxetine, and reboxetine, and GBR 12909 dihydrochloride (1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine) were purchased from SIGMA-ALDRICH (St. Louis, MO, U. S. A). All reagents used to perform

microdialysis (i.e. for aCSF) and saline solutions, as well as LC-MS reagents were purchased from Sigma Chemical Co (St Louis, MO, U. S. A.).

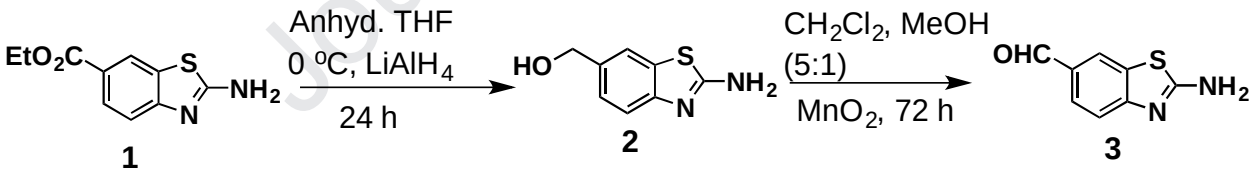
2.2. Animals

Male Sprague-Dawley rats (200-225 g) were purchased from Harlan (Indianapolis, IN, U. S. A.). Animals were group housed in a temperature and humidity controlled room with 12 h light /dark cycle with lights on at 6 AM. Food and water were accessible to animals freely throughout the duration of study except during behavioral testing. All testing occurred during the light component of the light/dark cycle. All animal procedures were reviewed and approved by Wayne State University Institutional Animal Care and Use Committee consistent with AALAC guidelines.

2.3 Synthesis of D-578

Synthesis of 2-aminobenzo[d]thiazole-6-carbaldehyde

Scheme 1



Commercially available ester **1** (1.0 g, 4.5 mmol) was dissolved in anhydrous THF and the RB flask was cooled to 0 ^{o}C. Then $LiAlH_4$ was added slowly and the flask was stirred at 0 ^{o}C for additional 10 min. The flask was then stirred at room temperature for 24 h. The reaction was then cooled, quenched with methanol, NH_4Cl Rochelle's salt and diluted with ethyl acetate (10 ml). The organic layer was separated and the aqueous layer was extracted with additional ethyl acetate (3 × 10 ml). The organic layers were combined,

dried over Na_2SO_4 and concentrated under vacuo on a rotary evaporator to obtain a yellow solid. The crude product was purified via gradient silica gel column chromatography using a mixture of CH_2Cl_2 and methanol (100:1 to 5:1) to obtain the desired alcohol as yellow solid **2** (420 mg, 52%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.57 (d, *J* = 0.9 Hz, 1 H), 7.35-7.46 (m, 1 H), 7.25 (dd, *J* = 8.2, 1.8 Hz, 1 H), 6.45 (br s, 1 H), 4.53 (s, 2 H), 4.0 (br s, 1 H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 167.6, 150.5, 134.9, 130.6, 125.1, 119.4, 117.7, 64.2,.

The alcohol **2** (420 mg, 2.33 mmol) was dissolved in a mixture of CH_2Cl_2 and methanol (5:1) and MnO_2 (81 mg, 9.32 mmol). The mixture was then stirred at room temperature for 48 h following which additional amount of MnO_2 (40 mg) was added after TLC showed incompletion of the reaction. The mixture was stirred for additional 24 h and then filtered through a whatman filter paper (grade 8) and the filtrate was concentrated under vacuo on a rotary evaporator to obtain the desired aldehyde as a yellow solid **3** (400 mg, 95%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.81 (s,1 H), 8.01 (d, *J* = 1.5 Hz, 1 H), 7.70 (dd, *J* = 8.2, 1.5 Hz, 1 H), 7.42 (d, *J* = 8.5 Hz, 1 H), 3.70 (br s, 1 H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 191.3, 164.5, 157.1, 130.3, 128.7, 122.6, 118.0

Synthesis of (2S,4R,5R)-5-(((2-aminobenzo[d]thiazol-6-yl)methyl)amino)-2-(bis(4-fluorophenyl)methyl)tetrahydro-2H-pyran-4-ol**, D-578**

Scheme 2

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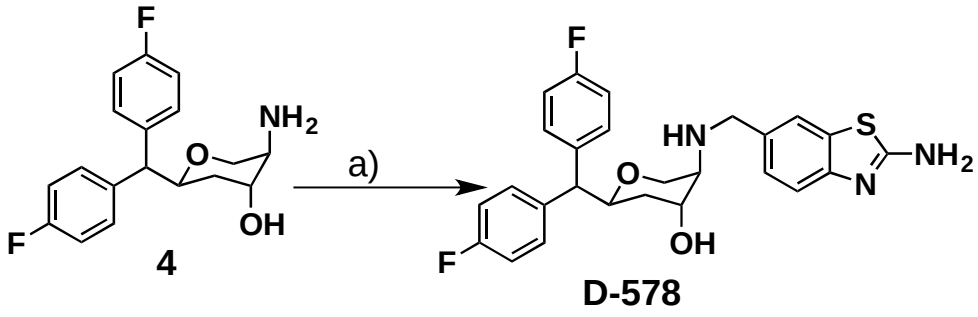

Reagents and conditions: a) RCHO, 1,2-dichloroethane, MeOH, AcOH, Na $(OAc)_3BH$, rt.

Amine **4** (60 mg, 0.19 mmol), which was synthesized by following our published procedure (Santra et al., 2012), was reacted with 2-aminobenzo[d]thiazole-6-carbaldehyde (37 mg, 0.21 mmol), glacial acetic acid (16 µl, 0.22 mmol), and $Na(OAc)_3BH$ (113 mg, 0.38 mmol) in a mixture of 1,2-dichloroethane (4.5 ml) and methanol (1.5 ml). The residue was purified by gradient silica gel column chromatography using a mixture of dichloromethane and methanol (100:1 to 6:1) to afford corresponding compound **(D-578)** as light yellow solid syrup (45 mg, 50%)

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.54 (s, 1 H), 7.30 (d, *J* = 8.2 Hz, 1 H), 7.20 (dd, *J* = 8.5, 6.1 Hz, 2 H), 7.16 (dd, *J* = 8.2, 1.1 Hz, 1 H), 7.09 (dd, *J* = 8.6, 5.5 Hz, 2 H), 6.79-6.96 (m, 4 H), 4.38 (dt, *J* = 10.1, 2.4 Hz, 1 H), 4.06 (s, 1 H), 3.98 (d, *J* = 13.1 Hz, 1 H), 3.92 (dd, *J* = 12.8, 1.5 Hz, 1 H), 3.82-3.92 (m, 1 H), 3.79 (d, *J* = 12.5 Hz, 1 H), 3.34 (br s, 2 H), 2.64 (br s, 1 H), 1.56-1.71 (m, 1 H), 1.35-1.47 (m, 1 H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 168.0, 162.4, 162.3, 160.5, 160.4, 151.4, 137.4, 137.2, 131.3, 129.7, 129.6, 126.9, 121.3, 118.2, 115.4, 115.3, 115.2, 115.0, 73.9, 64.5, 62.9, 55.8, 54.7, 50.2, 32.6.

