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(54) Title: PROPHYLACTIC EFFICACY OF SEROTONIN 4 RECEPTOR AGONISTS AGAINST STRESS

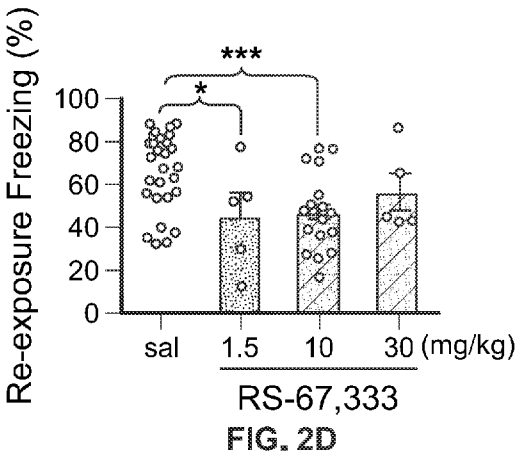


FIG. 2D

(57) Abstract: Methods for prophylactically treating a stress-induced affective disorder or stress-induced psychopathology in a subject are provided. Also provided are methods for inducing and/or enhancing stress resilience in a subject. In certain embodiments, an effective amount of an agonist of the serotonin 4 receptor (5-HT4R), or a pharmaceutically acceptable salt or derivative thereof, is administered to a subject prior to a stressor.

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PROPHYLACTIC EFFICACY OF SEROTONIN 4 RECEPTOR AGONISTS AGAINST STRESS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. Provisional Patent Application No. 62/831,517 filed on April 9, 2019, U.S. Provisional Patent Application No. 62/857,075 filed on June 4, 2019, and U.S. Provisional Patent Application No. 62/910,859 filed on October 4, 2019, each of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to serotonin 4 receptor (5-hydroxytryptamine (serotonin) receptor 4, or 5-HT_4R) agonist compositions and their use in methods of treatment or prevention of stress-induced affective disorders such as post-traumatic stress disorder (PTSD). In certain aspects, the present composition can be administered prior to a stressor.

BACKGROUND OF THE INVENTION

Stress exposure is a significant factor for the development of major depressive disorder (MDD) and post-traumatic stress disorder (PTSD). According to the National Comorbidity Study, approximately 60% of men and 51% of women have been exposed to one or more traumatic events during their lifetime. It is estimated that 7.8% of the overall population experiences PTSD at some point in their lives, with females (10.4%) experiencing the disorder at significantly higher rates than males (5.0%) [1]. Women are 2-3 times more at risk of suffering from stress-related anxiety or depressive disorders than men [61]. Traditionally, affective disorders have been treated from a symptom-suppression approach. Existing drugs aim to mitigate the impact of these chronic diseases, but do not cure or prevent the disease itself. However, if drugs were developed that enhance stress resilience, they could potentially be used in at-risk populations to protect against stress-induced psychiatric disorders.

Anxiety disorders are among the most common psychiatric disorders, with a lifetime prevalence of over 25 % (A1) and an annual financial burden of more than $40 billion (A2). Benzodiazepines (BZDs) are effective at treating most anxiety disorders and have been the standard treatment for years, with over an 80% response in reducing acute anxiety in patients (A3). However, their long-term daily use has been associated with a risk for dependency and amnesia. Consequently, they are often replaced by chronic treatment with serotonergic agents such as Selective Serotonin Reuptake Inhibitors (SSRIs) pointing out a role of serotonin (5-

HT) to treat anxiety. However, as SSRIs have a delayed onset of action of several weeks and a 40% non-responders rate in anxious patients (A4), there is a need to develop novel fast-acting anxiolytics.

We and others have recently reported that (*R,S*)-ketamine acts as a resilience enhancing drug (e.g., prophylactic) against stress when administered 1 week before stress in mice [2-6]. In addition, limited data in human patients have demonstrated (*R,S*)-ketamine's potential in preventing psychiatric disorders such as PTSD [7] and, perhaps in a dose-specific manner, post-partum depression (PPD) [8,9]. Prophylactic drug efficacy has been limited to (*R,S*)-ketamine until recently when Gould and colleagues reported that group II metabotropic glutamate receptor ($mGlu_{2/3}$) antagonists are also protective [10]. We have previously reported that the SSRI Flx is ineffective as a prophylactic, but it remains to be determined if other serotonergic drugs could be effective prophylactics and/or if the serotonergic system is involved in prophylactic efficacy.

The 5-HT_4Rs are a promising target for treating depression and anxiety. 5-HT_4Rs are metabotropic G-protein coupled receptors that stimulate the G_s/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway in response to 5-HT [11-15]. 5-HT_4Rs are highly expressed in the periphery, including the heart and adrenal gland, as well as in the brain in areas such as the amygdala (AMG), medial prefrontal cortex (mPFC), nucleus accumbens (NAc), and hippocampus (HPC) [16,17]. 5-HT_4R knockout mice display increased anxiety-like behavior and depressive-like behavior, while activation of 5-HT_4Rs stimulates neurogenesis in the HPC and produces rapid-acting antidepressant-like effects [18-21]. However, if and how 5-HT_4Rs are involved in stress resilience has yet to be determined.

There is an unmet need for effective prophylactic therapies to prevent the onset of stress-induced affective disorders.

SUMMARY OF THE INVENTION

The present disclosure provides for a method for preventing or delaying a stress-induced affective disorder or stress-induced psychopathology in a subject in need thereof. The method may comprise administering an effective amount of a pharmaceutic composition comprising an activator of serotonin 4 receptor (5-HT_4R) (e.g., an agonist of serotonin 4 receptor (5-HT_4R)), or a pharmaceutically acceptable salt, analog, derivative, or metabolite thereof, to a subject prior to a stressor.

The present disclosure also provides for a method for inducing and/or enhancing stress resilience in a subject in need thereof. The method may comprise administering an effective amount of a pharmaceutic composition comprising an activator of serotonin 4 receptor (5-HT_4R) (e.g., an agonist of serotonin 4 receptor (5-HT_4R)), or a pharmaceutically acceptable salt, analog, derivative, or metabolite thereof, to a subject prior to a stressor.

The agonist of 5-HT_4R may comprise 1-(4-amino-5-chloro-2-methoxyphenyl)-3-[1(n-butyl)-4-piperidinyl]-1-propanone HCl (RS-67,333 or RS67333), 4-amino-5-chloro-2,3-dihydro-N-[1-3-methoxypropyl)-4-piperidinyl]-7-benzofuran carboxamide monohydrochloride (prucalopride), 4-[4-[4-tetrahydrofuran-3-yloxy)-benzo[d]isoxazol-3-yloxymethyl]-piperidin-1-ylmethyl]-tetrahydropyran-4-ol (PF-04995274), or combinations thereof.

The pharmaceutic composition may be administered to the subject about 48 hours to about 3 weeks prior to a stressor. In certain embodiments, the pharmaceutic composition is administered to the subject about 72 hours to about 2 weeks prior to a stressor. In certain embodiments, the pharmaceutic composition is administered to the subject about 1 week prior to a stressor.

In certain embodiments, the pharmaceutic composition is administered to the subject once prior to a stressor.

In certain embodiments, the pharmaceutic composition is administered orally, intravenously, intranasally, or via injection to the subject.

The stress-induced affective disorder may comprise major depressive disorder (MDD) and/or posttraumatic stress disorder (PTSD). In certain embodiments, the stress-induced affective disorder is selected from the group consisting of: depressive-like behavior and associated affective disorders, anhedonic behavior and associated affective disorders, anxiety and associated affective disorders, cognitive impairments and deficits and associated disorders, stress-induced fear, and combinations thereof.

In additional embodiments, the stress-induced affective disorder comprises stress-induced psychopathology. In certain embodiments, the stress-induced psychopathology comprises depressive and/or anxious behavior.

The present method may prevent or delay stress-induced cognitive impairment and/or decline.

The present method may further comprise administering to the subject an effective amount of an anti-depressant, an anxiolytic, or combinations thereof.

The present method may further comprise administering an effective amount of a selective serotonin reuptake inhibitor (SSRI), or a pharmaceutically acceptable salt or derivative thereof.

The present method may further comprise administering an effective amount of fluoxetine, paroxetine, sertraline, lithium, riluzole, prazosin, lamotrigine, ifenprodil, or combinations thereof.

The subject may be a mammal. In certain embodiments, the subject is a human. The subject may be female or male.

In certain embodiments, the pharmaceutical composition is administered in a booster series.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1L. RS-67,333 protects against depressive- and anxiety-like behavior induced with a neuroendocrine model in male C57BL/6NTac mice. (1A) Experimental design. (1B) Behavioral assays to test anxiety-like behavior (EPM, NSF) and depressive-like behavior (ST). (1C-1F) By Week 6, CORT administration resulted in increased body weight when compared with VEH administration. RS and Flx administration resulted in decreased body weight in CORT-treated mice. (1G) All groups of mice exhibited comparable amounts of time in the open arms of the EPM. (1H) CORT + Veh mice had significantly less entries into the open arms of the EPM when compared with VEH + Veh mice. RS, but not Flx administration increased the number of entries into the open arms of the EPM in CORT-treated mice. (1I) All groups of mice traveled a similar distance in the EPM. (1J-1K) CORT administration increased the latency to feed in the NSF when compared with the VEH administration. RS, but not Flx administration decreased the latency to feed in CORT-treated mice. (1L) CORT administration decreased grooming duration in the ST when compared with VEH administration. RS, but not Flx administration increased the grooming duration in CORT-treated mice. (n = 9-14 male mice per group). Error bars represent ± SEM. *, $p < 0.05$; ** $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. VEH, vehicle; Veh, vehicle; CORT, corticosterone; Flx, fluoxetine; RS, RS-67,333; EPM, elevated plus maze; NSF, novelty suppressed feeding; ST, splash test; sec, seconds; no., number; cm, centimeters; g, grams.

Figures 2A-2O. A single, prophylactic injection of RS-67,333 attenuates learned fear and prevents novelty-induced hypophagia in male 129S6/SvEv mice. (2A) Experimental design. (2B) Mice administered 30, but not 1.5 or 10 mg/kg of RS-67,333 exhibited significantly less freezing during CFC training when compared with mice administered saline. (2C-2D) Mice administered 1.5 or 10, but not 30 mg/kg of RS-67,333 at exhibited significantly less freezing when compared with mice administered saline. (2E) Mice administered 10, but not 1.5 or 30 mg/kg of RS-67,333, exhibited reduced immobility when compared with mice administered saline during day 1 of the FST. (2F-2G) All groups of mice had comparable amounts of immobility during day 2 of the FST. (2H-2I) RS-67,333 (10 mg/kg) did not alter distance travelled or time spent in the center of the OF when compared to saline mice. (2J-2K) Both groups of mice had comparable time spent in the open arms and entries into the open arms of the EPM. (2L-2M) Mice administered RS-67,333 (10 mg/kg) exhibited a significantly reduced latency to feed when compared to saline mice. (2N) Mice in both groups ate a comparable amount of food in the home cage following the NSF. (2O)

Following food deprivation, mice in both groups lost a comparable amount of weight. (n = 5-29 male mice per group). Error bars represent ± SEM. *, $p < 0.05$; ***, $p < 0.001$. Sal, saline; CFC, contextual fear conditioning; FST, forced swim test; OF, open field; EPM, elevated plus maze; NSF, novelty suppressed feeding; min, minutes; sec, seconds; g, grams; mg, milligram; kg, kilogram; no., number; cm, centimeter.

Figures 3A-3O. A single, prophylactic administration of RS-67,333 prevents novelty-induced hypophagia, but does not alter fear- or depressive-like behavior, in female 129S6/SvEv mice. (3A) Experimental design. (3B) All mice exhibited comparable levels of freezing during CFC training. (3C-3D) All groups exhibited comparable levels of freezing during re-exposure. (3E) All groups of mice had comparable amounts of immobility during day 1 of the FST. (3F-3G) All groups of mice had comparable amounts of immobility during day 2 of the FST. (3H-3I) RS-67,333 did not alter distance travelled or time spent in the center of the OF. (3J) Time spent in the open arms of the EPM was comparable between all groups of mice. (3K) Entries into the open arms of the EPM was comparable between all groups of mice. (3L-3M) A single, prophylactic dose of RS-67,333 (10 mg/kg) significantly reduced latency to feed in the NSF. (3N) A single, prophylactic dose of RS-67,333 (10 mg/kg) did not alter the amount of food eaten in the home cage or (3O) body weight loss. (n = 6-11 female mice per group). Error bars represent ± SEM. *, $p < 0.05$; ***, $p < 0.001$. Sal, saline; CFC, contextual fear conditioning; FST, forced swim test; OF, open field; EPM, elevated plus maze; NSF, novelty suppressed feeding; min, minutes; sec, seconds; cm, centimeters; no., number; g, grams; mg, milligram; kg, kilogram.

Figures 4A-4M. A single, prophylactic administration of prucalopride or PF-04995274 attenuates learned fear and decreases depressive-like behavior in male 129S6/SvEv mice. (4A) Experimental design. (4B) All mice exhibited comparable levels of freezing during CFC training. (4C-4D) (*R*,*S*)-ketamine (30 mg/kg), prucalopride (3 mg/kg), and PF-04995274 (10 mg/kg), but not prucalopride (10 mg/kg) or PF-04995274 (3 mg/kg), administration attenuated learned fear when compared with saline administration. (4E) All groups of mice had comparable amounts of immobility during day 1 of the FST. (4F-4G) (*R*,*S*)-ketamine (30 mg/kg), prucalopride (3 mg/kg), and PF-04995274 (10 mg/kg), but not prucalopride (10 mg/kg) or PF-04995274 (3 mg/kg) significantly decreased immobility time during day 2 of the FST. (4H) All groups of mice traveled a comparable amount of distance in the OF. (4I) All groups of mice spent a comparable amount of time in the open arms of the EPM. (4J) All groups of mice had a comparable number of entries into the open arms of the EPM. (4K-4L) All groups of mice had a comparable latency to approach the pellet in the

NSF. (4M) All groups of mice lost a comparable amount of weight following food deprivation for the NSF. (n = 5-10 male mice per group). Error bars represent ± SEM. *, $p < 0.05$; ** $p < 0.01$; ***, $p < 0.001$. Sal, saline; K, (*R,S*)-ketamine; Prucal, prucalopride; PF, PF-04995274; CFC, contextual fear conditioning; FST, forced swim test; OF, open field; EPM; elevated plus maze; NSF, novelty suppressed feeding; min, minutes; sec, seconds; cm, centimeters; no, number; mg, milligram; kg, kilogram.

Figures 5A-5F. (*R,S*)-ketamine and prucalopride exhibit a common mechanism by reducing large AMPA-driven synaptic bursts in CA3. (5A) Experimental design. (5B) The average EPSC amplitude did not differ between the groups. (5C) The average number of EPSCs (within a 20-second recording window) did not differ between the groups. (5D) Saline-treated mice typically displayed large bursts of EPSCs (-590.8±13.85 pA), which were blocked by the AMPA receptor antagonist NBQX. These large AMPA receptor-mediated signals were not present in either (5E) (*R,S*)-ketamine- or (5F) prucalopride-treated mice. (n = 5-7 mice per group). Error bars represent ± SEM. Sal, saline; K, (*R,S*)-ketamine; Prucal, prucalopride; CA3, Cornu Ammonis 3; pA, picoamps; EPSCs, excitatory postsynaptic currents; no., number; 00NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline; mg, milligram; kg, kilogram; ms, millisecond.

Figures 6A-6J. Acute 5-HT$_4$ receptor stimulation induces fast anxiolytic-like effects in an anxious BALB/cJRj mouse strain. (6A, 6B) Experimental design. (6A) In a first cohort of animals, vehicle (V), fluoxetine (F, 18 mg/kg), diazepam (D, 1.5 mg/kg), or RS67333 (RS, 1.5 mg/kg) were administered via intraperitoneal (i.p.) injection 45 minutes before behavioral testing. (6B) In a second cohort of animals, treatments (V or RS, 0.5 µg/side) were infused in medial prefrontal cortex (mPFC) and diazepam (D, 1.5 mg/kg) administered via i.p. injection 45 minutes before the start of behavioral paradigms. (6C-6D and 6G-6H) Anxiolytic-like effect was measured in the elevated plus maze (EPM) as mean time or percent time spent in open arms (6C, 6G and inset), or as mean ratio ambulatory distance in open arms/total distance (6D, 6H) and as mean total ambulatory distance (inset) (n = 10 mice per group and n = 5-9 mice per group for systemic and local injection study respectively). (6E-6F and 6I-6J) In the Novelty Suppressed Feeding (NSF) paradigm, anxiolytic-like effect is expressed as fraction of animals that have not eaten over 10 minutes (6E, 6I), as mean of the food consumption (inset) or as mean of latency to feed (6F, 6J) (n = 10 mice per group, n = 4-8 mice per group). (6C-6D and 6E-6F) Systemic administration. (6G-6H and 6I-6J) local administration. Values plotted are mean ± SEM. *$p<0.05$, **$p<0.01$ *vs.* vehicle group.

Figures 7A-7G. Acute 5-HT$_4$ receptor stimulation induces fast anxiolytic-like effects on 5-HT function through a modulation of the mPFC in an anxious BALB/cJRj mouse strain. (7A) Before the administration of RS67333 (RS, 1.5 mg/kg), different tracks were performed to record 5-HT neurons for 30 min. At the end of this period, RS67333 was administered i.p. and 30 min after, two or three subsequent tracks were realized. (7B) Discharge frequency of DRN 5-HT neurons is assessed as mean firing rate. The number of neurons tested is indicated in each histogram (n = 25 and 26 before and after RS injection for a total of n = 5 mice). Data are mean ± frequency (Hz) of DRN 5-HT neurons determined before the administration of RS67333. (7C) Typical recordings of DRN 5-HT neurons in the different experimental conditions. Histograms in the upper panels represent the number of action potentials (APs) per 10 seconds (scale bar). Lower panels represent the well-characterized regular discharge of serotonergic neurons in both conditions. **p<0.01 *vs.* before RS67333 administration. (7D) p-CPA was injected i.p. twice a day during 3 days and treatments [vehicle (V), diazepam (D, 1.5 µg/side) and RS (0.5 µg/side)] were infused 24 hours after the final p-CPA administration in the medial prefrontal cortex (mPFC) and 45 minutes before the start of behavioral paradigms. (7E) Cortical 5-HT depletion by p-CPA pre-treatment is measured as mean 5-HT levels (n = 6-8 mice per group). (7F and 7G) Anxiety is measured in the elevated plus maze (EPM) as mean time or percent time spent in the open arms (F and inset), as mean ratio ambulatory distance in open arms/total distance (7G) and as mean total ambulatory distance (inset) (n = 8-11 mice per group). Values plotted are mean ± SEM. *p<0.05, **p<0.01 *vs.* vehicle group, #p<0.05, ##p<0.01 *vs.* appropriate group.

Figures 8A-8D. Effects of cortical terminals stimulation in the dorsal raphe nucleus of anxious BALB/cJRj mouse strain. (8A) Timeline regarding the behavioral consequences after stimulation of glutamatergic terminals in the DRN. AAV5-CamKIIα-ChR2-eYFP or AAV5-CamKII-eYFP virus were bilaterally injected in the medial prefrontal cortex (mPFC) and an optic fiber was implanted in the dorsal raphe nucleus (DRN), respectively 7 weeks and 1 week before testing in the Elevated Plus Maze (EPM). (8B) Expression of virus was confirmed in the mPFC (left) and in the DRN (right). (8C-8D) After optogenetic stimulation (3-min ON and 3-min OFF), anxiolytic-like effect is measured in the EPM as time or percent time spent in the open arms between laser ON and laser OFF (8C and inset), the distribution of time spent in open arms during and following cortical terminals stimulation in the dorsal raphe nucleus (inset), as mean ratio of ambulatory distance in the open arms divided by total

distance (8D) and as mean total ambulatory distance (inset) or (eYFP: n = 12 mice per group; ChR2: n = 19 mice per group). Values plotted are mean ± SEM. *p<0.05, **p<0.01 between laser ON and laser OFF; #p<0.05, ##p<0.01 between CHR2 and eYFP group.

Figures 9A-9F. Modulation of anxiolytic-like activity after optogenetic inhibition of glutamatergic terminals in the dorsal raphe nucleus (DRN) of the anxious BALB/cJRj mouse strain. (9A) Timeline regarding the behavioral consequences after inhibition of glutamatergic terminals in the DRN after medial prefrontal cortex (mPFC) infusion (diazepam [D] [1.5 mg/side] or RS67333 [RS] [0.5 mg/side]) or systemic administration of diazepam (1.5 mg/kg intraperitoneally [i.p.]), RS67333 (1.5 mg/kg i.p.) or vehicle (V). AAV5-CamKII-ArchT-GFP virus was injected bilaterally in the mPFC 7 weeks before testing. An optic fiber was implanted in the DRN 1 week before testing. For the local injection protocol, 2 injection cannulae were also implanted in the mPFC. Drug treatments were infused in the mPFC or injected i.p. 45 minutes before testing. (9B) Expression of control CaMKII-ArchT-GFP virus was confirmed in the mPFC (left) and in the DRN (right). (9C–9F) For the behavioral consequences of a local infusion (9C, 9D) or systemic administration (9E, 9F) with vehicle, diazepam, or RS, the anxiolytic-like effect is measured in the elevated plus maze (EPM) as time (9C, 9E) or percent time (insets in panels 9C and 9E) spent in the open arms across 2-period stimulation (3-minute light/dark cycle), as the distribution of time spent in open arms before and after cortical terminals inhibition in the DRN (insets in panels 9C and 9E), as mean time in open arms (OA) divided by total time (insets in panels 9C and 9E), as mean total ambulatory distance (insets in panels 9D and 9F), or as mean ratio of ambulatory distance in the OA divided by total distance (9D, 9F) (n = 7–11 mice per group and n = 7–9 mice per group for local and systemic administration, respectively). Values plotted are mean ± SEM. *p < .05 and **p < .01 vs. vehicle group; #p < .05 and ##p < .01 vs. the appropriate group during lights off. $^{\$}$p < .05 and $^{\$\$}$p < .01 vs. vehicle group during lights on.

Figure 10. The neuronal circuits involved in fast anxiolytic-like effects induced by acute systemic RS67333 and diazepam administration. Acute systemic administration with RS67333, a serotonin type 4 receptor (5-HT4R) agonist, induces fast anxiolytic-like effects in BALB/cJRj mice through at least activation of the cortical glutamatergic terminals in the dorsal raphe nucleus (DRN) confirming previous studies (19). Similarly, diazepam, a benzodiazepine (BZD), induces fast anxiolytic-like effects through a similar neuronal circuit recruitment. Our data also demonstrated that other brain structures might be involved in the fast anxiolytic-like activity of a 5-HT_4R agonist. Previous studies demonstrated that the

hippocampus (HPC)/medial prefontal cortex (mPFC) (46) circuit and the mPFC/amygdala (Amy) pathway (48) are recruited to modulate anxiety-like phenotypes, suggesting that these circuits should be also investigated for fast anxiolytic-like effects induced by 5-HT4R. GABA, gamma-aminobutyric acid.

Figures 11A-11E. Acute 5-HT$_4$R antagonist administration prevents RS67333-induced fast anxiolytic-like effects. (11A) Treatments (Fluoxetine 18 mg/kg, F; Diazepam 1.5 mg/kg, D; RS67333 1.5 mg/kg, RS; Vehicle, V) were administered i.p. 45 minutes before behavioral testing, except for GR125487 (GR, 1 mg/kg, i.p.) administered 15 minutes before RS67333 administration. (11B and 11C) After treatment administration, anxiety is measured in the EPM as mean time or percent time spent in the open arms (11B and inset), as mean ratio of ambulatory distance in the open arms divided by total distance or as mean ambulatory distance (11C and inset) (n = 5-6 mice per group). (11D and 11E) In the NSF, anxiety parameter is expressed as fraction of animals that have not eaten over 10 minutes (11D), as mean of the food consumption (inset) or mean of latency to feed (E) (n = 4-6 mice per group). All statistical tests and p values are mean ± SEM. *p<0.05, **p<0.01 *vs.* vehicle group (V), ##p<0.01 *vs.* RS group.

Figures 12A-12C. Acute systemic 5-HT$_4$R stimulation induces fast anxiolytic-like effects in the Open Field Paradigm. (12A) Experimental design. Vehicle (V), fluoxetine (F, 18 mg/kg), diazepam (D, 1.5 mg/kg), or RS67333 (RS, 1.5 mg/kg) were administered i.p. 45 minutes before behavioral testing in BALB/cJRj mouse strain. (12B-12C) Anxiolytic-like effect was measured in the OF as mean percent time spent in center (12B), as mean ratio ambulatory distance in center/total distance (12C) and as mean total ambulatory distance (inset) (n = 5-9 mice per group for systemic). All statistical tests and p values are mean ± SEM. *p<0.05, **p<0.01 *vs.* vehicle group (V).

Figures 13A-13C. Optogenetic stimulation of mPFC terminals in the DRN induces fast anxiolytic-like effects in the Open Field Paradigm. (13A) Timeline regarding the behavioral consequences after stimulation of cortical glutamatergic terminals in the DRN. AAV5-CamKIIα-ChR2-eYFP or AAV5-CamKII-eYFP virus were bilaterally injected in the medial prefrontal cortex (mPFC) and an optic fiber was implanted in the dorsal raphe nucleus (DRN), respectively 7 weeks and 1 week before testing in the OF. (13B-13C) Anxiolytic-like effect is measured in the OF as percent time spent in the center between laser ON and laser OFF (inset: time in seconds) (13B), as mean ratio of ambulatory distance in the center divided by total distance (inset: total ambulatory distance) (13C) (eYFP: n = 8 mice per group; ChR2: n = 13

mice per group). Values plotted are means ± SEM. **p<0.01 between laser ON and laser OFF; ##p<0.01 between CHR2 and eYFP group during laser ON.

Figures 14A-14C. Optogenetic inhibition of mPFC terminals in the DRN blocks anxiolytic activity of mPFC infusion with RS67333 and Diazepam. (14A) Timeline regarding the behavioral consequences of inhibition of medial prefrontal cortex (mPFC) terminals in the dorsal raphe nucleus (DRN) after mPFC infusion of diazepam (D, 1.5 µg/side), RS672333 (RS, 0.5 µg/side) or vehicle (V). AAV5-CamKII-ArchT-GFP virus was bilaterally injected in the mPFC 7 weeks before testing. An optic fiber was implanted in the DRN, 1 week before testing. (14B-14C) For the behavioral consequences of a local infusion with V, D or RS, anxiolytic-like effect is measured in the open field (OF) as percent time spent in the center across 2 epoch stimulation (3-min OFF and 3-min ON) (14B), the distribution of time spent in center prior to and following inhibition of glutamatergic axon terminals arising from the mPFC to the dorsal raphe nucleus (inset), as mean ratio of ambulatory distance in the center divided by total distance (14C) and as mean total ambulatory distance (inset) (n = 6-8 mice per group). Values plotted are mean ± SEM. *p<0.05, **p<0.01 *vs.* vehicle group, #p<0.05, ##p<0.01 *vs.* appropriate group during light ON.

Figures 15A-15I. Single stimulation of the 5-HT4 receptor could lead to long-lasting anxiolytic and antidepressant effects in the BALB/cJRj mice. (15A) The BALB/cJRj mice were systemically injected with a single dose of RS67333 (1.5 mg/kg) or diazepam (1.5 mg/kg), 45 minutes before performing the Splash Test. The following day, the mice underwent the EPM without having received another dose of RS67333, and then at the open field 24 hours later, and, finally, to NSF 24 hours after the open field. (15B-15C) RS67333 increased the grooming time ($t = 2.294$; $p<0.05$) in the Splash Test, without affecting the number of episodes ($t = 1.546$; $p = 0.1531$), following single administration. (15D-15E) We have not identified the anxiolytic effects expected of RS67333 in the EPM ($t = 0.4990$; $p = 0.6286$), 24 hours after the injection. However, the RS67333 has increased the time spent in the center of the OF ($t = 1.924$; $p<0.05$), without affecting the ratio of the distance in the center to the total walking distance ($t = 1.281$; $p = 2292$) (15F-15G), 48 hours after the injection, and reduced the lag for feeding in the NSF ($t = 2.520$; $p<0.05$), without affecting the consumption of food in a familiar environment ($t = 0.2203$; $p = 0.4151$) (15H-15I), 72 hours after the injection. (15B-15C) Splash test (acute effects). (15D-15E) Elevated plus maze (long-lasting effects). (15F-15G) Open field (long-lasting effects). (15H-15I) Novelty suppressed feeding (long-lasting effects). Abbreviations: *p<0.05 versus carrier; D: diazepam 1.5 mg/kg; i.p.: intraperitoneal injection RS: RS 67333 1.5 mg/kg; V: carrier.

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure provides methods for prophylactically treating a stress-induced affective disorder or stress-induced psychopathology in a subject. Also encompassed by the present disclosure are methods for inducing and/or enhancing stress resilience in a subject. In certain embodiments, an effective amount of an activator of serotonin 4 receptor (5-HT_4R) (e.g., an agonist of serotonin 4 receptor (5-HT_4R)), such as RS-67,333 (RS67333), prucalopride, PF-04995274, or a pharmaceutically acceptable salt, analog, derivative, or metabolite thereof, is administered to a subject prior to a stressor.

The present agent/composition may be administered therapeutically to achieve a therapeutic benefit or prophylactically to achieve a prophylactic benefit. By therapeutic benefit is meant eradication or amelioration of the underlying stress-induced affective disorder being treated, and/or eradication or amelioration of one or more of the symptoms associated with the underlying disorder. By prophylactic benefit is meant prevention or delay of the onset of a stress-induced affective disorder, and/or prevention or delay of the onset of one or more of the symptoms associated with a stress-induced affective disorder. In certain embodiments, an effective amount of the present agent/composition to be administered prevents stress-related disorders from developing or being exacerbated into more serious conditions.

In certain embodiments, for prophylactic administration, the present agent/composition may be administered to a patient at risk of developing a stress-induced affective disorder, or to a patient reporting one or more of the physiological symptoms of a stress-induced affective disorder, even though a diagnosis of a stress-induced affective disorder may not have yet been made. In certain embodiments, prophylactic administration is applied to avoid the onset of the physiological symptoms of the underlying disorder, before the symptom manifests cyclically. In this latter embodiment, the therapy is prophylactic with respect to the associated physiological symptoms instead of the underlying indication. In certain embodiments, the present agent/composition is administered prior to recurrence of a stressor. In certain embodiments, the present agent/composition is administered prior to the onset of a particular symptom.

In a further embodiment, the present invention provides for the use of the present agent or a pharmaceutically acceptable salt or solvate thereof, a physiologically functional derivative or analog thereof, or a metabolite thereof, in the preparation of a medicament for the treatment of a stress-induced affective disorder.

"Treating" or "treatment" of a state, disorder or condition includes: (1) preventing or delaying the appearance of clinical symptoms of the state, disorder, or condition developing in a person who may be afflicted with or predisposed to the state, disorder or condition but does not yet experience or display clinical symptoms of the state, disorder or condition; or (2) inhibiting the state, disorder or condition, i.e., arresting, reducing or delaying the development of the disease or a relapse thereof (in case of maintenance treatment) or at least one clinical symptom, sign, or test, thereof; or (3) relieving the disease, *i.e.*, causing regression of the state, disorder or condition or at least one of its clinical or sub-clinical symptoms or signs.

The benefit to a subject to be treated is either statistically significant or at least perceptible to the patient or to the physician.

The present agents include 5-HT_4R agonists, such as RS-67,333 (RS67333), prucalopride, PF-04995274, pharmaceutically acceptable salts or solvates thereof, analogs thereof, derivatives thereof (e.g., physiologically functional derivatives or analogs thereof), metabolites thereof, and combinations thereof.

A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. In certain embodiments, since a prophylactic dose is used in subjects prior to or at an earlier stage of a disorder, the prophylactically effective amount is less than the therapeutically effective amount. In certain embodiments, the prophylactically effective amount is similar to, identical to, or more than, the therapeutically effective amount.

A therapeutically effective amount, or an effective amount, of a drug is an amount effective to demonstrate a desired activity of the drug. A "therapeutically effective amount" will vary depending on the compound, the disorder and its severity and the age, weight, physical condition and responsiveness of the subject to be treated. In certain embodiments, an effective amount of the 5-HT_4R agonist, or a pharmaceutically acceptable salt or solvate thereof, or a physiologically functional derivative or analog thereof, or a metabolite thereof, is an amount effective to prevent or delay the onset of a stress-induced affective disorder, and/or effective to alleviate, one or more of the symptoms of a stress-induced affective disorder.

The present disclosure provides for a method for preventing or delaying a stress-induced affective disorder or stress-induced psychopathology in a subject in need thereof. The method may comprise administering an effective amount of a pharmaceutic composition comprising an activator of serotonin 4 receptor (5-HT_4R) (e.g., an agonist of serotonin 4 receptor (5-HT_4R)), or a pharmaceutically acceptable salt, analog, derivative, or metabolite thereof, to a subject prior to a stressor.

The present disclosure also provides for a method for inducing and/or enhancing stress resilience in a subject in need thereof. The method may comprise administering an effective amount of a pharmaceutic composition comprising an activator of serotonin 4 receptor (5-HT_4R) (e.g., an agonist of serotonin 4 receptor (5-HT_4R)), or a pharmaceutically acceptable salt, analog, derivative, or metabolite thereof, to a subject prior to a stressor.

The present composition may be administered by any method known in the art, including, without limitation, intranasal, oral, transdermal, ocular, intraperitoneal, inhalation, intravenous, intracerebroventricular (ICV), intracisternal injection or infusion, subcutaneous, implant, vaginal, sublingual, urethral (e.g., urethral suppository), subcutaneous, intramuscular, intravenous, rectal, sub-lingual, mucosal, ophthalmic, spinal, intrathecal, intra-articular, intra-arterial, sub-arachinoid, bronchial and lymphatic administration. Topical formulation may be in the form of gel, ointment, cream, aerosol, etc.; intranasal formulation can be delivered as a spray or in a drop; transdermal formulation may be administered via a transdermal patch or iontorphoresis; inhalation formulation can be delivered using a nebulizer or similar device. Compositions can also take the form of tablets, pills, capsules, semisolids, powders, sustained release formulations, solutions, suspensions, elixirs, aerosols, or any other appropriate compositions.

In certain embodiments, a subject is treated with the present agent/composition, via intravenous, oral, transdermal or intranasal administration. In certain embodiments, a subject is injected with the present agent/composition.

In certain embodiments, a subject is treated with a single dose of an effective amount of the present agent/composition, prior to, during, and/or after a stressor. In some aspects, a subject is treated with multiple doses of an effective amount of the present agent/composition, prior to, during, and/or after a stressor.

In certain embodiments, a 5-HT_4R agonist (such as RS-67,333 (RS67333), prucalopride, and PF-04995274), or a pharmaceutically acceptable salt or solvate thereof, an analog thereof, a derivative thereof, or a metabolite thereof, is administered in a composition comprising a pharmaceutically acceptable carrier, excipient or diluent. Also provided herein is a pharmaceutical composition that comprises a 5-HT_4R agonist (such as RS-67,333 (RS67333), prucalopride, and PF-04995274), or a pharmaceutically acceptable salt or solvate thereof, an analog thereof, a derivative thereof, or a metabolite thereof, and a pharmaceutically acceptable carrier, excipient or diluent, for use in the prophylactic treatment of a stress-induced affective disorder.

"Patient" or "subject" refers to mammals and includes human and veterinary subjects. In certain embodiments, the subject is mammalian.

The present agent (e.g., a 5-HT_4R activator) may activate 5-HT_4R through any mechanism, including, but not limited to, activating/increasing 5-HT_4R activity, activating/increasing 5-HT_4R level, and/or activating/increasing 5-HT_4R gene expression. The terms "activator of 5-HT_4R", "activator of the 5-HT4 receptor", "5-HT4 receptor activator", and "5-HT_4R activator" are used interchangeably herein.

By "activation", "up-regulation" or "increase" is meant any positive effect on the condition being studied; this may be total or partial. Thus, where the level or activity of a protein (e.g., 5-HT4 receptor or 5-HT_4R) is being detected, the present agent/composition is capable of activating, up-regulating, or increasing the level or activity of the protein (e.g., 5-HT4 receptor or 5-HT_4R). The activation or up-regulation of the level or activity of the protein achieved by the present agent may be at least 10%, such as at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more compared to the level or activity of the protein (e.g., 5-HT4 receptor or 5-HT_4R) in the absence of the present agent/composition.

Half maximal effective concentration (EC50) refers to the concentration of an agent which induces a response halfway between the baseline and maximum after a specified exposure time. The pEC50 is defined as the negative logarithm of the EC50:

$$pEC50 = -\log_{10}(EC50).$$

In certain embodiments, the present agent has a pEC50 in activating the 5-HT4 receptor activity ranging from about 3 to about 13, from about 4 to about 12, from about 5 to about 11, from about 6 to about 10, from about 6 to about 9, from about 6 to about 8, from about 6 to about 7, from about 7 to about 10, from about 7 to about 9, from about 7 to about 8, from about 8 to about 10, from about 8 to about 9, from about 9 to about 10, from about 5 to about 10, about 6, about 6.5, about 7, about 7.5, about 8, about 8.5, about 9, about 9.5, or about 10.

Ki denotes the affinity of an agent (e.g., an activator such as an agonist) for a receptor. When measured using a radioligand competition binding assay, it is the molar concentration of the competing ligand that would occupy 50% of the receptors if no radioligand was present. The pKi is the negative logarithm of the Ki value.

In certain embodiments, the present agent has a pKi for the 5-HT4 receptor ranging from about 3 to about 13, from about 4 to about 12, from about 5 to about 11, from about 6 to

about 10, from about 6 to about 9, from about 6 to about 8, from about 6 to about 7, from about 7 to about 10, from about 7 to about 9, from about 7 to about 8, from about 8 to about 10, from about 8 to about 9, from about 9 to about 10, from about 5 to about 10, about 6, about 6.5, about 7, about 7.5, about 8, about 8.5, about 9, about 9.5, or about 10.

5-HT$_4$R Agonists

The 5-HT$_4$R is a G-protein coupled receptor (GPCR) that activates G protein Gs and stimulates the cAMP/PKA signaling pathway, resulting in the phosphorylation of cAMP response element binding protein (CREB) and as a consequence the expression of a number of genes involved in neuroplasticity (A10). The majority of 5-HT$_4$Rs are expressed in the brain of primates and rodents specifically in the medium spiny neurons of the striatum, the ammon's horns (CA1 and CA3) of the hippocampus, the granule cells of the dentate gyrus and glutamatergic neurons in the cortex and amygdala (A11). In addition, 5-HT$_4$Rs are also found in hypothalamus, ventral pallidum, olfactory bulbs, septal area, and substantia nigra. Mice lacking the 5-HT$_4$R display anhedonia and a context-dependent anxiety-like behavior (A12) and various 5-HT$_4$R agonists can exert an antidepressant and anxiolytic-like activity (A6).

Whether in humans or in rodents, the expression of the serotoninergic type 4 receptor (5-HT$_4$) is found in the limbic regions ($_m$PFC, HPC and NAc). In addition, the basal ganglia, i.e., the caudate nucleus and the lenticular nucleus (putamen and pallidum), the black matter, and the amygdala, also express the 5-HT$_4$ receptor. The 5-HT$_4$ receptor is expressed at the somatodendritic level and at the level of the axon terminals of efferent spinal GABAergic neurons of the striatum, the Amon horns (CA1 and CA3) of the hippocampus, the granular cells of the dentate gyrus, and glutamatergic neurons of the cortex, the hippocampus and the amygdala.

5-HT$_4$ receptor is also found at the peripheral level, in particular at the cardiac level, where activation thereof exerts a positive inotropic effect, at the level of the gastro-intestinal tract where it is involved in intestinal motility, at the level of the adrenal glands where it plays a role in secretion of corticosterone, and at the level of the bladder where it causes contraction of the smooth muscles.

The 5-HT$_4$ receptor is a receptor having seven transmembrane domains. The N-terminal region faces towards the extracellular environment, whereas the C-terminal domain, coupled to a Gs protein, faces towards the cytoplasm. The activation of the 5-HT$_4$ receptor, e.g., by an agonist, can lead to the recruitment of the Gs protein which stimulates adenylate cyclase (AC) which is responsible for the production of cAMP. Protein kinase A (PKA),

activated by the cAMP, modulates different ionic currents and in particular potassium currents, the inhibition of which results in neuronal hyperexcitability. The PKA is also capable of phosphorylating the protein binding the response element to the cAMP (CREB – cAMP response element binding protein), which results in an increase in the transcription of neurotrophic brain factor (BDNF, brain-derived neurotrophic factor), involved in cognition, mood and cell survival.