$[\alpha]^{25}_D$ = (-) 30.6°, *c* = 1 in MeOH. The product was converted into the corresponding hydrochloride salt; mp: 210-215 °C. Anal. Calcd for [$C_{26}H_{25}F_2N_3O_2S.3HCl.3H_2O$] C, H, N.

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Mesylate salt of D-578 was used for in vivo forced swimming study.

2.4 Inhibition of uptake by monoamine transporters in brain synaptosomes and heterologous cells

Inhibition of substrate uptake by monoamine transporters in synaptosome-enriched fractions from rat brain was measured as we described previously (Santra et al., 2012; Santra et al., 2015; Sharma et al., 2014). DAT in rat striatum and NET in rat cerebral cortex was monitored with [3H]DA ([ring 2,5,6-3H]dopamine (45.0 Ci/mmol, Perkin-Elmer, Boston, MA, USA), and SERT in rat cerebral cortex with [^{3}H]5-HT ([1,2-^{3}H]serotonin (27.9 Ci/mmol, Perkin-Elmer). Inhibition of substrate uptake by cloned human transporters was measured with stably transfected human embryonic kidney (HEK) 293 cells as described in our previous work (Dutta et al., 2014; Reith et al., 2012). The cell lines were obtained and used in uptake assays as described in the same papers (Dutta et al., 2014; Reith et al., 2012), again with [^{3}H]DA for DAT and NET and [3H]serotonin for SERT.

The use of [3H]DA instead of {3H]NE greatly reduced nonspecific uptake values for rat synaptosomes; additionally, it is well established that DA is an excellent substrate for NET, both in cells with cloned transporters (Buck and Amara, 1994; Gu et al., 1994; Pacholczyk et al., 1991) and in rat tissue (Masserano et al., 1994; Snyder and Coyle, 1969; Williams and Steketee, 2004). Control experiments with various test compounds did not show significant differences between potencies measured with [3H]DA and [3H]NE.

Drug stocks contained an additional 0.01% (w/v) bovine serum albumin in order to reduce absorption of drug to the walls of the assay plates. At least five triplicate concentrations of each test compound were studied, spaced evenly around the IC_{50} value which was converted to K_i with the Cheng-Prusoff equation (Cheng and Prusoff, 1973)

With respective K_m values and total free [^{3}H]ligand concentrations, the conversion factors (multipliers applied to IC_{50} for calculating K_i) were > 0.84.

2.5. Evaluation of broad receptors activity

Compound D-578 was characterized in several CNS receptor binding assays to assess selective and specific interactions in the CNS. The assays were carried out generously by the National Institute of Mental Health's Psychoactive Drug Screening Program (NIMH PDSP). The NIMH PDSP is directed by Bryan L. Roth MD, PhD at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscol at NIMH, Bethesda MD, USA (http://pdsp.med.unc.edu/).

Compound D-578 was first evaluated in primary binding assays targeting, among others, cloned human dopamine receptor subtypes, serotonin receptor subtypes, α– and β-adrenergic receptors, and opioid receptors. The description of all receptors targeted and corresponding radioligand used, is provided in Table 3. The default concentration for primary binding experiments was 10 μM. When observed inhibition under those conditions was greater than 50%, secondary assays were conducted with full concentration curves of the test compound in order to calculate the Ki value for inhibition. For experimental details please refer to the PDSP web site <http://pdsp.med.unc.edu/> and click on "Binding Assay" or "Functional Assay" on the menu bar. We have also added a brief description of assay protocols in the supplementary material section.

2.6. hERG Binding Assay

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hERG channel inhibition activity was carried out by following the Thallium Flux Assays by using the FluxOR Potassium Ion Channel Assay kit (Invitrogen). The assay was carried out by the National Institute of Mental Health's Psychoactive Drug Screening Program, The protocol of the assay is posted on the PDSP website (https://pdspdb.unc.edu/pdspWeb/content/PDSP%20Protocols%20II%202013-03-28.pdf and was also published in detail (Huang et al., 2010).

2.7. Forced swimming test in rats after oral administration

The experiment was carried out in the same way as described in our previous publications according to the Porsolt protocol (Dutta et al., 2011; Porsolt et al., 1977). The rats weighing 200-225 g housed in cages for at least 1 week prior to testing. Animals were maintained in a temperature-controlled environment under a 12 hr light-dark cycle. All subjects were naive and were used only once.

Rats were transported to the testing room at least for one hour prior to testing for acclimatization and adaptation purposes. Experimental sessions were conducted between 9 AM to 2 PM daily. Animals were assigned randomly and were placed individually in a glass cylinder (24.5 cm X 35.5 cm) filled with water at room temperature to a depth of 22 cm. All the test sessions were recorded by video cameras. The water was changed in the beginning of each session and the temperature was maintained constant at 24-25 ^{o}C.

The test procedure consisted of a pretest and test session separated by 24 h (Porsolt et al., 1977). During the pretest period, rats were placed in the swim chamber for 15 min. Followed by the initial swim exposure; rats were patted dry and were transferred to the individual cages. Drugs or vehicle were then orally administered (p.o) 15 min after the

initial swim exposure and were then transported to their home cages. On the following day the rats were brought back to the testing room at least 1 h before the beginning of test session. Rats were administered either drugs or vehicle 1 h before the swim test. Each rat underwent a 5-min swim session, which was videotaped and scored later.

Drug solution was prepared freshly on the test days. Compound **D-578** was dissolved in 3% beta-hydroxy propyl cyclodextrin (BHCD) vehicle solution. The vehicle was prepared by dissolving required amount of BHCD in saline. The drug and vehicle preparations were administered orally. **D-578** was administered at a dose of 10, 15 and 25 mg/kg. Depending upon the weight of the rats a slight variation of the administered drug solution volume e.g. 1.2 ml/ rat, was maintained. Drug or vehicle was administered 1 h prior to testing for forced swimming. Individuals, blinded to the treatment, scored the videotapes for immobility. Rats exhibiting no activity other than that necessary to keep the rat's head above the water was considered as immobile. Immobility scores were analyzed by one way ANOVA test.

2.8. Locomotor Activity

Sprague Dawley Rats were tested at 25 mg/kg oral dose of D-578 to monitor changes in any locomotor activity in acrylic Auto-Track/Opto-Varimex-4 System (Columbus Instrument; Columbus, Ohio). The purpose was to evaluate locomotor activity of the same doses of drug that were used in the forced swimming test. Rats were acclimated in the test chambers for 1 h prior to oral (gavage) administration of either D-578 or vehicle. Locomotor activity of the drug was measured for a total of two hours post administration of

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the drug which corresponded to the time of measurement in the forced swimming test experiment.

2.9. Single Prolonged Stress and Fear Conditioning

In order to determine whether D-578 has effects on maladaptive alterations in fear memory following exposure to traumatic stress, a cohort of rats were exposed to single prolonged stress (SPS) or a control treatment, and then subjected to a cued fear conditioning and extinction procedure with or without the administration of D-578. SPS is a well-validated rodent model of changes in neuroendocrine function (Liberzon et al., 1997; Liberzon et al., 1999) and fear behavior (Knox et al., 2012) commonly seen in PTSD(Lisieski et al., 2018; Yamamoto et al., 2009).

After acclimating to the laboratory, male Sprague Dawley rats (Charles River Laboratories, Kingston, NY) were given SPS as previously described (Eagle et al., 2015). Rats were restrained for 2 h in cylindrical clear plastic restrainers. Immediately following this restraint, they were put into 48 cm-diameter tub filled with 30 cm of room temperature water for a 20-min group swim (6-8 rats at a time). Rats were then dried and allowed to rest in a clean cage for 15 min. Finally, rats were exposed to diethyl ether vapor as a group (6-8 rats) until loss of consciousness, which was confirmed by toe and tail pinch method. Control animals were held in a separate room for an equal period of time, during which they were weighed and handled for ~2 min. Following SPS or control exposure, rats were returned to the vivarium and left undisturbed (except for normal care) for 7 days; this undisturbed period is necessary for the development of SPS-related behavioral(Knox et al., 2012) and neuroendocrine changes (Liberzon et al., 1997; Liberzon et al., 1999).

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A timeline of fear conditioning experiments is shown in Fig. 4A. Cued fear conditioning, extinction, and extinction retention tests took place on the 8th, 9th, and 10th days, respectively, following SPS exposure. Rats were given i.p. injections of D-578 (10 mg/kg), paroxetine (5 mg/kg), or vehicle 90 minutes prior to each testing session. During acquisition, rats were placed in a fear conditioning chamber (Coulbourn Instruments, Whitehall, PA) and after a 180 s baseline period rats were exposed to 5 tones (10 s long, 2 kHz, 80 dB sine wave) each of which co-terminated with a 1 mA foot shock. Inter-trial intervals were 50 s. Each rat was returned to its home cage at the conclusion of the session. Digital videos were scored by two blinded raters for fear-related (i.e. freezing and rearing) behaviors; data were averaged between raters (inter-rater reliability: Pearson r>0.8 and quantified as percentage of time spent freezing and rearing during the 180s baseline period and during each 10 s tone presentation. For extinction and extinction-retention sessions, rats were placed in a different context, and after a 180 s baseline period, 20 tones with properties identical to those played during acquisition were played with inter-trial intervals of 50 s. FreezeFrame software (Coulbourn Instruments) was used to detect freezing in the videos, which was quantified as percentage of time spent freezing during the 180s baseline period and during each tone presentation. Data from the extinction and extinction-retention, but not acquisition, sessions were collapsed into bins of two-trials. Rater-observations were used for quantification of data for acquisition phase because FreezeFrame does not include fear related rearing behavior which was present during acquisition. Rearing behavior not related to fear observed during acquisition was not included in the freezing data (see supplemental data). However, because fear related rearing behavior was not observed during extinction or extinction retention (data not

shown), FreezeFrame was used to quantify data for these phases of conditioned fear testing. Five animals that showed aberrantly high or low freezing behavior during multiple sessions, defined as having values greater than +/- 2 standard deviations from the grand mean for 5 or more time points in each of two or more sessions were removed from the analysis; therefore, group sizes were control = 9, SPS+saline = 8, SPS+paroxetine = 10, and SPS+D-578 = 9.

Shock-reactivity to the five 1 mA shocks (tone-shock pairings) presented during the acquisition phase of fear learning was determined. The shock response ratings ranged from 1 to 4, where: 1 - Flinch involving only head and/or forepaw, 2 - Whole body flinch, 3 - Whole body flinch followed by ambulation, 4 - Whole body flinch and jump (all four feet in air - usually followed by ambulation(Perrine et al., 2006) (Menard, Champagne, Meaney,2004; Perrine, Hoshaw, Unterwald, 2006). The shock response for each animal was averaged across the five shock presentations, and data from two blinded raters was average (Pearson r>0.8).

Fear conditioning data from each session were analyzed using two-way repeated-measures ANOVAs (between-subjects factor: treatment group, within-subjects factor: bin); significant effects were followed up by post-hoc comparisons using Tukey's HSD test. To confirm that SPS caused changes in fear behavior, per-bin comparisons between the control and SPS+saline groups were conducted for all sessions. To determine whether administration of D-578 ameliorated any SPS-induced changes, per-bin comparisons between the SPS+D-578 and the SPS+saline groups were conducted. Per-bin two-group comparisons were conducted by t-test, using the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995; www.jstor.org/stable/2346101) to limit the family-wise

false discovery rate (FDR) to 0.1 for each session. All tests were two-tailed, except for one-tailed posthoc tests that were used for per-bin comparisons between SPS and control groups in the extinction-retention session, as we hypothesized *a priori* on the basis of previous findings that SPS-exposed animals would show persistent freezing during extinction-retention. The shock-reactivity data were analyzed by two-tailed, one-way ANOVA (between-subjects factor: treatment group) on shock response ratings.