The term "agonist" may refer to a substance, an agent or a compound capable of binding to and activating one or more receptors, such as 5-HT_4R. The term "agonist" may refer to a compound having the ability to initiate or enhance a biological function of a target protein (e.g., one or more receptors, such as 5-HT_4R), whether by enhancing or initiating the activity or expression of the target protein. 5-HT_4R agonists may be compounds that activate the action of the 5-HT4 receptor. The term "agonist" may be defined in the context of the biological role of the target protein. In one embodiment, an agonist is an agent that binds to a receptor (e.g., 5-HT_4R) and activates the receptor to produce a biological response. While agonists provided herein can specifically interact with (e.g., bind to) the target protein, compounds that initiate or enhance a biological activity of the target protein by interacting with other members of the signal transduction pathway of which the target protein is a member are also specifically included within this definition. A 5-HT_4R agonist may be a compound or an agent that activates the action of 5-HT_4R. A 5-HT_4R agonist may be any agent that acts directly or indirectly through or upon 5-HT_4R to produce a pharmacological effect. The terms “agonist of 5-HT_4R”, “agonist of the 5-HT4 receptor”, “5-HT4 receptor agonist”, and “5-HT_4R agonist” are used interchangeably herein.

The 5-HT_4R agonist may be selective for 5-HT4 receptors or it may be non-selective, exhibiting agonist or antagonist activity at other serotonin receptors. In one embodiment, the 5-HT_4R agonist is selective for 5-HT4 receptors.

The 5-HT_4R agonists may include full agonists, partial agonists, mixed 5-HT_4R agonists/antagonists, etc.

“Full agonists” may refer to agents bind to and activate a receptor with the maximum response that an agonist can elicit at the receptor. An agent may act as a full agonist in some tissues and as a partial agonist in other tissues, depending upon the relative numbers of receptors and differences in receptor coupling.

"Partial agonists" may refer to compounds able to bind and activate a given receptor, but having only partial efficacy at the receptor relative to a "full agonist" or complete agonist. Partial agonists can act as antagonists when competing with a full agonist for receptor

occupancy and producing a net decrease in the receptor activation compared to the effects or activation observed with the full agonist alone. Partial agonists may refer to mixed agonists/antagonists, which differentially affect a receptor function within different dose ranges. For example, partial agonists may serve as agonists at lower doses, and as antagonists at higher doses. Partial agonists may be compounds that have reduced efficacy for inducing conformational change in receptors (typically 40-80%) relative to full agonists, and which may induce agonist effects at low dose but antagonist effects at high dose.

The 5-HT_4R agonist may be an indole, a benzamide, a benzoate, an arylketone or a benzamide.

Non-limiting examples of 5-HT_4R agonists include: 1-(4-amino-5-chloro-2-methoxyphenyl)-3-[1(n-butyl)-4-piperidinyl]-1-propanone HCl (RS-67,333 or RS67333), 4-amino-5-chloro-2,3-dihydro-*N*-[1-3-methoxypropyl)-4-piperidinyl]-7-benzofuran carboxamide monohydrochloride (prucalopride), 4-[4-[4-Tetrahydrofuran-3-yloxy)-benzo[d]isoxazol-3-yloxymethyl]-piperidin-1-ylmethyl]-tetrahydropyran-4-ol (PF-04995274), and combinations thereof. Non-limiting examples of 5-HT_4R agonists also include: 2-[1-(4-Piperonyl)piperazinyl]benzothiazole (PPB), 5-methoxytryptamine, PRX-03140, cisapride ((±)-cis-4-amino-5-chloro-N-[1-[3-(4-fluorophenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxybenzamide monohydrate), BIMU-8 (2,3-Dihydro-N-[(3-endo)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl]-3-(1-methylethyl)-2-oxo-1H-benzimidazole-1-carboxamide, RS67506 (methylsulphonylamino)ethyl-4-piperidinyl]-1-propanone hydrochloride), mosapride (4-amino-5-chloro-2-ethoxy-N-[[4-[(4-fluorophenyl)methyl]-2-morpholinyl]methyl]benzamide citrate), tegaserod (2-[(5-Methoxy-1H-indol-3-yl)methylene]-N-pentyl-hydrazinecarboximidamide maleate), ML10302 (4-Amino-5-chloro-2-methoxybenzoic acid 2-(1-piperidinyl)ethyl ester hydrochloride), velusetrag (TD-5108) (N-[(1R,3R,5S)-8-[(2R)-2-hydroxy-3-(N-methylmethanesulfonamido)propyl]-8-azabicyclo[3.2.1]octan-3-yl]-2-oxo-1-(propan-2-yl)-1,2-dihydroquinoline-3-carboxamide), naropride (ATI-7505) ([(3R)-1-azabicyclo[2.2.2]octan-3-yl] 6-[(3S,4R)-4-[(4-amino-5-chloro-2-methoxybenzoyl)amino]-3-methoxypiperidin-1-yl]hexanoate, cinitapride (4-amino-N-[1-(cyclohex-3-en-1-ylmethyl)piperidin-4-yl]-2-ethoxy-5-nitrobenzamide), metoclopramide (4-amino-5-chloro-N-(2-(diethylamino)ethyl)-2-methoxybenzamide), renzapride (ATL-1251, BRL 24924, (±)-endo-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo [3.3.1]non-4-yl) benzamide), RQ-00000010 (4-{[4-({[4-(2,2,2-trifluoroethoxy)-1,2-benzisoxazol-3-yl]oxy}methyl)piperidin-1-yl]methyl}tetrahydro-2H-pyran-4-carboxylic acid), SUVN-D4010 (1-isopropyl-3-{5-[1-(3-methoxy propyl) piperidin-4-yl]-

[1,3,4]oxadiazol-2-yl}-1H-indazole), TD-8954 (4-{(4-[(2-isopropyl-1H-benzoimidazole-4-carbonyl)amino]methyl}- piperidin-1-ylmethyl)piperidine-1-carboxylic acid methyl ester), SC53116 (4-Amino-5-chloro-N-[[(1S,7aS)-hexahydro-1H-pyrrolizin-1-yl]methyl]-2-methoxy-benzamide), BIMU-1 (3-ethyl-2,3-dihydro-N-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2-oxo-1H- benzimidazole-1-carboxamide hydrochloride), donecopride (MR31147, which is: 1-(4-amino-5-chloro-2-methoxyphenyl)-3-[1-(cyclohexylmethyl)-4-piperidinyl]propan-1-one); LS 650155 (Caeserod, which is: 5-(8-amino-7-chloro-2,3-dihydrobenzo[b][1,4]dioxin-5-yl)-3-(1-phenethylpiperidin-4-yl)-1,3,4-oxadiazol-2(3H)-one hydrochloride); PF-00885706: N-[2-[(1R,8S)-4-[[4-(cyclobutylamino)-5-(trifluoromethyl)pyrimidin-2-yl]amino]-11-azatricyclo[6.2.1.02,7]undeca-2(7),3,5-trien-11-yl]-2-oxoethyl]acetamide, and combinations thereof.

RS-67,333 is a high-affinity 5-HT_4R partial agonist [22]. This drug is effective in improving behavioral deficits, decreasing the number of amyloid plaques as well as level of amyloid beta (Aβ) species, and decreasing hippocampal astrogliosis and microgliosis in the 5xFAD mouse model of Alzheimer's disease (AD) [23]. RS67333 is an arylketone. Incorporating an n-butyl group on the piperidine has increased the agonist activity with great effectiveness, optimal selectivity, and excellent bioavailability. Its increased hydrophobicity helps pass the blood-brain barrier, allowing for penetration into the brain (Eglen *et al.*, Pharmacological characterization of two novel and potent 5-HT4 receptor agonists, RS 67333 and RS 67506, in vitro and in vivo. Br. J. Pharmacol. 1995;115(8):1387-92).

Prucalopride is a selective, high affinity 5-HT_4R agonist [24]. Prucalopride is a derivative of the family of benzofurans which exhibits increased selectivity for 5-HT_4 receptor but no affinity for the hERG (human Ether-a-go-go Related Gene) channels. In 2018, it was approved by the FDA for chronic constipation and is currently being tested for chronic intestinal pseudo-obstruction. Prucalopride has also been tested in two separate clinical trials to investigate its effects on emotional processing in health volunteers after an acute (e.g., single dose) or chronic (e.g., 1 week) administration [25,26].

PF-04995274 is a potent, partial 5-HT_4R agonist [27]. A clinical trial was conducted to evaluate PF-04995274, alone or in combination with donepezil, on scopolamine-induced deficits in psychomotor and cognitive function in healthy adults; however, this trial was terminated, but not due to safety concerns [28]. Currently, a clinical trial is underway to test whether adjunctive administration of PF-04995247 has positive effects on emotional processing and neural activity in mediated, treatment-resistant (TRD) depressed patients compared to placebo [29].

Tegaserod is a partial agonist of the 5-HT_4 receptor, with moderate affinity for the 5-HT_1 (agonist) and 5-HT_{2A-C} (antagonist) receptors.

Cisapride is a parasympathomimetic which, by activating the 5-HT_4 receptor, increases the acetylcholine liberated in the enteric nervous system.

Cinitapride is a benzamide which acts as a 5-HT_{1A} and 5-HT_4 receptor agonist, and a 5-HT_{2A} receptor antagonist.

Mosapride is a selective 5-HT_4 receptor agonist, the main active metabolite of which acts as a 5-HT_3 receptor antagonist.

Metoclopramide is a 5-HT_4 and 5-HT_{3A} receptor agonist. It is a D2 receptor antagonist. It is also an M1 muscarinic receptor agonist, and an acetylcholinesterase inhibitor.

SUVN-D4010 is a powerful, selective and effective 5-HT4 receptor partial agonist, having good bioavailability via the oral route.

Mixed 5-HTR agonists/antagonists include, but are not limited to: buspirone, mianserin, trazodone, and mirtazapine.

The terms "serotonin," "5-hydroxytryptamine" and "5-HT" refers to a phenolic amine neurotransmitter produced from tryptophan by hydroxylation and decarboxylation in serotonergic neurons of the central nervous system and enterochromaffin cells of the gastrointestinal tract. Serotonin is a precursor of melatonin.

The term "pharmaceutically acceptable derivative" refers to any pharmaceutically acceptable salt, solvate, prodrug, e.g. ester, or other precursors, of a compound which upon administration to the recipient is capable of providing (directly or indirectly) the active compound or an active metabolite or residue thereof. Such salts include pharmaceutically acceptable basic or acid addition salts as well as pharmaceutically acceptable metal salts, ammonium salts and alkylated ammonium salts. Such derivatives are recognizable to those skilled in the art, without undue experimentation. Derivatives are described, for example, in Burger's Medicinal Chemistry and Drug Discovery, 5th Edition, Vol 1: Principles and Practice, which is incorporated herein by reference. In certain embodiments, pharmaceutically acceptable derivatives include salts, solvates, esters, carbamates, and phosphate esters.

The present agent/composition may be administered by various routes, including oral, intravenous (i.v. or IV), intranasal (i.n. or IN), intramuscular (i.m. or IM), caudal, intrathecal, and subcutaneous (s.c.) routes.

Pharmaceutical Compounds

The agents used in the present methods include all hydrates, solvates, and complexes of the compounds described herein. If a chiral center or another form of an isomeric center is present in a present compound, all forms of such isomer or isomers, including enantiomers and diastereomers, are intended to be covered herein. Compounds containing a chiral center may be used as a racemic mixture, an enantiomerically enriched mixture, or the racemic mixture may be separated using well-known techniques and an individual enantiomer may be used alone. The compounds described in the present disclosure may be in racemic form or as individual enantiomers. The enantiomers can be separated using known techniques, such as those described in Pure and Applied Chemistry 69, 1469-1474, (1997) IUPAC. In cases in which compounds have unsaturated carbon-carbon double bonds, both the cis (Z) and trans (E) isomers are within the scope of this disclosure. In cases wherein compounds may exist in tautomeric forms, such as keto-enol tautomers, each tautomeric form is contemplated as being included within this disclosure whether existing in equilibrium or predominantly in one form.

When the structure of the compounds used in this disclosure includes an asymmetric carbon atom such compound can occur as racemates, racemic mixtures, and isolated single enantiomers. All such isomeric forms of these compounds are expressly included in this disclosure. Each stereogenic carbon may be of the R or S configuration. It is to be understood accordingly that the isomers arising from such asymmetry (e.g., all enantiomers and diastereomers) are included within the scope of this disclosure, unless indicated otherwise. Such isomers can be obtained in substantially pure form by classical separation techniques and by stereochemically controlled synthesis, such as those described in "*Enantiomers, Racemates and Resolutions*" by J. Jacques, A. Collet and S. Wilen, Pub. John Wiley & Sons, NY, 1981. For example, the resolution may be carried out by preparative chromatography on a chiral column.

The present disclosure is also intended to include use of all isotopes of atoms occurring on the compounds disclosed herein. Isotopes include those atoms having the same atomic number but different mass numbers. Isotopically-labeled compounds can generally be prepared by conventional techniques known to those skilled in the art or by processes analogous to those described herein using an appropriate isotopically-labeled reagents in place of the non-labeled reagents employed.

The compounds of the instant disclosure may be in a salt form. As used herein, a "salt" is a salt of the instant compound which has been modified by making acid or base, salts of the compounds. In the case of compounds used for treatment of mammals, the salt is

pharmaceutically acceptable. Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as phenols. The salts can be made using an organic or inorganic acid. Such acid salts are chlorides, bromides, sulfates, nitrates, phosphates, sulfonates, formates, tartrates, maleates, malates, citrates, benzoates, salicylates, ascorbates, and the like. Phenolate salts are the alkaline earth metal salts, sodium, potassium or lithium. The term "pharmaceutically acceptable salt" in this respect, refers to the relatively non-toxic, inorganic and organic acid or base addition salts of compounds of the present invention. These salts can be prepared in situ during the final isolation and purification of the compounds of the invention, or by separately treating a purified compound of the invention in its free base or free acid form with a suitable organic or inorganic acid or base, and isolating the salt thus formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, phosphate, nitrate, acetate, valerate, oleate, palmitate, stearate, laurate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, napthylate, mesylate, glucoheptonate, lactobionate, and laurylsulphonate salts and the like. (See, e.g., Berge et al. (1977) "Pharmaceutical Salts", *J. Pharm. Sci.* 66:1-19).

The present methods also encompass administering a physiologically functional derivative of the present compound. As used herein, the term "physiologically functional derivative" refers to a compound (e.g., a drug precursor) that is transformed *in vivo* to yield the present compound or its active metabolite, or a pharmaceutically acceptable salt, hydrate or solvate of the compound. The transformation may occur by various mechanisms (*e.g.*, by metabolic or chemical processes), such as, for example, through hydrolysis in blood. Prodrugs are such derivatives, and a discussion of the use of prodrugs is provided by T. Higuchi and W. Stella, "Pro-drugs as Novel Delivery Systems," Vol. 14 of the A.C.S. Symposium Series, and in Bioreversible Carriers in Drug Design, ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987.

Dosages

In certain embodiments, the effective amount of the present agent is a dose of about 0.01 to about 3 mg per kilogram of body weight of the subject (mg/kg), i.e., from about 0.01 mg/kg to about 3 mg/kg body weight. In certain embodiments, the effective amount of the present compound ranges 0.001 to approximately 3 mg/kg body weight, 0.001 to approximately 2 mg/kg body weight, from about 0.01 mg/kg to about 3 mg/kg body weight, from about 0.01 to about 2 mg/kg of body weight, about 0.01 to about 1.5 mg/kg of body weight, about 0.05 to about 1.4 mg/kg of body weight, about 0.05 to about 1.3 mg/kg of body

weight, about 0.05 to about 1.2 mg/kg of body weight, about 0.05 to about 1.1 mg/kg of body weight, about 0.01 to about 1 mg/kg of body weight, or about 0.05 to about 0.7 mg/kg of body weight. In some aspects, the dose is about 0.05 to about 0.5 mg/kg. In some aspects, the dose is less than about 0.5 mg/kg, less that about 0.4 mg/kg, or less than about 0.3 mg/kg body weight. In some aspects, the effective amount of the present compound is a dose in the range of from about 0.01 mg/kg to about 1.5 mg/kg body weight. In some aspects, the effective amount of the present compound is a dose in the range of from about 0.01 mg/kg to about 1 mg/kg body weight. In some aspects, the effective amount of the present compound is a dose in the range of from about 0.01 mg/kg to about 0.75 mg/kg body weight. In some aspects, the effective amount of the present compound is a dose in the range of from about 0.75 mg/kg to about 1.5 mg/kg body weight. In some aspects, the effective amount of the present compound is a dose in the range of from about 0.5 mg/kg to about 1.2 mg/kg body weight. In some aspects, the effective amount of the present compound is a dose in the range of from about 0.05 mg/kg to about 0.5 mg/kg. In some aspects, the effective amount of the present compound is a dose of about 0.2 mg/kg or about 0.4 mg/kg body weight. In some aspects, the dose of the present compound is, about 0.01 to about 1 mg/kg, about 0.1 to about 0.5 mg/kg, about 0.8 to about 1.2 mg/kg, about 0.7 to about 1.1 mg/kg, about 0.05 to about 0.7 mg/kg, about 0.01 mg/kg, about 0.05 mg/kg, about 0.1 mg/kg, about 0.2 mg/kg, about 0.3 mg/kg, about 0.4 mg/kg, about 0.5 mg/kg, about 0.6 mg/kg, about 0.7 mg/kg, about 0.8 mg/kg, about 0.9 mg/kg, about 1.0 mg/kg, about 1.1 mg/kg, about 1.2 mg/kg, about 1.3 mg/kg, about 1.4 mg/kg, about 1.5 mg/kg, about 1.6 mg/kg, about 1.7 mg/kg, about 1.8 mg/kg, about 1.9 mg/kg, about 2.0 mg/kg, or about 3 mg/kg body weight.

In certain embodiments, the dose of the present compound per administration is from about 1 to about 250 mg, from about 10 mg to about 300 mg, about 10 mg to about 250 mg, about 10 to about 200 mg, about 15 to about 175 mg, about 20 to about 175 mg, about 8 mg to about 32 mg, about 50 mg to about 75 mg, about 25 to about 150 mg, about 25 to about 125 mg, about 25 to about 100 mg, about 50 to about 100 mg, about 50 mg to about 75 mg, about 75 mg to about 100 mg, or about 75 mg to about 200 mg, about 1 mg, 2 mg, 4 mg, 5 mg, 10 mg, 15 mg, 20 mg, 25 mg, 30 mg, 35 mg, 40 mg, 45 mg, 50 mg, 55 mg, 60 mg, 65 mg, 70 mg, 75 mg, 80 mg, 85 mg, 90 mg, 95 mg, 100 mg, 110 mg, 120 mg, 130 mg, 140 mg, 150 mg, 160 mg, 170 mg, 180 mg, 190 mg, 200 mg, 210 mg, 220 mg, 230 mg, 240 mg, and 250 mg. In some aspects, the dose of the present compound is about 50 mg. In some aspects, the dose of the present compound is about 75 mg. In some aspects, the total dose of the present compound is about 100 mg.

In certain embodiments, the therapeutically effective amount of the present agent is below the level that results in one or more side effects of the agent.

In some aspects, the (therapeutically) effective amount of the present agent d is about 0.01 mg to about 1000 mg, from about 0.01 mg to about 500 mg, from about 0.1 mg to about 250 mg, or any amount or range therein. In another aspect, the (therapeutically) effective amount of the present agent is, e.g., 0.01 mg, 0.025 mg, 0.05 mg, 0.1 mg, 0.5 mg, 1 mg, 5 mg, 10 mg, 25 mg, 50 mg, 55 mg, 60 mg, 65 mg, 70 mg, 75 mg, 80 mg, 90 mg, 95 mg, 100 mg, 150 mg, 200 mg, 250 mg, or 500 mg.

In certain embodiments, a therapeutically effective dose of the present agent may be adjusted depending on conditions of the disease/disorder to be treated or prophetically treated, the age, body weight, general health conditions, sex, and diet of the subject, dose intervals, administration routes, excretion rate, and combinations of drugs.

An initial dose of the present agent may be larger, followed by one or more smaller maintenance doses. Other ranges are possible, depending on the subject's response to the treatment. An initial dose may be the same as, or lower or higher than subsequently administered doses.

The present agent/composition may be administered daily, weekly, biweekly, several times daily, semi-weekly, every other day, bi-weekly, quarterly, several times per week, semi-weekly, monthly etc. The duration and frequency of treatment may depend upon the subject's response to treatment.

In certain embodiments, a subject may be administered 1 dose, 2 doses, 3 doses, 4 doses, 5 doses, 6 doses or more of the present agent/composition. In certain embodiments, a single dose of the present agent/composition is administered in the present method. In certain embodiments, multiple doses of the present agent/composition (e.g., 2 doses, 3 doses, 4 doses, 5 doses, 6 doses, 7 doses, 8 doses, 9 doses, 10 doses or more) are administered in the present method.

In certain embodiments, when there are more than one doses of the present agent/composition administered to a subject, the second dose is lower than the first dose. In certain embodiments, the second dose is an amount that is at most one-half, one-quarter, or one-tenth the amount of the first dose.

The number and frequency of doses may be determined based on the subject's response to administration of the composition, e.g., if one or more of the patient's symptoms improve and/or if the subject tolerates administration of the composition without adverse reaction.

In certain embodiments, the present agent/composition is administered at least once a day, at least twice a day, at least three times per day, or more. In certain embodiments, the present agent/composition is administered at least once a week, at least twice a week, at least three times per week, or more frequently. In certain embodiments, the present agent/composition is administered at least twice per month, or at least once per month.

Treatment using the present method can continue as long as needed.

Dosing Time Frame

In certain embodiments, the present agent/composition is administered to a subject prior to a stressor. In certain embodiments, the present agent/composition is administered to a subject both prior to and after a stressor. In certain embodiments, the present agent/composition is administered to a subject after a stressor. In certain embodiments, the present agent/composition is administered to a subject prior to a stressor, and again prior to a recurrence of the stressor or a different stressor.

In certain embodiments, the present agent/composition is administered to the subject about 12 hours to about 4 weeks, about 18 hours to about 4 weeks, about 1 day to about 3.5 weeks, about 2 days to about 3 weeks, about 3 days to about 3 weeks, about 4 days to about 3 weeks, about 5 days to about 3 weeks, about 6 days to about 3 weeks, about 2 days to about 2.5 weeks, about 3 days to about 2.5 weeks, about 4 days to about 2.5 weeks, about 5 days to about 2.5 weeks, about 6 days to about 2.5 weeks, about 1 week to about 2.5 weeks, about 1 week to about 2.5 weeks, about 1 week to about 2 weeks, about 5 minutes to about 3 days, about 10 minutes to about 2 days, about 15 minutes to about 24 hours, about 20 minutes to about 12 hours, about 30 minutes to about 8 hours, about 45 minutes to about 5 hours, about 1 hour to about 12 hours, about 2 hours to about 5 hours, about 5 minutes, about 10 minutes, about 15 minutes, about 20 minutes, about 30 minutes, about 45 minutes, about 1 hour, about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 8 hours, about 10 hours, about 12 hours, about 15 hours, about 1 day, about 1.5 days, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 1 week, about 8 days, about 9 days, about 10 days, about 11 days, about 12 days, about 13 days, about 2 weeks, about 2.5 weeks, about 3 weeks, about 3.5 weeks, or about 4 weeks, prior to, and/or after a stressor.

In certain embodiments, the administration of the present agent/composition is continued over a period of up to 2 days, up to 3 days, up to 4 days, up to 5 days, up to 6 days, up to 1 week, up to 2 weeks, up to 3 weeks, up to 4 weeks, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 1 week, about 2 weeks, about 3 weeks, about 4

weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 9 weeks, about 10 weeks, or longer.

In certain embodiments, the present agent/composition is administered once, twice, at least twice, at least three times, at least four times, at least five time, at least six times, at least seven times, at least eight times, at least nine times, or more per treatment.

In certain embodiments, the present agent/composition is administered at least once a day, at least twice a day, at least three times per day, at least once a week, at least twice a week, at least three times a week, at least once per month, at least twice per month, or more frequently. Treatment can continue as long as needed. The present agent/composition may be administered daily, weekly, biweekly, several times daily, semi-weekly, every other day, bi-weekly, quarterly, several times per week, semi-weekly, monthly etc. The duration and frequency of treatment may depend upon the subject's response to treatment.

Stressors

A stressor is a stimulus that causes stress. It can be an event or other factor that disrupts the body's homeostasis of temperature, blood pressure, and/or other functions. In certain embodiments, a stressor is a traumatic or stressful event. Because humans have sophisticated brains and thought processes, anticipating a disruption can also be a stressor. In certain embodiments, a stressor is injury, trauma, combat, warfare, surgery, an accident, a criminal assault, child abuse, natural or human-caused disasters, a crash, grief, hunger, heat, cold, chemical exposure, autoimmune disease, infectious disease, viral infection, cancer, exhaustion, physical distress, neuropathy, hyperalgesia, allodynia, emotional distress, or depression. A traumatic event may be an event or something that threatens the person's life or the life of a close one or it could be something witnessed. U.S. Patent Application No. 20140018339.

A stressor may be acute, or may be chronic.

There are numerous physiological processes that are altered in response to stress. Among these are altered cortisol, corticotropin, catecholamine and serotonin levels. These levels return to baseline after an acute stressor is removed (McEwen N Eng J Med 1998 338(3):171-179). These biochemical markers of stress in turn lead to ill health and psychosocial disorders. Consequently, stress plays a major role in physical and mental health. Stress can affect the onset of, or susceptibility to disease. It can also affect the progression or course of disease even when there is another underlying pathophysiology of the disease. Recovery from an existing disease can also be delayed due to stress. For example, stress is a contributing factor to high blood pressure, heart disease, headaches,

colitis, irritable bowel syndrome, temporo-mandibular joint disorder, cancer, peptic ulcers, insomnia, skin disorders and asthma. Stress can also aggravate other conditions such as multiple sclerosis, diabetes, herpes, mental illness, substance abuse and psychiatric disorders characterized by the presence of violent or aggressive tendencies. Particularly, stress contributes to functional somatic disorders, affective disorders and major depressive disorder (MDD). These include disorders such as chronic fatigue syndrome (CFS), fibromyalgia (FMS), Gulf War Syndrome, anxiety and post-traumatic stress disorder (PTSD). Stressors that disrupt normal exercise or sleep patterns.

Additional examples of use include administration prior to military deployment to protect Service members (active combat soldiers, battlefield surgeons, etc.) and even military working dogs against stress. Potential non-military use cases include, but are not limited to: police, firefighters, first responders, emergency medical technicians (EMTs), emergency room (ER) doctors, prison guards (and prisoners), humanitarian aid workers, and refugees.

In certain embodiments, a subject may be administered the present agent or composition prior to a situation in which the subject (such as an early responder or military personnel) is likely to be exposed to traumatic stress, immediately after exposure to traumatic stress, and/or when the subject feels that his or her PTSD symptoms are likely to appear.

Resilience to Stress

Resilience to stress refers to the capacity of a subject to adapt or change successfully, and/or to maintain physiological, neurological, or psychological homeostasis, in the face of a stressor (e.g., adversity). As used herein, the term "enhancing resilience" refers to increasing the ability of a subject to experience a stressor (e.g., a traumatic event) without suffering a stress-induced affective disorder, and/or with less post-event symptomatology or disruption of homeostasis and/or normal activities of daily living. In certain embodiments, improving resilience can prevent a stress-induced affective disorder. In certain embodiments, improving resilience can reduce at least one of the signs, symptoms, or symptom clusters of a stress-induced affective disorder. In certain embodiments, the present method enhances a subject's resilience to stress, helps protect against developing stressor-related psychopathology, decrease the functional consequences of stressor-induced disorders (e.g., PTSD, etc.), and reduce medical morbidity and mortality.

The Connor-Davidson Resilience Scale (CD-RISC) is a 25-item self-report scale, each rated on a 5-point scale (0-4), with higher scores reflecting greater resilience (Connor K M & Davidson, J R T. Development of a new resilience scale: the Connor-Davidson Resilience Scale (CD-RISC). Depression and Anxiety, 2003: 18: 71-82).

Resilience, psychological growth and life satisfaction may be measured with the CD-RISC, the Purpose in Life Scale, the abbreviated MOS Social Support Survey, the PTGI, and the Q-LES-Q.

Combination Therapy

The present agent or composition may be administered to a subject alone, or may be administered to a subject in combination with one or more other treatments/agents.

In certain embodiments, the second agent is an anti-depressant, an anxiolytic, or combinations thereof. In certain embodiments, the second agent is a serotonin reuptake inhibitor (SRI), or a selective serotonin reuptake inhibitor (SSRI). In certain embodiments, the second agent is fluoxetine, paroxetine, sertraline, lithium, riluzole, prazosin, lamotrigine, ifenprodil, or combinations thereof. In certain embodiments, the second agent is a dual serotonin norepinephrine reuptake inhibitor compound (DRI). In certain embodiments, the second agent is venlafaxine, duloxetine, milnacipran, or combinations thereof. In certain embodiments, the second agent is a non-tricyclic triple reuptake inhibitor (TRI).

In certain embodiments, the present agent or composition is administered to a subject in combination with one or more treatments/agents such as antidepressants, analgesics, muscle relaxants, anorectics, stimulants, antiepileptic drugs, and sedative/hypnotics. Non-limiting examples of compounds that can be administered in combination with the present compound or composition include, neurontin, pregabalin, pramipexole, L-DOPA, amphetamine, tizanidine, clonidine, tramadol, morphine, tricyclic antidepressants, codeine, carbamazepine, sibutramine, amphetamine, valium, trazodone and combinations thereof.

In certain embodiments, combination therapy means simultaneous administration of the agents in the same dosage form, simultaneous administration in separate dosage forms, or separate administration of the agents.

In certain embodiments, the second agent/treatment is used as adjunctive therapy to the present agent or composition. In certain embodiments, the treatment includes a phase wherein treatment with the second agent/treatment takes place after treatment with the present agent or composition has ceased. In certain embodiments, the treatment includes a phase where treatment with the present agent or composition and treatment with the second agent/treatment overlap.

Combination therapy can be sequential or can be administered simultaneously. In either case, these drugs and/or therapies are said to be "co-administered." It is to be understood that "co-administered" does not necessarily mean that the drugs and/or therapies

are administered in a combined form (i.e., they may be administered separately (e.g., as separate compositions or formulations) or together (e.g., in the same formulation or composition) to the same or different sites at the same or different times).

In certain embodiments, a subject is treated concurrently (or concomitantly) with the present agent or composition and a second agent. In certain embodiments, a subject is treated initially with the present agent or composition, followed by cessation of the present compound or composition treatment and initiation of treatment with a second agent. In certain embodiments, the present agent or composition is used as an initial treatment, e.g., by administration of one, two or three doses, and a second agent is administered to prolong the effect of the present agent or composition, or alternatively, to boost the effect of the present agent or composition. A person of ordinary skill in the art will recognize that other variations of the presented schemes are possible, e.g., initiating treatment of a subject with the present compound or composition, followed by a period wherein the subject is treated with a second agent as adjunct therapy to the present compound or composition treatment, followed by cessation of the present compound or composition treatment.

The present compound and the other pharmaceutically active agent(s) may be administered together or separately and, when administered separately this may occur simultaneously or sequentially in any order. The amounts of the present agent and the other pharmaceutically active agent(s) and the relative timings of administration will be selected in order to achieve the desired combined therapeutic effect.

In various embodiments, the therapies (e.g., a composition provided herein and a second agent in a combination therapy) are administered less than 5 minutes apart, less than 30 minutes apart, 1 hour apart, at about 1 hour apart, at about 1 to about 2 hours apart, at about 2 hours to about 3 hours apart, at about 3 hours to about 4 hours apart, at about 4 hours to about 5 hours apart, at about 5 hours to about 6 hours apart, at about 6 hours to about 7 hours apart, at about 7 hours to about 8 hours apart, at about 8 hours to about 9 hours apart, at about 9 hours to about 10 hours apart, at about 10 hours to about 11 hours apart, at about 11 hours to about 12 hours apart, at about 12 hours to 18 hours apart, 18 hours to 24 hours apart, 24 hours to 36 hours apart, 36 hours to 48 hours apart, 48 hours to 52 hours apart, 52 hours to 60 hours apart, 60 hours to 72 hours apart, 72 hours to 84 hours apart, 84 hours to 96 hours apart, or 96 hours to 120 hours part. In certain embodiments, the therapies are administered no more than 24 hours apart or no more than 48 hours apart. In certain embodiments, two or more therapies are administered within the same patient visit. In other embodiments, the composition provided herein and the second agent are administered concurrently. In other

embodiments, the composition provided herein and the second agent are administered at about 2 to 4 days apart, at about 4 to 6 days apart, at about 1 week part, at about 1 to 2 weeks apart, or more than 2 weeks apart. In certain embodiments, administration of the same agent may be repeated and the administrations may be separated by at least 1 day, 2 days, 3 days, 5 days, 10 days, 15 days, 30 days, 45 days, 2 months, 75 days, 3 months, or 6 months. In other embodiments, administration of the same agent may be repeated and the administration may be separated by at least at least 1 day, 2 days, 3 days, 5 days, 10 days, 15 days, 30 days, 45 days, 2 months, 75 days, 3 months, or 6 months. In certain embodiments, a composition provided herein and a second agent are administered to a subject in a sequence and within a time interval such that the composition provided herein can act together with the other agent to provide an increased benefit than if they were administered otherwise. For example, the second active agent can be administered at the same time or sequentially in any order at different points in time; however, if not administered at the same time, they should be administered sufficiently close in time so as to provide the desired therapeutic or prophylactic effect. In one embodiment, the composition provided herein and the second active agent exerts their effect at times which overlap. Each second active agent can be administered separately, in any appropriate form and by any suitable route. In other embodiments, the composition provided herein is administered before, concurrently or after administration of the second active agent. The term "about" refers to $\pm$10% of the referenced value. In other embodiments, courses of treatment are administered concurrently to a patient, i.e., individual doses of the second agent are administered separately yet within a time interval such that the compound provided herein can work together with the second active agent. For example, one component can be administered once per week in combination with the other components that can be administered once every two weeks or once every three weeks. In other words, the dosing regimens are carried out concurrently even if the therapeutics are not administered simultaneously or during the same day. The second agent can act additively or synergistically with the compound provided herein. In one embodiment, the composition provided herein is administered concurrently with one or more second agents in the same pharmaceutical composition. In another embodiment, a composition provided herein is administered concurrently with one or more second agents in separate pharmaceutical compositions. In still another embodiment, a composition provided herein is administered prior to or subsequent to administration of a second agent. Also contemplated are administration of a composition provided herein and a second agent by the same or different routes of administration, e.g., oral and parenteral. In certain embodiments, when the

composition provided herein is administered concurrently with a second agent that potentially produces adverse side effects including, but not limited to, toxicity, the second active agent can advantageously be administered at a dose that falls below the threshold that the adverse side effect is elicited.

Encompassed by the present disclosure are methods to prophylactically treat a subject prior to a stressor. In certain embodiments, the present agent/composition and method prevent or delay a stress-induced affective disorder or stress-induced psychopathology in a subject. In certain embodiments, stress-induced affective disorders include major depressive disorder and posttraumatic stress disorder.

Stress-Induced Affective Disorders

There are numerous disorders that are either caused by or exacerbated by stress. The present agent/composition and method may prevent or delay a stress-induced affective disorder or stress-induced psychopathology. Stress-induced affective disorders or stress-induced psychopathologies which may be prevented or treated by the present agent/composition and method include, but are not limited to, addictive disorders such as substance abuse, anorexia, bulimia, obesity, smoking addiction, and weight addiction; anxiety disorders such as agoraphobia, anxiety disorder, obsessive compulsive disorder, panic attacks, performance anxiety, phobias, and post-traumatic stress disorder (PTSD); psychiatric disorders such as stress-induced psychiatric disorders; autoimmune diseases such as allergies, arthritis, fibromyalgia, fibromytosis, lupus, multiple sclerosis, rheumatoid arthritis, Sjogren's syndrome, and vitiligo; cancer such as bone cancer, brain cancer, breast cancer, cervical cancer, colon cancer, Hodgkin's disease, leukemia, liver cancer, lung cancer, lymphoma, multiple myeloma, ovarian cancer, pancreatic cancer, and prostate cancer; cardiovascular disorders such as arrhythmia, arteriosclerosis, Burger's disease, essential hypertension, fibrillation, mitral valve prolapse, palpitations, peripheral vascular disease, Raynaud's disease, stroke, tachycardia, and Wolff-Parkinson-White Syndrome; and developmental disorders such as attention deficit disorder, concentration problems, conduct disorder, dyslexia, hyperkinesis, language and speech disorders, and learning disabilities.

Anxiety Disorders

The present agent/composition and method may prevent or delay an anxiety disorder. The five major types of anxiety disorders are: panic disorder, obsessive-compulsive disorder, post-traumatic stress disorder, generalized anxiety disorder and phobias (including social phobia, also called social anxiety disorder). Each anxiety disorder has its own distinct features, but they are all bound together by the common theme of excessive, irrational fear

and dread. It is common for an anxiety disorder to accompany depression, eating disorders, substance abuse, or another anxiety disorder.

Panic disorder is characterized by repeated episodes of intense fear that strike often and without warning. Physical symptoms include chest pain, heart palpitations, shortness of breath, dizziness, abdominal distress, feelings of unreality, and fear of dying. Obsessive-compulsive disorder is characterized by repeated, unwanted thoughts or compulsive behaviors that seem impossible to stop or control. Generalized Anxiety Disorder is characterized by exaggerated worrisome thoughts and tension about everyday routine life events and activities, lasting at least six months. Almost always anticipating the worst even though there is little reason to expect it; accompanied by physical symptoms, such as fatigue, trembling, muscle tension, headache, or nausea. Phobias are characterized into two major types of phobias, social phobia and specific phobia. People with social phobia have an overwhelming and disabling fear of scrutiny, embarrassment, or humiliation in social situations, which leads to avoidance of many potentially pleasurable and meaningful activities. People with specific phobia experience extreme, disabling, and irrational fear of something that poses little or no actual danger; the fear leads to avoidance of objects or situations and can cause people to limit their lives unnecessarily.

Posttraumatic Stress Disorder (PTSD)

Typically, a subject suffering from PTSD was exposed to a traumatic event in which the person experienced, witnessed, or was confronted with an event or events that involved actual or threatened death or serious injury, or a threat to the physical integrity of self or others and the person's response involved intense fear, helplessness, or horror.

Having repeated intrusive memories of the trauma exposure is one of the core symptoms of PTSD. Patients with PTSD are known to display impairments in learning and memory during neuropsychological testing. Other core symptoms of PTSD include heightened stress sensitivity (startle), tension and anxiety, memory disturbances, and dissociation.

In certain embodiments, the present method prevents or inhibits the development of post-traumatic stress disorder (PTSD) in a subject. In certain embodiments, the present method prevents or inhibits the development of one or more PTSD-like symptoms. In certain embodiments, a subject may be administered the present agent or composition prior to a situation in which the subject (such as an early responder or military personnel) is likely to be exposed to traumatic stress, immediately after exposure to traumatic stress, and/or when the subject feels that his or her PTSD symptoms are likely to appear.

Typically, the traumatic event is persistently re-experienced in one or more of the following ways: recurrent and intrusive distressing recollections of the event, including images, thoughts, or perceptions, recurrent distressing dreams of the event, acting or feeling as if the traumatic event were recurring (includes a sense of reliving the experience, illusions, hallucinations, and dissociative flashback episodes, including those that occur on awakening or when intoxicated), intense psychological distress at exposure to internal or external cues that symbolize or resemble an aspect of the traumatic event, physiological reactivity on exposure to internal or external cues that symbolize or resemble an aspect of the traumatic event. An individual suffering from PTSD also has persistent avoidance of stimuli associated with the trauma and numbing of general responsiveness (not present before the trauma), as indicated by 3 or more of the following: efforts to avoid thoughts, feelings, or conversations associated with the trauma, efforts to avoid activities, places, or people that arouse recollections of the trauma, inability to recall an important aspect of the trauma, significantly diminished interest or participation in significant activities, feeling of detachment or estrangement from others, restricted range of affect (e.g., unable to have loving feelings), sense of a foreshortened future (e.g., does not expect to have a career, marriage, children, or a normal life span), persistent symptoms of increased arousal (not present before the trauma), as indicated by 2 or more of the following: difficulty falling or staying asleep, irritability or outbursts of anger, difficulty concentrating, hypervigilance, exaggerated startle response. The disturbance, which has lasted for at least a month, causes clinically significant distress or impairment in social, occupational, or other important areas of functioning.

In certain embodiments, the present compound or composition prevents, reduces, eliminates or delays one or more of the symptoms including, but not limited to, re-experiencing of the traumatic experience in the form of intrusive memories, nightmares, flashbacks; emotional and physical reactions triggered by reminders of the trauma; distancing from others; decreased interest in activities and other people; numbing of feelings; avoidance of trauma reminders; hyperarousal symptoms, including disrupted sleep, irritability, hypervigilance, decreased concentration; increased startle reflex; and combinations thereof.