3. Results

3.1. Inhibition of monoamine uptake into rat brain synaptosomes and cells heterologously expressing DAT, SERT and NET

Tables 1 and 2 show the potency of D-578 and other drugs in inhibiting uptake of radiolabeled monoamines by their respective transporters, either measured in rat brain synaptosomes or in cell lines expressing cloned human transporters. As shown in Table 1, in rat brain synaptosomes, D-578 displays equipotency for DAT and SERT (Ki; 16.2 and 16.2 nM, respectively) whereas it exhibits an almost five-fold higher potency at NET (3.23 nM). The potency at SERT is comparable to that of fluoxetine, an SSRI. Compared to the tricyclic antidepressant imipramine, D-578 is far more potent at DAT and NET. The well known TRI DOV 21,947 under our assay conditions exhibited potencies at DAT and NET comparable to previously reported values. However, we found DOV 21,947 to be somewhat less potent at SERT in our assay condition than previously described (Skolnick et al., 2003).

In the cell assays with cloned human transporters, the potency of D-578 is very similar to the data found from rat tissue. Again D-578 is 3-4 times more potent at NET

compared to DAT and SERT (Ki; 6,10 vs. 29.6 and 20.6, respectively). This cross validation reaffirms a similar potency of D-578 across species.

Table 1. Uptake Inhibitory Potency at DAT, SERT, and NET in Rat Brain Synaptosomes.

Drugs	DAT uptake, Ki, nM, [^{3}H]DA [a]	SERT uptake, Ki, nM, [^{3}H]5-HT[a]	NET uptake, Ki, nM [^{3}H]DA[a]
D-578	**16.2 ± 2.0**	**16.2 ± 1.5**	**3.23 ± 0.99**
Fluoxetine	1,092 ± 98	12.2 ± 2.4	770 ± 100[b]
Reboxetine	2,908 ± 136	503 ± 61	0.79 ± 0.25
Desipramine	1,567 ± 260 [b]	106 ± 17 [b]	1.30 ± 0.22 [b]
Imipramine	1,696 ± 246 [b]	20.0 ± 7.2 [b]	170 ± 48 [b]
DOV 21,947	97.1 ± 19.6	53.1 ± 5.5	32.5 ± 6.2
DOV 21,947	96 ± 20[c]	12 ± 2.8[c]	23 ± 3[c]

[a] Uptake into rat brain synaptosomes was measured as described in Methods. Where indicated (superscript b & c), values are from previous publications. Results are average ± S.E.M. for 3 to 6 independent determinations.

[b] Data from (Dutta et al., 2008); c Data from(Skolnick et al., 2003)

Table 2. Uptake Inhibitory Potency In Cells Expressing Human Transporters.

Drugs	DAT uptake, Ki, nM, [^{3}H]DA [a]	SERT uptake, Ki, nM, [^{3}H]5-HT[a]	NET uptake, Ki, nM [^{3}H]DA[a]
D-578	**29.6 ± 6.95**	**20.6 ± 3.8**	**6.10 ± 1.14**
Fluoxetine		14.4 ± 1.1	
Desipramine			**0.86 ± .04**

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Citalopram	**3.66 (6) ± 0.64**

[a] Uptake into cells was measured as described in Methods.

3.2 Broad screening CNS receptors data

Table 3: Binding affinity of D-578 for CNS receptors. The default concentration of drugs for primary binding experiments was 10 μM, and % inhibition (mean of 4 determinations) is shown. The study was carried out by the National Institute of Mental Health's Psychoactive Drug Screening Program,

Target Receptor	Radioligand	% Inhibition of Binding at 10 μM **D-578**	Ki for D-578 (nM)
D_1	[^{3}H]SCH 23390	11	
D_2	[^{3}H]N-methylspiperone	62.3	773
D_3	[^{3}H]N-methylspiperone	56.4	2615
D_4	[^{3}H]N-methylspiperone	20.9	
D_5	[^{3}H]SCH 23390	37.4	
$5HT_{1a}$	[^{3}H]8-OH-DPAT	19.9	
$5HT_{1b}$	[^{3}H]GR-125743	13.6	
$5HT_{1d}$	[^{3}H]GR-125743	72	702
$5HT_{2a}$	[^{3}H]ketanserin	62.5	1,639.00
$5HT_{2b}$	[^{3}H]LSD	80.3	425

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$5HT_{2c}$	[^{3}H]Mesulergine	96.3	73
$5HT_3$	[^{3}H] LY 278,584	8.5	
$5HT_{5a}$	[^{3}H]LSD	35.2	
$5HT_6$	[^{3}H]LSD	12.8	
$5HT_7$	[^{3}H]LSD	28.9	
GABA $_A$	[^{3}H]Muscimol	2.3	
$Alpha_{1A}$	[^{3}H]Prazosin	18.6	
$Alpha_{1B}$	[^{3}H]Prazosin	38.3	
$Alpha_{1D}$	[^{3}H]Prazosin	24.7	
$Alpha_{2A}$	[^{3}H]Clonidine	22.6	
$Alpha_{2B}$	[^{3}H]Clonidine	6.3	
$Alpha_{2C}$	[^{3}H]Clonidine	72	2014
$Beta_1$	[^{125}I]Iodopindolol	45.6	
$Beta_2$	[^{125}I]Iodopindolol	59.4	>10,000
$Beta_3$	[^{125}I]Iodopindolol	59.8	2703
BZP Rat Brain Site	^{3}H-Flunitrazepam	28.8	
Ca^{2+} Channel	[^{3}H]Nitrendipine		
δ-opioid	[^{3}H]DADLE	24.1	
κ-opioid	[^{3}H]Bremazocine	68.9	1387
H_1	[^{3}H]Pyrilamine	11	

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H_2	[^{3}H]Tiotodine	94.1	42
H_3	[^{3}H]Alpha-methyl Histamine	19.6	
H_4	[^{3}H]Histamine	2.4	
μ-Opioid	[^{3}H]Diprenorphine	11.7	
$M_{□}$	[^{3}H]QNB	97.2	>10,000
M_2	[^{3}H]QNB	20.9	
M_3	[^{3}H]QNB	7.5	
M_4	[^{3}H]QNB	55	>10,000
M_5	[^{3}H]QNB	84.4	>10,000
NMDA	MK801		
PCP Site			
PBR		10.6	
mGlur5		52.8	600
Oxytocin		53.1	>10,000
V1A		44.6	
V1B		7.8	
V2		21	

D-578 was evaluated for its interaction with various CNS GPCR receptors and ion channels with various radioligands as shown in Table 3. The compound did not exhibit any appreciable affinity for dopamine receptor subtypes at 10 μM. However, for serotonin receptor subtypes, full dose dependent binding inhibition experiment indicated moderate affinity for 5HT2c (Ki = 73 nM) and very weak affinity for 5HT2b (Ki = 425 nM). For other

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serotonin receptor subtypes, the compound did not show any activity. When evaluated at alpha adrenergic and beta adrenergic receptors, the compound was found to be inactive. Similarly, when evaluated at muscarinic subtype receptors, the compound did not exhibit any affinity for these receptors. The compound did not show any activity at three opioid receptors and was also devoid of any activity at the GABA and benzazepine sites. The only appreciable affinity uncovered was for the histamine receptor subtype 2 (H2): a Ki of 42 nM. In general, D-578, beyond interacting with the three monoamine transporters DAT, SERT and NET, does not display appreciable affinity for other brain targets as assessed in this screen.