Whatever the source of the problem, some people with PTSD repeatedly relive the trauma in the form of nightmares and disturbing recollections during the day. They may also experience other sleep problems, feel detached or numb, or be easily startled. They may lose interest in things they used to enjoy and have trouble feeling affectionate. They may feel irritable, more aggressive than before, or even violent. Things that remind them of the trauma may be very distressing, which could lead them to avoid certain places or situations that bring

back those memories.

The disorder may be accompanied by depression, substance abuse, or one or more other anxiety disorders. In severe cases, the person may have trouble working or socializing.

Major Depressive Disorder

Major depressive disorder refers to a class of syndromes characterized by negative affect and repeated episodes of depression without any history of independent episodes of mood elevation and over-activity that fulfill the criteria of mania. Multiple subtypes of major depressive disorders are recognized, including these with atypical characteristics, psychotic components, etc. The age of onset and the severity, duration and frequency of the episodes of depression are all highly variable. The disorder may begin at any age. The symptoms of major depressive disorder typically develop over days to weeks. Prodromal symptoms include generalized anxiety, panic attacks, phobias or depressive symptoms and may occur during several months preceding the episode. Individual episodes also last between 3 and 12 months but recur less frequently. Most patients are asymptomatic between episodes, but a minority of patients may develop a persistent depression, mainly in old age. Individual episodes of any severity are often precipitated by stressful life events. Common symptoms of a depressive episode include reduced concentration and attention; reduced self-esteem and self-confidence; ideas of guilt and unworthiness, ideas or acts of self-harm or suicide; disturbed sleep; and diminished appetite. In certain embodiments, a major depressive episode follows a psychosocial stressor, e.g., death of a loved one, marital separation, childbirth or the end of an important relationship.

The lowered mood varies little from day to day and is often unresponsive to circumstances, yet may show a characteristic diurnal variation as the day goes on. As with manic episodes, the clinical presentation shows marked individual variations, and atypical presentations are particularly common in adolescence. In some cases, anxiety, distress, and motor agitation may be more prominent at times that the depression, and the mood change may also be masked by added features such as irritability, excessive consumption of alcohol, histrionic behavior, and exacerbation of pre-existing phobic or obsessional symptoms, or by hypochondria.

Psychiatric evaluations

In certain embodiments, the effects or efficacy of treatment with the present agent/composition are evaluated by the subject and/or a medical professional, e.g., the subject's physician. In certain embodiments, the evaluation is conducted within about 10 minutes, within about 15 minutes, within about 20 minutes, within about 25 minutes, within

about 0.5 hours, within about 1 hour, within about 2 hours, within about 2.5 hours, within about 3 hours, within about 3.5 hours, within about 4 hours, within about 4.5 hours, within about 5 hours, within about 5.5 hours, within about 6 hours, within about 6.5 hours, within about 7 hours, within about 7.5 hours, within about 8 hours, within about 8.5 hours, within about 9 hours, within about 9.5 hours, within about 10 hours, within about 10.5 hours, within about 11 hours, within about 11.5 hours, within about 12 hours, within about 18 hours, within about 1 day, within about 2 days, within about 3 days, within about 4 days, within about 5 days, within about 6 days, within about 1 week, within about 2 weeks, within about 3 weeks, within about 4 weeks, within about 1 month, within about 2 months, within about 3 months, within about 4 months, within about 5 months, within about 6 months, within about 1 year, within about 2 years, or longer, following a stressor and/or administration of the present agent/composition.

Psychiatric evaluations of a patient being treated with the present method can be conducted to determine whether the method is effective. In certain embodiments, the psychiatric evaluation may be carried out before treatment, at the time of treatment, during treatment, and/or after treatment. When the psychiatric evaluation is carried out both before treatment and after (and/or during) treatment with the present method, the results of the evaluation before treatment can provide a baseline for comparison to the results of the evaluation during and/or after treatment. In certain embodiments, psychiatric evaluation is conducted only after treatment.

Psychophysiological stress tests can be performed to measure the amount of stress-induced anxiety present in the various systems of the body (i.e. muscular, cardiovascular, digestive, respiratory and neurological systems). These stress tests are routinely used in the art. Test results are compared to both local and national norms, to determine if the individual is exhibiting an excessive amount of physiological anxiety and whether or not they are able to recover from a standardized stressful stimuli in an appropriate length of time.

Psychiatric testing can be used to monitor a subject to determine the emotional and/or social etiology of the stress disorder. These tests are known in the art and include health-related assessments, mental health assessments, personality tests, and personality type assessment.

In certain embodiments, clinician-administered evaluation and/or self-report instruments are used, with the aim of measuring baseline symptomatology as well as drug actions on (1) the overall severity of the disorder, (2) the core symptoms, and (3) depressed mood.

Non-limiting examples of psychiatric evaluation tools and questionnaires include the following measures.

The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) includes the revised diagnostic criteria for PTSD. See, American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition. Arlington, Va., American Psychiatric Association, 2013. See also ptsd.va.gov/professional/PTSD-verview/dsm5_criteria_ptsd.asp.

The Structured Clinical Interview for DSM-IV Axis I Disorders, Patient Edition (SCID-P) is a semi-structured interview that provides probe questions as well as follow-up questions to be asked by the clinician to assist in diagnosis. First et al., Structured Clinical Interview for DSM-IV TR Axis I Disorders, Research Version, Patient Edition (SCID-I/P). New York: New York State Psychiatric Institute, Biometrics Research; 2001. It includes an overview to obtain information about demographics, work, chief complaint, history of present illness, past history, treatment history, and current functioning. The main body of SCID-P includes 9 modules that are designed to diagnose 51 mental illnesses in all.

The SCID-P for DSM-5 is the SCID--Patient version, and is the next edition of the SCID modified to incorporate the new DSM-5 criteria.

The Clinician-Administered PTSD Scale (CAPS) is a structured clinical interview designed to assess the essential features of PTSD as defined by the DSM-IV. Weathers et al., Clinician-administered PTSD scale: a review of the first ten years of research. Depress Anxiety. 2001; 13(3):132-156. The CAPS can be used to provide categorical ratings of diagnostic status as well as a quantitative index of symptom severity. Both frequency and intensity scores are derived for each individual symptom. The CAPS total score is based on an individual's response to the 17 items that assess the frequency and intensity of current PTSD symptoms. Subscales of the CAPS are utilized to assess specific symptom clusters. The total score can range from 0 to 136.

The Clinician-Administered PTSD Scale for DSM-5 (CAPS-5) is a 30-item structured interview that can be used to make current (past month) diagnosis of PTSD, make lifetime diagnosis of PTSD, and to assess PTSD symptoms over the past week. CAPS-5 is a 30-item questionnaire, corresponding to the DSM-5 diagnosis for PTSD. The language of the CAPS-5 reflects both changes to existing symptoms and the addition of new symptoms in DSM-5. Weathers, F. W., et al (2013). The Clinician-Administered PTSD Scale for DSM-5 (CAPS-5).

The Treatment Outcome PTSD Scale (TOP-8) is a brief interviewer-administered scale designed specifically for the assessment of commonly occurring signs and symptoms of

PTSD that are subject to change in response to treatment (Davidson, J. R., & Colket, J. T. (1997). The eight-item treatment-outcome post-traumatic stress disorder scale: A brief measure to assess treatment outcome in post-traumatic stress disorder. International Clinical Psychopharmacology, 12(1), 41-45). The TOP-8 is comprised of eight items, each measured on a scale of 0-4, with defined anchors given for each item. The items are representative of the three core features of PTSD with a maximum possible score of 32.

The Hamilton Psychiatric Rating Scale for Anxiety (HAM-A) is a widely used observational rating measure of anxiety severity. The scale consists of 14 items. Each item is rated on a scale of 0 to 4. This scale is administered to assess the severity of anxiety and its improvement during the course of treatment. The HAM-A total score is the sum of the 14 items and the score ranges from 0 to 56. Hamilton M. The Assessment of Anxiety-States by Rating. Br J Med Psychol. 1959; 32(1):50-55.

The Montgomery-Asberg Depression Rating Scale (MADRS) is a 10-item instrument used for the evaluation of depressive symptoms in adults and for the assessment of any changes to those symptoms. Montgomery S. A., et al., A new depression scale designed to be sensitive to change. Br J Psychiatry. 1979 April; 134:382-389. Each of the 10 items is rated on a scale of 0 to 6, with differing descriptors for each item. These individual item scores are added together to form a total score, which can range between 0 and 60 points.

The Young Mania Rating Scale, item 1 (YMRS-1) used to assess mood elevation on the infusion days. Young R C, et al. Rating-Scale for Mania--Reliability, Validity and Sensitivity. Br J Psychiatry. 1978; 133(NOV):429-435.

The Brief Psychiatric Rating Scale (BPRS) is used to assess acute behavioral changes during the infusions. Overall J E et al., The Brief Psychiatric Rating-Scale. Psychol. Rep. 1962; 10(3):799-812 Four key BPRS items for the positive (+) symptoms of psychosis are used: conceptual disorganization, hallucinatory behavior, suspiciousness, and unusual thought content. Three items representing the negative (-) symptoms of psychosis will also be used: blunted affect, emotional withdrawal, and motor retardation.

The Clinician-Administered Dissociative States Scale (CADSS) is used to measure dissociative effects during the infusions. Bremner J D, et al., Measurement of Dissociative States with the Clinician-Administered Dissociative States Scale (CADSS). J Trauma Stress. 1998; 11(1):125-136 The scale includes 19 questions and 8 observer ratings scored from 0 (not at all) to 4 (extremely). The CADSS measures impairment in body perception, environmental perception, time perception, memory impairment, and feelings of unreality.

The Patient Rating Inventory of Side Effects (PRISE) is a patient self-report used to

qualify side effects by identifying and evaluating the tolerability of each symptom. Levine J, Schooler N R. SAFTEE: A technique for the systematic assessment of side effects in clinical trials. Psychopharmacol Bull. 1986; 22(2):343-381.

The Clinical Global Impression (CGI) scale assesses treatment response in psychiatric patients. The administration time is 2 minutes. This scale consists of three items: Severity of Illness (item 1); Global Improvement (item 2); and Efficacy Index (item 3). Item 1 is rated on a seven-point scale (1=normal, 7=among the most extremely ill patients) as is item 2 (1=very much improved, 7=very much worse). Each includes an additional response of "not assessed." Item 3 is rated on a four-point scale (from "none" to "outweighs therapeutic effect").

The Impact of Events Scale (IES) is one of the most widely used self-report measures of stress reactions to traumatic events. Horowitz et al., Impact of Event Scale: a measure of subjective stress. Psychosom Med. 1979 May; 41(3):209-218. See also, Weiss et al., The Impact of Event Scale--Revised In: Wilson J, Keane T M, eds. Assessing psychological trauma and PTSD. New York: Guilford; 1996:399-411. It measures both intrusion and avoidance. Sundin et al., Impact of Event Scale: psychometric properties. Br J Psychiatry. 2002 March; 180:205-209. Joseph S. Psychometric evaluation of Horowitz's Impact of Event Scale: a review. J Trauma Stress. 2000 January; 13(1):101-113. The total score can range from 0 to 75.

The Posttraumatic Stress Disorder Checklist (PCL-5) is a 17-item self-report measure reflecting DSM-5 symptoms of PTSD. The PCL-5 measures symptoms in response to stressful situations (Weathers, F., et al. (1993). The PTSD checklist (PCL): Reliability, validity, and diagnostic utility. Annual Convention of the International Society for Traumatic Stress Studies, San Antonio, Tex.).

The Quick Inventory of Depressive Symptomatology, Self Report (QIDS-SR) is a 16-item self-rated instrument designed to assess the severity of depressive symptoms present in the past seven days. Rush A J, Trivedi M H, Ibrahim H M et al. The 16-Item quick inventory of depressive symptomatology (QIDS), clinician rating (QIDS-C), and self-report (QIDS-SR): a psychometric evaluation in patients with chronic major depression. Biol. Psychiatry. 2003; 54(5):573-583. The 16 items cover the nine symptom domains of major depression, and are rated on a scale of 0-3. Total score ranges from 0 to 27, with ranges of 0-5 (normal), 6-10 (mild), 11-15 (moderate), 16-20 (moderate to severe), and 21+ (severe).

The Childhood Trauma Questionnaire (CTQ) is a 28-item self-report instrument that assesses childhood trauma in the following areas: physical, sexual and emotional abuse and

physical and emotional neglect. Bernstein D P, Stein J A, Newcomb M D et al. Development and validation of a brief screening version of the Childhood Trauma Questionnaire. Child Abuse Negl. 2003 February; 27(2):169-190. Each item is rated on a scale of 1 (never true) to 5 (very often true). The 5 subscales are then totaled, with scores ranging from 5-25 for each traumatic category.

Visual Analogue Scales (VAS) are used to assess subjective state changes. Bond A, Lader M. The use of analogue scales in rating subjective feelings. Br J Med Psychol. 1974; 47(3):211-218. They are 100-mm horizontal lines marked proportionately to the perceived intensity of the subjective experience (0=not at all, to 10=extremely) for the following states: anxious, depressed, drowsy, high, hungry, and nauseous.

The Sheehan Disability Scale (SDS) is a self-report disability measure. It has demonstrated sensitivity to impairment and changes as a result of treatment across a wide range of psychiatric disorders. The SDS asks only about current levels of impairment, providing no indication of whether the person has done better or worse in the past, thus making it a reasonable short-term outcome measure that is un-confounded by historical impressions. The dependent variable is the total score, which is based on the sum of three 10-point items (work, social life, and family life), with higher scores reflecting greater disability. Sheehan D. The Anxiety Disease. New York, N.Y.: Scribner; 1983.

The Wechsler Abbreviated Scale of Intelligence 2-Subtest (WASI-2) is a reliable brief measure of IQ for 6 to 89 year-olds that includes Vocabulary (an estimate of verbal fluid abilities) and Matrix Reasoning (an estimate of nonverbal fluid abilities). Wechsler D. Wechsler Abbreviated Scale of Intelligence San Antonio, Tex.: Psychological Corporation; 1999. It is extensively used in clinical, educational, and research settings. Average reliability coefficient is 0.96 and test-retest reliability is 0.88.

The Hopkins Verbal Learning Test (HVLT) is a repeatable test of memory acquisition and delayed recall of words. Subjects are presented with the same 12-item list for 3 learning trials and asked each time to repeat the items on each list. Delayed recall and recognition conditions are administered later. Dependent variables used in this study include total learning over the 3 trials (for the acquisition variable) and total delayed recall score (for the recall component). Brandt J, Benedict R. Hopkins Verbal Learning Test, Revised. Odessa, Fla.: Psychological Assessment Resources; 1997.

The Profile of Mood States-Bipolar (POMS-Bi) scale measures moods and feelings primarily in clinical rather than nonclinical settings. It can help to determine an individual's psychiatric status for therapy, or be used to compare mood profiles associated with various

personality disorders. It is also a useful instrument in identifying the effects of drug treatments.

The Post-Traumatic Cognitions Inventory (PTCI) is a 33-item scale, which is rated on a Likert-type scale ranging from 1 (totally disagree) to 7 (totally agree). Scale scores are formed for the three subscales, which show a high degree of intercorrelation (rs=0.57-0.75).

The New Cognitions scale is a 6-item pilot scale, which is rated on a Likert-type scale ranging from 1 (not at all) to 4 (a lot). The scale is based on the Post Traumatic Growth Inventory (PTGI) from which items have been directly selected (new items were added to the scale as well), and on the Brief-COPE (see Carver, C. S. (1997) "You want to measure coping but your protocol's too long: Consider the brief COPE." International Journal of Behavioral Medicine 4; 92-100).

The Medical Outcomes Study (MOS) Social Support Survey is a 19-item self-report measure designed to assess levels of functional social support. The MOS-SS has two subscales (emotional and instrumental social support) to identify potential social support deficits (Sherbourne, C. D. & Stewart, A. L. (1991). "The MOS Social Support Survey." Soc Sci Med 32(6): 705-714).

The Purpose in Life test-Short Form (PIL-SF) is a brief, 4-item form of the 20-item Purpose in Life test. This scale asks respondents to report to what extent they have achieved their goals in life, and to what extent they perceive their life to be meaningful or purposeful. (Schulenberg et al 2010; Psychotherapy (Chic). 2008 December; 45(4):447-63).

Posttraumatic Growth Inventory (PTGI)-Short Version is a 10-item shortened version of the PTGI self-report questionnaire (ref). It asks respondents to rate the extent to which they have changed as the result of experiencing a highly stressful life event. Items span positive changes in five domains: relating to others, new possibilities, personal strength, spiritual change, and appreciation of life (Cann, A., et al. (2010). A short form of the Posttraumatic Growth Inventory. Anxiety, Stress & Coping, 23, 127-137).

The Quality of Life Enjoyment and Satisfaction Questionnaire (Q-LES-Q) is a self-report scale measuring the degree of enjoyment and satisfaction experienced by subjects in various areas of daily functioning. The summary scores are reliable and valid measures of these dimensions in a group of depressed subjects (Endicott J, et al. Quality of Life Enjoyment and Satisfaction Questionnaire: A New Measure. Psychopharmacology Bulletin; 1993; 29:321-326).

In certain embodiments, self-evaluation of the subject being treated is conducted.

Pharmaceutical Compositions

While it is possible that the present agent, as well as salts, solvates and physiological functional derivatives thereof, may be administered as the raw chemical, it is possible to present the active ingredient as a pharmaceutical composition. Accordingly, the invention further provides a pharmaceutical composition, which comprises the present compound and/or salts, solvates and physiological functional derivatives thereof, and one or more pharmaceutically acceptable carriers, diluents, or excipients. The carrier(s), diluent(s) or excipient(s) must be acceptable in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. In accordance with another aspect of the invention there is also provided a process for the preparation of a pharmaceutical composition including admixing the present compound, or salts, solvates and physiological functional derivatives thereof, with one or more pharmaceutically acceptable carriers, diluents or excipients.

The term "composition", as in pharmaceutical composition, is intended to encompass a product comprising the active ingredient(s), and the inert ingredient(s) (pharmaceutically acceptable excipients) that make up the carrier, as well as any product which results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. Accordingly, the pharmaceutical compositions of the present invention encompass any composition made by admixing compound 20, and pharmaceutically acceptable excipients.

Acceptable excipients, diluents, and carriers for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington: The Science and Practice of Pharmacy. Lippincott Williams & Wilkins (A. R. Gennaro edit. 2005). The choice of pharmaceutical excipient, diluent, and carrier can be selected with regard to the intended route of administration and standard pharmaceutical practice.

As used herein, the phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are "generally regarded as safe", e.g., that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopoeia or other generally recognized pharmacopeias for use in animals, and more particularly in humans.

Pharmaceutical compositions of the present invention may be presented in unit dose forms containing a predetermined amount of active ingredient per unit dose. Such a unit may contain, for example, 5 μg to 1 g, preferably 1 mg to 700 mg, more preferably 5 mg to 100 mg

of the present compound, depending on the condition being treated, the route of administration and the age, weight and condition of the patient. Such unit doses may therefore be administered more than once a day. Preferred unit dosage compositions are those containing a daily dose or sub-dose (for administration more than once a day), as herein above recited, or an appropriate fraction thereof, of an active ingredient. Furthermore, such pharmaceutical compositions may be prepared by any of the methods well known in the pharmacy art.

Pharmaceutical compositions of the present invention may be adapted for administration by any appropriate route, for example by the oral (including buccal or sublingual), inhaled, nasal, ocular, or parenteral (including intravenous and intramuscular) route. The present composition may be injected. Such compositions may be prepared by any method known in the art of pharmacy, for example by bringing into association the active ingredient with the carrier(s) or excipient(s).

In a further embodiment, the present invention provides a pharmaceutical composition adapted for administration by the oral route, the treatment of stress-induced affective disorder.

Pharmaceutical compositions of the present invention which are adapted for oral administration may be presented as discrete units such as capsules or tablets; powders or granules; solutions or suspensions in aqueous or non-aqueous liquids; edible foams or whips; or oil-in-water liquid emulsions or water-in-oil liquid emulsions.

For instance, for oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic pharmaceutically acceptable inert carrier such as ethanol, glycerol, water and the like. Powders are prepared by comminuting the compound to a suitable fine size and mixing with a similarly comminuted pharmaceutical carrier such as an edible carbohydrate, as, for example, starch or mannitol. Flavoring, preservative, dispersing and coloring agent can also be present.

Capsules are made by preparing a powder mixture, as described above, and filling formed gelatin sheaths. Glidants and lubricants such as colloidal silica, talc, magnesium stearate, calcium stearate or solid polyethylene glycol can be added to the powder mixture before the filling operation. A disintegrating or solubilizing agent such as agar-agar, calcium carbonate or sodium carbonate can also be added to improve the availability of the medicament when the capsule is ingested.

Moreover, when desired or necessary, suitable binders, lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium alginate, carboxymethylcellulose, polyethylene

glycol, waxes and the like. Lubricants used in these dosage forms include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum and the like. Tablets are formulated, for example, by preparing a powder mixture, granulating or slugging, adding a lubricant and disintegrant and pressing into tablets. A powder mixture is prepared by mixing the compound, suitably comminuted, with a diluent or base as described above, and optionally, with a binder such as carboxymethylcellulose, an aliginate, gelatin, or polyvinyl pyrrolidone, a solution retardant such as paraffin, a resorption accelerator such as a quaternary salt and/or an absorption agent such as bentonite, kaolin or dicalcium phosphate. The powder mixture can be granulated by wetting with a binder such as syrup, starch paste, acadia mucilage or solutions of cellulosic or polymeric materials and forcing through a screen. As an alternative to granulating, the powder mixture can be run through the tablet machine and the result is imperfectly formed slugs broken into granules. The granules can be lubricated to prevent sticking to the tablet forming dies by means of the addition of stearic acid, a stearate salt, talc or mineral oil. The lubricated mixture is then compressed into tablets. The compounds of the present invention can also be combined with a free flowing inert carrier and compressed into tablets directly without going through the granulating or slugging steps. A clear or opaque protective coating consisting of a sealing coat of shellac, a coating of sugar or polymeric material and a polish coating of wax can be provided. Dyestuffs can be added to these coatings to distinguish different unit dosages.

Oral fluids such as solution, syrups and elixirs can be prepared in dosage unit form so that a given quantity contains a predetermined amount of the compound. Syrups can be prepared by dissolving the compound in a suitably flavored aqueous solution, while elixirs are prepared through the use of a non-toxic alcoholic vehicle. Suspensions can be formulated by dispersing the compound in a non-toxic vehicle. Solubilizers and emulsifiers such as ethoxylated isostearyl alcohols and polyoxy ethylene sorbitol ethers, preservatives, flavor additive such as peppermint oil or natural sweeteners or saccharin or other artificial sweeteners, and the like can also be added.

It should be understood that, in addition to the ingredients particularly mentioned above, the compositions may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavoring agents.

A therapeutically effective amount of a compound of the present invention will depend upon a number of factors including, for example, the age and weight of the subject, the precise

condition requiring treatment and its severity, the nature of the formulation, and the route of administration, and will ultimately be at the discretion of the attendant physician or veterinarian.

Kits

Also provided are kits for use in the present methods of prophylactically treating a stress-induced affective disorder.

The kits can include an agent or composition provided herein, and instructions providing information to a health care provider regarding usage in accordance with the present methods. The kit may optionally contain a second agent or composition. Instructions may be provided in printed form or in the form of an electronic medium such as a floppy disc, CD, or DVD, or in the form of a website address where such instructions may be obtained. A unit dose of a compound or composition provided herein, or a second agent or composition, can include a dosage such that when administered to a subject, a therapeutically or prophylactically effective plasma level of the compound or composition can be maintained in the subject for at least 1 days. In some embodiments, a compound or composition can be included as a sterile aqueous pharmaceutical composition or dry powder (e.g., lyophilized) composition. In some embodiments, suitable packaging is provided. As used herein, "packaging" includes a solid matrix or material customarily used in a system and capable of holding within fixed limits a compound provided herein and/or a second agent suitable for administration to a subject. Such materials include glass and plastic (e.g., polyethylene, polypropylene, and polycarbonate) bottles, vials, paper, plastic, and plastic-foil laminated envelopes and the like.

The kits described herein contain one or more containers, which contain compounds, signaling entities, biomolecules and/or particles as described. The kits also contain instructions for mixing, diluting, and/or administrating the compounds. The kits also include other containers with one or more solvents, surfactants, preservative and/or diluents (e.g., saline (0.9% NaCl), or 5% dextrose) as well as containers for mixing, diluting or administering the components to the sample or to the patient in need of such treatment.

The compositions of the kit may be provided as any suitable form, for example, as liquid solutions or as dried powders. When the composition provided is a dry powder, the powder may be reconstituted by the addition of a suitable solvent, which may also be provided. In embodiments where liquid forms of the composition are used, the liquid form may be concentrated or ready to use. The solvent will depend on the compound and the mode of use or administration. Suitable solvents for drug compositions are well known and

are available in the literature. The solvent will depend on the compound and the mode of use or administration.

The kits comprise a carrier being compartmentalized to receive in close confinement one or more container such as vials, tubes, and the like, each of the container comprising one of the separate elements to be used in the method. For example, one of the container may comprise a positive control in an assay. Additionally, the kit may include containers for other components, for example, buffers useful in the assay.

Unless otherwise indicated, all numbers expressing quantities of ingredients, properties and so forth used in the present disclosure and associated claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in this disclosure and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the examples of the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claim, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. It should be noted that when "about" is at the beginning of a numerical list, "about" modifies each number of the numerical list. Further, in some numerical listings of ranges some lower limits listed may be greater than some upper limits listed. One skilled in the art will recognize that the selected subset will require the selection of an upper limit in excess of the selected lower limit. The term "about" refers to $\pm$10% of the referenced value. In other words, the numeric value can be in a range of 90% of the stated value to 110% of the stated value.

This invention will be better understood from the Examples, which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims that follow thereafter.

EXAMPLES

EXAMPLE 1: Prophylactic efficacy of 5-HT4R agonists against stress

Enhancing stress resilience could protect against stress-induced psychiatric disorders in at-risk populations. We have previously reported that (*R*,*S*)-ketamine acts as a prophylactic against stress when administered 1 week before stress. While we have shown that the selective 5-hydroxytryptamine (5-HT) (serotonin) reuptake inhibitor (SSRI) fluoxetine (Flx) is ineffective as a prophylactic, we hypothesized that other serotonergic compounds such as serotonin 4 receptor (5-HT_4R) agonists could act as prophylactics. We tested if three 5-HT_4R

agonists with varying affinity could protect against stress in two mouse strains by utilizing chronic corticosterone (CORT) administration or contextual fear conditioning (CFC). Mice were administered saline, (*R*,*S*)-ketamine, Flx, RS-67,333, prucalopride, or PF-04995274 at varying doses and then 1 week later were subjected to chronic CORT or CFC. In C57BL/6N mice, chronic Flx administration attenuated CORT-induced weight changes and increased open arm entries in the elevated plus maze (EPM). Chronic RS-67,333 administration attenuated CORT-mediated weight changes and protected against depressive- and anxiety-like behavior. In 129S6/SvEv mice, RS-67,333 attenuated learned fear in male, but not female mice. RS-67,333 was ineffective against stress-induced depressive-like behavior in the forced swim test (FST) but prevented anxiety-like behavior in both sexes. Prucalopride and PF-04995274 attenuated learned fear and decreased stress-induced depressive-like behavior. Electrophysiological recordings following (*R*,*S*)-ketamine or prucalopride administration revealed that both drugs alter AMPA receptor-mediated synaptic transmission in CA3. These data show that in addition to (*R*,*S*)-ketamine, 5-HT_4R agonists are also effective prophylactics against stress, suggesting that the 5-HT_4R may be a novel target for prophylactic drug development.

Here, we hypothesized that since 5-HT_4Rs have been heavily implicated in depression and anxiety, they may have a role in stress resilience. We focused our studies on three 5-HT_4R agonists with varying affinity. First, RS-67,333 (1-(4-amino-5-chloro-2-methoxyphenyl)-3-[1(n-butyl)-4-piperidinyl]-1-propanone HCl) is a high-affinity 5-HT_4R partial agonist [22]. This drug is effective in improving behavioral deficits, decreasing the number of amyloid plaques as well as level of amyloid beta (Aβ) species, and decreasing hippocampal astrogliosis and microgliosis in the 5xFAD mouse model of Alzheimer's disease (AD) [23]. Second, prucalopride (4-amino-5-chloro-2,3-dihydro-*N*-[1-3-methoxypropyl)-4-piperidinyl]-7-benzofuran carboxamide monohydrochloride) is a selective, high affinity 5-HT_4R agonist [24]. In 2018, it was approved by the FDA for chronic constipation and is currently being tested for chronic intestinal pseudo-obstruction. Prucalopride has also been tested in two separate clinical trials to investigate its effects on emotional processing in health volunteers after an acute (e.g., single dose) or chronic (e.g., 1 week) administration [25,26]. Third, PF-04995274 (4-[4-[4-Tetrahydrofuran-3-yloxy)-benzo[d]isoxazol-3-yloxymethyl]-piperidin-1-ylmethyl]-tetrahydropyran-4-ol) is a potent, partial 5-HT_4R agonist [27]. A clinical trial was conducted to evaluate PF-04995274, alone or in combination with donepezil, on scopolamine-induced deficits in psychomotor and cognitive function in healthy adults; however, this trial was terminated, but not due to safety concerns [28]. Currently, a clinical trial is underway to test

whether adjunctive administration of PF-04995247 has positive effects on emotional processing and neural activity in mediated, treatment-resistant (TRD) depressed patients compared to placebo [29].

To determine if 5-HT$_4$R agonists may be potential prophylactics against stress, we utilized two different stress models (acute and chronic) in two different strains of mice (C57BL/6NTac and 129S6/SvEv). We found that RS-67,333, prucalopride, and PF-04995274 attenuate learned fear. RS-67,333 prevents depressive-like behavior when administered chronically and stress-induced anxiety-like behavior in both sexes when administered acutely. Prucalopride and PF-04995274 decrease stress-induced depressive-like behavior in the FST. To investigate shared or distinct mechanisms of prophylactic (*R,S*)-ketamine and 5-HT$_4$R agonists, we utilized slice electrophysiology to investigate spontaneous glutamatergic transmission in CA3. We found that (*R,S*)-ketamine and prucalopride attenuate bursts of large amplitude AMPA receptor-mediated synaptic currents. These data suggest that in addition to (*R,S*)-ketamine, 5-HT$_4$R agonists are also effective prophylactics against stress and may alter AMPA-related glutamatergic transmission to enhance stress resilience.

MATERIALS AND METHODS

Mice: All mice were housed in a 12-h (06:00-18:00) light-dark colony room at 22°C. Food and water were provided *ad libitum*. Behavioral testing was performed during the light phase.

C57BL/6NTac mice: Male C57BL/6NTac mice were purchased from Taconic Farms (Lille Skensved, Denmark) at 8 weeks of age and were housed 5 per cage before the start of CORT treatment. All testing was conducted in compliance with the laboratory animal care guidelines and with protocols approved by the Institutional Animal Care and Use Committee (IACUC) (European Directive, 2010/63/EU for the protection of laboratory animals, permissions # 92-256B, authorization ethical committee CEEA n°26 2012_098).

129S6/SvEv mice: Male and female 129S6/SvEvTac mice were purchased from Taconic (Hudson, NY) at 7-8 weeks of age. The procedures described herein were conducted in accordance with the National Institutes of Health (NIH) regulations and approved by the IACUC of the New York State Psychiatric Institute (NYSPI).

Stress models:

Corticosterone (CORT) model: In this model, glucocorticoid levels are exogenously increased in C57BL/6NTac mice. This chronic CORT elevation dysregulates the hypothalamic-pituitary-adrenal axis (HPA) in a manner similar to that observed in clinical depression. The dose and duration of CORT treatment was selected based on previous studies

[20,30]. CORT (35 μg/ml, equivalent to about 5 mg/kg/day) dissolved in 0.45% hydroxypropyl-β-cyclodextrin (β-CD) or vehicle (VEH) (0.45% β-CD) was available *ad libitum* in the drinking water in opaque bottles to protect it from light. VEH- and CORT-treated water was changed every 3 days to prevent possible degradation.

Contextual Fear Conditioning (CFC): A 3-shock CFC procedure was administered as previously published [31,32]. Briefly, mice were placed into context A and administered 3 2-s shocks (0.75 mA) at 180 s, 240 s or 300 s following placement into context A. Mice were removed from the context 15 s following the termination of shock (at 317 s). For context retrieval, mice were placed back into context A for 300 s.

Electrophysiology: Electrophysiology was conducted as previously described [33].

Statistical Analysis: Results from data analyses are expressed as means ± SEM. Alpha was set to 0.05 for all analyses. Data were analyzed using GraphPad Prism v7.0 or v8.0. For all experiments, unless otherwise noted, one- or two-way ANOVAs with repeated-measures were applied to the data as appropriate. Significant main effects and/or interactions were followed by Fisher's PLSD post hoc analysis or unpaired *t*-tests. All main effects, interactions, and p values are listed in **Table 2**.

Drugs: All drugs were prepared in physiological saline and all injections were administered intraperitoneally (i.p.) in volumes of 0.1 cc per 10 mg body weight unless otherwise noted.

Fluoxetine hydrochloride (Flx): Flx (BioTrend Chemicals AG, BG197, Zurich, Germany) was administered in the drinking water (18 mg/kg/day) for 3 weeks before the start of CORT.

RS-67,333 (RS): RS-67,333 (Tocris Bioscience, 0989, Bristol, United Kingdom) was administered chronically or in a single injection. For the chronic experiment, RS-67,333 (1.5 mg/kg/day) was administered via ALZET osmotic minipumps (ALZET, Model 2004, Cupertino, CA) [30]. For the acute experiment, RS-67,333 was administered in a single dose of 1.5, 10, or 30 mg/kg of body weight 1 week before the start of CFC. RS-67,333 was dissolved in saline using an ultrasonic homogenizer (BioLogics, Model 3000, Manassas, VA).

(*R*,*S*)-ketamine (K): (*R*,*S*)-ketamine (Ketaset III, Ketamine HCl injection, Fort Dodge Animal Health, Fort Dodge, IA) was administered in a single dose at 30 mg/kg of body weight 1 week before the start of CFC. A dose of 30 mg/kg of body weight was chosen in the 129S6/SvEv experiments, as previous studies indicated that is the effective dose for prophylactic efficacy [S1].

Prucalopride: Prucalopride (Sigma, 179474-81-8, St. Louis, MO) was administered a single dose at 3 or 10 mg/kg of body weight 1 week before the start of CFC. Prucalopride was dissolved in saline using an ultrasonic homogenizer (BioLogics, Model 3000, Manassas, VA).

PF-04995274: PF-04995274 (Sigma, Catalog No. 1331782-27-4, St. Louis, MO) was administered a single dose at 3 or 10 mg/kg of body weight 1 week before the start of CFC. PF-04995274 was dissolved in saline using an ultrasonic homogenizer (BioLogics, Model 3000, Manassas, VA).

Osmotic minipump implantation: ALZET osmotic minipumps (Model 2004, 0.25 µl/hr, 28 days) were implanted subcutaneously under isoflurane anesthesia as previously described [S2]. Osmotic minipumps were rotated under the skin two to three times per week.

Behavioral Assays: All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the New York Psychiatric Institute (NYSPI).

Elevated Plus Maze (EPM): Testing was performed as previously described [S3]. Briefly, the maze is a plus-cross-shaped apparatus consisting of four arms, two open and two enclosed by walls, linked by a central platform at a height of 50 cm from the floor. Mice were individually placed in the center of the maze facing an open arm and were allowed to explore the maze for 5 min. The time spent in and the number of entries into the open arms was used as an anxiety index. Videos were scored using ANY-maze behavior tracking software (Stoelting, Wood Dale, IL).

Novelty-Suppressed Feeding: The NSF is a conflict test that elicits competing motivations: the drive to eat and the fear of venturing into the center of a brightly lit arena. The latency to feed is used as an index of anxiety -like behavior, because classical anxiolytic drugs decrease this measure. The NSF test was carried out during an 8 min period as previously described [S3]. Briefly, the testing apparatus consisted of a plastic box (50x50x20 cm), the floor of which was covered with approximately 2 cm of bedding. For 129S6/SvEv experiments, mice were food restricted for 12 h. For the C57BL/6N experiments, mice were food restricted for 24 h. At the time of testing, a single pellet of food (regular chow) was placed on a paper platform positioned in the center of the box. Each animal was placed in a corner of the box, and a stopwatch was immediately started. The latency to feed (defined as the mouse biting the pellet) was timed. Immediately afterwards, the animal was transferred to its home cage, and the amount of food consumed by the mouse in the subsequent 5 min was measured, serving as a control for change in appetite as a possible confounding factor.

Splash Test: This test consisted of squirting 200 µl of a 10% sucrose solution on the mouse's snout. The grooming duration was quantified using Stopwatch+ (Center for Behavioral Neuroscience, Georgia State University).

Forced Swim Test (FST): The FST is typically used in rodents to screen for potential human antidepressants [S4,S5]. In fact, many papers examining ketamine in mouse models only observe effects in the FST [S6-S8]. In the FST, time spent immobile, as opposed to swimming, is used as a measure of depressive behavior. The FST was administered as previously described [1]. Briefly, mice were placed into clear plastic buckets 20 cm in diameter and 23 cm deep filled 2/3 of the way with 22°C water. Mice were videotaped from the side for 6 min and were exposed to the swim test on 2 consecutive days. Immobility time was scored by an experimenter blind to the experimental groups.

Open Field (OF): The OF assay was administered as previously described [3]. Briefly, motor activity was quantified in four Plexiglas open field boxes 43x43 cm^2 (MED Associates, Georgia, VT). Two sets of 16 pulse-modulated infrared photobeams on opposite walls 2.5-cm apart recorded x–y ambulatory movements. Activity chambers were computer interfaced for data sampling at 100-ms resolution. The computer defined grid lines that dividing center and surround regions, with the center square consisting of four lines 11 cm from the wall.

Electrophysiology: One week after saline, (*R,S*)-ketamine (30 mg/kg), or prucalopride (3 mg/kg) injection, mice were anesthetized by isoflurane inhalation, decapitated, and brains rapidly removed. CA3 slices (350 µm) were cut on a vibratome (Leica VT1000S) in ice cold partial sucrose artificial cerebrospinal fluid (ACSF) solution (in mM): 80 NaCl, 3.5 KCl, 4.5 $MgSO_4$, 0.5 $CaCl_2$, 1.25 H_2PO_4, 25 $NaHCO_3$, 10 glucose, and 90 sucrose equilibrated with 95% O_2 / 5% CO_2 and stored in the same solution at 37°C for 30 minutes, then at room temperature until use. Recordings were made at 30-32°C (TC324-B; Warner Instrument Corp) in ACSF (in mM: 124 NaCl, 8.5 KCl, 1 NaH_2PO_4, 25 $NaHCO_3$, 20 glucose, 1 $MgCl_2$, 2 $CaCl_2$). Whole-cell voltage clamp recordings (-70 mV) were obtained using a patch pipette (4-6 M MΩ) containing (in mM): 135 K-Gluconate, 5 KCl, 0.1 EGTA-Na, 10 HEPES, 2 NaCl, 5 ATP, 0.4 GTP, 10 phosphocreatine (pH 7.2; 280–290 mOsm). Bicuculline (5 µM) was also included in the bath solution to inhibit $GABA_AR$s. NBQX (20 µM) was added later in recordings to inhibit AMPAR synaptic currents. Patch pipettes were made from borosilicate glass (A-M Systems, Sequium, WA) using a micropipette puller (Model P-1000; Sutter Instruments). Recordings were made without correction for junction potentials. Pyramidal cells were visualized and

targeted via infrared-differential interference contrast (IR-DIC; 40x objective) optics on an Axioskop-2 FS (Zeiss).

RESULTS

Chronic administration of RS-67,333 is prophylactic against stress in male mice

We have previously reported that chronic Flx administration (3 weeks of administration) is not prophylactic in 129S6/SvEv mice [3]. However, it remained to be determined if other serotonergic drugs could act as prophylactics. Here, we administered Flx (18 mg/kg/day) in the drinking water or RS-67,333 (1.5 mg/kg/day) in osmotic minipumps for 3 weeks prior to CORT administration in C57Bl/6NTac male mice followed by a series of behavioral assays, including the EPM, novelty-suppressed feeding (NSF), and sucrose splash test (ST) (**Fig. 1A-1B**). CORT increased body weight over the 6-week behavioral protocol, as previously observed [34], (**Fig. 1C-1F**), but this was attenuated by Flx and RS-67,333 administration.

In the EPM, CORT + Veh, CORT + Flx, and CORT + RS-67,333 administration did not alter the time spent in the open arms when compared with VEH + Veh administration (**Fig. 1G**). However, CORT + Veh mice exhibited a significantly decreased number of entries into the open arms of the EPM when compared with VEH + Veh mice (**Fig. 1H**). CORT + Flx and CORT + RS-67,333 mice had significantly more entries into the open arms of the EPM when compared with CORT + Veh mice. The total distance traveled in the EPM did not differ between any of the groups (**Fig. 1I**).

Next, the NSF task was administered to assay anxiety-like behavior (**Fig. 1J-1K**). CORT + Veh mice exhibited an increased latency to approach the food pellet when compared with VEH + Veh mice. CORT + RS-67,333, but not CORT + Flx mice exhibited a significantly decreased latency to approach the pellet when compared with CORT + Veh mice.

Finally, in the ST, CORT + Veh mice exhibited decreased grooming duration when compared with VEH + Veh mice (**Fig. 1L**). CORT + RS-67,333, but not CORT + Flx mice exhibited increased grooming duration when compared with CORT + Veh mice. These data suggest that chronic RS-67,333, but not chronic Flx administration is prophylactic against a wide range of CORT-induced behavioral abnormalities.

A single injection of RS-67,333 attenuates learned fear and protects against stress-induced hypophagia in male mice

Previously, we have shown that a single injection of (*R*,*S*)-ketamine is prophylactic against stress-induced depressive-like behavior and attenuates learned fear in 129S6/SvEv mice [3]. Here, we sought to determine if a single injection of RS-67,333 could also prevent a variety of maladaptive behaviors following a single, acute stressor. Male 129S6/SvEv mice were injected with saline or RS-67,333 (1.5, 10, or 30 mg/kg) (**Fig. 2A**). One week later, mice were administered 3-shock CFC. Mice administered 30, but not 1.5 or 10 mg/kg, of RS-67,333 exhibited significantly less freezing during CFC training when compared with mice administered saline (**Fig. 2B**). Five days later, mice were re-exposed to the training context. Mice administered 1.5 or 10, but not 30 mg/kg of RS-67,333 exhibited significantly less freezing when compared with mice administered saline (**Fig. 2C-2D**).

Following CFC, mice were administered the FST. On Day 1, mice administered 10, but not 1.5 or 30 mg/kg, of RS-67,333 were significantly less immobile when compared with saline mice (**Fig. 2E**). However, on Day 2, immobility time was comparable between all groups (**Fig. 2F-2G**).

Next, mice administered saline or RS-67,333 (10 mg/kg) were tested in the OF. Both groups of mice travelled a comparable distance (**Fig. 2H**) and spent a comparable amount of time in the center of the arena (**Fig. 2I**). Subsequently, mice were tested in the EPM, and neither in the open arms nor entries into the open arms of the maze was significantly different between saline or RS-67,333 mice (**Fig. 2J-2K**).

Finally, mice were administered the NSF. Mice given prophylactic RS-67,333 (10 mg/kg) exhibited a significantly reduced latency to approach the pellet (**Fig. 2L-2M**). However, neither food eaten in the home cage nor weight loss following food deprivation differed between the groups (**Fig. 2N-2O**). Together, these data indicate that a single injection of RS-67,333 is effective as a prophylactic in attenuating learned fear and preventing stress-induced hypophagia, but not depressive-like behavior, as measured by the FST, in male 129S6/SvEv mice.

A single prophylactic injection of RS-67,333 protects against stress-induced anxiety-like behavior in female mice

We next sought to determine if a single injection of RS-67,333 could also be prophylactic in female mice. Female 129S6/SvEv mice were injected with saline or RS-67,333 (1.5 or 10 mg/kg) (**Fig. 3A**). One week later, mice were administered 3-shock CFC. All groups of mice exhibited comparable levels of freezing during CFC training (**Fig. 3B**). Five days later, mice were re-exposed to the training context. Again, all groups of mice exhibited comparable levels

of freezing (**Fig. 3C-3D**). Following CFC, mice were administered the FST. During days 1 (**Fig. 3E**) and 2 (**Fig. 3F-3G**) of the FST, all groups of mice had comparable levels of immobility.

Next, mice were tested in the OF and the EPM. Mice in all groups travelled comparable distances in the OF and spent a comparable amount of time in the center of the arena (**Fig. 3H-3I**). Similarly, in the EPM, mice spent a comparable amount of time in the open arms of the maze (**Fig. 3J**) and had a comparable number of entries into the open arms (**Fig. 3K**).

Finally, mice were assayed in the NSF paradigm. Prophylactic RS-67,333 (10 mg/kg), but not RS-67,333 (1.5 mg/kg), significantly reduced latency to feed (**Fig. 3L-3M**). Neither food eaten in the home cage nor weight loss following food deprivation differed between the groups (**Fig. 3N-3O**). Together, these data indicate that RS-67,333 does not attenuate learned fear or protect against stress-induced depressive-like behavior, but may prevent stress-induced hypophagia in the NSF in female 129S6/SvEv mice.

A single prophylactic injection of prucalopride or PF-04995274 is prophylactic against stress in male mice

We next sought to determine if other 5-HT_4R agonists could also be prophylactic in male 129S6/SvEv mice. Male 129S6/SvEv mice were injected with saline, (*R,S*)-ketamine (30 mg/kg), prucalopride (3 or 10 mg/kg), or PF-04995274 (3 or 10 mg/kg) (**Fig. 4A**). One week later, mice were administered 3-shock CFC. All groups of mice exhibited comparable levels of freezing during CFC training (**Fig. 4B**). Five days later, mice were re-exposed to the training context. As we have previously published, (*R,S*)-ketamine attenuated learned fear (**Fig. 4C-4D**). Interestingly, prucalopride at 3 mg/kg, but not 10 mg/kg, and PF04995274 at 10 mg/kg, but not 3 mg/kg, attenuated learned fear when compared with saline administration.

Following CFC, mice were administered the FST. During day 1, all groups of mice had comparable levels of immobility (**Fig. 4E**). During day 2, (*R,S*)-ketamine administration decreased immobility time when compared with saline administration (**Fig. 4F-4G**). Moreover, prucalopride at 3 mg/kg, but not 10 mg/kg, and PF04995274 at 10 mg/kg, but not 3 mg/kg, decreased immobility time when compared with saline administration.

Stress-induced anxiety-like behavior was next quantified. In the OF, all groups of mice traveled a comparable distance (**Fig. 4H**). In the EPM, all groups of mice spent comparable time in the open arms (**Fig. 4I**) and entered into the open arms a comparable number of times (**Fig. 4J**). In the NSF paradigm, all groups of mice approached the pellet in a comparable amount of time (**Fig. 4K-4L**). Finally, all mice lost a comparable amount of weight during the

NSF paradigm (**Fig. 4M**). In summary, these data indicate that a single injection of prucalopride or PF0499574 results in prophylactic efficacy by attenuating learned fear and decreasing stress-induced depressive-like behavior. However, these drugs are not prophylactic against stress-induced anxiety-like behavior.

(R,S)-ketamine and prucalopride exhibit a common mechanism by reducing bursts of large AMPA receptor-driven synaptic currents in CA3

We next sought to elucidate potential common mechanisms between (*R,S*)-ketamine and a 5HT$_4$R agonist such as prucalopride. Specifically, we hypothesized that there may be similarities between the effects of (*R,S*)-ketamine and prucalopride on glutamatergic transmission in CA3 since we previously reported that prophylactic (*R,S*)-ketamine alters activity in ventral CA3 (vCA3), but not in the DG [6]. To test this, mice were injected with saline, (*R,S*)-ketamine (30 mg/kg), or prucalopride (3 mg/kg) and were euthanized 1 week later (**Fig. 5A**). We performed whole-cell voltage clamp recordings of spontaneous excitatory postsynaptic currents (EPSCs) in CA3 pyramidal cells. We found there were no differences in the average EPSC amplitude (**Fig. 5B**) or the number of EPSCs (**Fig. 5C**) between the groups. However, we did find that saline-treated mice typically displayed large bursts of EPSCs (-590.8±13.85 pA), which were completely blocked by the AMPA receptor blocker NBQX (**Fig. 5D**). These large AMPA receptor-mediated signals were not present in either (*R,S*)-ketamine- (**Fig. 5E**) or prucalopride-treated mice (**Fig. 5F**), suggesting that although these drugs target different receptors, they both alter AMPA-mediated synaptic transmission in a similar manner.

DISCUSSION

Here, we hypothesized that 5-HT$_4$R agonists could be prophylactic against fear, depressive-like, and/or anxiety-like behavior. We tested if three 5-HT$_4$R agonists with varying affinity could protect against stress. Chronic administration of RS-67,333 was prophylactic against CORT stress. A single injection of RS-67,333 attenuated learned fear in male, but not female, 129S6/SvEv mice, and prevented stress-induced hypophagia in the NSF in both sexes. Acute administration of RS-67,333 was ineffective against stress-induced depressive-like behavior. A single injection of either prucalopride or PF-04995274 attenuated learned fear and decreased depressive-like behavior but had no effect on anxiety-like behavior. Moreover, a single injection of (*R,S*)-ketamine or prucalopride reduced large, spontaneous AMPA receptor-

driven bursts in CA3, indicating a common mechanism by which either drug may protect against stress-induced maladaptive behavior.

The 5-HT_4R is widely distributed throughout the brain and heavily expressed in areas related to emotional regulation and cognitive function. The 5-HT_4R is also heavily expressed throughout the periphery and plays a crucial role in regulating ENS activity and function.

The three 5-HT_4R agonists chosen in this study have differential affinity to the 5-HT_4R (Table 1). RS-67,333, Prucalopride, and PF-04995274 have varying selectivity and affinity for the 5-HT_4R. These differences may contribute to the drugs' prophylactic efficacy in preventing fear, depressive-like, or anxiety-like behavior following stress. RS-67,333 and PF-04995274 are high-affinity 5-HT_4R partial agonists, whereas prucalopride is a selective, high-affinity 5-HT_4R agonist. RS-67,333 attenuated learned fear and protected against novelty-induced hypophagia, but did not decrease stress-induced depressive-like behavior. Prucalopride and PF-04995274 attenuated learned fear and decreased depressive-like behavior but had no effect on various measures of anxiety-like behavior. These data suggest that the unique combination of high pK_i and partial selectivity for the 5-HT_4R exhibited by RS-67,333 is sufficient to prevent against anxiety-like behavior whereas the differential activity of prucalopride and PF-04995274 at the 5-HT_4R protect against stress-induced depressive-like behavior. Further study is necessary to determine if and how the 5-HT_4R may contribute to the neurobiological mechanisms underlying stress resilience.

Table 1. Summary of behavioral results in male mice

Activity of 5HT$_4$R agonists							
Drug	Selectivity	Affinity (pKi)	Behavioral efficacy				
			Fear	Depressive-like	Anxiety-like	Dose-specific?	Activity at other receptors?
RS-67,333	partial	8.7	+	–	+	Y	5HT$_{1a, 1d, 2a, 2c}$, D1, D2 and M1-3
Prucalopride	selective	8.1-8.6	+	+	–	Y	>290-fold selectivity for 5HT$_4$R
PF-04995274	partial	0.34-0.82	+	+	–	Y	(data not available)

The expression and activity of 5-HT$_4$Rs within the central nervous system (CNS) and periphery may provide insight into these mechanisms. In the brain, 5-HT$_4$Rs are expressed in areas of the brain involved in processing emotion, including the HPC, AMG, and PFC [11,16,21,35,36]. In addition to a multitude of other functions, such as modulating dopamine and acetylcholine release [36], as well as facilitating synaptic plasticity [36], 5-HT$_4$Rs are known to interact with the calcium effector protein p11 [37]. 5-HT$_4$Rs are highly co-expressed with p11, which increases surface expression of the receptor in the HPC and AMG, facilitates its downstream signaling pathways, and is necessary for the antidepressant effects of 5-HT$_4$R stimulation [37,38]. Levels of p11 are correlated with measures of suicidality and PTSD, indicating its potential as a biomarker for suicidal ideation and PTSD [39-41]. Additionally, 5-HT$_4$R expression and activity in the PFC is regulated by casein kinase 2 (CK2), which may be an important modulator of depressive- and anxiety-like behaviors [42]. Further studies examining 5-HT$_4$R agonists and their effects on these cellular regulators of 5-HT$_4$R expression and activity could yield further insight into prophylactic efficacy.

Because all three 5-HT$_4$R agonists exhibited prophylactic properties similar to (*R,S*)-ketamine, we investigated whether these compounds had comparable effects on neural activity in CA3. We found that a single injection of (*R,S*)-ketamine or prucalopride eliminated large bursts of AMPA receptor-mediated synaptic currents typically seen in saline controls without significantly altering the overall amplitude or number of EPSCs. Therefore, although these compounds target distinct receptors, they may achieve similar behavioral effects by altering AMPA receptor-dependent glutamatergic transmission in a convergent manner. Although (*R,S*)-ketamine is known to inhibit NMDA receptors [43-45], emerging evidence indicates that (*R,S*)-ketamine may also act on AMPA receptors to exert its *antidepressant* effects [46,47]. Our results are congruent with these data and suggest that (*R,S*)-ketamine's actions on AMPA receptor-mediated glutamatergic activity may contribute to the compound's *prophylactic* effects. Additionally, previous studies show that pharmacological activation of 5-HT$_4$Rs results in the long-term potentiation (LTP) of CA3-CA1 synapses along the Schaffer collaterals [48]. In combination with these data, our results suggest that (*R,S*)-ketamine and 5-HT$_4$R agonists, by attenuating large, spontaneous AMPA receptor-driven synaptic events in the CA3 autoassociative network, may reduce overall noise in the hippocampal circuit which may allow for a greater signal-to-noise ratio of relevant stimuli [49]. However, further research is necessary to confirm this hypothesis and to examine whether this potential mechanism directly contributes to enhanced resilience.

In addition to the actions of 5-HT$_4$R agonists within the brain, it is likely that these compounds exert additional changes within the periphery. 5-HT$_4$Rs are expressed in the periphery, such as the enteric nervous system (ENS), adrenal glands, and heart [17]. Importantly, 5-HT$_4$Rs play a major role in maintaining communication along the gut-brain axis. Recent data indicate that microbiota in the ENS communicate with the CNS by stimulating 5-HT$_4$Rs present throughout the gut to stimulate serotonin release in the brain [50]. Concurrently, numerous previous studies have shown that activation of 5-HT$_4$Rs is neuroprotective against oxidative stress, reduces inflammation, and stimulates neurogenesis in the brain and ENS [50-52]. Our manipulations may have stimulated gut-brain communication to promote neuroprotection and neurogenesis and thereby, enhance resilience against stress. We hypothesize that this action may have had an additive effect on the numerous, well-characterized consequences of 5-HT$_4$R stimulation in the brain, such as increasing neuronal firing in the medial PFC (mPFC) and enhancing mitogenesis in the HPC [19,35], although this remains to be determined.

To develop safe and efficacious pharmacological methods of enhancing stress resilience it will be necessary to determine the toxicity of 5-HT$_4$R agonists. Because 5-HT$_4$Rs are so widely expressed throughout the periphery, chronic exposure to these drugs could result in negative outcomes [17]. We found that chronic administration of RS-67,333 did not result in adverse side effects. However, because we did not conduct additional assays, such as assessing changes in cardiovascular activity or liver toxicity, it is impossible to know if chronic 5-HT$_4$R administration would negatively impact peripheral organs. Nonetheless, the drugs that we tested were efficacious in enhancing stress resilience even after a single dose, obviating chronic administration.

Here, we utilized two strains - C57BL/6NTac and 129S6/SvEv – in order to validate our effects of RS,67-333 in both a neuroendocrine model of stress and a fear-based stressor. In the C57BL/6NTac mice, we found prophylactic RS-67,333 was effective at decreasing depressive- and anxiety-like behavior, whereas in the 129S6/SvEv mice, we found prophylactic RS-67,333 was effective at attenuating learned fear and preventing anxiety-like behavior in the NSF, but not decreasing depressive-like behavior.

Previous research examining 5-HT$_4$R agonists as rapid-acting *antidepressants* have exclusively used male subjects [19,20,54]. However, previous studies indicate that *antidepressant* (*R,S*)-ketamine exhibits sex-specific behavioral and neurobiological effects. Across the estrous cycle, the efficacy of *antidepressant* (*R,S*)-ketamine varies in female mice, and this variability may be attributed to changing levels of neurotrophic factors (e.g. BDNF)

or changes in NMDA receptor activity across the estrous cycle [55,56]. Additionally, acute (*R,S*)-ketamine administration may lead to a sustained increase in GluR1 and GluR2 AMPA receptor subunits in the mPFC and HPC of male, but not female mice [45,46,57,58]. Despite numerous studies showing *prophylactic* efficacy in male rodents, only one study to date has examined female rodents [59]. Maier and colleagues showed that *prophylactic* (*R,S*)-ketamine reduced stress-induced activation of the dorsal raphe nucleus (DRN) and eliminated DRN-dependent social exploration deficits in female rats. However, this study did not measure fear, depressive-like, and anxiety-like behavior as done here. Nonetheless, we show that RS-67,333 does not attenuate learned fear or prevent depressive-like behavior but does protect against stress-induced anxiety-like behavior in female 129S6/SvEv mice. Thus, our data indicate that 5-HT_4R agonists may exclusively target the neural circuits underlying anxiety-like, but not depressive- or fear-related, behaviors in female mice. We did not utilize female C57BL/6NTac mice, as a previous study of our own has shown that female C57BL/6NTac mice are insensitive to chronic CORT [60].

Overall, the present study has identified three novel compounds to be effective prophylactics against two types of stress and in both sexes. These data suggest that the 5-HT_4R can be a novel target for prophylactic development and future studies can be directed to how 5-HT_4R agonists administered prior to a stressor result in stress resiliency.

Table 2. Statistical analysis summary

Cohort	Behavioral Paradigm	Abbrev	Measurement	Statistical Test	Comparison	F	° of freedom	p	*	Fig.
Figure 1 Chronic RS67333	Body Weight	BW	Body Weight Change (g)	RMANOVA	Drug	3.086	3,43	0.0371	*	1C
					Week	73.890	1,43	<0.0001	****	
					Drug x Time	3.102	3,43	0.0364	*	
			Week 3	Fisher's LSD	Vehicle vs. CORT/Vehicle	-	-	0.8643	ns	
					Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.1100	ns	
					Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.6933	ns	
					CORT/Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.0838	ns	
					CORT/Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.5717	ns	

					CORT/Fluoxetine 18mg/kg/day vs. CORT/RS67333 1.5mg/kg/day	-	-	0.1852	ns	
			Week 6	Fisher's LSD	Vehicle vs. CORT/Vehicle	-	-	0.0216	*	
					Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.6945	ns	
					Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.2218	ns	
					CORT/Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.0048	**	
						-	-	0.0004	***	

					CORT/Vehicle vs. CORT/RS67333 1.5mg/kg/day					
					CORT/Fluoxetine 18mg/kg/day vs. CORT/RS67333 1.5mg/kg/day	-	-	0.3620	ns	
			Body Weight Change (g)	RMANOVA	Drug	3.523	3,43	0.0227	*	Figures 1D, 1E, 1F
					Time	132.3	6,258	<0.0001	****	
					Drug x Time	5.728	18,258	<0.0001	****	
				Fisher's LSD	Vehicle vs. CORT/Vehicle	-	-	0.5826	ns	
					Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.0631	ns	
					Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0417	*	
					CORT/Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.0187	*	
					CORT/Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0118	*	
					CORT/Fluoxetine 18mg/kg/day vs. CORT/RS67333 1.5mg/kg/day	-	-	0.8353	ns	

			Body Weight Change Week 0 (g)	Fisher's LSD	Vehicle vs. CORT/Vehicle	-	-	0.6022	ns	
					Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.1509	ns	
					Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0064	**	
					CORT/Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.4047	ns	
					CORT/Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0366	*	
					CORT/Fluoxetine 18mg/kg/day vs. CORT/RS67333 1.5mg/kg/day	-	-	0.1536	ns	
			Body Weight Change Week 1 (g)	Fisher's LSD	Vehicle vs. CORT/Vehicle	-	-	0.6476	ns	
					Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.0087	**	
					Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.6994	ns	
					CORT/Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.0396	*	
					CORT/Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.9065	ns	
					CORT/Fluoxetine 18mg/kg/day vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0141	*	

			Body Weight Change Week 2 (g)	Fisher's LSD	Vehicle vs. CORT/Vehicle	-	-	0.8980	ns	
					Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.0115	*	
					Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.6015	ns	
					CORT/Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.0097	**	
					CORT/Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.5197	ns	
					CORT/Fluoxetine 18mg/kg/day vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0277	*	
			Body Weight Change Week 3 (g)	Fisher's LSD	Vehicle vs. CORT/Vehicle	-	-	0.8286	ns	
					Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.0420	*	
					Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.6172	ns	
					CORT/Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.0277	*	
					CORT/Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.4734	ns	
					CORT/Fluoxetine 18mg/kg/day vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0923	ns	

			Body Weight Change Week 4 (g)	Fisher's LSD	Vehicle vs. CORT/Vehicle	-	-	0.8109	ns	
					Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.3207	ns	
					Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0008	***	
					CORT/Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.4805	ns	
					CORT/Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0026	**	
					CORT/Fluoxetine 18mg/kg/day vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0087	**	
			Body Weight Change Week 5 (g)	Fisher's LSD	Vehicle vs. CORT/Vehicle	-	-	0.2904	ns	
					Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.5013	ns	
					Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0055	**	
					CORT/Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.0742	ns	
					CORT/Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0001	***	
					CORT/Fluoxetine 18mg/kg/day vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0206	*	

			Body Weight Change Week 6 (g)	Fisher's LSD	Vehicle vs. CORT/Vehicle	-	-	0.0033	**	
					Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.6186	ns	
					Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.1208	ns	
					CORT/Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.0003	***	
					CORT/Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	<0.0001	****	
					CORT/Fluoxetine 18mg/kg/day vs. CORT/RS67333 1.5mg/kg/day	-	-	0.2475	ns	
	Elevated Plus Maze	EPM	Open Arm Time	ANOVA	Drug	2.554	3,43	0.0678	ns	Fig. 1G
			Open Arm Entries (No.)	ANOVA	Drug	5.548	3,43	0.0026	**	Fig. 1H
				Fisher's LSD	Vehicle vs. CORT/Vehicle	-	-	0.0003	***	
					Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.2031	ns	
					Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0684	ns	
					CORT/Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.0043	**	

					CORT/Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0182	*	
					CORT/Fluoxetine 18mg/kg/day vs. CORT/RS67333 1.5mg/kg/day	-	-	0.5307	ns	
			Total Distance	ANOVA	Drug	1.840	3,43	0.1542	ns	Fig. 1I
	Novelty Suppressed Feeding	NSF	Fraction of mice eating	Log-rank (Mantel-Cox) test	Drug	-	-	<0.0001	***	Fig. 1J
			Latency to Feed (sec)	ANOVA	Drug	7.528	3,43	0.0004		Fig. 1K
				Fisher's LSD	Vehicle vs. CORT/Vehicle			0.0002	***	
					Vehicle vs. CORT/Fluoxetine 18mg/kg/day			0.0003	***	
					Vehicle vs. CORT/RS67333 1.5mg/kg/day			0.0388	*	
					CORT/Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.5809	ns	
					CORT/Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0267	*	
					CORT/Fluoxetine 18mg/kg/day vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0556	ns	
	Splash Test	ST	Grooming Duration (sec)	ANOVA	Drug	4.909	3,43	0.0051	**	Fig. 1L

				Fisher's LSD	Vehicle vs. CORT/Vehicle	-	-	0.0021	**	
					Vehicle vs. CORT/Fluoxetine 18mg/kg/day	-	-	0.0784	ns	
					Vehicle vs. CORT/RS67333 1.5mg/kg/day	-	-	0.8398	ns	
					CORT/Vehicle vs.	-	-	0.0847	ns	
					CORT/Vehicle vs.	-	-	0.0019	**	
					CORT/Fluoxetine 18mg/kg/day vs. CORT/RS67333 1.5mg/kg/day	-	-	0.0868	ns	
Figure 2 Acute RS67333 Male	Contextual Fear Conditioning	CFC	Training Freezing (%)	RMANOVA	Drug	2.5420	3,55	0.0656	ns	Fig. 2B
					Time	45.9700	4,220	<0.0001	****	
					Drug x Time	2.064	12,220	0.0204	*	
			Training Freezing (min 1)		Saline vs. RS67333 (1.5 mg/kg)	-	-	0.9785	ns	
					Saline vs. RS67333 (10 mg/kg)	-	-	0.9758	ns	

					Saline vs. RS67333 (30 mg/kg)	-	-	0.9378	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.9933	ns	
				Fisher's LSD	RS67333 (1.5 mg/kg) vs. RS67333 (30 mg/kg)	-	-	0.9359	ns	
					RS67333 (10 mg/kg) vs. RS67333 (30 mg/kg)	-	-	0.9257	ns	
			Training Freezing (min 2)		Saline vs. RS67333 (1.5 mg/kg)	-	-	0.9521	ns	
					Saline vs. RS67333 (10 mg/kg)	-	-	0.9351	ns	
				Fisher's LSD	Saline vs. RS67333 (30 mg/kg)	-	-	0.9521	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.9160	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (30 mg/kg)	-	-	>0.9999	ns	
					RS67333 (10 mg/kg) vs. RS67333 (30 mg/kg)	-	-	0.9160	ns	
			Training Freezing (min 3)	Fisher's LSD	Saline vs. RS67333 (1.5 mg/kg)	-	-	0.7649	ns	
					Saline vs. RS67333 (10 mg/kg)	-	-	0.9687	ns	

					Saline vs. RS67333 (30 mg/kg)	-	-	0.9978	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.7547	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (30 mg/kg)	-	-	0.8205	ns	
					RS67333 (10 mg/kg) vs. RS67333 (30 mg/kg)	-	-	0.9797	ns	
			Training Freezing (min 4)	Fisher's LSD	Saline vs. RS67333 (1.5 mg/kg)	-	-	0.1524	ns	
					Saline vs. RS67333 (10 mg/kg)	-	-	0.2793	ns	
					Saline vs. RS67333 (30 mg/kg)	-	-	0.0165	*	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.4480	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (30 mg/kg)	-	-	0.4553	ns	
					RS67333 (10 mg/kg) vs. RS67333 (30 mg/kg)	-	-	0.0892	ns	
			Training Freezing (min 5)	Fisher's LSD	Saline vs. RS67333 (1.5 mg/kg)	-	-	0.5384	ns	
					Saline vs. RS67333 (10 mg/kg)	-	-	0.0017	**	

					Saline vs. RS67333 (30 mg/kg)	-	-	<0.0001	****	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.2137	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (30 mg/kg)	-	-	0.0025	**	
					RS67333 (10 mg/kg) vs. RS67333 (30 mg/kg)	-	-	0.0096	**	
			Re-exposure Freezing (%)	RMANOVA	Drug	5.314	3,55	0.0027	**	Fig. 2C
					Time	11.400	4,220	<0.0001	****	
					Drug x Time	0.798	12,220	0.6528	ns	
				Fisher's LSD	Saline vs. RS67333 (1.5 mg/kg)	-	-	0.0185	*	
					Saline vs. RS67333 (10 mg/kg)	-	-	0.0006	***	
					Saline vs. RS67333 (30 mg/kg)	-	-	0.2625	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.8278	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (30 mg/kg)	-	-	0.3259	ns	

					RS67333 (10 mg/kg) vs. RS67333 (30 mg/kg)	-	-	0.3050	ns	
			Re-exposure Average Freezing (%)	ANOVA	Drug	5.314	3,55	0.0027	**	Fig. 2D
				Fisher's LSD	Saline vs. RS67333 (1.5 mg/kg)	-	-	0.0185	*	
					Saline vs. RS67333 (10 mg/kg)	-	-	0.0006	***	
					Saline vs. RS67333 (30 mg/kg)	-	-	0.2625	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.8278	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (30 mg/kg)	-	-	0.3259	ns	
					RS67333 (10 mg/kg) vs. RS67333 (30 mg/kg)	-	-	0.3050	ns	
	Forced Swim Test	FST	Day 1 Immobility Time (sec)	RMANOVA	Drug	3.381	3,55	0.0245	*	Fig. 2E
					Time	25.700	5,275	<0.0001	*** *	
					Drug x Time	0.484	15,275	0.9479	ns	
				Fisher's LSD	Saline vs. RS67333 (1.5 mg/kg)	-	-	0.3784	ns	

					Saline vs. RS67333 (10 mg/kg)	-	-	0.0028	**	
					Saline vs. RS67333 (30 mg/kg)	-	-	0.8437	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.3430	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (30 mg/kg)	-	-	0.5994	ns	
					RS67333 (10 mg/kg) vs.RS67333 (30 mg/kg)	-	-	0.1099	ns	
			Day 2 Immobility Time (sec)	RMANOVA	Drug	0.69	3,55	0.5620	ns	Fig. 2F
					Time	5.210	5,275	<0.0001	****	
					Drug x Time	0.720	15,275	0.7637	ns	
			Day 2 Immobility Time (min	ANOVA	Drug	0.942	3,55	0.4266	ns	Fig. 2G
	Open Field Test	OF	Distance Travelled (cm)	RMANOVA	Drug	1.368	1,18	0.2575	ns	Fig. 2H
					Time	1.513	9,162	0.1472	ns	
					Drug x Time	0.755	9,162	0.6578	ns	

			Time in Center (%)	*t* -test	Saline vs. RS67333 (10 mg/kg)	-	-	0.2475	ns	Fig. 2I
	Elevated Plus Maze	EPM	Time in Open Arms (sec)	*t* -test	Saline vs. RS67333 (10 mg/kg)	-	-	0.2069	ns	Fig. 2J
			Entries into Open Arms	*t* -test	Saline vs. RS67333 (10 mg/kg)	-	-	0.3584	ns	Fig. 2K
	Novelty Suppressed Feeding	NSF	Fraction of mice not eating	Log-rank (Mantel-Cox) test	Drug	-	-	0.0183	*	Fig. 2L
			Latency to feed (sec)	*t* -test	Saline vs. RS67333 (10 mg/kg)	-	-	0.0108	*	Fig. 2M
			Food Eaten (g)	*t* -test	Saline vs. RS67333 (10 mg/kg)	-	-	0.3871	ns	Fig. 2N
			Body Weight Loss (g)	*t* -test	Saline vs. RS67333 (10 mg/kg)	-	-	0.8829	ns	Fig. 2O
Figure 3 Acute RS67333 Female	Contextual Fear Conditioning	CFC	Training Freezing (%)	RMANOVA	Drug	4.0900	2,38	0.0246	*	Fig. 3B
					Time	104.4000	4,152	<0.0001	****	
					Drug x Time	3.130	8,152	0.0026	**	
				Fisher's PLSD	Saline vs. RS67333 (1.5 mg/kg)	-	-	0.0941	ns	

					Saline vs. RS67333 (10 mg/kg)	-	-	0.0076	**	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.3858	ns	
			Training Freezing (min 1)	Fisher's PLSD	Saline vs. RS67333 (1.5 mg/kg)	-	-	0.8847	ns	
					Saline vs. RS67333 (10 mg/kg)	-	-	0.9054	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.9715	ns	
			Training Freezing (min 2)	Fisher's PLSD	Saline vs. RS67333 (1.5 mg/kg)	-	-	0.8966	ns	
					Saline vs. RS67333 (10 mg/kg)	-	-	0.8719	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.9854	ns	
			Training Freezing (min 3)	Fisher's PLSD	Saline vs. RS67333 (1.5 mg/kg)	-	-	0.8999	ns	
					Saline vs. RS67333 (10 mg/kg)	-	-	0.9721	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.8746	ns	
			Training Freezing (min 4)	Fisher's PLSD	Saline vs. RS67333 (1.5 mg/kg)	-	-	0.0168	*	

					Saline vs. RS67333 (10 mg/kg)	-	-	0.0040	**	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.7882	ns	
			Training Freezing (min 5)	Fisher's PLSD	Saline vs. RS67333 (1.5 mg/kg)	-	-	0.0117	*	
					Saline vs. RS67333 (10 mg/kg)	-	-	<0.0001	****	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.0492	*	
			Re-exposure Freezing (%)	RMANOVA	Drug	2.257	2,38	0.1185	ns	Fig. 3C
					Time	8.836	4,152	<0.0001	****	
					Drug x Time	0.618	8,152	0.7620	ns	
			Re-exposure Average	ANOVA	Drug	2.257	2,38	0.1185	ns	Fig. 3D
	Forced Swim Test	FST	Day 1 Immobility Time (sec)	RMANOVA	Drug	1.354	2,38	0.2705	ns	Fig. 3E
					Time	16.760	5,190	<0.0001	****	
					Drug x Time	0.651	10,190	0.7685	ns	

			Day 2 Immobility Time (sec)	RMANOVA	Drug	2.688	2,38	0.0810	ns	Fig. 3F
					Time	18.900	5,190	<0.0001	****	
					Drug x Time	1.470	10,190	0.1534	ns	
			Day 2 Immobility Time (min 3-6) (sec)	ANOVA	Drug	3.957	2,38	0.0275	*	Fig. 3G
				Fisher's PLSD	Saline vs. RS67333 (1.5 mg/kg)	-	-	0.4916	ns	
					Saline vs. RS67333 (10 mg/kg)	-	-	0.1991	ns	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.0235	*	
	Open Field	OF	Distance Travelled (cm)	RMANOVA	Drug	2.046	2,25	0.1504	ns	Fig. 3H
					Time	3.358	9,225	0.0007	***	
					Drug x Time	1.165	18,225	0.2923	ns	
			Time in Center (%)	ANOVA	Drug	0.611	2,25	0.5508	ns	Fig. 3I
	Elevated Plus Maze	EPM	Time in Open Arms (sec)	ANOVA	Drug	0.4706	2,25	0.6301	ns	Fig. 3J

			Entries into Open Arms	ANOVA	Drug	1.396	2,25	0.2663	ns	Fig. 3K
	Novelty Suppressed Feeding	NSF	Fraction of mice not eating	Log-rank (Mantel-Cox) test	Drug	-	-	0.0010	**	Fig. 3L
			Latency to feed (sec)	ANOVA	Drug	-	-	0.0038	**	Fig. 3M
				Fisher's PLSD	Saline vs. RS67333 (1.5 mg/kg)	-	-		ns	
					Saline vs. RS67333 (10 mg/kg)	-	-	0.0010	***	
					RS67333 (1.5 mg/kg) vs. RS67333 (10 mg/kg)	-	-	0.0820	ns	
			Food Eaten (g)	ANOVA	Drug	2.774	2,25	0.0817	ns	Fig. 3N
			Body Weight Loss (g)	ANOVA	Drug	0.5534	2,25	0.5819	ns	Fig. 3O
Figure 4 Acute Prucalopride and PF04995274 Male	Contextual Fear Conditioning	CFC	Training Freezing (%)	RMANOVA	Drug	0.317	5,34	0.8992	ns	Fig. 4B
					Time	50.650	4,136	<0.0001	****	
					Drug x Time	0.736	20,136	0.7835	ns	

			Re-exposure Freezing (%)	RMANOVA	Drug	5.284	5,34	0.0011	**	Fig. 4C
					Time	45.700	4,136	<0.0001	****	
					Drug x Time	1.201	20,136	0.2632	ns	
				Fisher's LSD	Saline vs. (R,S)-ketamine	-	-	0.0448	*	
					Saline vs. Prucalopride (3 mg/kg)	-	-	0.0004	***	
					Saline vs. Prucalopride (10 mg/kg)	-	-	0.4723	ns	
					Saline vs. PF04995274 (3 mg/kg)	-	-	0.6871	ns	
					Saline vs. PF04995274 (10 mg/kg)	-	-	0.0255	*	
					(R,S)-ketamine (30 mg/kg) vs. Prucalopride (3 mg/kg)	-	-	0.1343	ns	
					(R,S)-ketamine (30 mg/kg) vs. Prucalopride (10 mg/kg)	-	-	0.1023	ns	
					(R,S)-ketamine (30 mg/kg) vs. PF04995274 (3 mg/kg)	-	-	0.1027	ns	
					(R,S)-ketamine (30 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.8015	ns	

					Prucalopride (3 mg/kg) vs. Prucalopride (10 mg/kg)	-	-	0.0004	***	
					Prucalopride (3 mg/kg) vs. PF04995274 (3 mg/kg)	-	-	0.0014	**	
					Prucalopride (3 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.2229	ns	
					Prucalopride (10 mg/kg) vs. PF04995274 (3 mg/kg)	-	-	0.7981	ns	
					Prucalopride (10 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.0568	ns	
					PF04995274 (3 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.0619	ns	
			Re-exposure Average Freezing (%)	ANOVA	Drug	5.284	5,34	0.0011	**	Fig. 4D
				Fisher's LSD	Saline vs. (R,S)-ketamine	-	-	0.0448	*	
					Saline vs. Prucalopride (3 mg/kg)	-	-	0.0004	***	
					Saline vs. Prucalopride (10 mg/kg)	-	-	0.4723	ns	
					Saline vs. PF04995274 (3 mg/kg)	-	-	0.6871	ns	
					Saline vs. PF04995274 (10 mg/kg)	-	-	0.0255	*	

					(R,S)-ketamine (30 mg/kg) vs. Prucalopride (3 mg/kg)	-	-	0.1343	ns	
					(R,S)-ketamine (30 mg/kg) vs. Prucalopride (10 mg/kg)	-	-	0.1023	ns	
					(R,S)-ketamine (30 mg/kg) vs. PF04995274 (3 mg/kg)	-	-	0.1027	ns	
					(R,S)-ketamine (30 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.8015	ns	
					Prucalopride (3 mg/kg) vs. Prucalopride (10 mg/kg)	-	-	0.0004	***	
					Prucalopride (3 mg/kg) vs. PF04995274 (3 mg/kg)	-	-	0.0014	**	
					Prucalopride (3 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.2229	ns	
					Prucalopride (10 mg/kg) vs. PF04995274 (3 mg/kg)	-	-	0.7981	ns	
					Prucalopride (10 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.0568	ns	
					PF04995274 (3 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.0619	ns	
		FST	Day 1 Immobility Time (sec)	RMANOVA	Drug	0.520	5,34	0.7596	ns	Fig. 4E
					Time	19.370	5,170	<0.0001	****	

					Drug x Time	0.990	25,170	0.4827	ns	
			Day 2 Immobility Time (sec)	RMANOVA	Drug	3.135	5,34	0.0197	*	Fig. 4F
					Time	3.161	5,170	0.0094	**	
					Drug x Time	0.859	25,170	0.6616	ns	
				Fisher's LSD	Saline vs. (R,S)-ketamine (30 mg/kg)	-	-	0.0218	*	
					Saline vs. Prucalopride (3 mg/kg)	-	-	0.0052	**	
					Saline vs. Prucalopride (10 mg/kg)	-	-	0.1772	ns	
					Saline vs. PF04995274 (3 mg/kg)	-	-	0.1259	ns	
					Saline vs. PF04995274 (10 mg/kg)	-	-	0.0023	**	
					(R,S)-ketamine (30 mg/kg) vs. Prucalopride (3 mg/kg)	-	-	0.8332	ns	
					(R,S)-ketamine (30 mg/kg) vs. Prucalopride (10 mg/kg)	-	-	0.1712	ns	
					(R,S)-ketamine (30 mg/kg) vs. PF04995274 (3 mg/kg)	-	-	0.4094	ns	

					(R,S)-ketamine (30 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.3813	ns	
					Prucalopride (3 mg/kg) vs. Prucalopride (10 mg/kg)	-	-	0.0568	ns	
					Prucalopride (3 mg/kg) vs. PF04995274 (3 mg/kg)	-	-	0.2475	ns	
					Prucalopride (3 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.4224	ns	
					Prucalopride (10 mg/kg) vs. PF04995274 (3 mg/kg)	-	-	0.6674	ns	
					Prucalopride (10 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.0209	*	
					PF04995274 (3 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.0941	ns	
			Day 2 Immobility Time (min 3-6) (sec)	ANOVA	Drug	2.940	5,34	0.0260	*	Fig. 4G
				Fisher's LSD	Saline vs. (R,S)-ketamine (30 mg/kg)	-	-	0.0330	*	
					Saline vs. Prucalopride (3 mg/kg)	-	-	0.0093	**	
					Saline vs. Prucalopride (10 mg/kg)	-	-	0.2443	ns	
					Saline vs. PF04995274 (3 mg/kg)	-	-	0.1050	ns	

					Saline vs. PF04995274 (10 mg/kg)	-	-	0.0027	**	
					(R,S)-ketamine (30 mg/kg) vs. Prucalopride (3 mg/kg)	-	-	0.8478	ns	
					(R,S)-ketamine (30 mg/kg) vs. Prucalopride (10 mg/kg)	-	-	0.1763	ns	
					(R,S)-ketamine (30 mg/kg) vs. PF04995274 (3 mg/kg)	-	-	0.5815	ns	
					(R,S)-ketamine (30 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.3207	ns	
					Prucalopride (3 mg/kg) vs. Prucalopride (10 mg/kg)	-	-	0.0622	ns	
					Prucalopride (3 mg/kg) vs. PF04995274 (3 mg/kg)	-	-	0.4089	ns	
					Prucalopride (3 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.3387	ns	
					Prucalopride (10 mg/kg) vs. PF04995274 (3 mg/kg)	-	-	0.4653	ns	
					Prucalopride (10 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.0156	*	
					PF04995274 (3 mg/kg) vs. PF04995274 (10 mg/kg)	-	-	0.1270	ns	
	Open Field	OF	Distance Traveled (cm)	RMANOVA	Drug	0.350	45,306	0.9139	ns	Fig. 4H

					Time	7.441	9,306	<0.0001	****	
					Drug x Time	0.7151	45,306	0.9139	ns	
			Time in Center (sec)	ANOVA	Drug	0.4761	5,34	0.7915	ns	data not shown
	Elevated Plus Maze	EPM	Time in Open Arms (sec)	ANOVA	Drug	1.870	5,34	0.1257	ns	Fig. 4I
			Entries Into Open Arms(sec)	ANOVA	Drug	1.599	5,34	0.1686	ns	Fig. 4J
	Novelty Suppressed Feeding	NSF	Fraction of mice not eating	Log-rank (Mantel-Cox) test	Drug	-	-	0.0316	*	Fig. 4K
			Latency to Feed (sec)	ANOVA	Drug	2.115	5,34	0.0874	ns	Fig. 4L
			Food Eaten (g)	ANOVA	Drug	1.853	5,34	0.1288	ns	data not shown
			Body Weight Loss (g)	ANOVA	Drug	1.293	5,34	0.2899	ns	Fig. 4M
Figure 5, Acute Prucalopride,	Electrophysiology	Ephys	Amplitude	ANOVA	Drug	0.1233	2,14	0.8849	ns	Fig. 5B
			EPSCs (No.)	ANOVA	Drug	3.295	2,14	0.0672	ns	Fig. 5C

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EXAMPLE 2 Rapid anxiolytic effects of RS67333, a serotonin type 4 receptor agonist, and diazepam, a benzodiazepine, are mediated by projections from the prefrontal cortex to the dorsal raphe nucleus

Summary

Background: Activating the serotonin (5-HT) 4 receptors (5-HT_4Rs) has been shown to have anxiolytic effects in a variety of animal models. Characterizing the circuits responsible for these effects should offer insights into new approaches to treat anxiety.