3.3 hERG inhibition

The hERG potassium channel (human ether-a-go-go related gene) is expressed in the human heart. The channel is a key effector of cardiac repolarization and contributes to the QT interval measured by the electrocardiogram. Inhibition of hERG can lead to a prolongation of the QT interval, widely considered a critical risk factor for torsades de pointes (TdP) arrhythmia in non-cardiac drugs. Therefore, evaluation of hERG blocking activity of test compounds early on in the drug discovery process is important to identify any potential issue related to cardiotoxicity.

The hERG channel inhibition assay was carried out by following Thallium Flux FluxOR Potassium Ion Channel Assay kit (Invitrogen). The assay was carried out by the National Institute of Mental Health's Psychoactive Drug Screening Program. The results from the assay show almost no inhibition of the hERG channel by D-578. On the other hand, the reference compound cisapride exhibited potent inhibition of the channel.

Table 4: Assessment of Inhibition of hERG channel by Tl+ flux assay. Potency is

expressed in EC50 (μM). Results represent best fit values + SE taken directly from curve-fittings in Prism. Multiple assays were normalized to percentage inhibition and pooled for analysis

Compound	Tl+ Flux Assay EC50 (μM)	Hill Slope
Cisapride	0.205 ± 0.034	1.01 ± 0.07
D-578	14.75 ± 3.310	1.471 ± 0.23

3.4 In Vivo data from forced swimming test (oral administration)

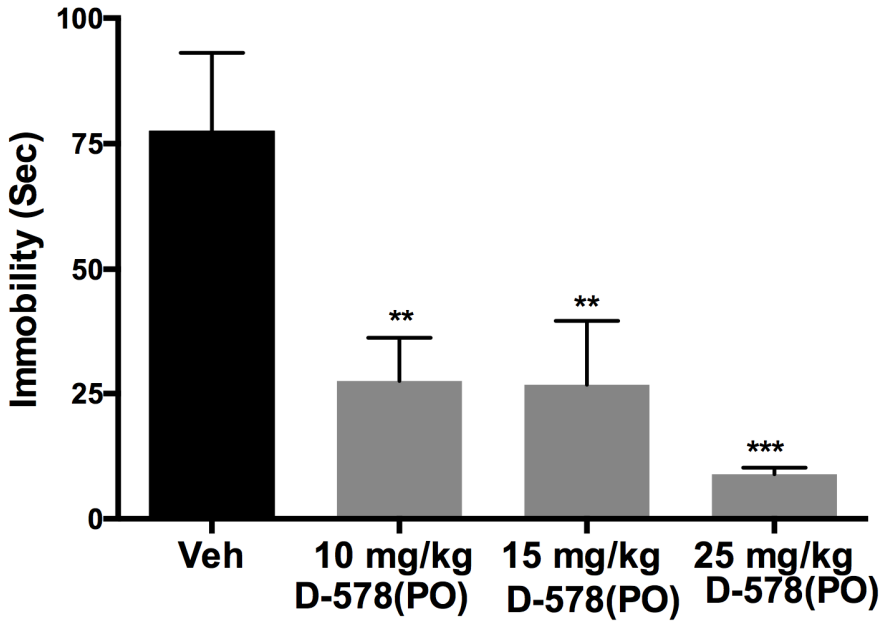

Fig. 2: Effect of sub-chronic oral administration of vehicle (n = 18) and **D-578** (n = 6-8 per group) on the duration of immobility in the forced swimming test in rats. One way ANOVA analysis demonstrates significant effect among treatments: F (3, 17) = 6.956 (P< 0.0029). Dunnett's analysis showed that the effect of **D-578** at three doses (10, 15 and 25 mg/kg)

on immobility was statistically significant different compared to vehicle (P< 0.01 and 0.001).

In order to evaluate dose dependent effect of D-578 on immobility, we decided to use 10, 15 and 25 mg/kg doses of the drug. As shown in Fig. 2, D-578 dose-dependently reduced immobility in the rat forced swimming test (FST) upon oral administration. The two lowest doses (10 and 15 mg/kg) produced similar, but significantly reduced, immobility compared to vehicle control. At the highest dose of 25 mg/kg, the compound produced the greatest effect with marked reduction of immobility. Thus, the compound shows high efficacy in the FST.

3.5 In Vivo data from locomotor activity test (oral administration)

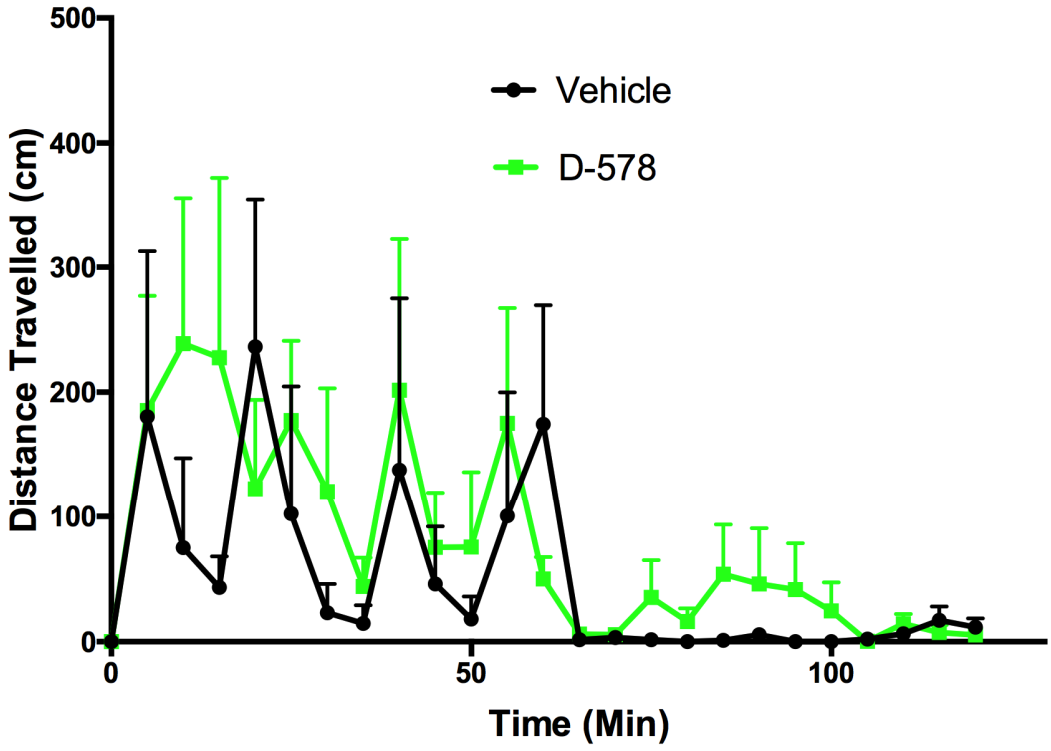

Fig. 3: Effects of D-578 (PO) and vehicle on locomotor activity (Distance travelled, n = 4-7

rats/group). D-578 and vehicle were administered orally to the rats. The distance travelled locomotor activities up to 120 min was measured and is represented by 5 min Bin block. Unpaired two-tailed t-test analysis demonstrates non-significant effect between control and D-578. Each treatment group contains four (vehicle) to seven (D-578 group) rats.

The dose which produced the highest efficacy in FST, was tested in locomotor activity test to observe any effect in motor stimulation. Thus, distance travelled data from oral administration of 25 mg/kg dose of D-578 is not significantly different from vehicle. It is important to note that in the final hour of locomotor activity measurement which corresponds to swim test measurement, neither D-578 or vehicle exhibited any locomotor activity at all.

3.6 In Vivo data from Fear Conditioning studies in rodent PTSD model

The timeline of the conditioned fear experiments in the rat SPS model of PTSD is shown in Fig. 4A. Fear-related behaviors from the acquisition, extinction, and extinction retention sessions are shown in Fig. 4B-D**.** Shock-reactivity behavior during acquisition learning is shown in Fig. 4E. The dose 10 mg/kg for D-578 was chosen as this dose produced significant reduction of immobility compared to control in the FST test which reflects sufficient behavioral alteration at that dose. For paroxetine, 5 mg/kg dose was chosen as previous studies with another SPS model indicated efficacy of the drug at a lower dose concentration administered chronically (Takahashi et al., 2006). Since D-578 at a 25 mg/kg dose did not behave any differently than vehicle control in the locomotor activity study (Fig. 3), it was not included as a control in a non-SPS animals group.

Acquisition of conditioned fear

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A repeated-measures ANOVA on rater-observed fear-behaviors during the acquisition session revealed a main effect of bin, $F(4,128) = 48.879$, $p < 0.001$, but no main effect of medication group, $F(3,32) = 0.153$, $p=0.927$ or bin x group interaction, $F(12,128) = 0.795$, $P =0.654$. No posthoc tests were conducted given that no interaction was observed. As expected, all animals showed good acquisition of conditioned fear behavior in a paradigm intended to maximize acquisition learning to allow focus on extinction learning (Fig. 4B).

Extinction of conditioned fear

A repeated-measures ANOVA on freezing behavior during the extinction session revealed a main effect of bin, $F(10,320) = 20.055$, $P < 0.001$; a main effect of medication group, $F(3,32) = 5.453$, $P = 0.004$; and a bin x group interaction, $F(30,320) = 2.0128$, $P = 0.001$. Tukey's HSD showed that rats given D-578 (i.p.) at a 10 mg/kg dose after SPS froze less during the extinction session than rats given either saline ($P = 0.007$) or paroxetine (i.p.) ($P = 0.015$) at a 5 mg/kg dose. Most importantly, false-discovery-rate-corrected per-bin comparisons showed that in a majority of bins (all bins except for bins 1 and 7), the SPS+saline group showed significantly more freezing behavior than the control group, and the SPS+D-578 group showed significantly less freezing behavior than the SPS+saline group (Fig. 4C).

Extinction Retention of conditioned fear

A repeated-measures ANOVA on freezing behavior during the extinction retention session revealed a main effect of bin, $F(10,320) = 22.612$, $P <0.001$; and a main effect of medication group, $F(3,32) = 3.229$, $P =0.035$; and a significant bin x group interaction, $F(30,320) = 1.495$, $P =0.05$. Tukey's HSD showed that rats given D-578 after SPS froze less during the retention session than rats given paroxetine ($P = 0.034$). One-tailed post-

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hoc tests between SPS+saline and control groups showed differences between these groups during bins 6 and 7, but only when not correcting for multiple comparisons. Most importantly, false-discovery-rate-corrected per-bin comparisons showed that the SPS+D-578 group froze significantly less than the SPS+saline group during the first two bins of the session (Fig. 4D).

Shock-Reactivity during Acquisition

An ANOVA on rater-observed shock response behaviors during the acquisition session revealed no main effects of treatment, $F(3, 32) = 1.486$, $P = 0.237$. As expected, SPS-alone (i.e. SPS+saline) or either drug group (i.e. SPS+paroxetine or SPS+D-578) had no effect on shock reactivity compared to the control group and therefor likely did not influence acquisition or extinction of conditioned fear behaviors (Fig. 4D).