Methods: We evaluated whether acute 5-HT_4R activation in glutamatergic axon terminals arising from the medial prefrontal cortex (mPFC) to the dorsal raphe nucleus (DRN) induced fast anxiolytic effects. Anxiolytic effects of an acute systemic administration (1.5 mg/kg, intraperitoneally, i.p.) or intra-mPFC infusion with the 5-HT_4R agonist, RS67333 (0.5 mg/side), were examined in mice. To provide evidences that anxiolytic effects of RS67333 recruited an mPFC-DRN neural circuit, *in vivo* recordings of firing rate of DRN serotonin (5-HT) neurons, cerebral 5-HT depletion, and optogenetic activation/silencing were performed.

Results: Acute systemic administration and intra-mPFC infusion of RS67333 produced fast anxiolytic effects and increased DRN 5-HT cell firing. Serotonin depletion prevented anxiolytic effects induced by mPFC infusion of RS67333. Surprisingly the anxiolytic effects of mPFC infusion diazepam (1.5 mg/side) were also blocked by 5HT depletion. Optogenetically activating mPFC terminals targeting the DRN reduced anxiety whereas silencing this circuit blocked RS67333 and diazepam mPFC infusion -induced anxiolytic effects. Finally, anxiolytic effects induced by an acute systemic RS67333 or diazepam

administration were partially blocked after optogenetically inhibiting cortical glutamatergic terminals in the DRN.

Conclusions: Our findings suggest that activating 5-HT_4R acutely in the mPFC or targeting mPFC pyramidal cell terminals in the DRN, may constitute a strategy to produce a fast-anxiolytic response.

Unlike SSRIs, treatment with RS67333, a 5-HT_4R agonist (A13), induced fast anxiolytic/antidepressant-like effects through a neurogenesis-independent mechanism (A5). Although a number of studies have assessed the anxiolytic/antidepressant-like activity of 5-HT_4R modulation after subchronic or chronic treatment, few have evaluated their anxiolytic-like profile acutely. Conflicting evidence suggests that 5-HT_4R antagonists have acute anxiolytic-like effects (A14, A15). Two reports showed an anxiolytic effect of 5-HT_4R antagonists SB 204070, GR 113808 (A15) and SB 207266A (A14, A15) in rats in the elevated plus maze (EPM). However, another study did not detect an effect of the antagonists SB 204070 and GR 113808 on the number of open arm entries in the EPM (A15). Similarly, a direct effect of the 5-HT_4R antagonists on anxiety-like behavior in the light/dark choice test was not detected (A16). The reasons for these discrepancies are unclear. In contrast, acute 5-HT_4R activation has been shown to be an encouraging pharmacological strategy to obtain a fast anxiolytic-like response. Recently, acute administration of RS67333, induced anxiolytic-like effects in mice (A17) and reversed the anxiogenic effects of chronic exposure to cannabinoids during adolescence (A18).

Interestingly, approximately 60% of pyramidal neurons recorded in the medial prefrontal cortex (mPFC) contain both the 5-HT_4R transcript and protein. Activation of these somatodendritic 5-HT_4R in the mPFC results in glutamate release in the DRN to stimulate the firing of 5-HT neurons (A19). A large body of evidence also suggests that mPFC projections to the DRN modulate anxiety and depression-related behaviors (A20-A22). Indeed, chronic optical stimulation of layer V pyramidal cells in the PFC induced a long-lasting anxiolytic-like effect in a mouse model of anxiety/depression (A23), and inhibition of mPFC terminals targeting to the DRN induces a long-lasting suppression of anxiety-like behavior in socially stressed mice (A24). Lastly, a recent study revealed a key role for DRN circuits in environment-specific adaptive behaviors (A25). As a result, it is possible that projection from the mPFC to DRN may mediate the anxiolytic effects of 5-HT_4R activation.

Here, using behavioral paradigms predictive of anxiolytic-like activity, we first evaluated the consequences of an acute systemic or intra-mPFC administration with RS67333

or the $GABA_A$ modulator diazepam in male BALB/cJRj anxious mice (A26). Then, using optogenetic techniques, we assessed the contribution of glutamatergic axon terminals arising from the mPFC to the DRN on fast anxiolytic-like effects.

MATERIALS AND METHODS

Subjects

Male BALB/cJRj mice (Janvier Labs, Le Genest-St-Isle, France) were 7-8 weeks old, weighed 25-30 g, and were maintained on a 12h light:12h dark schedule (lights on at 06:00 hours). Food and water were provided *ad libitum* except during behavioral observations. The protocols were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (Council directive #87-848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions # 92-256B to DJD, Institutional Animal Care and Use Committee 26 authorization #4074).

Drugs

1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-butyl-4piperidinyl)-1-propanone hydrochloride [RS67333, a serotonin 4 receptor ($5\text{-}HT_4R$) agonist] administered intraperitoneally (i.p.) at 1.5 mg/kg (A-S1), or locally in the medial Prefrontal Cortex (mPFC) (A-S2, A-S3) at 0.5 µg/side and 5-Fluoro-2-methoxy-[1-[2-[(methylsulfonyl)amino]ethyl]-4-piperidinyl]-1H-indole-3-methylcarboxylate sulfamate (GR125487, a $5\text{-}HT_4R$ antagonist) administered i.p. at 1 mg/kg (1) were dissolved in saline (0.9 % NaCl) solution and purchased from Tocris Bioscience (Bristol, United Kingdom). RS67333 shows high binding affinity for the $5\text{-}HT_4R$ with a pKi of 8.7 (A-S4, A-S5). Except for the sigma receptors, which are bound at affinities comparable to $5\text{-}HT_4R$ (sigma 1: pKi = 8.9; and sigma 2: pKi = 8.0), RS67333 has a pKi of less than 6.7 for other neurotransmitter receptors. Diazepam hydrochloride (dissolved in 0.5 % Tween®20 solution, Sigma-Aldrich, Saint-Quentin Fallavier, France) was administered i.p. at 1.5 mg/kg (A-S6) or locally in the mPFC at 1.5 µg/side (A-S7), 45 minutes before testing. Fluoxetine hydrochloride (dissolved in saline, Anawa Trading, Zurich, Switzerland) was administered at 18 mg/kg, i.p., 45 minutes before testing (A-S6). Para-chlorophenylalanine methyl ester (p-CPA, dissolved in Tween 1% solution, Sigma-Aldrich, Saint-Quentin Fallavier, France) was administered i.p. twice a day for 3 consecutive days at 150 mg/kg (A-S8, A-S9).

Treatments

Systemic administration with RS67333, diazepam or fluoxetine

RS67333 (1.5 mg/kg, i.p.), diazepam (1.5 mg/kg, i.p.), fluoxetine (18 mg/kg, i.p.) were injected 45 min before testing in the Elevated Plus Maze (EPM), the Novelty Suppressed Feeding (NSF) or the Open Field (OF) in three independent cohorts of male BALB/cJRj mice.

To ensure the selectivity of the anxiolytic-like effects of RS67333, in a new cohort of male BALB/cJRj mice, GR125487 (1.0 mg/kg, i.p.) dissolved in 0.9 % NaCl solution, was injected 15 minutes before RS67333 administration (1.5 mg/kg, i.p.). EPM or NSF occurred 45 min after RS67333 administration (A-S1). Behavioral consequences of the co-administration of GR125487+RS67333 were compared to RS67333 alone, diazepam (1.5 mg/kg, i.p.) fluoxetine (18 mg/kg, i.p.) and vehicle groups (0.9% saline solution, i.p.).

mPFC local infusion of RS67333

For mPFC drug infusion, two bilateral cannulae (75 µm-diameter silica capillary tubing inserted in 27G stainless steel catheter) were implanted in the mPFC [stereotaxic coordinates in mm from bregma: A = +2.10, L = ±0.50, V = -2.60, A, anterior; L, lateral; and V, ventral, according to (A-S10)] under anesthesia (chloral hydrate, 400 mg/kg, i.p.). The following day, RS67333 (0.5 µg/side) was continuously perfused in awake freely moving male BALB/cJRj mice at a flow rate of 0.2 µL/min for 2 minutes (LEGATO™ 180 syringe pump, KD Scientific Inc., Holliston, MA, USA), 45 min before testing in the EPM and in the NSF. Diazepam (1.5 mg/kg) was used as a positive control.

Serotonin depletion

In a new cohort of male BALB/cJRj mice, p-CPA was administered twice daily (at 0900 and 1700 h) for 3 consecutive days. RS67333 (0.5 µg/side) and diazepam (1.5 µg/side) were then intra-mPFC administered 24 h after the final p-CPA administration and behavioral test (EPM) occurred 45 minutes after local infusion.

For the p-CPA study, immediately after behavioral tests, animals were sacrificed and frontal cortex were dissected and reduced in cortical brain homogenates for 5-HT concentration measurements by ELISA method (Immusmol, France) to verify the 5-HT depletion of tissue content.

Behavioral tests

Elevated plus maze

The EPM is a widely used behavioral assay for rodents and it has been validated to assess the anti-anxiety effects of pharmacological agents (A-S11). This test was performed as described by (A-S1). The maze is a plus-cross-shaped apparatus, with two open arms and two

arms closed by walls linked by a central platform 50 cm above the floor. Mice were individually put in the center of the maze facing an open arm and were allowed to explore the maze during 5 min for the behavioral consequences of an acute systemic administration or mPFC infusion and during 6 min for the optogenetic experiments. The time spent in and the numbers of entries into the open arms were used as an anxiety index. All parameters were measured using a videotracker (EPM3C, Bioseb, Vitrolles, France).

Novelty suppressed feeding

The NSF is a conflict test that elicits competing motivations: the drive to eat and the fear of venturing into the center of a brightly lit arena. The latency to begin eating is used as an index of anxiety/depression-like behavior, because classical anxiolytic drugs as well as chronic antidepressants decrease this measure. The NSF test was carried out during a 10 min period as previously described (A-S12). Briefly, the testing apparatus consisted of a plastic box (50x50x20 cm), the floor of which was covered with approximately 2 cm of wooden bedding. Twenty-four hours prior to behavioral testing, all food was removed from the home cage. At the time of testing, a single pellet of food (regular chow) was placed on a white paper platform positioned in the center of the box. Each animal was placed in a corner of the box, and a stopwatch was immediately started. The latency to eat (defined as the mouse sitting on its haunches and biting the pellet with the use of forepaws) was timed. Immediately afterwards, the animal was transferred to its home cage, and the amount of food consumed by the mouse in the subsequent 5 min was measured, serving as a control for change in appetite as a possible confounding factor.

Open field paradigm (OF)

Motor activity was quantified in four 39 × 39 cm perpex plastic open field boxes (Vivo-tech/Ugo Basile, Salon de Provence, France). The apparatus was illuminated from the ground with special designed 40 x 40 cm Infra-red backlights (monochromatic wavelength 850 nm high homogeneity, Vivo-tech, Salon de Provence, France). Activity chambers were monitored by four black and white cameras with varifocal optics and polarizing filters (Vivo-tech, Salon de Provence, France). For optogenetic experiments, optical bandpass filters were specifically selected to improve tracking detection. The whole set-up was controlled using ANYMAZE version 6 video tracking software (Stoelting Co/Vivo-tech, Salon de Provence, France). Dependent measures were time in the center over a 10 min for systemic administration or 6

min test period for optogenetic experiments, total ambulatory distance and ambulatory distance traveled in the center divided by total distance.

***In vivo* electrophysiological recordings**

Dorsal Raphe Nucleus (DRN) 5-HT neurons were identified according to the following criteria: a slow (0.5–2.5 Hz) and regular firing rate and a long duration, positive action potential as previously reported (A-S13).

Optogenetic manipulations

Virus injection

To target opsin expression selectively to cortical glutamatergic terminals in the DRN, AAV5-CaMKIIα-ChR2-enhanced yellow fluorescent protein (eYFP), AAV5-CaMKII-ArchT-green fluorescent protein (GFP) or AAV5-CaMKII-eYFP, obtained from Karl Deisseroth and Ed Boyden (UNC Vector Core, NC, USA) were bilaterally injected into the mPFC (in mm from bregma, A = +2.10, L = ±0.50, V = -2.60). Mice injected with AAV5-CamKII-eYFP were used as control.

Optical fibers construction

As we previously described, for all experiments, a 200 µm core, 0.37 numerical aperture (NA) multimode fiber (ThorLabs, Maison Laffitte, France) was used for optical stimulation through a patch cable connected to a 100 mw 473 nm blue and 532 nm green laser diode (OEM laser systems, USA) (A-S14).

Fiber optics implantation and optogenetic procedure

BALB/cJRj mice were surgically implanted with fiber optics targeted to the DRN (in mm from bregma, A = -4.50, L = +1.20, V = -4.0, angle 15°). A 200 mm core, 0.37 NA fiber optic (ThorLabs, ~10-12 and 15-16 mW for ChR2 and Arch-T, respectively, at the tip of optic) was used for optical stimulation via a patch cable connected to either a 473 or 532 nm laser diode (OEM laser systems, USA) as previously described (A-S15). For behavioral experiments, AAV5-CaMKIIα-ChR2-eYFP mice and their controls received a 10 Hz stimulation, 20 ms pulses, over a 3-minute period whereas a green light was delivered continuously to AAV5-CaMKII-ArchT-GFP throughout the 3 min testing period. Similar doses of RS67333 (locally in the mPFC at 0.5 µg/side or at 1.5 mg/kg, i.p.) and diazepam (locally in the mPFC at 1.5 µg/side or at 1.5 mg/kg, i.p.) were infused in the mPFC or administered i.p. The stimulation or the inhibition of mPFC projections in the DRN occurred simultaneously to the behavioral paradigms.

Immunohistochemistry

To ensure opsin expression, mice were perfused transcardially (cold saline for 2 minutes, followed by 4% cold PFA) after anesthesia (100 mg/ml ketamine and 20 mg/ml xylazine, i.p.). Brains were removed and cryoprotected with 30% sucrose at 4°C. Thirty-five µm-thick coronal sections were cut through the entire brain and stored in 1X phosphate buffered saline (PBS) with 0.1% sodium azide. Free-floating sections were incubated in a blocking buffer (0.5% Triton X-100, 5% normal donkey serum (NDS), 1X PBS) for 2 hours at room temperature. eYFP and GFP were detected using rabbit GFP Tag polyclonal antibody (1:500, Thermo Fisher Scientific, catalog #A-11122) in the same buffer at 4°C overnight. Following washed in 1X PBS, secondary Cy3-AffiniPure donkey anti-rabbit antibody (1:250, Jackson Immunoresearch, 711-165-152) was added in 1X PBS with 10% NDS buffer for 2 hours at room temperature. After several rinses in 1X PBS, sections mounted on slide, air-dried, coverslipped with fluoromont and examined under confocal microscopy (Olympus BX51) using appropriate filters.

Statistical analysis

Results from data analyses, expressed as mean ± SEM were analyzed using Prism 8.1.2 software (Graphpad, San Diego, CA, USA). For all experiments, Student's test, one-way or two-way ANOVAs were applied to the data as appropriate. Significant main effects and/or interactions were followed by Fisher's PLSD post-hoc analysis. In the NSF, we used the Kaplan-Meier survival analysis due to the lack of normal distribution of the data and Mantel-Cox log rank test to evaluate differences between experimental groups. Statistical significance was set at $p<0.05$. All statistical tests and p values are listed in Tables 3-6.

RESULTS

Acute systemic 5-HT$_4$R stimulation induced fast anxiolytic-like effect.

To assess putative fast anxiolytic 5-HT$_4$R activation, vehicle, fluoxetine (18 mg/kg), diazepam (1.5 mg/kg), or RS67333 (1.5 mg/kg) were administered i.p., 45 minutes before behavioral testing in the EPM or NSF (Figure 6A). In the EPM, acute systemic injection of RS67333 and diazepam induced a fast anxiolytic-like effect when compared with vehicle and fluoxetine administration in BALB/cJRj mice. RS67333 and diazepam increased time and the percent time spent in the open arms (one-way ANOVA, $^{**}p<0.01$ *vs.* vehicle group, Figure 1C and inset). It is unlikely that this effect was the consequence of a change in locomotor activity, since no change in this parameter was detected and the ratio of ambulatory distance

in the open arms divided by total distance was significantly increased for both drugs (one-way ANOVA, **p<0.01 *vs.* vehicle group, Figure 6D and inset).

To assess the selectivity of RS67333-induced anxiolytic-like effects, we also tested whether the 5-HT_4R antagonist GR125487 (1 mg/kg, i.p.) influenced the response of RS67333 (1.5 mg/kg) on anxiety-like behavior. Here, GR125487 was administered 15 minutes before RS administration (Figure 11A). In the EPM, GR125487 administration prevented RS67333-induced increase in time and the percent time spent in the open arms, or the increase of ambulatory distance in the open arms divided by total distance without affecting locomotor activity (one-way ANOVA, *p<0.05, **p<0.01 or ##p<0.01 *vs.* vehicle group and *vs.* RS67333 group respectively, Figures 11B-11C and insets).

In another anxiety-related test, the NSF, we found that RS67333 and diazepam, unlike fluoxetine that induced an anxiogenic-like effect, decreased the latency to feed when compared with saline administration (Kaplan–Meier survival analysis and one-way ANOVA, **p<0.01 *vs.* vehicle group, inset, Figures 6E-6F and inset) without affecting the home-cage food consumption. Moreover, GR125487 occluded the effect of RS67333 on the latency to feed without affecting food consumption (Kaplan–Meier survival analysis and one-way ANOVA, **p<0.01 or ##p<0.01 *vs.* vehicle group and *vs.* RS67333 group respectively (Figures 11D-11E and inset).

To further validate these results, we next tested the effect of RS67333, fluoxetine and diazepam in another anxiety-related test, the Open Field (OF) (Figures 12A-12C). We found that, unlike fluoxetine, acute RS67333 and diazepam increase the percent time spent in Center (one-way ANOVA, *p<0.05, **p<0.01, Figures 12B) without affecting locomotor activity. Indeed, the ratio of ambulatory distance in the center divided by total distance was significantly increased for diazepam and a trend was observed for RS67333 (one-way ANOVA, *p<0.05, **p<0.01 *vs.* vehicle group, Figures 12C and inset). In summary, these data indicate that RS67333-induced fast anxiolytic-like effects through 5-HT_4R activation.

Acute cortical 5-HT_4R activation induces fast anxiolytic-like effects.

Since 5-HT_4Rs are expressed in the mPFC (A27), a brain region involved in the physiopathology of mood disorders related to central 5-HT dysfunction (A19, A28, A29), we examined the contribution of 5-HT_4R activation in the mPFC to fast anxiolytic-like activity (Figure 6B). In the EPM, as observed with a systemic administration of diazepam (1.5 mg/kg), a local infusion of RS67333 (1 µg) significantly increased time and percent time spent in the open arms without affecting locomotion (one-way ANOVA, *p<0.05, **p<0.01

vs. vehicle group, Figure 6G and insets), as a significant increase in ratio of ambulatory distance in the open arms divided by total distance was observed (One Way ANOVA, *p<0.05 *vs.* vehicle group, Figures 6H). In the NSF, RS67333 and systemic administration of diazepam decreased latency to feed without affecting the home-cage food consumption (Kaplan–Meier survival analysis and one-way ANOVA, *p<0.05, **p<0.01 *vs.* vehicle group, Figures 6I-6J and inset) confirming the anxiolytic-like effects of 5-HT_4R activation in the mPFC.

Serotonin from the dorsal raphe nucleus is involved in fast anxiolytic-like effects of acute RS67333 and diazepam administration.

Here, we set out to test whether an acute administration of RS67333 could induce persistent changes in serotonergic activity (Figure 7A). Indeed, we found that acute systemic administration of RS67333 (1,5 mg/kg) increased the discharge frequency of DRN 5-HT neurons by 63 % (Student's test, **p<0.01 *vs.* before RS6733, Figures 2B-2C).

To further confirm that anxiolytic-like effects of mPFC 5-HT_4R stimulation depend on an intact 5-HT system, mice were pre-treated with p-CPA for 3 days before RS67333 (0.5 µg/side) or diazepam (1.5 µg/side) intra-mPFC infusion (Figure 7D). p-CPA induced an average decrease of 86 % in the 5-HT content in the mPFC of vehicle mice (two-way ANOVA, #p<0.05, ##p<0.01 *vs.* appropriate vehicle group, Figure 7E). Acute intra-mPFC infusion with RS67333 or diazepam increased time, percent time spent in the open arms of the EPM, and the ratio of ambulatory distance in the open arms / total distance were abolished in 5-HT-depleted p-CPA mice (two-way ANOVA, **p<0.01 *vs.* vehicle/vehicle or or #p<0.05, ##p<0.01 *vs.* vehicle/appropriate group, Figures 7F-7G). p-CPA –induced 5-HT depletion did not affect locomotor activity (Inset Figure 7G). These results point out the critical role of the 5-HT neurotransmission in fast anxiolytic-like effects. In summary, RS67333, as suggested previously (A29), and diazepam act on 5-HT function through a modulation of the mPFC.

Fast anxiolytic-like effects of 5-HT_4R agonist recruit medial prefrontal cortex-brainstem neural circuit.

While emotional behaviors are mediated by mPFC pyramidal neurons projecting to the DRN (A22), no direct evidence suggests that these projections are involved in anxiolytic-like effects. Thus, using optogenetic strategies, we began an examination of the specific

contribution of the incoming cortical glutamatergic terminals in the DRN to fast anxiolytic-like effects induced by acute diazepam or RS67333 administration.

First, to target opsin expression selectively to cortical glutamatergic projections to the DRN, we employed an AAV5-CaMKIIα-virus, that specifically expresses ChR2 in mPFC pyramidal cell terminals in the DRN (Figures 8A-8B). AAV5-CaMKIIα-ChR2-eYFP injected mice were compared to AAV5-CaMKII-eYFP-injected control. In the EPM, illumination of mPFC projections in the DRN in CamKII-ChR2-injected BALB/cJRj mice induced a significant increase in time, percent time or change in the distribution of time spent in the open arms in comparison to light OFF and also to control group (two-way ANOVA, **p<0.01 or ##<p0.01 *vs.* CaMKIIα-ChR2-eYFP or CaMKII-eYFP respectively during light ON, Figures 8C and insets). It is unlikely that this effect was the consequence of a change in locomotor activity, as even if the total ambulatory distance was decrease in CamKII-ChR2-injected mice during light ON, the ratio of ambulatory distance in open arms divided by total distance was increased in comparison to light OFF (two-way ANOVA, **p<0.01 or ##<p0.01 *vs.* CaMKIIα-ChR2-eYFP or CaMKII-eYFP respectively during light ON, Figures 3D and inset). To further confirm that the fast anxiolytic-like effects of the 5-HT_4R agonist recruit the mPFC-brainstem neural circuit, we evaluated the behavioral consequences of an optogenetic stimulation of mPFC terminals in the DRN in the OF paradigm (Figures 13A-13C). We found that illumination of mPFC projections in DRN in CamKII-ChR2-injected BALB/cJRj mice induced a significant increase in time spent in the center in comparison to light OFF and also to control group (two-way ANOVA, **p<0.01 *versus* during light OFF, ##p<0.01 *versus* eYFP during light ON, Figures 13B and inset). It is unlikely that this effect was the consequence of a change in locomotor activity, as the total ambulatory distance was not affected and the ratio of ambulatory distance in center divided by total distance was increased in comparison to light OFF (two-way ANOVA, **p<0.01 or ##<p0.01 vs. CaMKIIα-ChR2-eYFP or CaMKII-eYFP during light ON, Figure 13C and inset).

Next, we probed the effects of optogenetic inhibition of mPFC projections to the DRN after cortical infusion of RS67333 or diazepam (Figure 9A). AAV5-CaMKII-ArchT injected mice in the mPFC showed robust expression of ArchT-GFP in mPFC but also in cortical glutamatergic terminals in the DRN (Figures 9B). In the EPM, RS67333 (0.5 µg/side) and diazepam (1.5 µg/side) injected in the mPFC of CamKII-ArchT mice increased significantly time, percent time or change in the distribution of time spent in the open arms during light OFF and was reversed during a 3-min green light illumination (70 ±8 % and 85 ±5 % of

inhibition for RS67333 and diazepam respectively, two-way ANOVA, **p<0.01 or ##<p0.01 *vs.* CaMKIIα-ArchT or CaMKII-GFP respectively during light ON for appropriate treatment Figures 9C and inset). Similarly, acute RS67333 and diazepam administration increased the ratio of ambulatory distance in open arms divided by total distance during light OFF in comparison to controls and was blocked during light ON, confirming the anxiolytic effects of both drugs (two-way ANOVA, **p<0.01 *vs.* CaMKIIα-ArchT or #<p0.05 CaMKII-GFP respectively during light OFF for appropriate treatment, Figure 9D). No changes in ambulatory distance were observed during light OFF or light ON (Figures 9D and inset). These results were also confirmed in the OF (Figure 14A-14C). Indeed, RS67333 (0.5 µg/side) and diazepam (1.5 µg/side) infused in mPFC of CamKII-ArchT mice, induced anxiolytic effects that were blocked by optogenetic inhibition of mPFC terminals in the DRN. Specifically, RS67333 and diazepam injected in the mPFC of CamKII-ArchT mice increased significantly the time spent in the center during light OFF, and this effect was reversed during a 3-min green light illumination (85 ±20 % and 80 ±22 % of inhibition for RS67333 and diazepam respectively, two-way ANOVA, *p<0.05, **p<0.01 or #p<0.05, ##<p0.01 *vs.* CaMKIIα-ArchT or CaMKII-GFP during light ON, Figures 14B-14C and insets). These data support that the mPFC-DRN neural circuit is recruited for both RS67333 and diazepam to induce anxiolytic-like effects.

We then proceeded to investigate whether mPFC terminals targeting to the DRN circuit could be sufficient for fast anxiolytic-like effects induced by acute systemic diazepam or RS67333 treatment (Figures 9A-9B). In the EPM paradigm, as previously shown, acute systemic administration of RS67333 (1.5 mg/kg) or diazepam (1.5 mg/kg) in CaMKII-ArchT -mPFC injected mice increased time, percent time spent in open arms and ratio ambulatory distance in open arms divided by total distance eliciting an anxiolytic-like effect without affecting locomotor activity during the OFF epoch (two-way ANOVA, **p<0.01 *vs.* vehicle group during light OFF, Figures 9E-9F and inset). During a 3-minute green-light illumination of cortical glutamatergic terminals in the DRN, even though acute RS67333 or diazepam induced an anxiolytic-like effect, the size of the effect was attenuated in comparison to light OFF (two-way ANOVA, §§p<0.01 *vs.* vehicle group during light ON, #p<0.05 *vs.* appropriate group during light OFF, Figures 9E-9F and inset). Indeed, a significant decrease of 24 % for RS67333 and a 17 % decrease (p<0.09), for diazepam, in time spent in the open arms was observed in the EPM. The distribution of time spent in the open arms and the decrease in ambulatory distance in open arms divided by total distance between light ON

after acute systemic RS67333 or diazepam administration confirmed that inhibition of mPFC pyramidal cell terminals in the DRN significantly reduced anxiolytic effects of these two compounds (two-way ANOVA, #p<0.05, ##p<0.01 *vs.* appropriate group during light OFF, Figures 9E-9F and inset). Overall, these data suggest that the mPFC terminals in the DRN are recruited for fast anxiolytic effects of RS67333 and diazepam.

Evaluation of the long-term activity of a 5-HT$_4$ receptor agonist in BALB/cJRj mice

Having shown that RS 67333 was provided with prophylactic properties, we subsequently sought to gain knowledge of this molecule in the long term, i.e. to find out whether the anxiolytic response brought about by RS 67333 is sustainable over time. In order to achieve this, the BALB/cJRj mice were systemically injected with a single dose of RS 67333 (1.5 mg/kg) or diazepam (1.5 mg/kg), 45 minutes before performing the Splash Test. The following day, the mice underwent the EPM without having received another dose of RS, and then at the open field 24 hours later, and, finally, to NSF 24 hours after the open field (Fig. 15A).

As anticipated, RS 67333 increased the grooming time ($t = 2.294$; $p<0.05$) in the Splash Test, without affecting the number of episodes ($t = 1.546$; $p = 0.1531$) (Fig. 15B-15C), following single administration. We have not identified the anxiolytic effects expected of RS 67333 in the EPM ($t = 0.4990$; $p = 0.6286$) (Fig. 15D-15E), 24 hours after the injection. However, the RS 67333 has increased the time spent in the center of the OF ($t = 1.924$; $p<0.05$), without affecting the ratio of the distance in the center to the total walking distance ($t = 1.281$; $p = 2292$) (Fig. 15F-15G), 48 hours after the injection, and reduced the lag for feeding in the NSF ($t = 2.520$; $p<0.05$), without affecting the consumption of food in a familiar environment ($t = 0.2203$; $p = 0.4151$) (Fig. 15H-15I), 72 hours after the injection. Since diazepam does not exhibit any antidepressant activity, it is therefore normal to not identify any effect in the Splash Test. We likewise have not been able to observe any long-term activity for diazepam either.

Therefore, although this study should be repeated, the RS could have persistent effects up to 72 hours after injection.

DISCUSSION

Acute 5-HT$_4$R activation and fast anxiolytic-like effects.

Our study provides evidence in BALB/cJRj, a mouse strain with a high anxiety level, that 5-HT$_4$R stimulation induced fast anxiolytic-like effects similar to diazepam in three

different anxiety paradigms, namely EPM, NSF and OF. Interestingly, unlike RS67333, acute systemic administration of fluoxetine did not affect anxiety-like behavior, confirming previous observations (A30). These data also suggest that rapid anxiolytic-like activity requires selective activation of some key postsynaptic receptors such as the 5-HT_4R or the 5-HT_{1A}R (A31) more than a global increase in 5-HT neurotransmission.

Involvement of mPFC-DRN circuit for the 5-HT_4R activation-mediated rapid anxiolytic-like activity.

In both humans and also in rodents, 5-HT_4R are mainly localized in limbic areas involved in psychiatric disorders, such as anxiety (A27, A32). We explored the role of the 5-HT_4R activation expressed in mPFC in fast anxiolytic-like effects. Indeed, 5-HT_4R is expressed in excitatory pyramidal neurons of the mPFC, a region showing glutamate dysregulation in patients with generalized anxiety disorders (A33, A34). Interestingly, fast anxiolytic-like effects observed after acute systemic administration of RS67333 were reproduced by an acute infusion of this 5-HT_4R agonist in mPFC. Indeed, after a bilateral infusion with RS67333, similar to diazepam, there was an increase in time spent in open arms in the EPM and a decrease in latency to feed was observed in the NSF. Our results are in line with previous results showing that 5-HT_4R overexpression in the mPFC yields a robust anxiolytic-like behavioral phenotype (A7, A19).

Anatomical studies have shown that the prelimbic/cingulate cortices also project abundantly to DRN 5-HT neurons (A35). This connectivity has attracted great interest as a potential circuit involved in modulating stress and depressive behaviors (A22). For example, stressor exposure to inescapable shock in rodents increased cFos expression in 5-HT neurons in the middle and caudal regions of the DRN, suggesting an increased neural activation of this structure in anxiogenic situations (A36). There is also evidence that 5-HT_4R activation in the mPFC controls the firing rate of midbrain serotonergic neurons via descending inputs (A19, A29, A37). A reduction in the spontaneous activity of 5-HT neurons and a decrease in 5-HT content in the DRN of 5-HT_4R-null mice were observed (A38). Conversely, administration of RS67333 in rat, at different time points, drives effects on DRN 5-HT neuronal activity (A19, A29, A39), and increase 5-HT release at projection sites (A40). We showed that acute systemic injection of RS67333 enhances the firing rate of DRN 5-HT neurons in mice, suggesting that the fast anxiolytic-like activity of the 5-HT_4R agonist is dependent on activation of this neuronal population, despite the fact that DRN does not express 5-HT_4R (A27). In fact, the fast onset of action of the 5-HT_4R agonist could be a

consequence of an increase in serotonergic output to projection areas including the mPFC (A19, A41). These results are supported by the fact that the depletion of whole brain 5-HT content by pre-treatment with systemic p-CPA prevented RS67333-induced anxiolytic-like phenotype, while p-CPA alone did not affect behavior as previously reported (A42). Interestingly, despite different pharmacological targets, the 5-HT_4R agonist and BZD shared a common anxiolytic-like activity with similar efficiency suggesting activation of 5-HT neurotransmission and possibly common neural circuit recruitment by these two drugs. Under our experimental conditions, anxiolytic-like activity of intra-mPFC infusion of diazepam was blocked by a pre-treatment with p-CPA, suggesting a participation of 5-HT system in this activity.

Knowing that 5-HT-producing neurons in the DRN are preferentially modulated by monosynaptic glutamatergic inputs from the mPFC (A35), we used a CamKII promoter to target glutamatergic pyramidal neurons and evaluate this neuronal brain circuit involved in fast anxiolytic-like effects. Illumination of ChR2-expressing terminals of mPFC neurons projecting to the DRN of BALB/cJRj mice induced an anxiolytic-like effect, measured as an increase in the time spent in the open arms, an effect similarly to intra-mPFC RS67333 and diazepam. This result emphasizes the role of the mPFC-brainstem DRN neural circuit in fast anxiolytic-like effects. Multiple studies have implicated the mPFC-DRN circuit in the regulation of behavioral response to aversive challenges. For example, deep brain stimulation (DBS) in the ventromedial PFC of chronic social defeat (SD) mice restored social interaction (A43). At the same time, 1-hour DBS in naïve mice and chronic DBS in chronic SD mice, increased cFos immunoreactivity in the DRN and reversed SD-induced hypoexcitability of DRN 5-HT neurons, respectively (A43). In SD mice, chronic photoactivation of mPFC pyramidal cells increased the time spent in the open arms in the EPM (A23), whereas stimulation had no effect on anxiety-related behavior in non-stressed animals (A23, A44). Conversely, photosilencing mPFC terminals in the DRN prevented a decrease in social interaction in SD mice, suggesting a contributing role in anxiety-like behavior (A24).

To ensure that the behavioral response to local 5-HT_4R agonist infusion is in line with the idea that glutamatergic mPFC pyramidal neurons are mediators of 5-HT_4R agonist -driven effects on DRN 5-HT neuronal activity, we optogenetically silenced incoming cortical glutamatergic terminals in the DRN. Inhibition of these projections reversed the anxiolytic-like behavior induced by intra-mPFC RS67333 and diazepam administration, confirming that the cortex-raphe circuit recruitment is essential for rapid anxiolytic-like activity (A7). Interestingly, in line with our results, a recent study show that rescuing the mPFC-5-HT_4R

expression in 5-HT_4R KO mice partly reduced stress levels (A28). Although these results were not surprising for the 5-HT_4R agonist, they were unexpected for diazepam. However, cortical $GABA_A$ receptor activation through intra-mPFC muscimol infusion (a direct agonist of $GABA_A$ receptor) has been proposed to attenuate anxiety-related behavior in adult Wistar rats (A45). These findings indicate that despite different pharmacological targets, 5-HT_4R agonist and BZD share common mechanisms to induce fast anxiolytic-like effect through prefrontal cortex-DRN brainstem neural circuit recruitment. Whether or not these glutamatergic projections might also regulate DRN activity via an effect on local interneurons should be investigated. Anatomical studies using viral anterograde tracing revealed that 5-HT neurons and GABA interneurons in the DRN receive excitatory inputs from the prelimbic part of the mPFC, with a larger proportion of inputs to DRN 5-HT neurons compared to GABAergic neurons (A35) suggesting that GABA interneurons influence may be secondary (A37).

In order to evaluate whether mPFC-DRN circuit recruitment is not only necessary but also sufficient in the fast anxiolytic-like activity related to 5-HT_4R activation, we investigated the consequences of optogenetic inhibition of the mPFC terminals in the DRN after acute systemic administration of diazepam and RS67333. Inhibition of cortical glutamatergic terminals in the DRN attenuates but does not prevent the anxiolytic effect induced by acute systemic administration with RS67333 or diazepam suggesting that other brain structures might also be involved in the fast anxiolytic-like activity of BZD and 5-HT_4R agonist. These results are not surprising since other circuits are also involved in anxiety-related behaviors such as the ventral hippocampus to prefrontal cortex (46), prefrontal cortex to basolateral amygdala (A47), basolateral amygdala to ventral hippocampus (A48) or DRN to bed nucleus of the stria terminalis (31) inputs (Figure 10). For instance, RS67333 administration into the basolateral amygdala, hippocampus, or nucleus basalis magnocellularis modulated also emotional memory formation and consolidation (A49-A51), suggesting that the anxiolytic-like effect of 5-HT_4R agonist might depend on these different limbic areas.

Interestingly, we found that silencing cortical glutamatergic terminals in the DRN attenuates also the anxiolytic-like activity of diazepam. Many other structures express the $GABA_A$ receptor and the 5-HT_4R, but the mPFC-DRN circuit appears necessary but not sufficient for diazepam and RS67333-mediated fast anxiolytic-like activity. Future studies should examine how brain structures also involved in anxiety phenotype interact with the mPFC-DRN circuit for fast anxiolytic-like activity and also whether 5-HT_4R expression in the mPFC is responsible for fast anxiolytic-like effect of diazepam since diazepam have been

shown to be dose-dependently inhibited by antagonists of the 5-HT_4R (A16).

Taken together, our study reveals the importance the mPFC-DRN circuit in mediating the fast anxiolytic-like effects of both 5-HT_4R agonists and BZD. Stimulating the 5-HT_4R in the mPFC or more generally the mPFC-brainstem DRN neural circuit facilitates anxiolytic effect and could represent an innovative and rapid onset therapeutic approach to treat anxiety. However, the use of 5-HT_4R agonists as a fast-acting anxiolytics may be hampered by the fact that the 5-HT_4R are expressed outside the central nervous system in the heart, gastrointestinal tract, adrenal gland, and urinary bladder (A52). It may be worth identifying other components of the mPFC-DRN circuits that are more specific and amenable to drug development.

Table 3. Overall statistical results

			ANALYSES							
	Test	**Parameters**	**One-Way ANOVA F-value**	**One-Way ANOVA P-value**	**Log-rank (Mantel-Cox) test P-value**	**Fisher's test: fluoxetine vs vehicle**	**Fisher's test: diazepam vs vehicle**	**Fisher's test: RS67333 vs vehicle**	**Fisher's test: RS67333 1µg vs vehicle**	**Fisher's test: GR125487 vs vehicle**	**Fisher's test: RS67333 vs GR125487+RS67333**
Figure 6											
6B	**Elevated Plus Maze**	Time in Open Arms	F(3,36) = 9.627	**<0.0001**		0.5364	**<0.0001**	**0.001**			
		Time in Open Arms (%)	F(3,36) = 9.627	**<0.0001**		0.5364	**<0.0001**	**0.001**			

6C			Total Amb. Dist.	F(3,36) = 3.313							
			Amb. Dist. Open Arms/Total Amb.	F(3,36) = 8.269							
			Fraction of animals not eating								
6D	**Novelty Suppressed Feeding**		Food consumption	F(3,36) = 1.852							
6E			Latency to Feed	F(3,36) = 16.85							
6G	**Elevated Plus Maze**		Time in Open Arms	F(2,16) = 5.789							
			Time in Open Arms (%)	F(2,16) = 5.789							
			Total Amb. Dist.	F(2,16) = 2.944							
6H			Amb. Dist. Open Arms/Total Amb.	F(2,16) = 3.529							
6I	**Novelty Suppressed Feeding**		Fraction of animals not eating								

6C			Total Amb. Dist.	0.3325							
			Amb. Dist. Open Arms/Total Amb.	**0.0046**							
			Fraction of animals not eating								
6D	**Novelty Suppressed Feeding**		Food consumption	0.4221							
6E			Latency to Feed								
6G	**Elevated Plus Maze**		Time in Open Arms								
			Time in Open Arms (%)								
			Total Amb. Dist.								
6H			Amb. Dist. Open Arms/Total Amb.								
6I	**Novelty Suppressed Feeding**		Fraction of animals not eating								

6C			Total Amb. Dist.	**0.0307**							
			Amb. Dist. Open Arms/Total Amb.	**0.0003**							
			Fraction of animals not eating	**<0.0001**							
6D	**Novelty Suppressed Feeding**		Food consumption	0.1552							
6E			Latency to Feed	**<0.0001**							
6G	**Elevated Plus Maze**		Time in Open Arms	**0.0128**							
			Time in Open Arms (%)	**0.0128**							
			Total Amb. Dist.	0.0815							
6H			Amb. Dist. Open Arms/Total Amb.	**0.0537**							
6I	**Novelty Suppressed Feeding**		Fraction of animals not eating	**0.0142**							

6C			Total Amb. Dist.								
			Amb. Dist. Open Arms/Total Amb.								
			Fraction of animals not eating								
6D	**Novelty Suppressed Feeding**		Food consumption								
6E			Latency to Feed								
6G	**Elevated Plus Maze**		Time in Open Arms	**0.0145**							
			Time in Open Arms (%)	**0.0145**							
			Total Amb. Dist.								
6H			Amb. Dist. Open Arms/Total Amb.								
6I	**Novelty Suppressed Feeding**		Fraction of animals not eating								

6C			Total Amb. Dist.								
			Amb. Dist. Open Arms/Total Amb.								
			Fraction of animals not eating								
6D	**Novelty Suppressed Feeding**		Food consumption								
6E			Latency to Feed								
6G	**Elevated Plus Maze**		Time in Open Arms								
			Time in Open Arms (%)	**0.0054**							
			Total Amb. Dist.								
6H			Amb. Dist. Open Arms/Total Amb.								
6I	**Novelty Suppressed Feeding**		Fraction of animals not eating								

6J		Food consumption	F(2,13) = 0.006	0.9937						
		Latency to Feed	F(2,13) = 5.852	**0.0154**			**0.0206**	0.996	**0.0067**	0.9991
Figure 11										
11B	**Elevated Plus Maze**	Time in Open Arms	F(4,22) = 5.797	**0.0024**		0.5965	0.5965	**0.0435**	**0.0093**	0.4337	**0.0009**
		Time in Open Arms (%)	F(4,22) = 5.797	**0.0024**		0.5965	0.5965	**0.0435**	**0.0093**	0.4337	**0.0009**
		Total Amb. Dist.	F(4,22) = 0.7574	0.564							
11C		Amb. Dist. Open Arms/Total Amb.	F(4,22) = 5.550	**0.0030**		0.6076		**0.0317**	**0.0233**	0.3156	**0.0015**
		Fraction of animals not eating			**<0.0001**						
11D	**Novelty Suppressed Feeding**	Food consumption	F(4,18) = 0.3668	0.8291							
11E		Latency to Feed	F(4,18) = 15.85	**<0.0001**		0.2392	**< 0.0001**	**0.0068**		0.9443	**0.0088**
Figure 12											

12C	**Open Field**	Amb. Dist. Cter/Total Amb.	F(3,35) = 5.567	**0.0031**		0.8302	**0.0015**	0.1531
		Total Amb. Dist.	F(3,35) = 4.114	**0.0134**		0.0649	**0.0014**	0.1467
12B		Time in Center (%)	F(3,35) = 10.13	**<0.0001**		0.7325	**< 0.0001**	**0.0585**

Table 4. Overall statistical results

			ANALYSES							POST HOC						
	Test	**Parameters**	**Student's test: after RS67333 vs before RS67333**	**Two-Way ANOVA F-value (Pretreatment effect)**	**Two-Way ANOVA P-value (Pretreatment effect)**	**Two-Way ANOVA F-value (Treatment effect)**	**Two-Way ANOVA P-value (Treatment effect)**	**Two-Way ANOVA F-value (Interaction)**	**Two-Way ANOVA P-value (Interaction)**	**Fisher's test: vehicle/diazepam vs vehicle/vehicle**	**Fisher's test: vehicle/RS67333 vs vehicle/vehicle**	**Fisher's test: pCPA/diazepam vs p-CPA/vehicle**	**Fisher's test: p-CPA/RS67333 vs p-CPA/vehicle**	**Fisher's test: p-CPA/vehicle vs vehicle/vehicle**	**Fisher's test: p-CPA/diazepam vs vehicle/diazepam**	**Fisher's test: p-CPA/RS67333 vs vehicle/RS67333**
Figure 7																

7G	7F			7E	7B
	Elevated Plus Maze			ELISA	Electrophysiology
Amb. Dist. Open Arms/To	Total Amb. Dist.	Time in Open Arms (%)	Time in Open Arms	Serotonin levels	Firing rate of DR 5-HT neurons
					0.0016
F(1,51) = 10.16	F(1,52) = 4.432	F(1,51) = 10.53	F(1,51) = 10.53	F(1,34) = 45.25	
0.0024	**0.0401**	**0.0021**	**0.0021**	**<0.0001**	
F(2,51) = 7.107	F(2,52) = 0.5300	F(2,51) = 4.568	F(2,51) = 4.568	F(2,34) = 0.1347	
0.0019	0.5918	**0.0150**	**0.0150**	0.8745	
F(2,51) = 3.920	F(2,52) = 0.09717	F(2,51) = 4.300	F(2,51) = 4.300	F(2,34) = 2.593	
0.0261	0.9076	**0.0188**	**0.0188**	0.0895	
0.0001		**0.0003**	**0.0003**		
0.0014		**0.0041**	**0.0041**		
0.2827		0.7780	0.7780		
0.8819		0.6954	0.6954		
0.6927	0.1625	0.6200	0.6200	**<0.0001**	
0.0118	0.1672	**0.0036**	**0.0036**	**0.0285**	
0.0023	0.4054	**0.0047**	**0.0047**	**0.0002**	

Table 5. Overall statistical results

								Test
								Parameters
Supplemental Figure 13						**Figure 8**		
13C	**13B**				**8D**	**8C**		**Elevated Plus Maze**
Open Field								
Total Amb. Dist.	Time in Center (%) or (sec, inset)	Amb. Dist. Open Arms/Total Amb.	Total Amb. Dist.	Time in Open Arms (%)	Amb. Dist. Open Arms/Total Amb.	Total Amb. Dist.	Time in Open Arms	
F(1,38) = 2.271	F(1,38) = 8.216	F(1,58) = 2.491	F(1,58) = 0.03346	F(1,58) = 2.779	0.1199	0.8555	0.1009	**Two-Way ANOVA F-value (Virus effect)**
0.1400	**0.0067**	0.1199	0.8555	0.1009	0.2023	**0.0098**	**0.0243**	**Two-Way ANOVA P-value (Virus effect)**
F(1,38) = 0.8561	F(1,38) = 4.505	F(1,58) = 1.663	F(1,58) = 7.137	F(1,58) = 5.348	F(1,58) = 4.512	F(1,58) = 0.04611	F(1,58) = 5.282	**Two-Way ANOVA F-value (Laser effect)**
0.3607	**0.0404**	0.2023	**0.0098**	**0.0243**	**0.0379**	0.8307	**0.0252**	**Two-Way ANOVA P-value (Laser effect)**
F(1,38) = 0.07317	F(1,38) = 5.565	F(1,58) = 4.512	F(1,58) = 0.04611	F(1,58) = 5.282	0.5961	0.1221	0.9928	**Two-Way ANOVA F-value (Interaction)**
0.7882	**0.0236**	**0.0379**	0.8307	**0.0252**	**0.0081**	**0.0239**	**0.0005**	**Two-Way ANOVA P-value (Interaction)**
	0.8813	0.5961	0.1221	0.9928	0.7009		0.6571	**Fisher's test: eYFP - laser ON vs laser OFF**
	0.0008	**0.0081**	**0.0239**	**0.0005**			**0.0069**	**Fisher's test: ChR2 - laser ON vs laser OFF**
	0.7218	0.7009		0.6571				**Fisher's test: laser OFF - eYFP vs ChR2**
	0.0007	**0.0113**		**0.0069**				**Fisher's test: laser ON - GFP vs ChR2**

		Amb. Dist. Cter/Total Amb.	F(1,38) = 4.677
			0.0369
			F(1,38) = 3.074
			0.0876
			F(1,38) = 4.154
			0.0485
			0.8573
			0.0039
			0.9304
			0.0051

Table 6. Overall statistical results

			ANALYSES	POST HOC
Figure 9		**Test**		
9C	**Elevated Plus Maze**	**Parameters**	**Two-Way ANOVA F-value (Virus or treatment effect)**	**Laser OFF: diazepam vs sham**
		Time in Open Arms	F(2,46) = 4.923	**< 0.0001**
			0.0116	**0.0016**
			0.0009	0.8843
			0.0098	0.8463
				0.6193
				0.0004
				0.0064
9D		Total Amb. Dist.	**Two-Way ANOVA P-value (Virus or treatment effect)**	**Laser OFF: RS67333 vs sham**
			F(2,46) = 4.139	0.479
			0.0222	0.2848
			0.2094	**0.0149**
			0.3595	0.5078
9D		Amb. Dist. Open Arms/Total Amb.	**Two-Way ANOVA F-value (Laser effect)**	**Laser ON: diazepam vs sham**
			F(1,46) = 15.34	**< 0.0001**
			0.0003	**0.0029**
			F(2,46) = 6.896	0.8924
			0.0024	0.9732
				0.5798
				< 0.0001
				0.0087
			Two-Way ANOVA P-value (Laser effect)	**Laser ON: RS67333 vs sham**
			F(1,46) = 1.621	**0.0039**
			0.0222	0.5078
			0.2094	0.9732
			0.3595	0.5798
				0.0087
				0.0149
				0.0024
			Two-Way ANOVA F-value (Interaction)	**sham: laser OFF vs laser ON**
			F(2,46) = 5.123	0.9304
			0.0098	**0.0051**
				0.6193
				0.8463
				0.8843
				0.0064
			Two-Way ANOVA P-value (Interaction)	**diazepam: laser OFF vs laser ON**
			0.0485	**0.0004**
			0.8573	**< 0.0001**
			0.0039	0.5798
			0.9304	0.9732
			0.0051	0.8924
				0.0087
				0.0029
				0.2848
				0.479
				0.0003
				F(2,46) = 6.896
				0.0039
				F(1,46) = 15.34
				0.0024
				F(2,46) = 1.046
				0.2094
				F(1,46) = 1.621
				0.0222
				F(2,46) = 4.139
				0.0039
				F(2,46) = 6.274

14C	14B	Open Field		9F	9F	9E
Amb. Dist. Cter/Total Amb.	Total Amb. Dist.	Time in Center (%) or (sec, inset)	Amb. Dist. Open Arms/Total Amb.	Total Amb. Dist.		Time in Open Arms
F(2,44) = 3.648	F(2,44) = 10.73	F(2,44) = 9.628	F(2,38) = 34.28	F(2,38) = 6.499		F(2,38) = 33.42
0.0342	**0.0002**	**0.0003**	**< 0.0001**	**0.0037**		**< 0.0001**
F(1,44) = 0.7221	F(1,44) = 13.33	F(1,44) = 15.64	F(1,38) = 7.893	F(1,38) = 1.762		F(1,38) = 4.601
0.400	0.0007	**0.0003**	**0.0078**	0.1924		**0.0384**
F(2,44) = 0.1913	F(2,44) = 5.703	F(2,44) = 3.332	F(2,38) = 1.688	F(2,38) = 0.04086		F(2,38) = 1.052
0.8217	**0.0063**	**0.0449**	0.1984	0.9600		0.3591
0.8507	0.6466	**0.0381**	**< 0.0001**	**0.0276**		**< 0.0001**
0.0188	**< 0.0001**	**0.0029**	**< 0.0001**	0.7185		**< 0.0001**
0.7949	0.5774	0.6922	**0.0007**	**0.0196**		**0.0003**
0.1713	0.3112	0.2134	**0.0001**	0.4541		**< 0.0001**
0.7695	0.3750	0.3251	0.9288			0.975
0.8519	0.4965	**0.0412**	**0.0298**			0.0901
0.2591	**< 0.0001**	**< 0.0001**	**0.0095**			0.0412

Figure 14

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The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the

accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Patents, patent applications, and publications are cited throughout this application, the disclosures of which, particularly, including all disclosed chemical structures, are incorporated herein by reference. Citation of the above publications or documents is not intended as an admission that any of the foregoing is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents. All references cited herein are incorporated by reference to the same extent as if each individual publication, patent application, or patent, was specifically and individually indicated to be incorporated by reference.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for preventing or delaying a stress-induced affective disorder or stress-induced psychopathology in a subject, comprising administering an effective amount of a pharmaceutic composition comprising an agonist of serotonin 4 receptor (5-HT_4R), or a pharmaceutically acceptable salt, analog, derivative, or metabolite thereof, to a subject prior to a stressor.

2. A method for inducing and/or enhancing stress resilience in a subject, comprising administering an effective amount of a pharmaceutic composition comprising an agonist of serotonin 4 receptor (5-HT_4R), or a pharmaceutically acceptable salt, analog, derivative, or metabolite thereof, to a subject prior to a stressor.

3. The method of claims 1 or 2, wherein the agonist of 5-HT_4R comprises 1-(4-amino-5-chloro-2-methoxyphenyl)-3-[1(n-butyl)-4-piperidinyl]-1-propanone HCl (RS-67,333), 4-amino-5-chloro-2,3-dihydro-*N*-[1-3-methoxypropyl)-4-piperidinyl]-7-benzofuran carboxamide monohydrochloride (prucalopride), 4-[4-[4-Tetrahydrofuran-3-yloxy)-benzo[d]isoxazol-3-yloxymethyl]-piperidin-1-ylmethyl]-tetrahydropyran-4-ol (PF-04995274), or combinations thereof.

4. The method of claims 1 or 2, wherein the pharmaceutic composition is administered to the subject about 48 hours to about 3 weeks prior to a stressor.

5. The method of claims 1 or 2, wherein the pharmaceutic composition is administered to the subject about 72 hours to about 2 weeks prior to a stressor.

6. The method of claims 1 or 2, wherein the pharmaceutic composition is administered to the subject about 1 week prior to a stressor.

7. The method of claims 1 or 2, wherein the pharmaceutic composition is administered to the subject once prior to a stressor.

8. The method of claims 1 or 2, wherein the pharmaceutic composition is administered orally, intravenously, intranasally, or via injection to the subject.

9. The method of claim 1, wherein the stress-induced affective disorder comprises major depressive disorder and/or posttraumatic stress disorder (PTSD).

10. The method of claim 1, wherein the stress-induced affective disorder is selected from the group consisting of: depressive-like behavior and associated affective disorders, anhedonic behavior and associated affective disorders, anxiety and associated affective disorders, cognitive impairments and deficits and associated disorders, stress-induced fear, and combinations thereof.

11. The method of claim 1, wherein the stress-induced affective disorder comprises stress-induced psychopathology.

12. The method of claim 11, wherein the stress-induced psychopathology comprises depressive and/or anxious behavior.

13. The method of any of the preceding claims, preventing or delaying stress-induced cognitive impairment and/or decline.

14. The method of any of the preceding claims, further comprising administering an effective amount of an anti-depressant, an anxiolytic, or combinations thereof.

15. The method of any of the preceding claims, further comprising administering an effective amount of a selective serotonin reuptake inhibitor (SSRI), or a pharmaceutically acceptable salt or derivative thereof.

16. The method of any of the preceding claims, further comprising administering an effective amount of fluoxetine, paroxetine, sertraline, lithium, riluzole, prazosin, lamotrigine, ifenprodil, or combinations thereof.

17. The method of any of the preceding claim, wherein the subject is a mammal.

18. The method of any of the preceding claim, wherein the subject is a human.

19. The method of any of the preceding claim, wherein the subject is female.

20. The method of any of the preceding claim, wherein the subject is male.

21. The method of any of the preceding claim, wherein the pharmaceutical composition is administered in a booster series.