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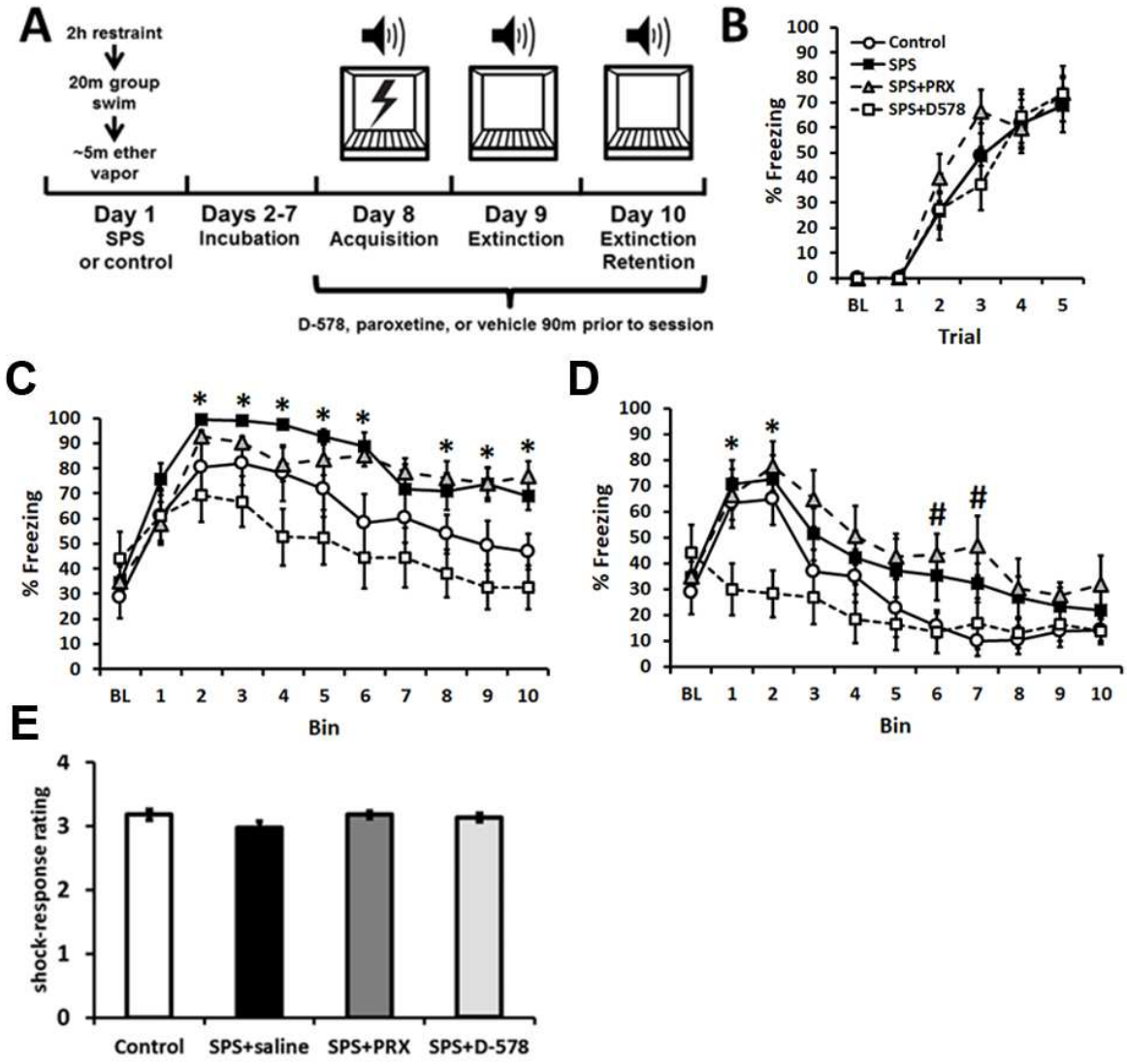

Fig. 4: A: Timeline of conditioning fear experiment in PTSD model. Rats were exposed to single prolonged stress (SPS), allowed a 7-day incubation period, and then subjected to a three-phase cued fear conditioning protocol with injections of D-578 (10 mg/kg), paroxetine (PRX, 5 mg/kg), or saline-vehicle (n = 8-10 per group). See Methods for more details. **B, C, and D: Effects of SPS and drugs on fear conditioning**

behaviors. Rats given SPS+vehicle showed increased freezing relative to Control rats throughout the extinction sessions **(C)** and during the later phase of extinction-retention testing **(D)**, but as expected did not affect fear-related behaviors during acquisition sessions **(B)**. Although paroxetine did not reverse the SPS-induced deficits during the extinction and extinction-retention sessions, D-578 significantly reduced SPS-induced freezing in response to the fear-conditioned cue in the extinction sessions, and reduced freezing during the initial stage of the extinction retention session. **E: Effects of SPS and drugs on shock reactivity during acquisition of conditioned fear learning.** Rats *****: significant difference between SPS+saline and control groups or SPS+saline and SPS+D-578 groups, family-wise FDR = 0.1. #: significant difference between SPS+saline and control groups without multiple comparison correction.

4. Discussion

The general consensus is that current treatments for both MDD and PTSD are not adequate, indicating significant unmet needs for development of effective therapies (Bandelow et al., 2012; Hoskins et al., 2015; Rush et al., 2006). The role of dopamine in depression has been uncovered in an adjunct therapy approach: the combination of bupropion and a SSRI turned out to be effective in patients refractory to SSRIs (Mischoulon et al., 2000). Efficacy in such treatment strongly points towards the involvement of a dopamine component since bupropion is a blocker of dopamine transporter (Ascher et al., 1995). In another study, pramipexole, an antiparkinsonian drug with D_3 dopamine receptor preferring agonist activity, exhibited effectiveness in both unipolar and bipolar depression (Sporn et al., 2000).

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It is established that innervation of dopaminergic neurons in the cortex, limbic region, and pituitary gland is linked with cognition, motivation and reward (D'Aquila et al., 2000; Dunlop and Nemeroff, 2007; Nutt et al., 2007; Papakostas, 2006; Sharma et al., 2015). Mesolimbic dopamine is associated with motivation and reward-related behavior and, therefore, inclusion of dopamine activity in a TRI should improve anhedonia that is the central component in depression (Nestler and Carlezon, 2006; Willner, 1983). As alluded above such anhedonia is also implicated in PTSD. Thus, a rational strategy of inhibiting the reuptake of all three monoamines with development of TRIs for the treatment of depression as well as PTSD is warranted (Guiard et al., 2009; Marks et al., 2008a).

A number of TRIs have been developed in the past with varying potencies for inhibition of monoamine transport (Sharma et al., 2015). Some of these compounds including DOV 21,947, JNJ-7925476 and BMS-820836 (Fig. 1) have been well characterized in animal models of depression (Risinger et al., 2014; Sharma et al., 2015; Skolnick et al., 2003). DOV 21,947 exhibited promising results in initial clinical trial and has undergone Phase IIb/IIIa studies (NCT01318434)(Tran et al., 2012). Another norepinephrine preferring TRI, EB-1020, produced positive results in all subtypes of ADHD population (NCT01939353)(Bymaster et al., 2012). In general TRIs have been shown to be safe in human clinical studies when no off targets issues are involved (Sharma et al., 2015). Thus, TRIs can potentially offer higher efficacy in these therapeutic areas compared to the existing antidepressants and pharmacotherapies for PTSD. Although a number of TRIs did not succeed in the clinical trials due to various issues including those related to transporter occupancy and non-specific interaction, there are other TRIs which showed

promising results in clinical trials, indicating a suitable TRI will have potential to provide higher efficacy (Bymaster et al., 2012; Sharma et al., 2015).