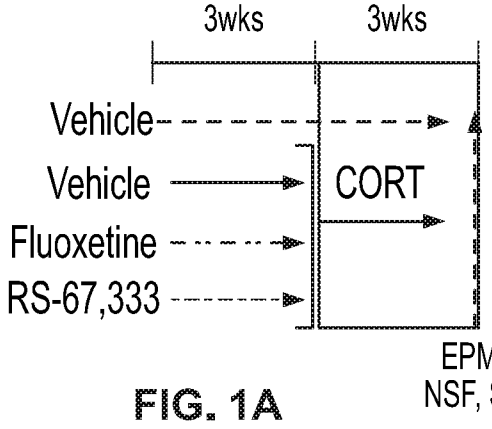


FIG. 1A

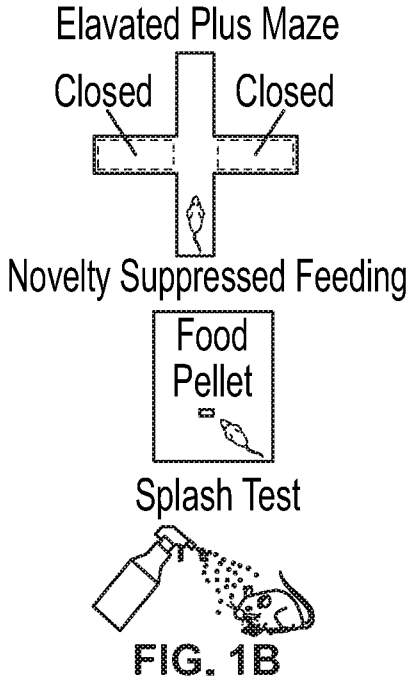


FIG. 1B

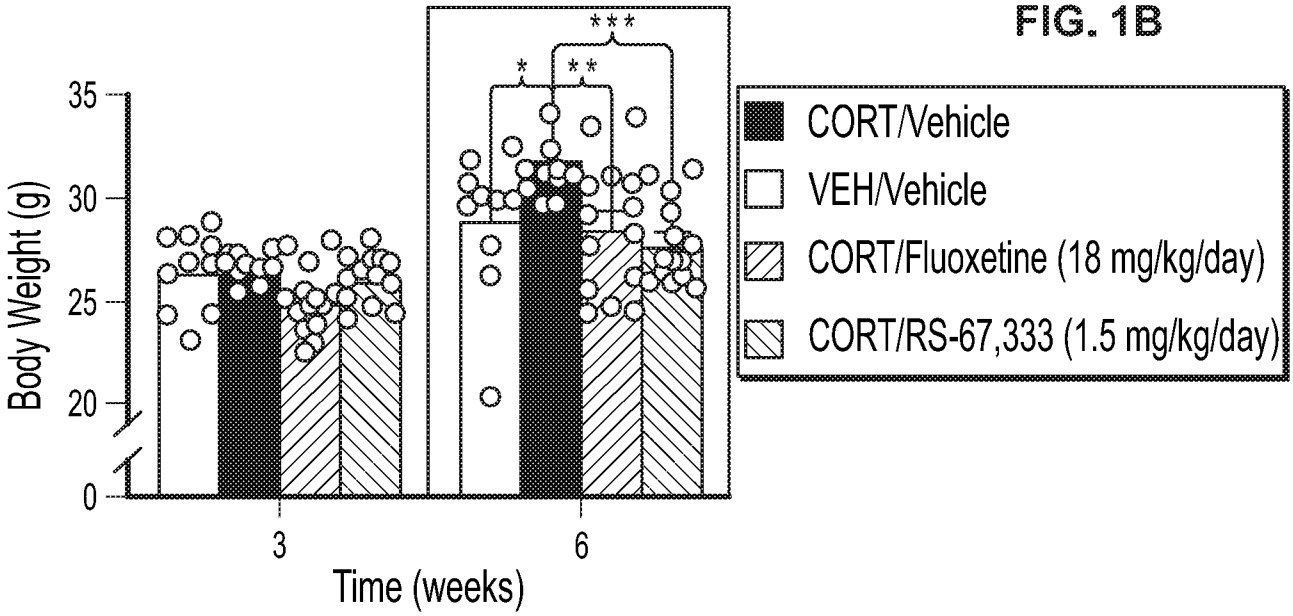


FIG. 1C

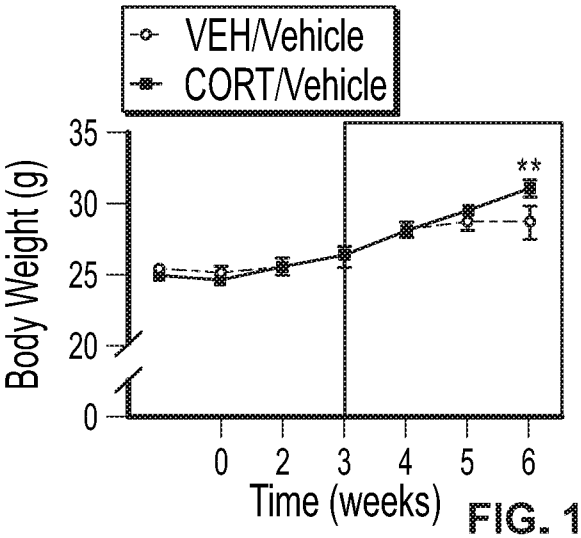


FIG. 1D

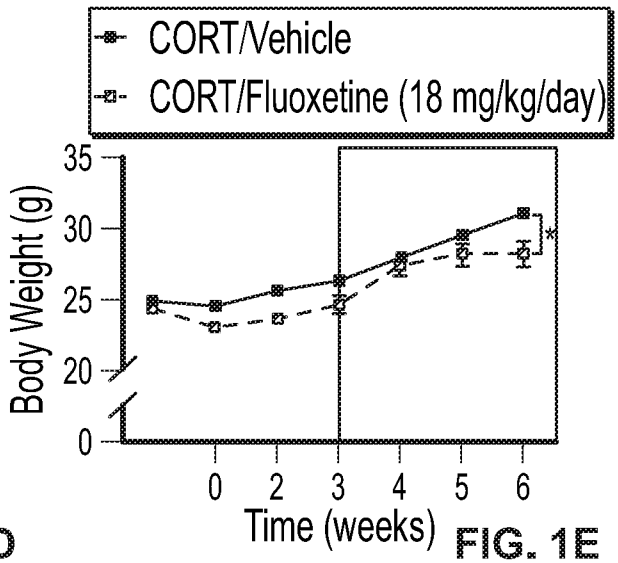


FIG. 1E

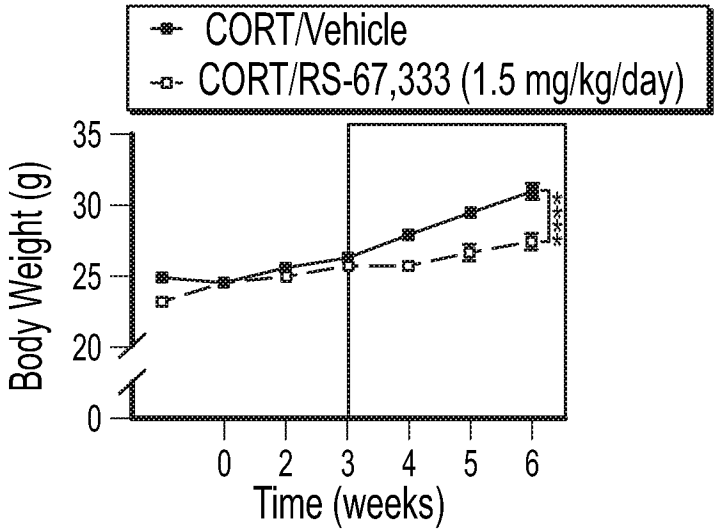


FIG. 1F

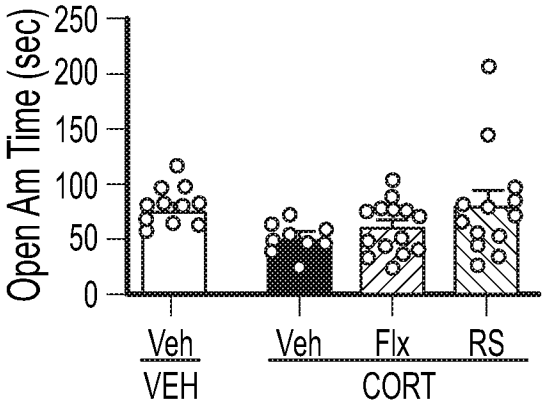


FIG. 1G

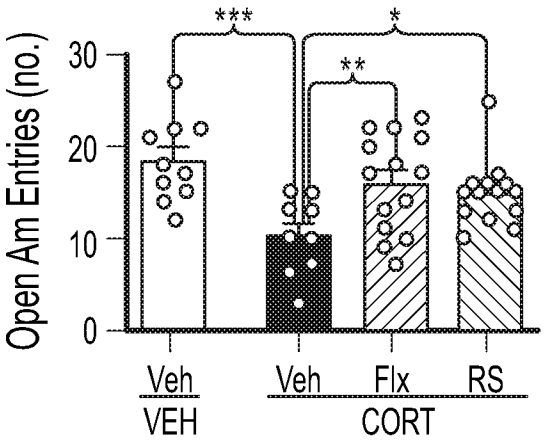


FIG. 1H

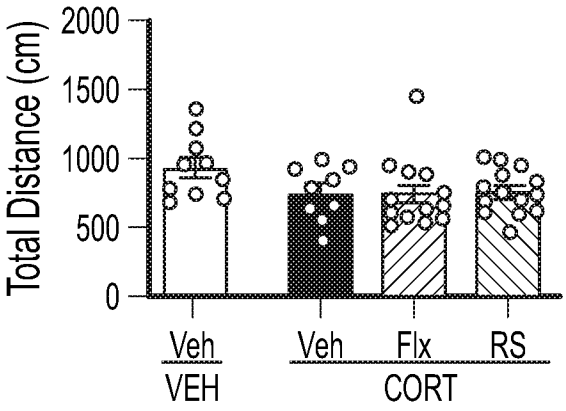


FIG. 1I

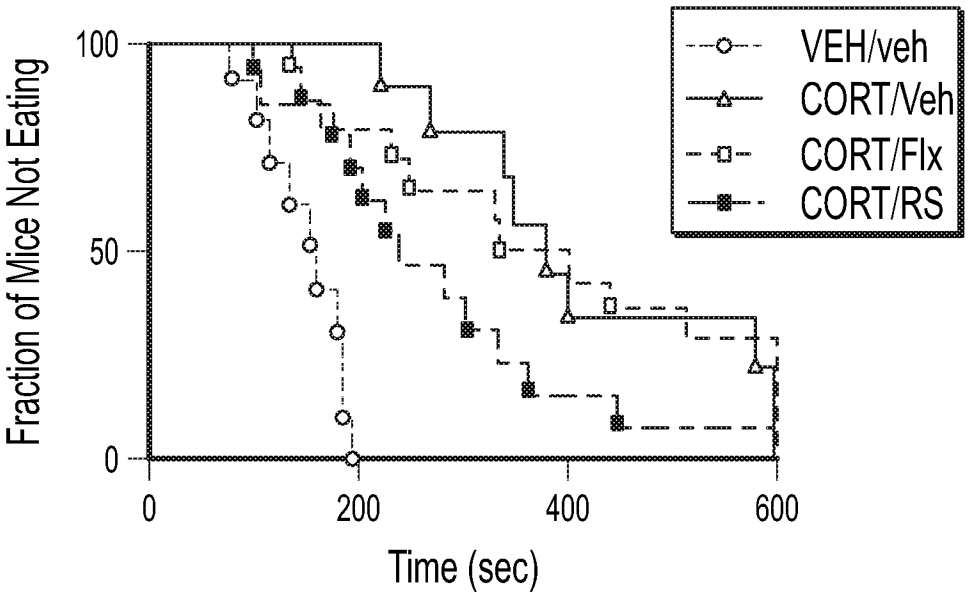


FIG. 1J

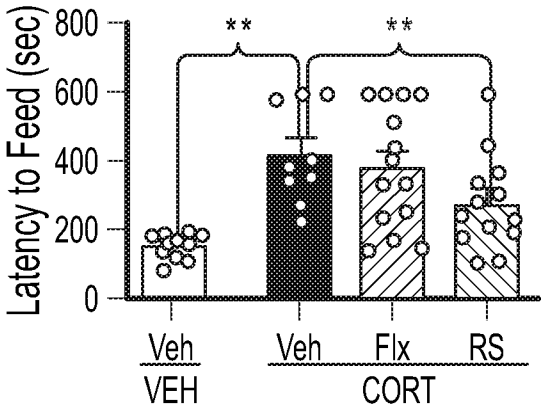


FIG. 1K

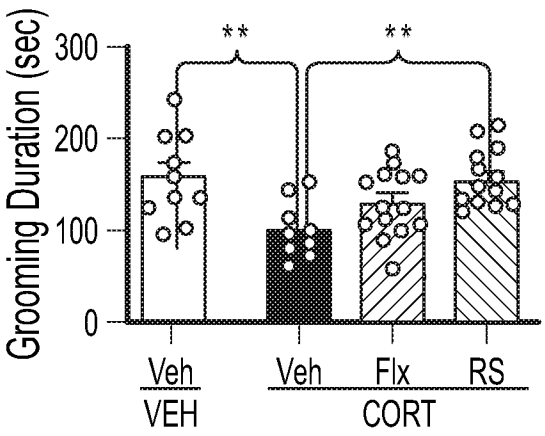


FIG. 1L

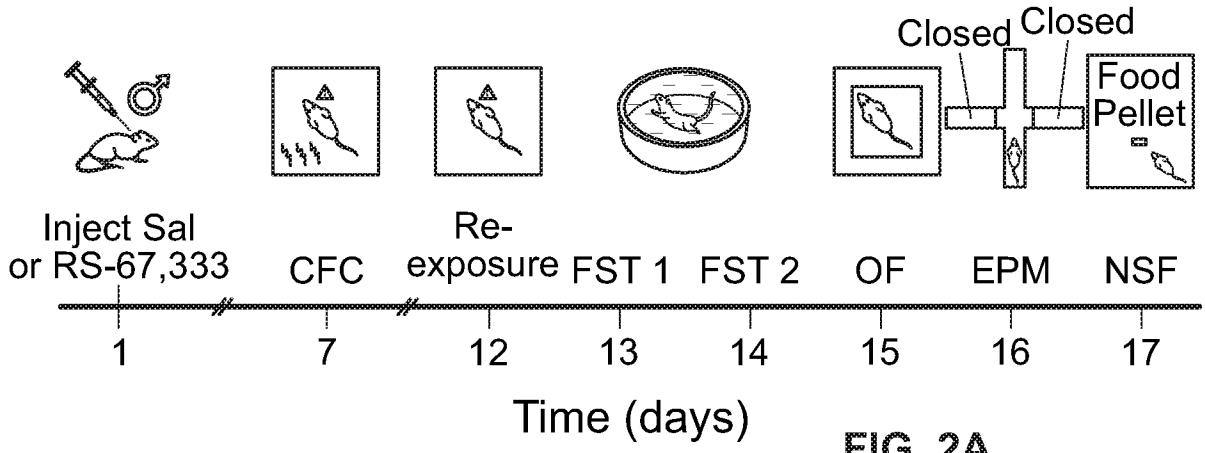


FIG. 2A

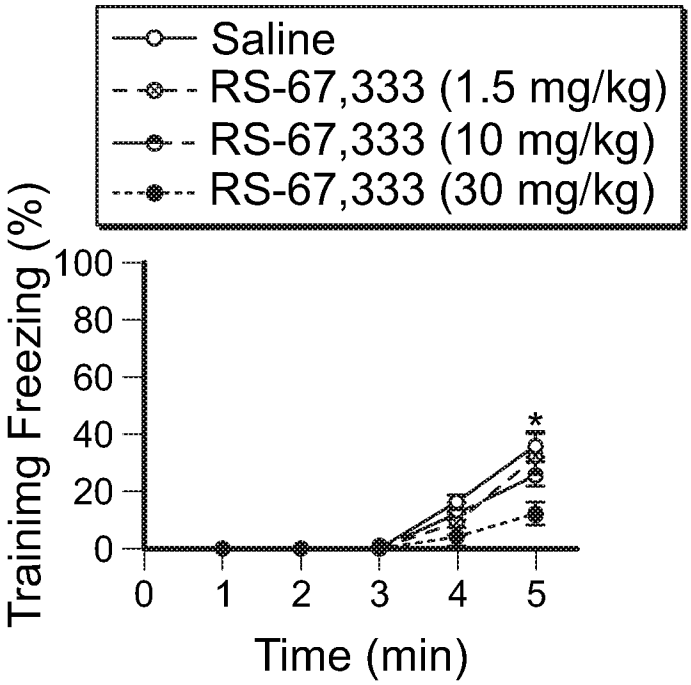


FIG. 2B

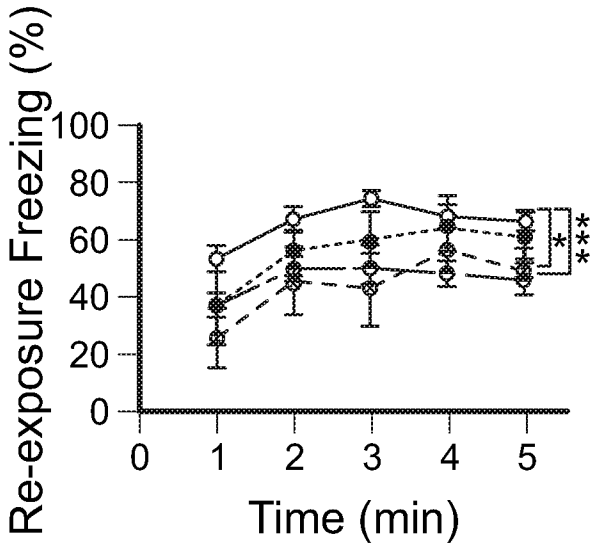


FIG. 2C

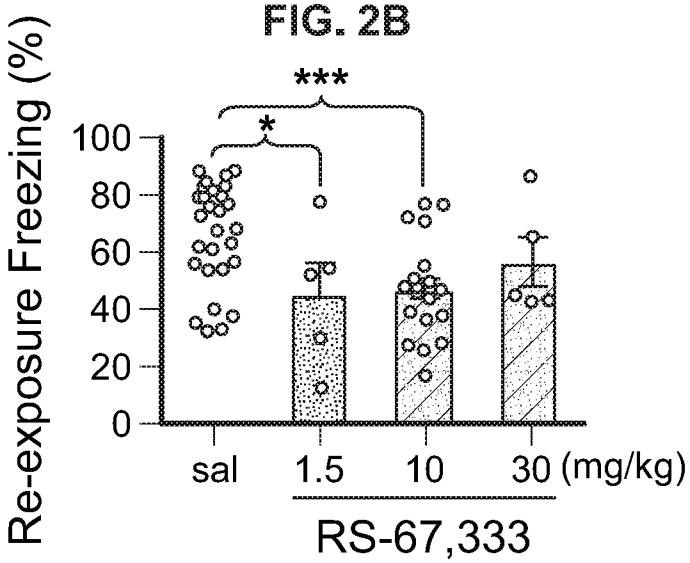


FIG. 2D

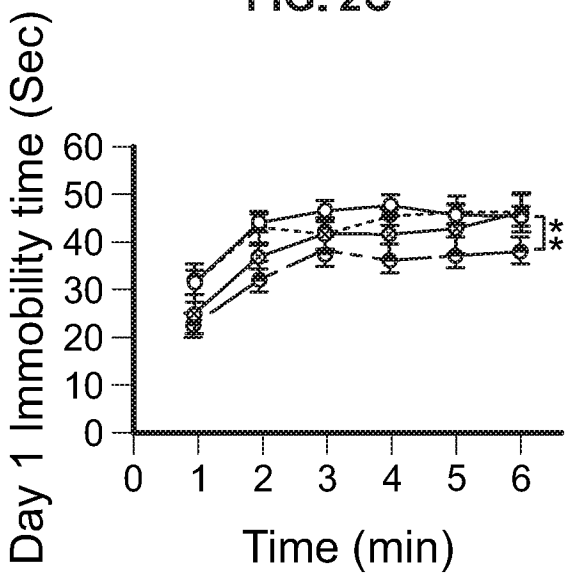


FIG. 2E

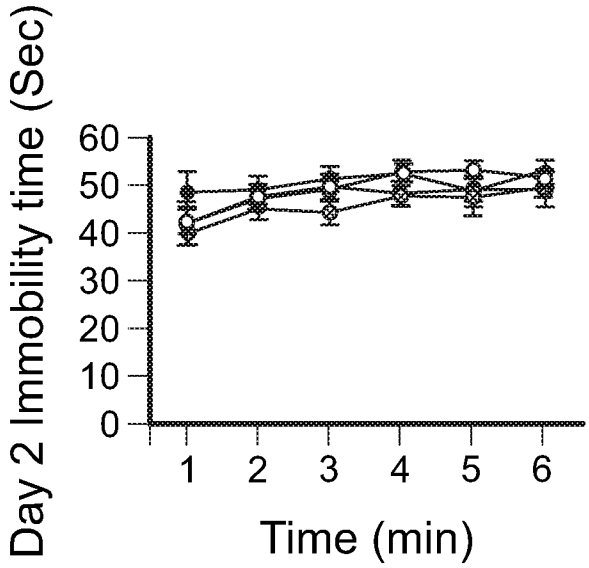


FIG. 2F

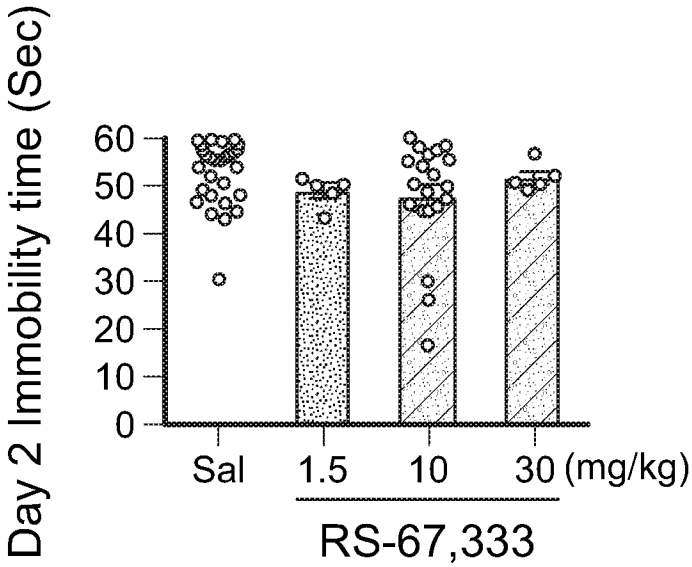


FIG. 2G

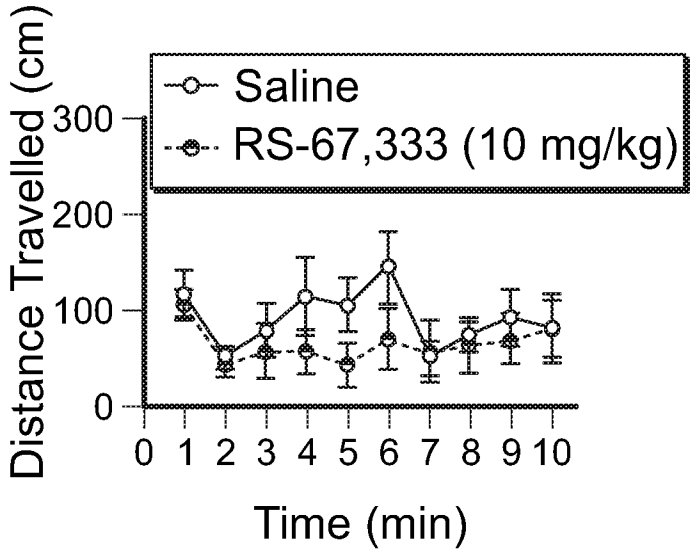


FIG. 2H

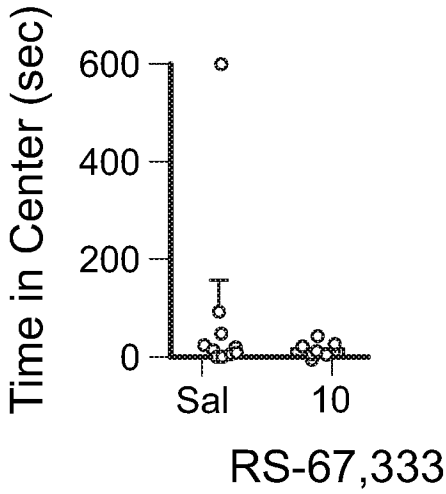


FIG. 2I

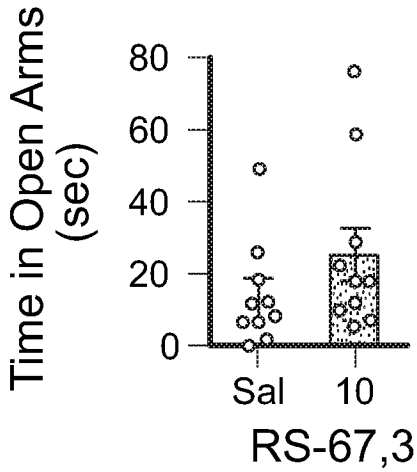


FIG. 2J

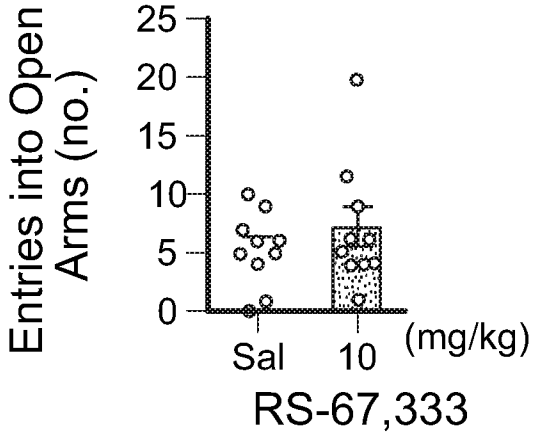


FIG. 2K

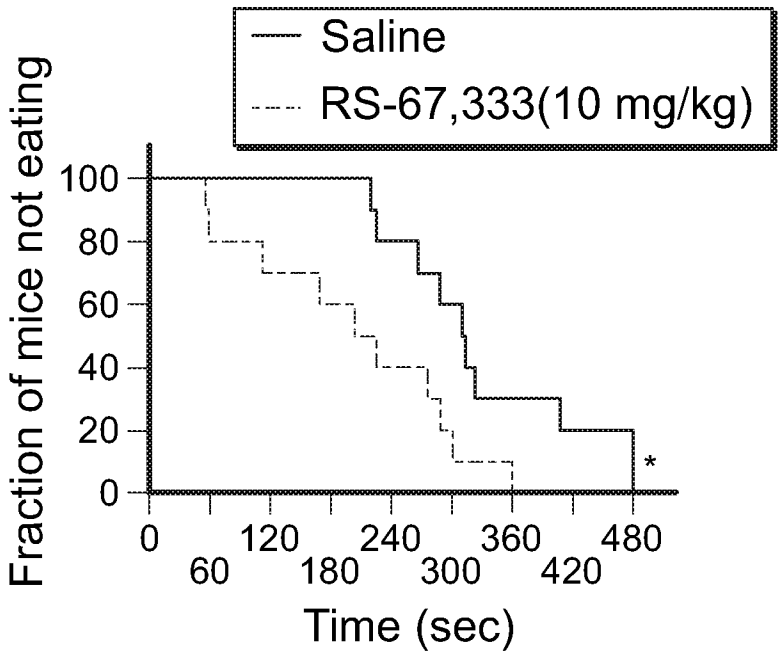


FIG. 2L

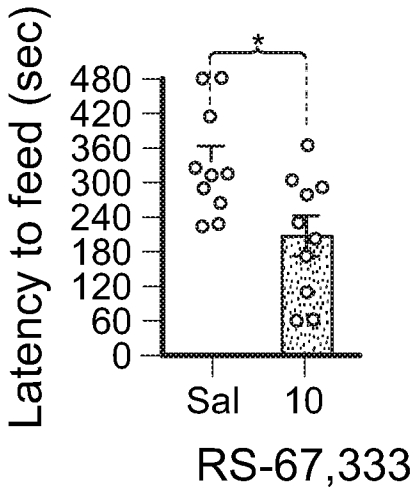


FIG. 2M

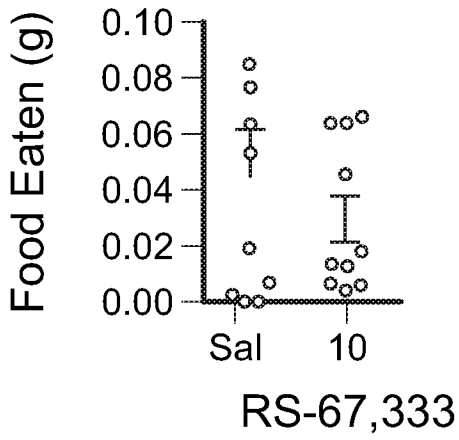


FIG. 2N

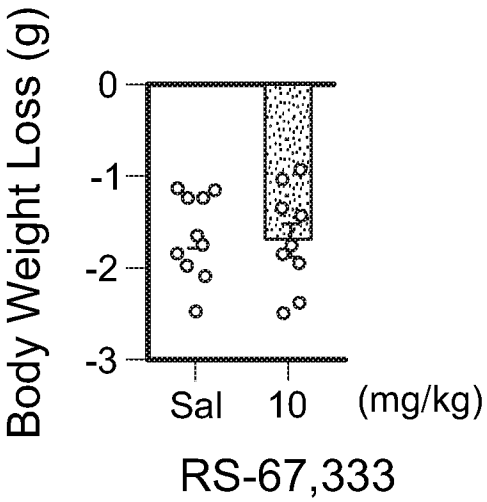


FIG. 2O

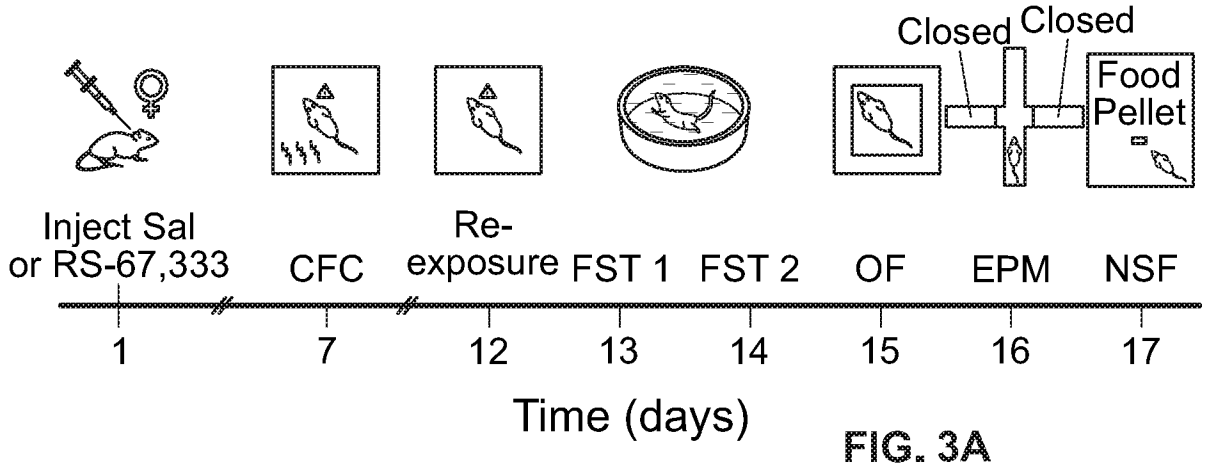


FIG. 3A

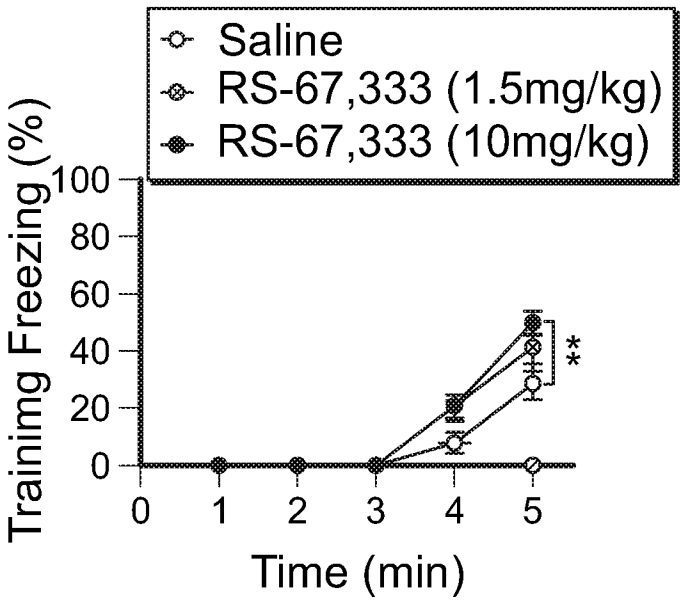


FIG. 3B

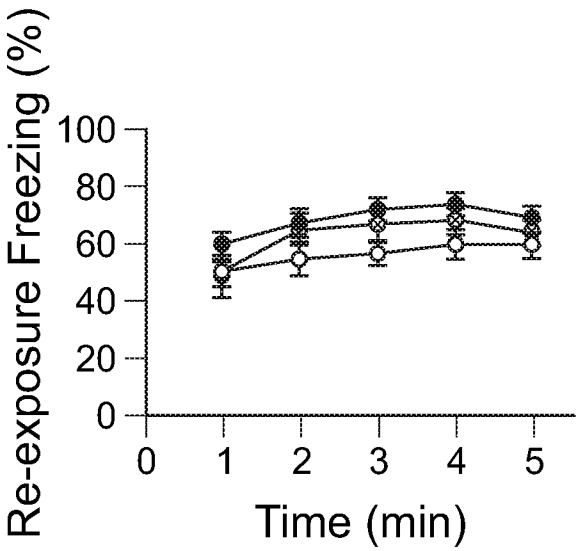


FIG. 3C

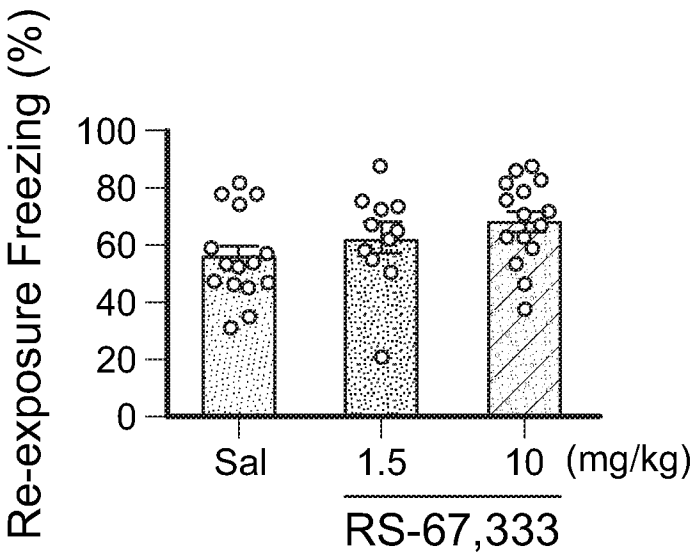


FIG. 3D

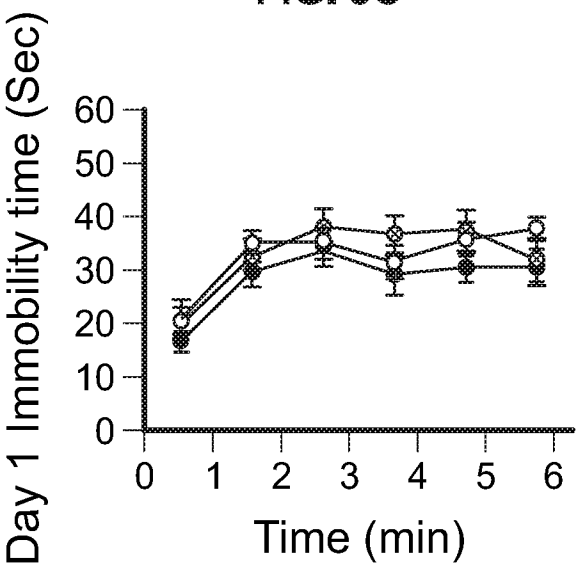


FIG. 3E

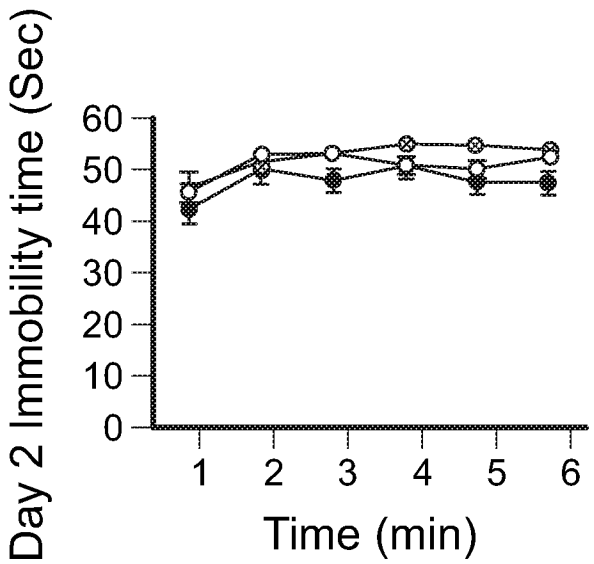


FIG. 3F

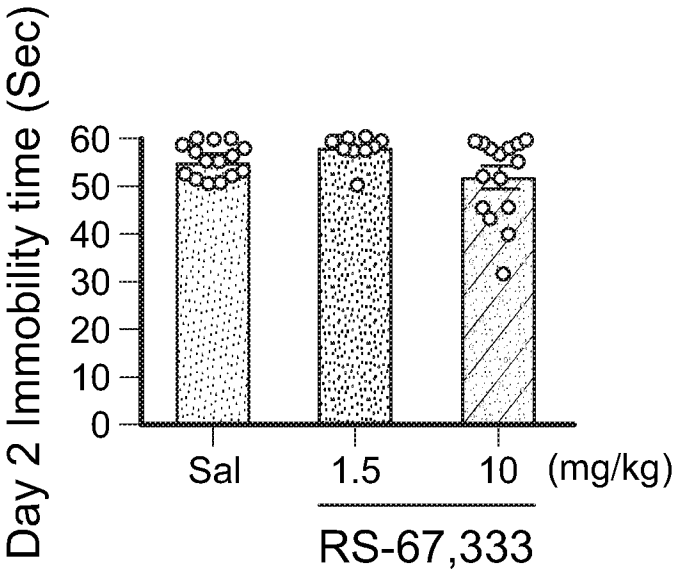


FIG. 3G

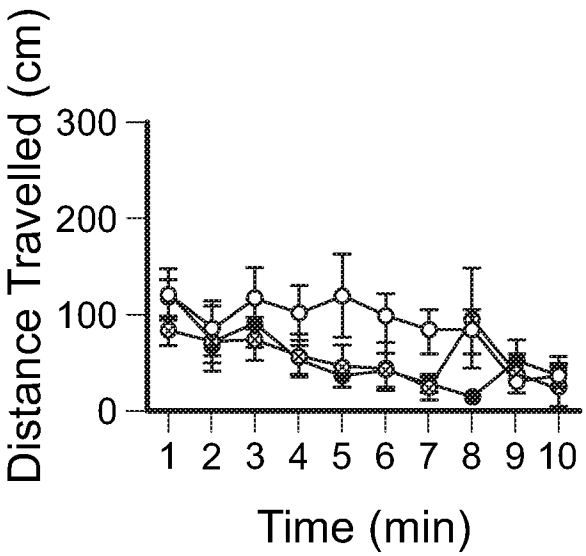


FIG. 3H

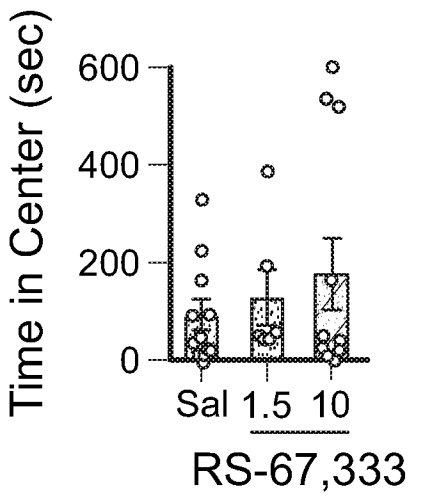


FIG. 3I

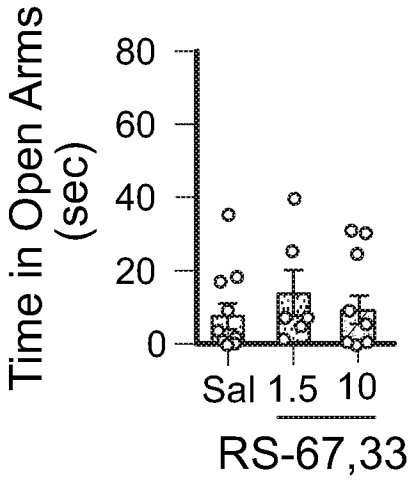


FIG. 3J

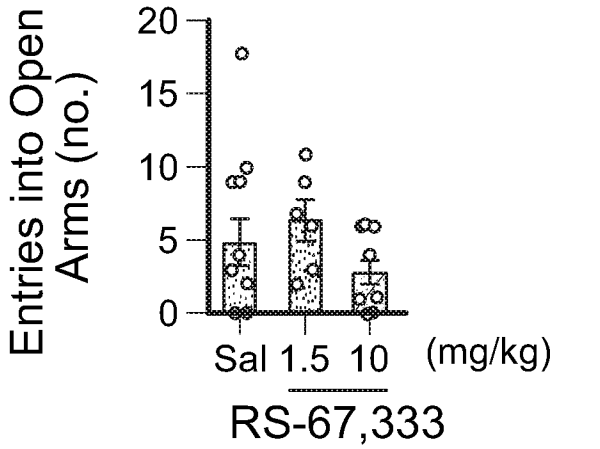


FIG. 3K

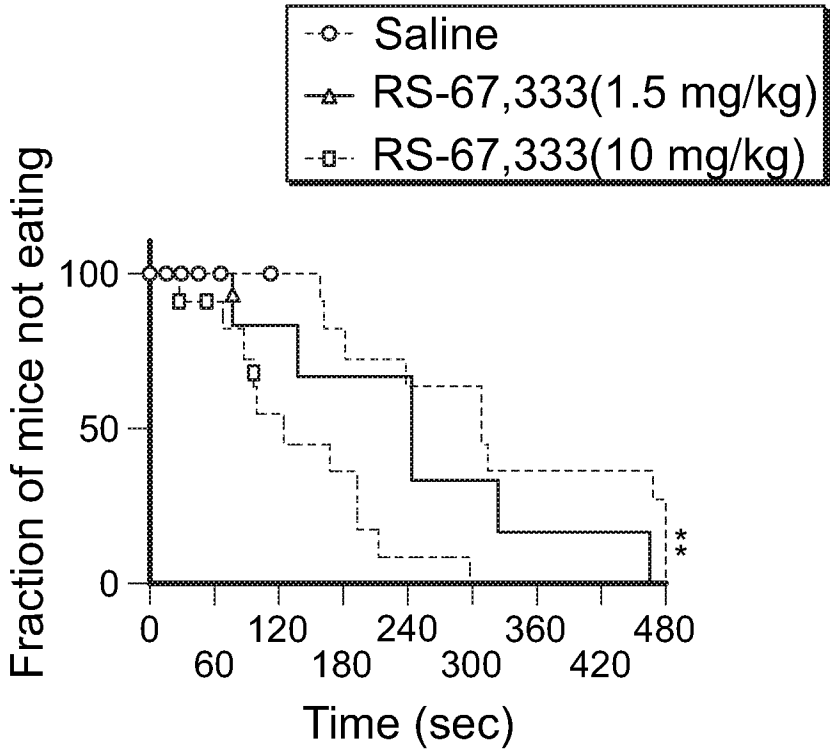


FIG. 3L

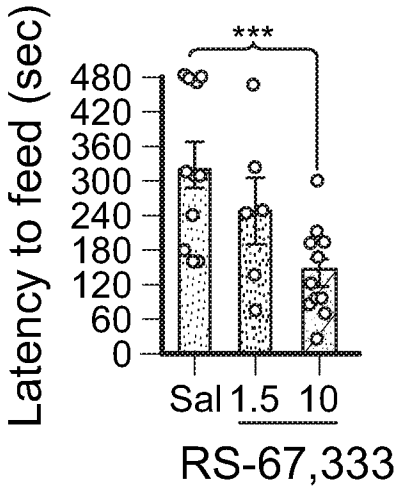


FIG. 3M

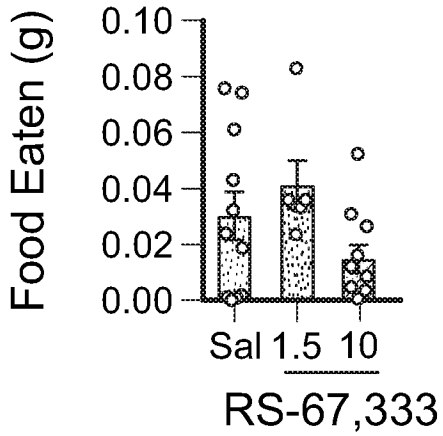


FIG. 3N

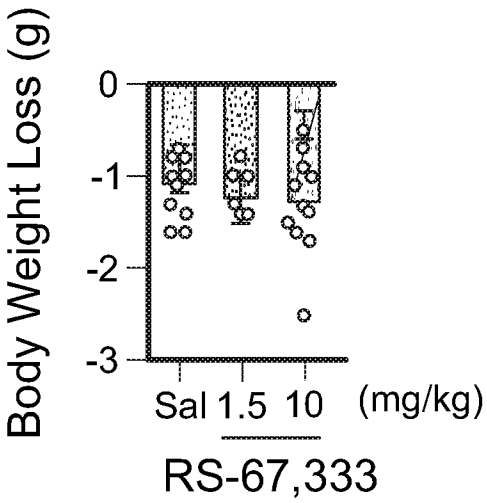


FIG. 3O

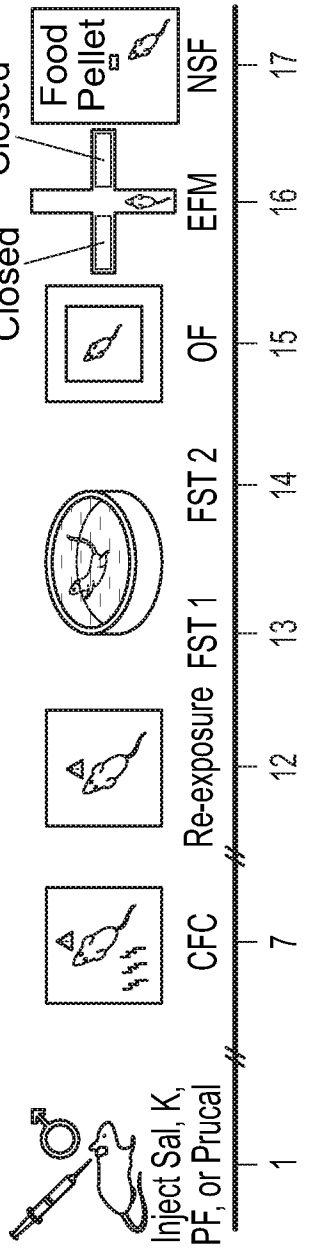

Inject Sal, K, PF, or Prucal
CFC
Re-exposure
FST 1
FST 2
OF
EFM
NSF
Closed
Closed
Food Pellet
1
7
12
13
14
15
16
17
Time (days)