D-578 exhibits high potency at three monoamine transporters with preferential affinity for norepinephrine transporter. In both rat and human transporter uptake inhibition assays (Table 1 & 2), D-578 exhibits similar potency at both DAT and SERT (16.2 and 16.2 vs. 29.6 and 20.6 nM, respectively) whereas at NET the potency was higher. In this regard, the potency for SERT is comparable to fluoxetine in both rat and human transporter. Overall, D-578 exhibits an uptake inhibition profile that favors the production of a synergistic pharmacological effect emanating from interaction with all three monoamines transporters. In regards to specificity of D-578 for monoamine transporters in the CNS, Table 3 indicates that the compound is mostly selective for the transporters as it does not exhibit much affinity for known major receptors. This indicates that D-578 may not exhibit many side effects arising from non-specific interactions in the CNS. Additionally, we have carried out hERG inhibition assay as it is imperative to evaluate any potential of cardiotoxicity early on in the development process. As shown in Table 4, D-578 showed almost no inhibition of hERG channel whereas the positive reference cisapride was potent in inhibiting the channel. This result indicates that D-578 is less likely to produce any arrhythmia related cardiotoxicity.

In our next goal, we wanted to evaluate the effect of D-578 on immobility of rats in forced swimming test which seems to correlate well with antidepressant activity of a given test drug. We evaluated the effect of D-578 under oral administration as it was determined that D-578 gets absorbed orally and penetrates into the brain well (unpublished data). As shown in the Fig. 2, the compound D-578 significantly reduced immobility right from the

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starting dose 10 mg/kg and produced the highest effect at 25 mg/kg (PO). In fact reduction of immobility at 25 mg/kg was greater than 90% compared to control. It is quite conceivable that D-578 would also be efficacious at doses lower than 10 mg/kg which we will investigate in the future. The results indicate high efficacy of D-578 to produce anti-depressant like activity under oral administration. In order to determine any contribution of locomotor activation in forced swim activity, we carried out locomotor activity testing under similar conditions as those used for FST. As shown in Fig. 3, at the highest dose tested, the compound was not able to significantly increase locomotor activity compared to the control. This indicates that efficacy of D-578 in FST was not influenced by locomotor activity.

To evaluate the efficacy of D-578 in PTSD, the single prolonged stress (SPS) rat model for PTSD was used (Knox et al., 2012). Using a cued fear conditioning paradigm following traumatic stress exposure, we demonstrated that D-578 reduces SPS-induced fear behavior during extinction and extinction retention sessions. D-578 was superior to paroxetine in reducing fear-related learning deficits during both the extinction and extinction retention sessions, where paroxetine had no effect. The result from the effect of paroxetine treatment in this case is different than reported earlier with another SPS model involving chronic exposure to paroxetine through out the study session (Takahashi et al., 2006). Our results indicate superior effect of D-578 in reducing maladaptive fear responses following traumatic stress, possibly by facilitating more rapid and complete extinction learning (Fig. 4).

5. Conclusion

In conclusion, D-578 is a novel TRI which displays potent low nanomolar uptake inhibition activity at all three monoamine transporters. The compound exhibits relatively higher affinity at NET than DAT and SERT. D-578 did not exhibit any appreciable affinity for known, important CNS receptors as inferred from extended receptor screening studies, and it also did not inhibit the hERG channel, indicating less propensity for producing side effects. In the FST, the compound exhibited high efficacy upon oral administration without producing any motor stimulation measured under the same experimental conditions. In a fear conditioning SPS animal PTSD model experiment, compound D-578 was far more efficacious than paroxetine, an SSRI currently used for treatment of PTSD. These results indicate the potential of novel TRI D-578 as a pharmacotherapeutic agent in MDD and PTSD.

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Author Contributions:

AKD was involved with all aspects of the studies including design of compounds and

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biological (in vitro and in vivo) experiments and writing of the manuscript. SS was involved with synthesis of the compounds. BD and LX participated in animal work. MEAR. and TA performed in vitro uptake inhibition assays. AKD, AH, ML, BD and SAP participated in PTSD study. MEAR and SAP contributed in editing the manuscript.

Declarations of interest: None

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Figure Legend:

Fig. 1: Molecular structures of triple reuptake inhibitors.

Fig. 2: Effect of sub-chronic oral administration of vehicle and **D-578** on the duration of immobility in the forced swimming test in rats (n = 6-8 per group). One way ANOVA analysis demonstrates significant effect among treatments: F (3, 17) = 6.956 (P< 0.0029). Dunnett's analysis showed that the effect of **D-578** at three doses (10, 15 and 25 mg/kg) on immobility was statistically significant different compared to vehicle (P< 0.05 and 0.01).

Fig. 3: Effects of drugs, D-578 (PO) and vehicle on locomotor activity (Distance travelled, n = 4-7 rats/group). D-578 and vehicle were administered orally to the rats. The distance travelled locomotor activities up to 120 min was measured and is represented by 5 min Bin block. Unpaired two-tailed t-test analysis demonstrates non-significant effect between control and D-578.

Fig. 4: A: Timeline of conditioning fear experiment in PTSD model. Rats were exposed to single prolonged stress (SPS), allowed a 7-day incubation period, and then subjected to a three-phase cued fear conditioning protocol with injections of D-578 (10 mg/kg), paroxetine (PRX, 5 mg/kg), or saline-vehicle (n = 8-10 per group). See Methods for more details. B, C, and D: Effects of SPS and drugs on fear conditioning behaviors. Rats given SPS+vehicle showed increased freezing relative to Control rats throughout the extinction sessions **(C)** and during the later phase of extinction-retention testing **(D)**, but as expected did not affect fear-related behaviors during acquisition sessions **(B)**. Although paroxetine

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did not reverse the SPS-induced deficits during the extinction and extinction-retention sessions, D-578 significantly reduced SPS-induced freezing in response to the fear-conditioned cue in the extinction sessions, and reduced freezing during the initial stage of the extinction retention session. E: Effects of SPS and drugs on shock reactivity during acquisition of conditioned fear learning. Rats *****: significant difference between SPS+saline and control groups or SPS+saline and SPS+D-578 groups, family-wise FDR = 0.1. #: significant difference between SPS+saline and control groups without multiple comparison correction.

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