FIG. 4A

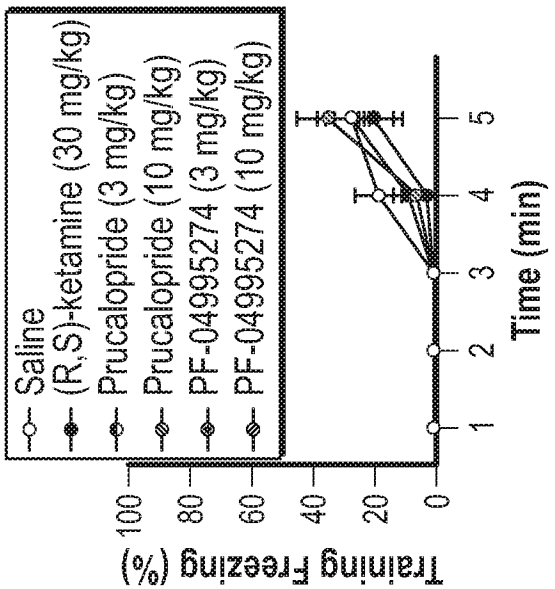


FIG. 4B

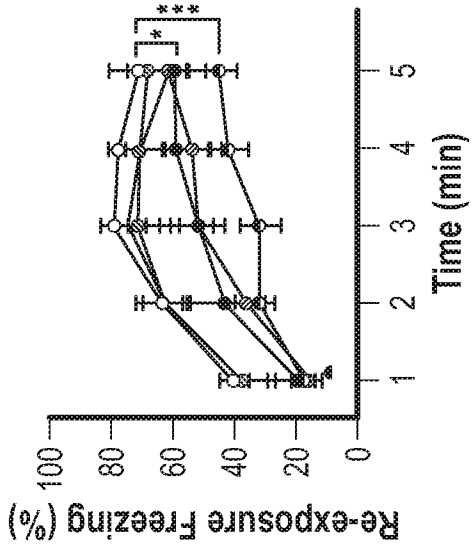


FIG. 4C

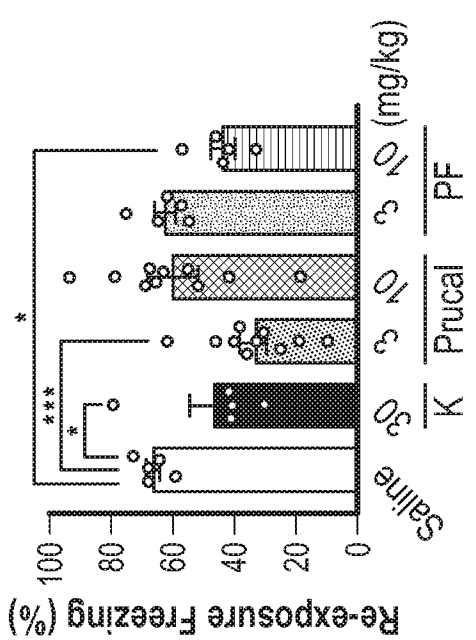


FIG. 4D

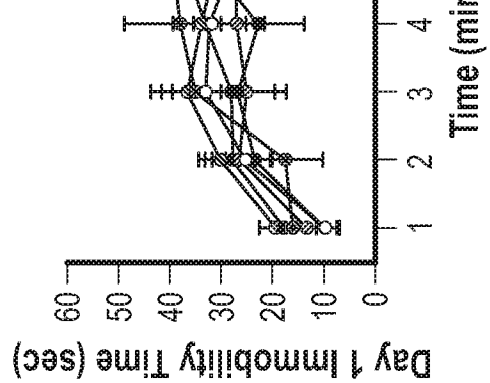


FIG. 4E

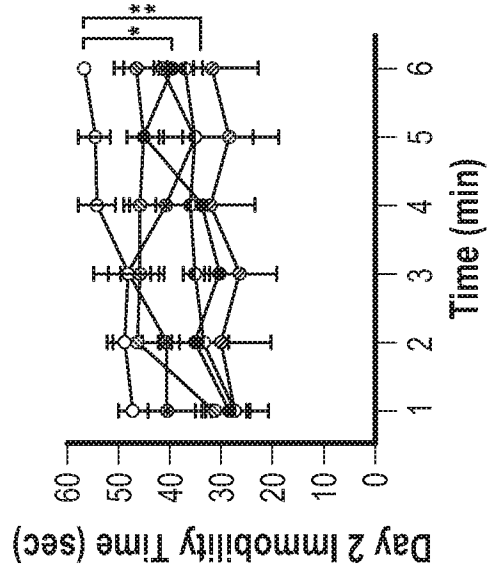


FIG. 4F

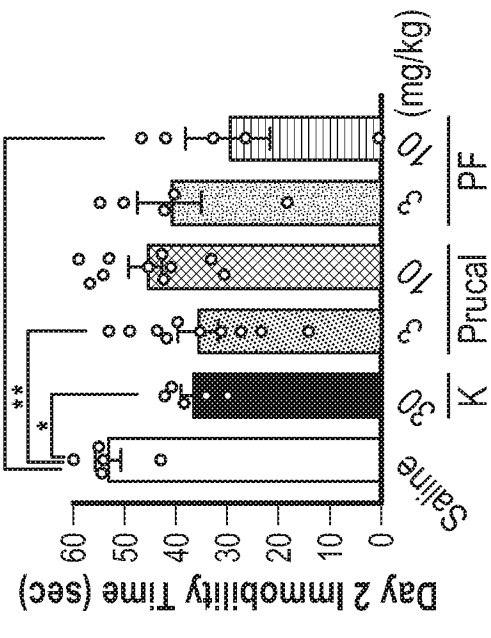


FIG. 4G

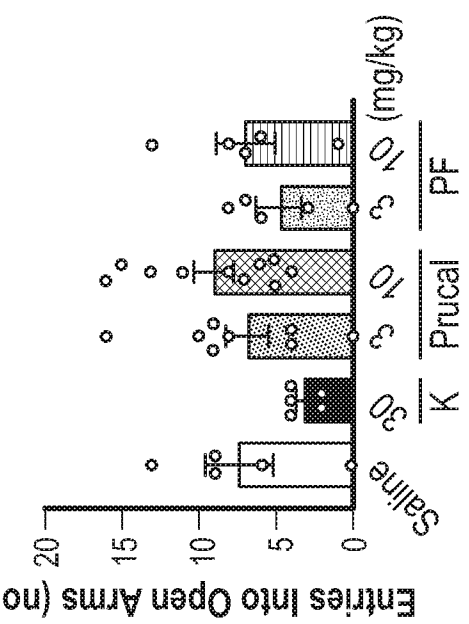


FIG. 4J

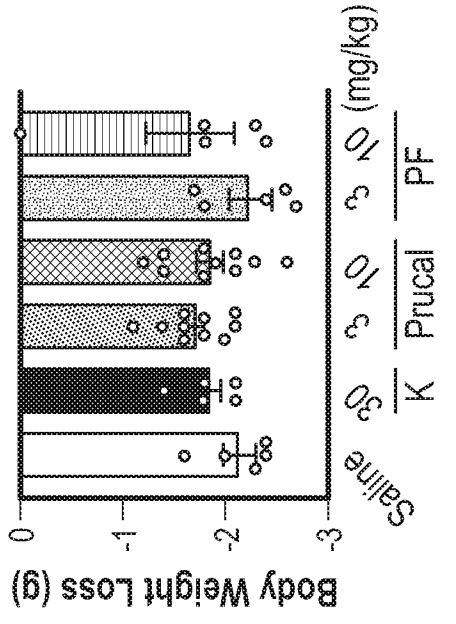


FIG. 4M

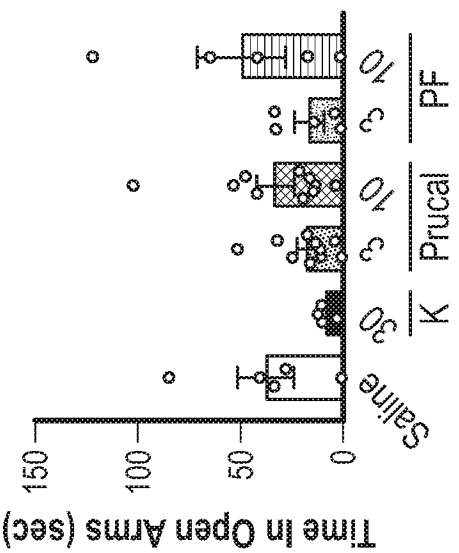


FIG. 4I

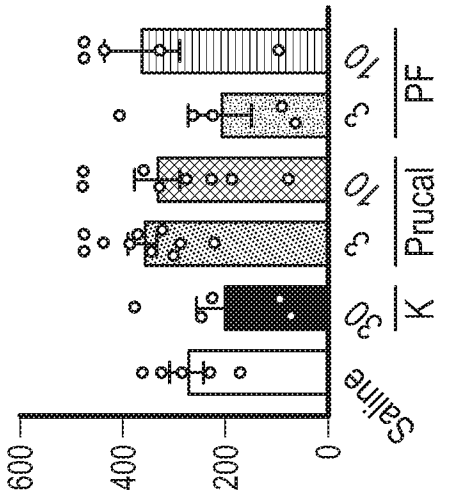


FIG. 4L

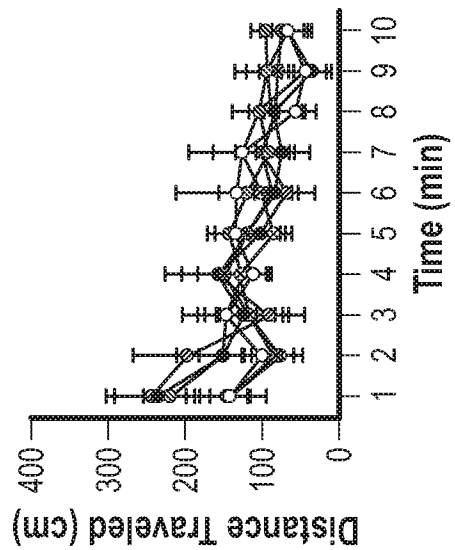


FIG. 4H

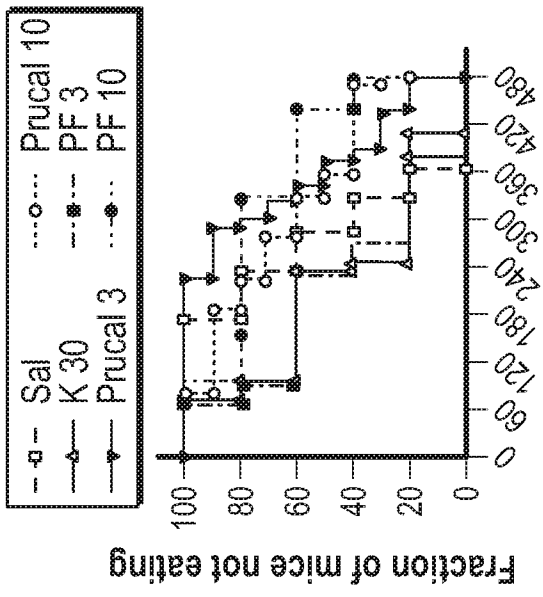


FIG. 4K

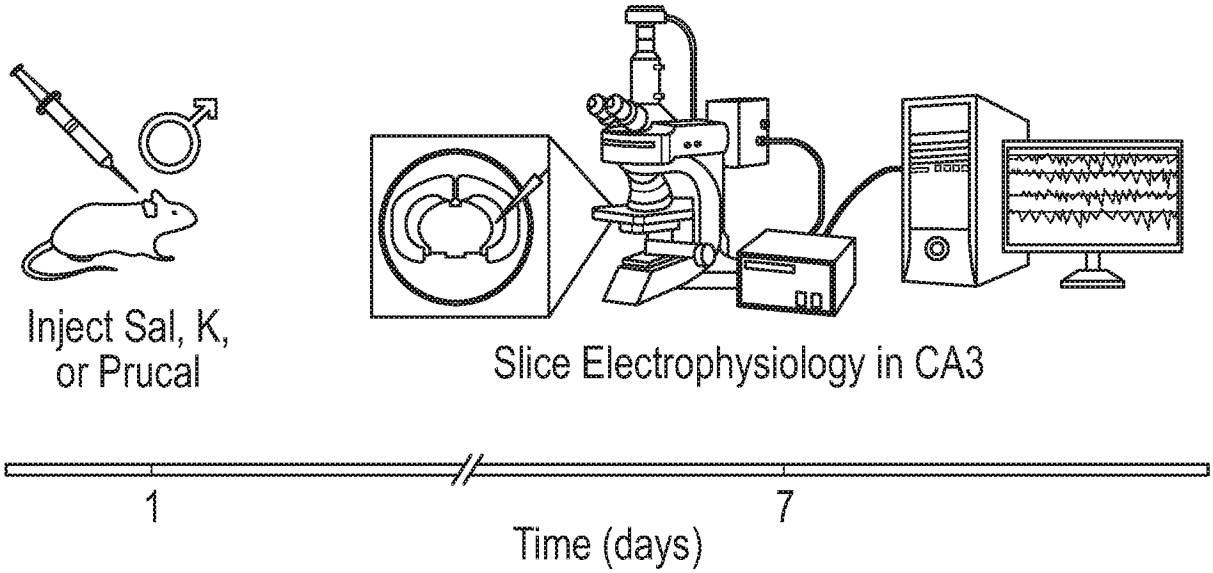


FIG. 5A

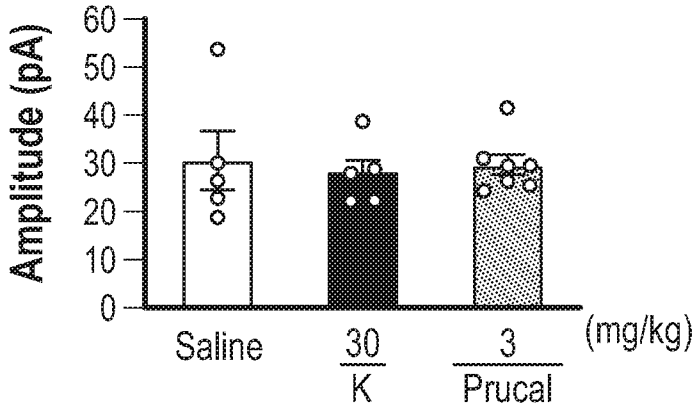


FIG. 5B

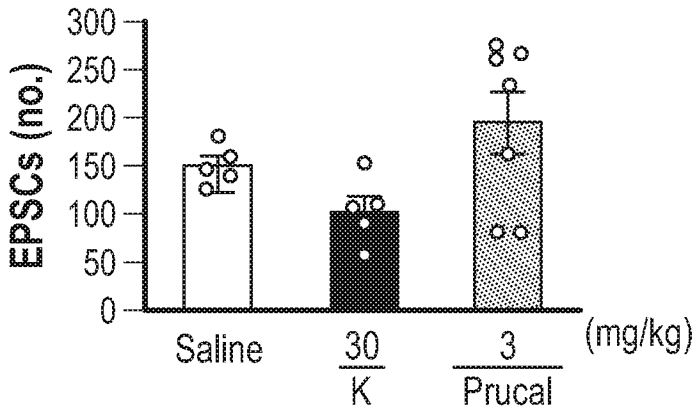


FIG. 5C

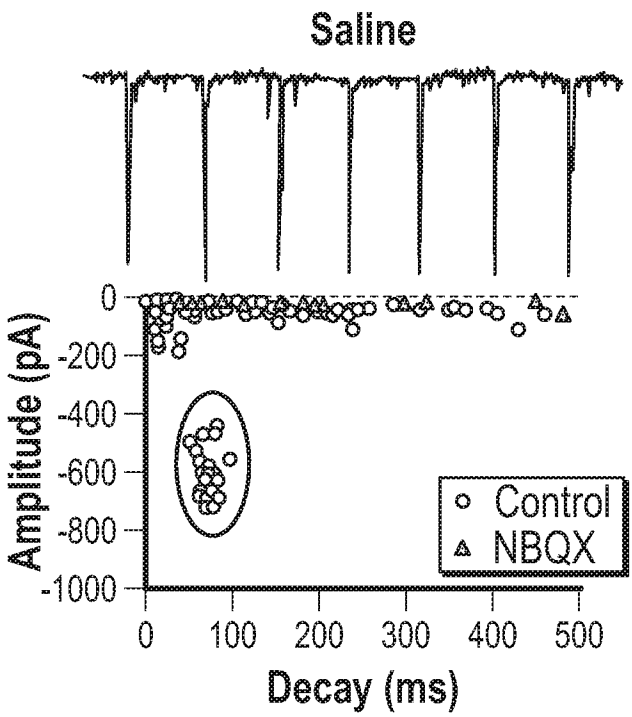


FIG. 5D

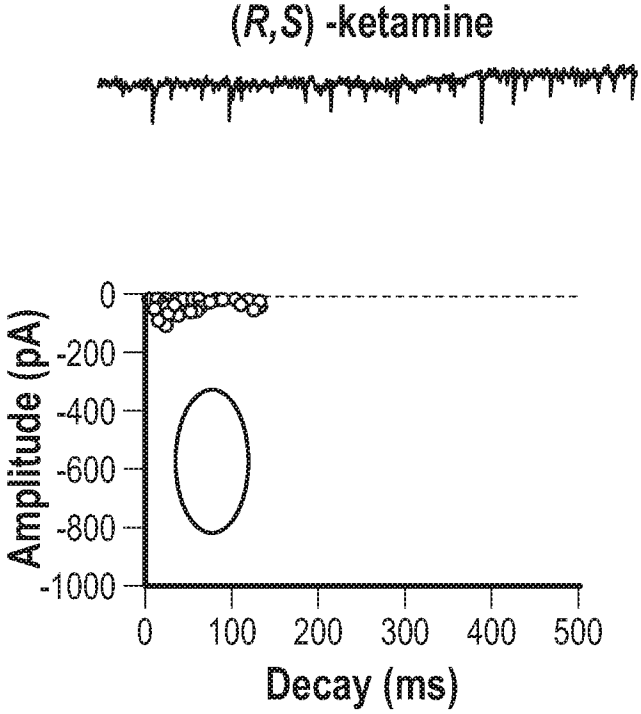


FIG. 5E

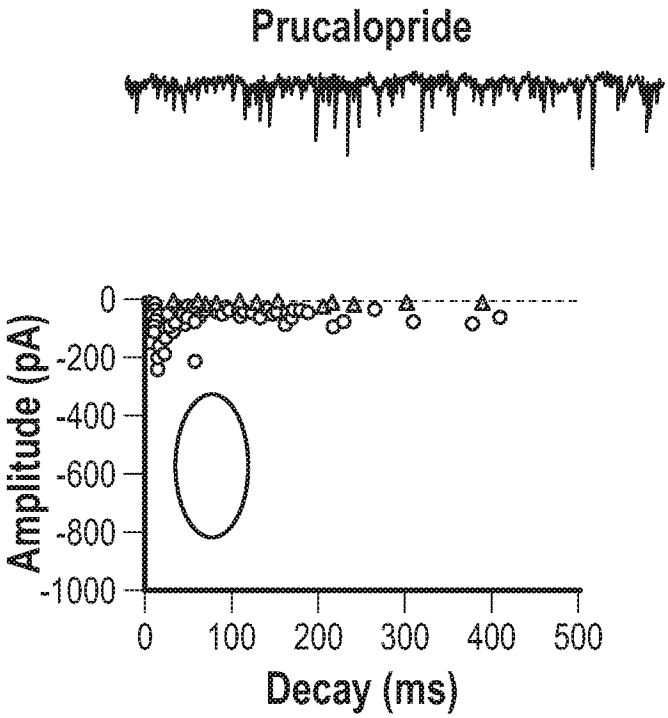


FIG. 5F

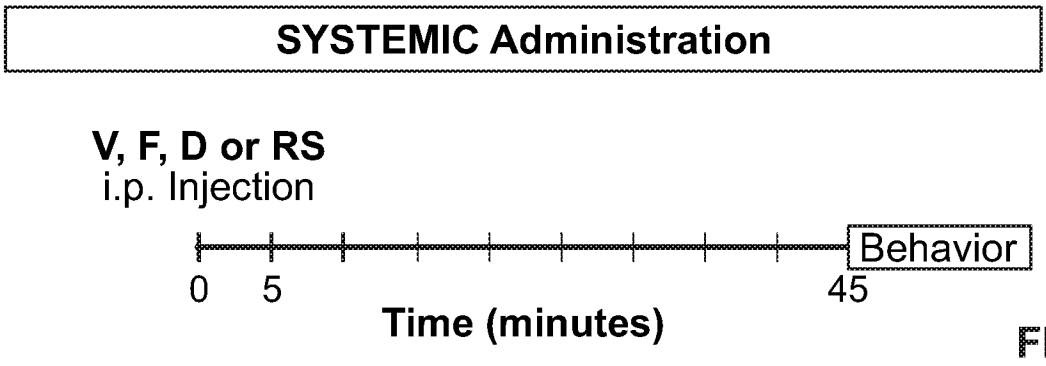


FIG. 6A

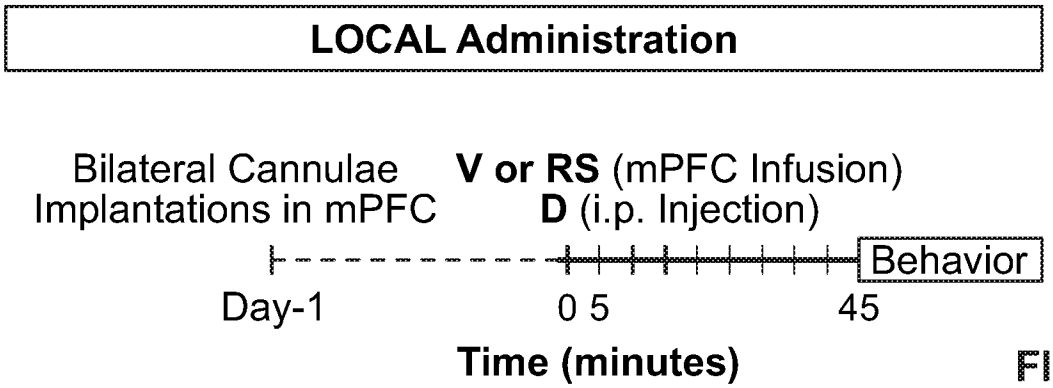


FIG. 6B

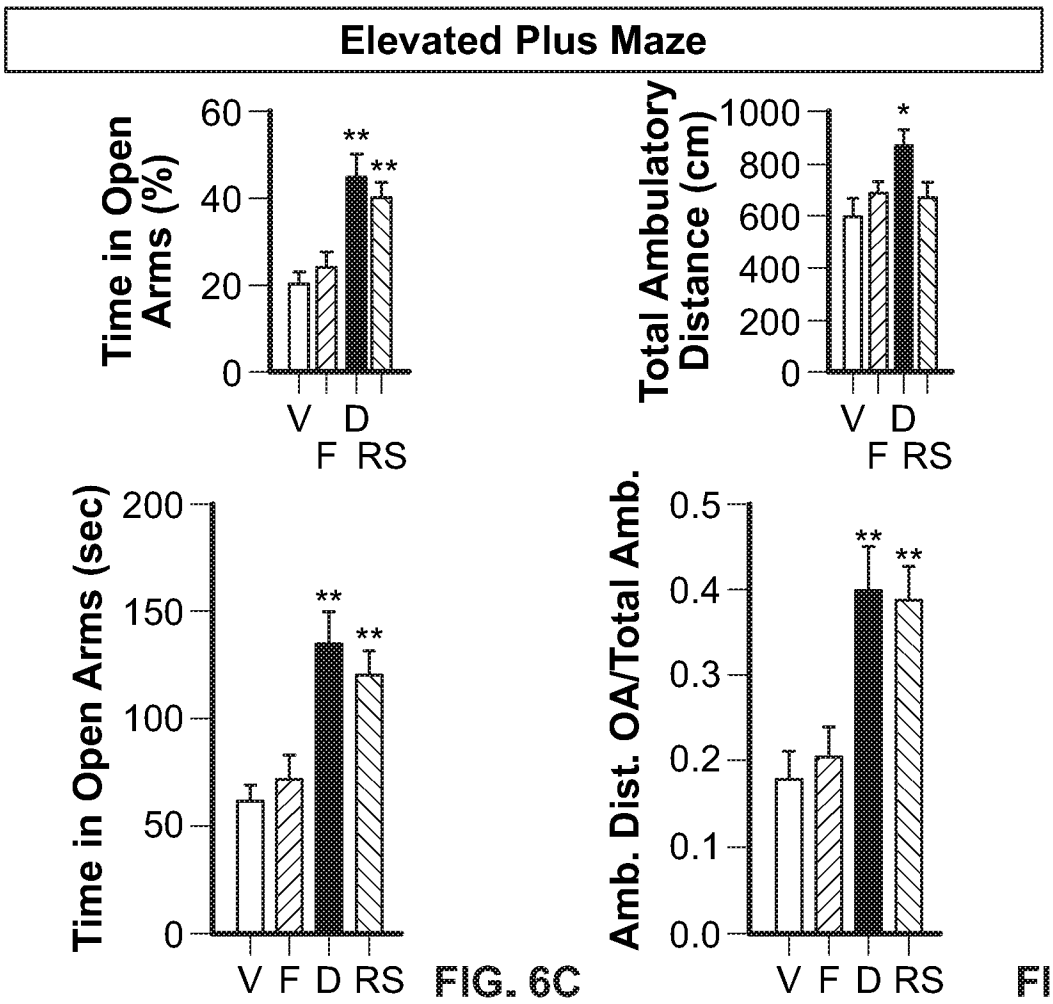


FIG. 6C

FIG. 6D

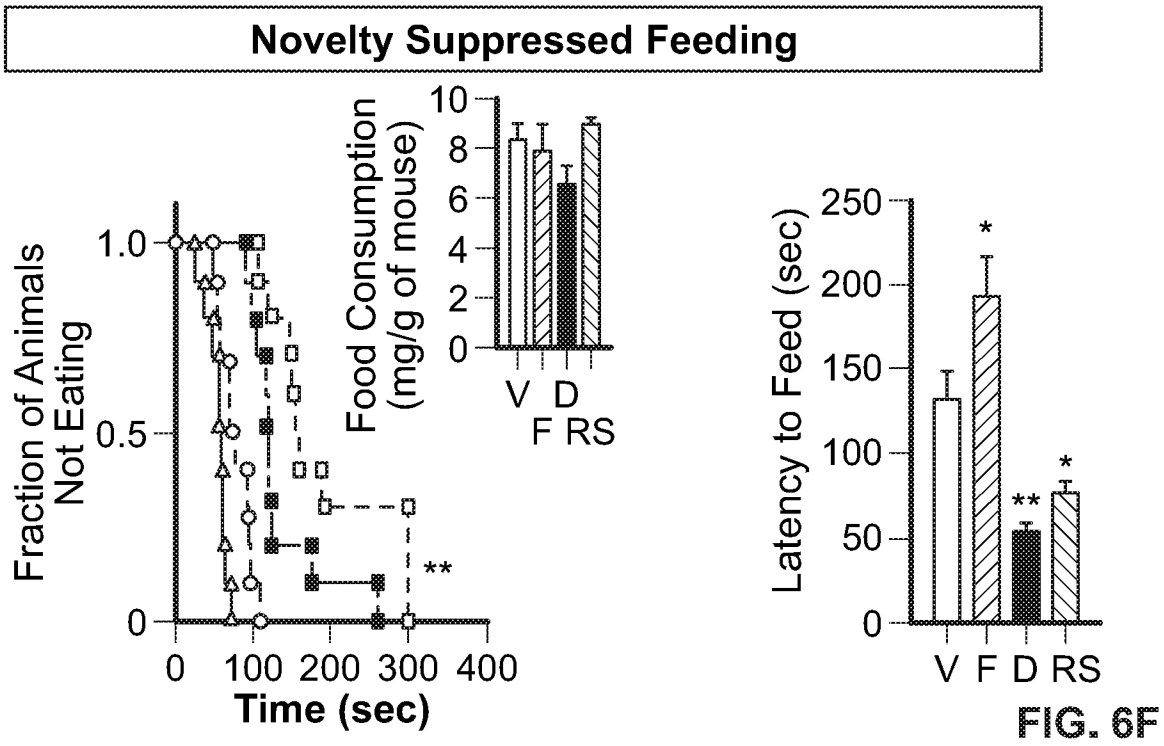


FIG. 6F

FIG. 6E



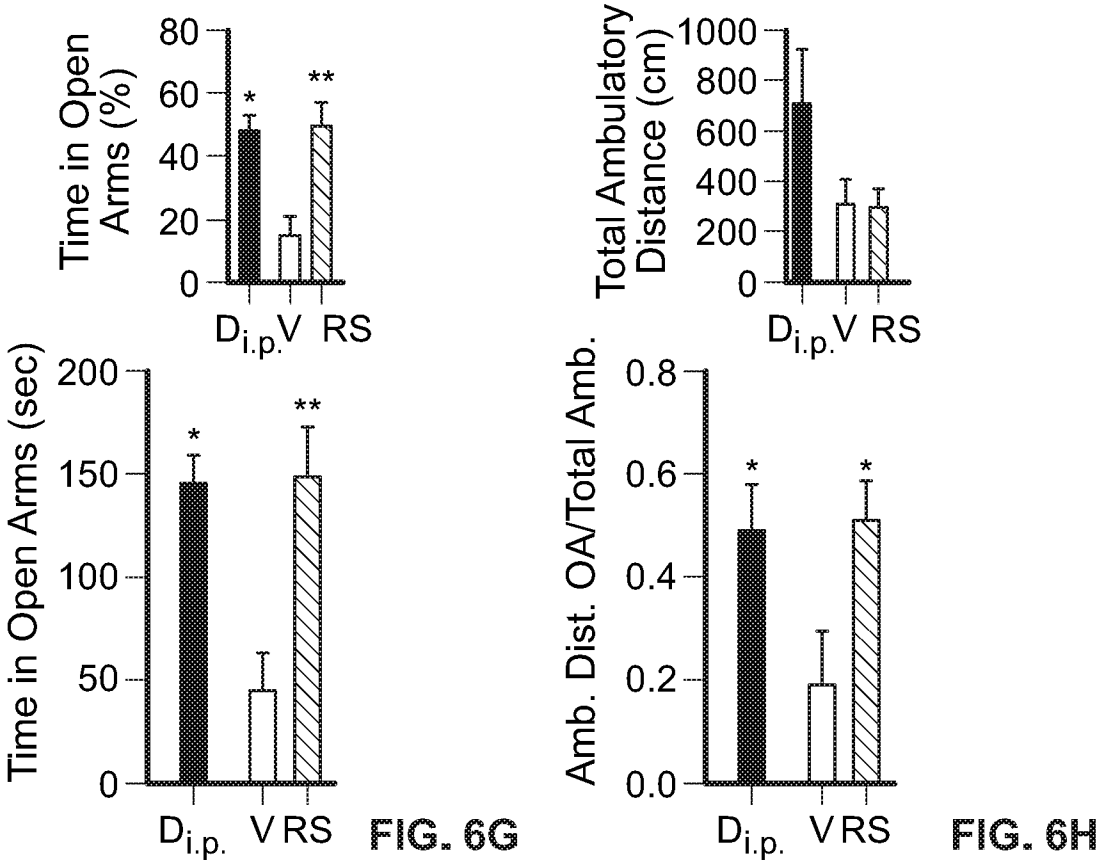


FIG. 6G

FIG. 6H

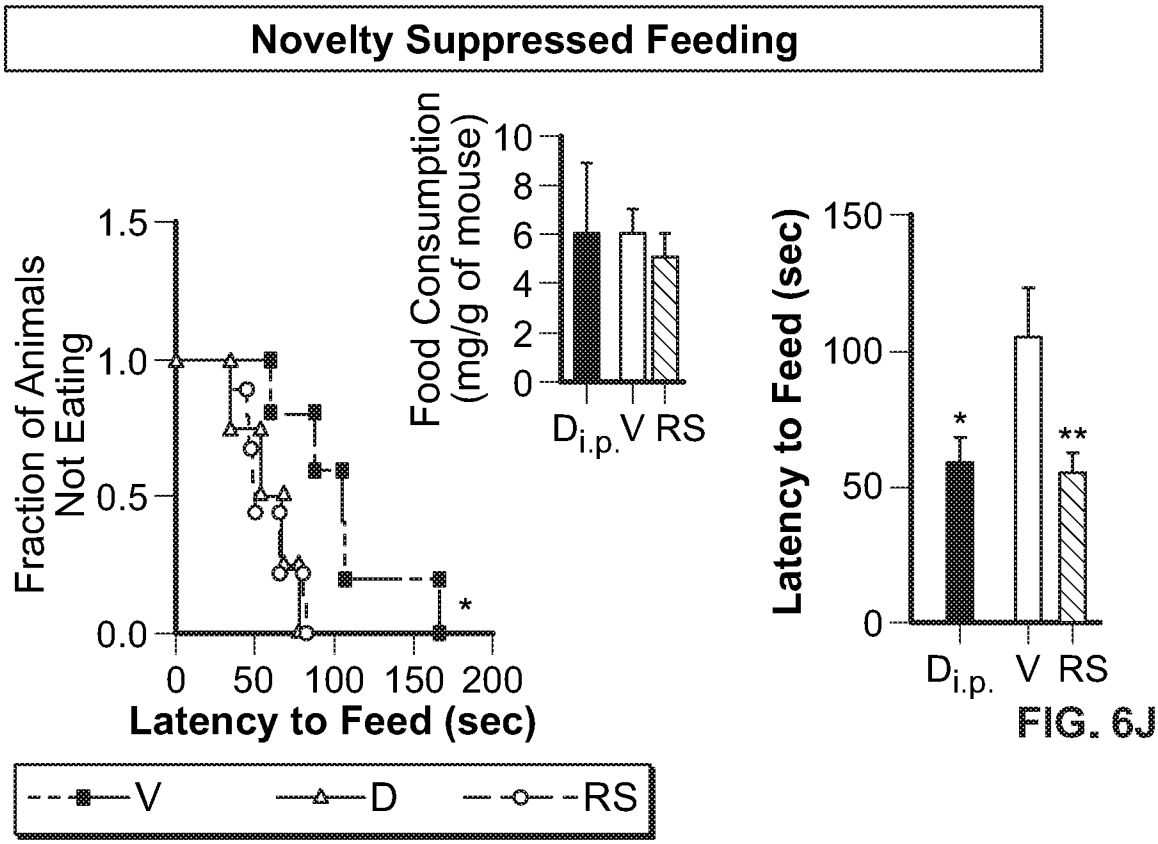

Novelty Suppressed Feeding
Fraction of Animals Not Eating
1.5
1.0
0.5
0.0
0
50
100
150
200
*
Latency to Feed (sec)
V
D
RS
Food Consumption (mg/g of mouse)
10
8
6
4
2
0
Di.p.
V
RS
Latency to Feed (sec)
150
100
50
0
*
**
Di.p.
V
RS

FIG. 6J

FIG. 6I

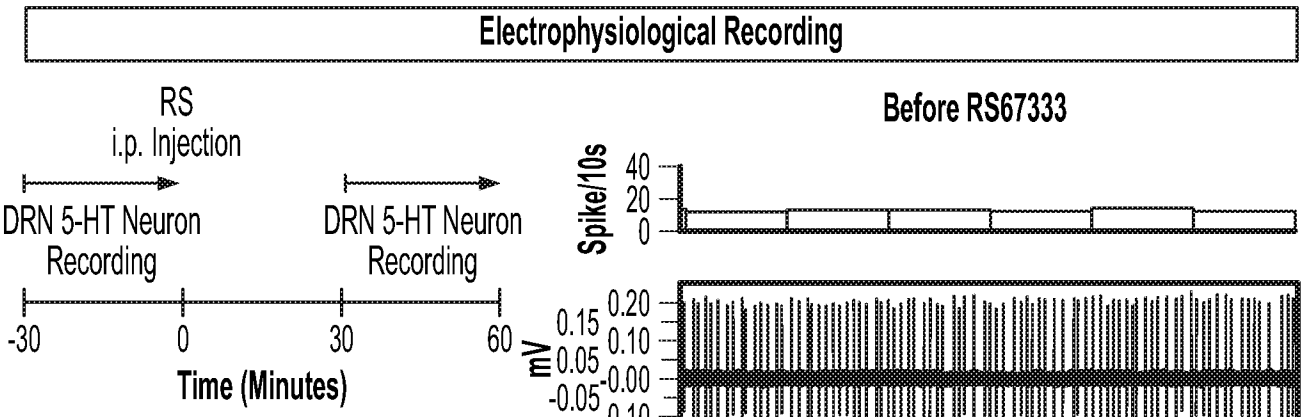


FIG. 7A

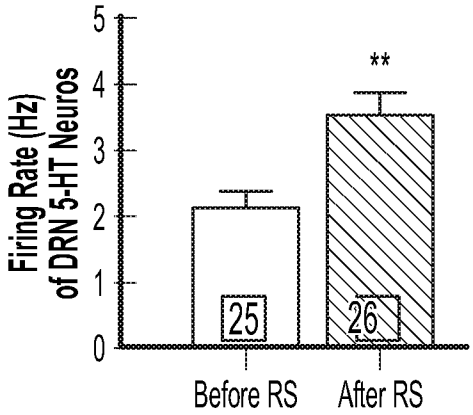


FIG. 7B

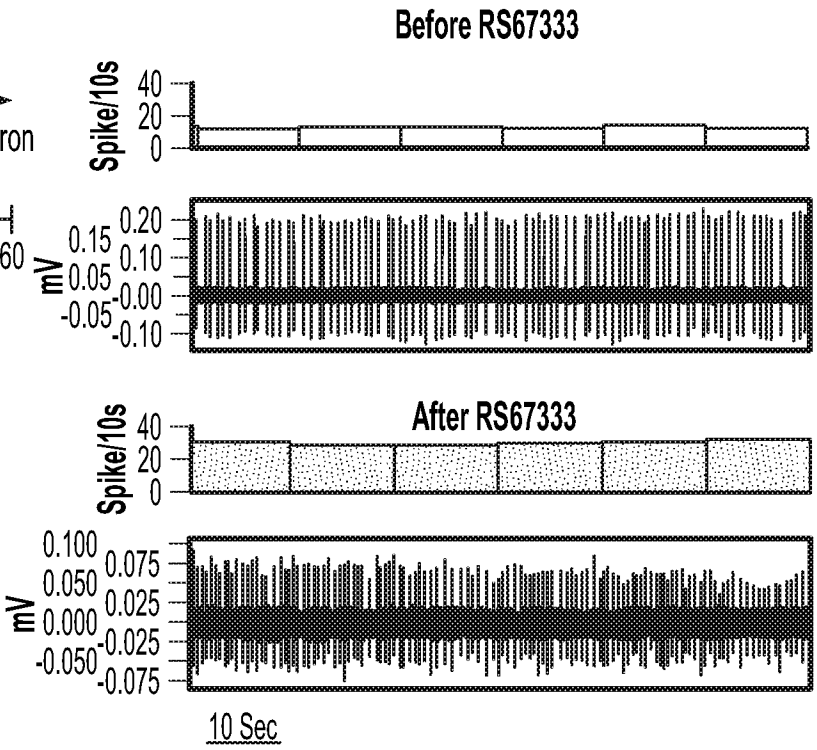


FIG. 7C

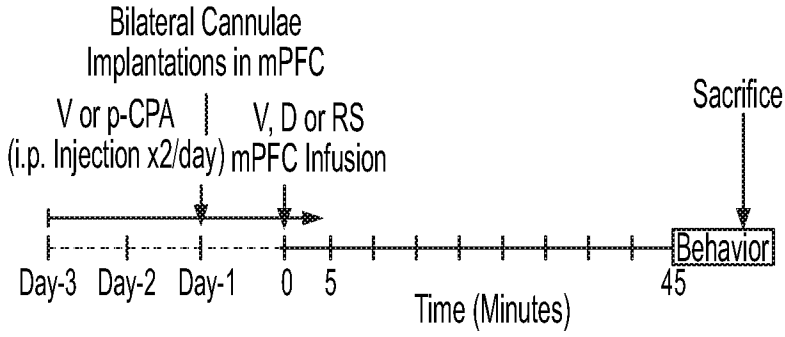


FIG. 7D

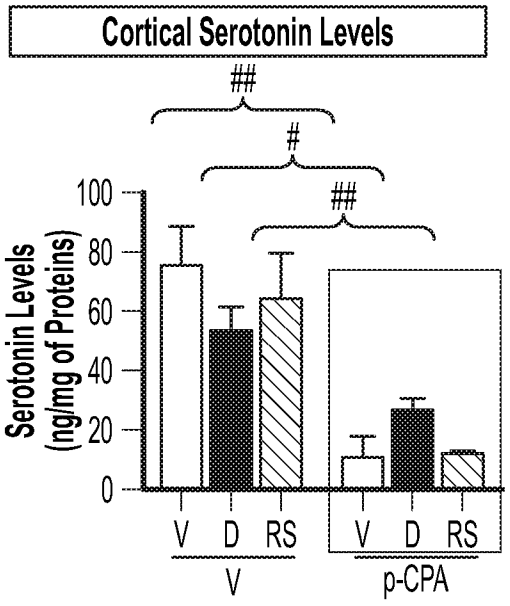


FIG. 7E

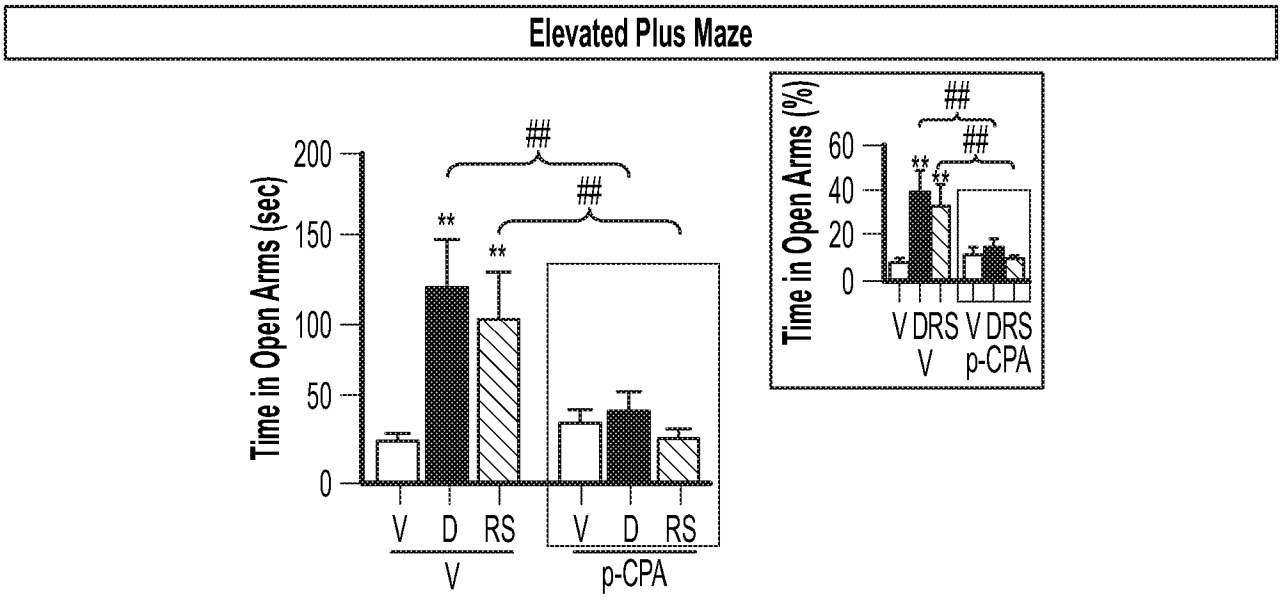

Elevated Plus Maze
Time in Open Arms (sec)
200
150
100
50
0
##
##
**
**
V D RS V D RS
V p-CPA
Time in Open Arms (%)
60
40
20
0
V DRS V DRS
V p-CPA

FIG. 7F

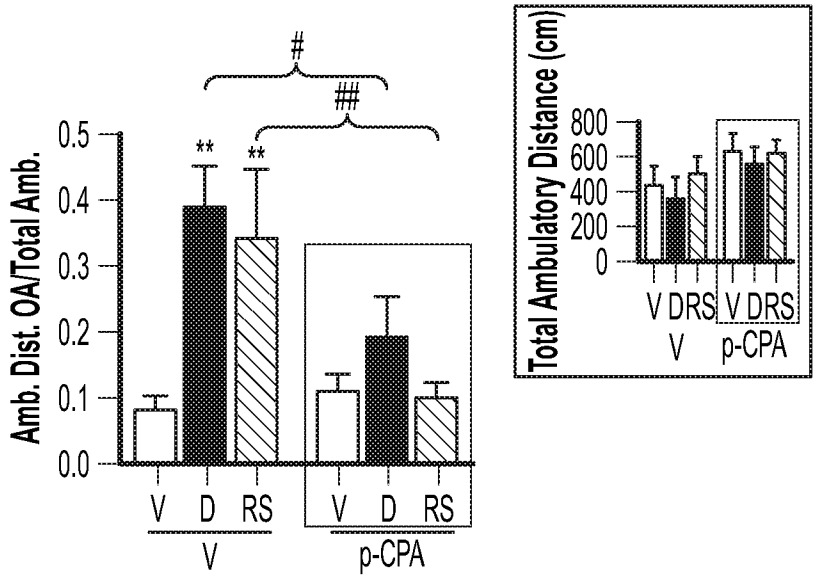

Amb. Dist. OA/Total Amb.
0.5
0.4
0.3
0.2
0.1
0.0
#
##
**
**
V D RS V D RS
V p-CPA
Total Ambulatory Distance (cm)
800
600
400
200
0
V DRS V DRS
V p-CPA

FIG. 7G

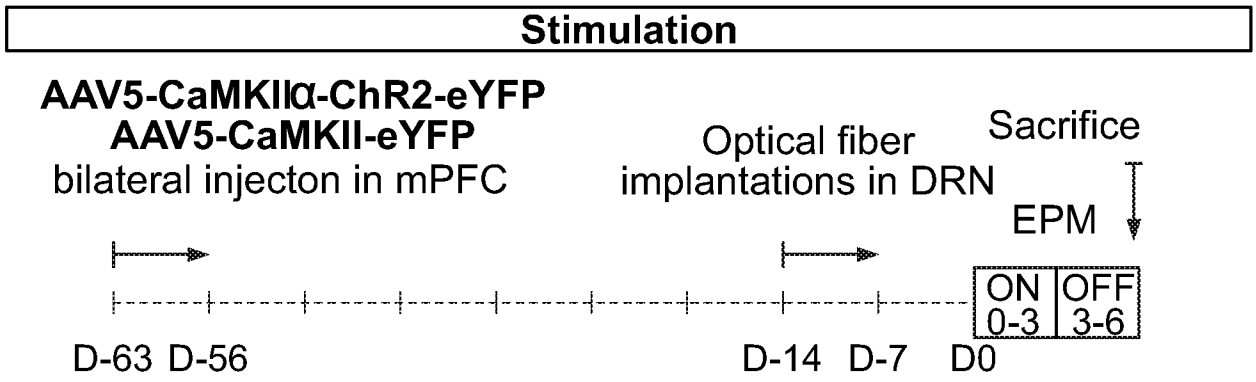

Stimulation
AAV5-CaMKIIα-ChR2-eYFP
AAV5-CaMKII-eYFP
bilateral injecton in mPFC
Optical fiber
implantations in DRN
Sacrifice
EPM
ON
0-3
OFF
3-6
D-63 D-56
D-14 D-7 D0

FIG. 8A

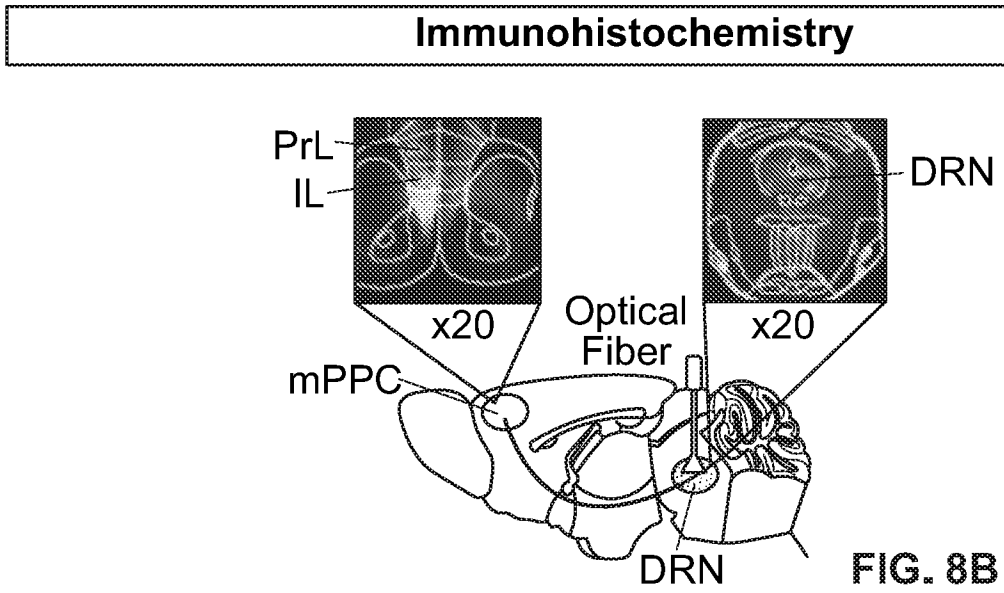

Immunohistochemistry
PrL
IL
x20
Optical
Fiber
x20
DRN
mPPC
DRN

FIG. 8B

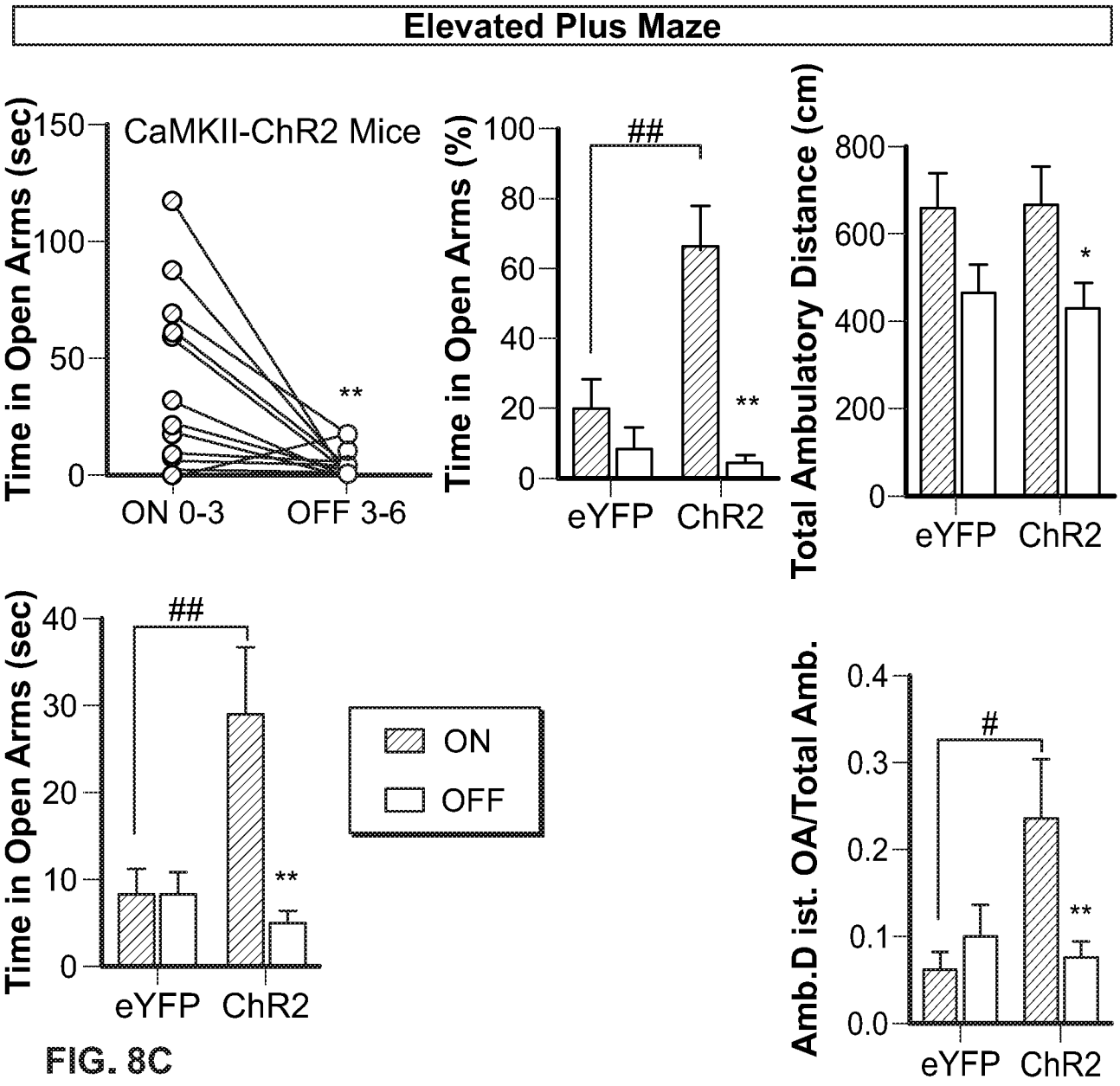

Elevated Plus Maze
CaMKII-ChR2 Mice
Time in Open Arms (sec)
150
100
50
0
ON 0-3
OFF 3-6
**
Time in Open Arms (%)
100
80
60
40
20
0
##
**
eYFP
ChR2
Total Ambulatory Distance (cm)
800
600
400
200
0
*
eYFP
ChR2
Time in Open Arms (sec)
40
30
20
10
0
##
**
eYFP
ChR2
ON
OFF
Amb.D ist. OA/Total Amb.
0.4
0.3
0.2
0.1
0.0
#
**
eYFP
ChR2

FIG. 8C

FIG. 8D

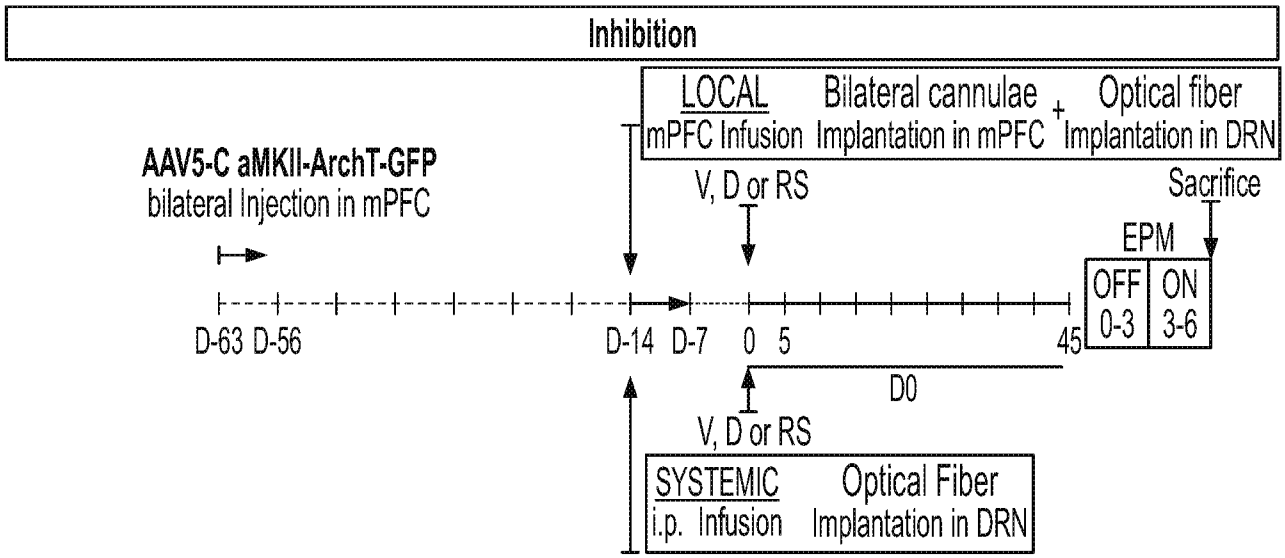

Inhibition
LOCAL
mPFC Infusion
Bilateral cannulae
Implantation in mPFC
+
Optical fiber
Implantation in DRN
AAV5-C aMKII-ArchT-GFP
bilateral Injection in mPFC
V, D or RS
Sacrifice
EPM
OFF
0-3
ON
3-6
D-63 D-56
D-14 D-7
0 5
45
D0
V, D or RS
SYSTEMIC
i.p. Infusion
Optical Fiber
Implantation in DRN

FIG. 9A

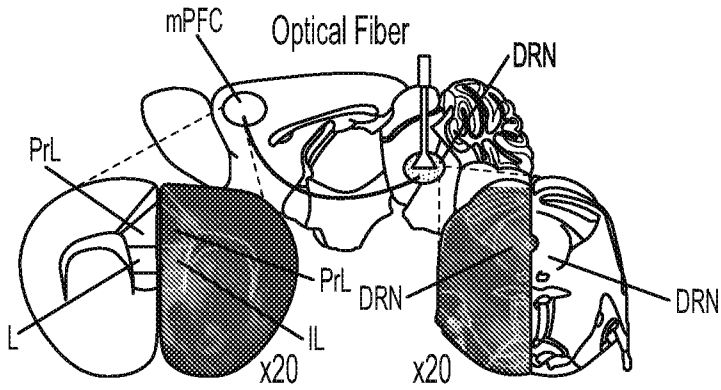

mPFC
Optical Fiber
DRN
PrL
PrL
DRN
DRN
L
IL
x20
x20

FIG. 9B

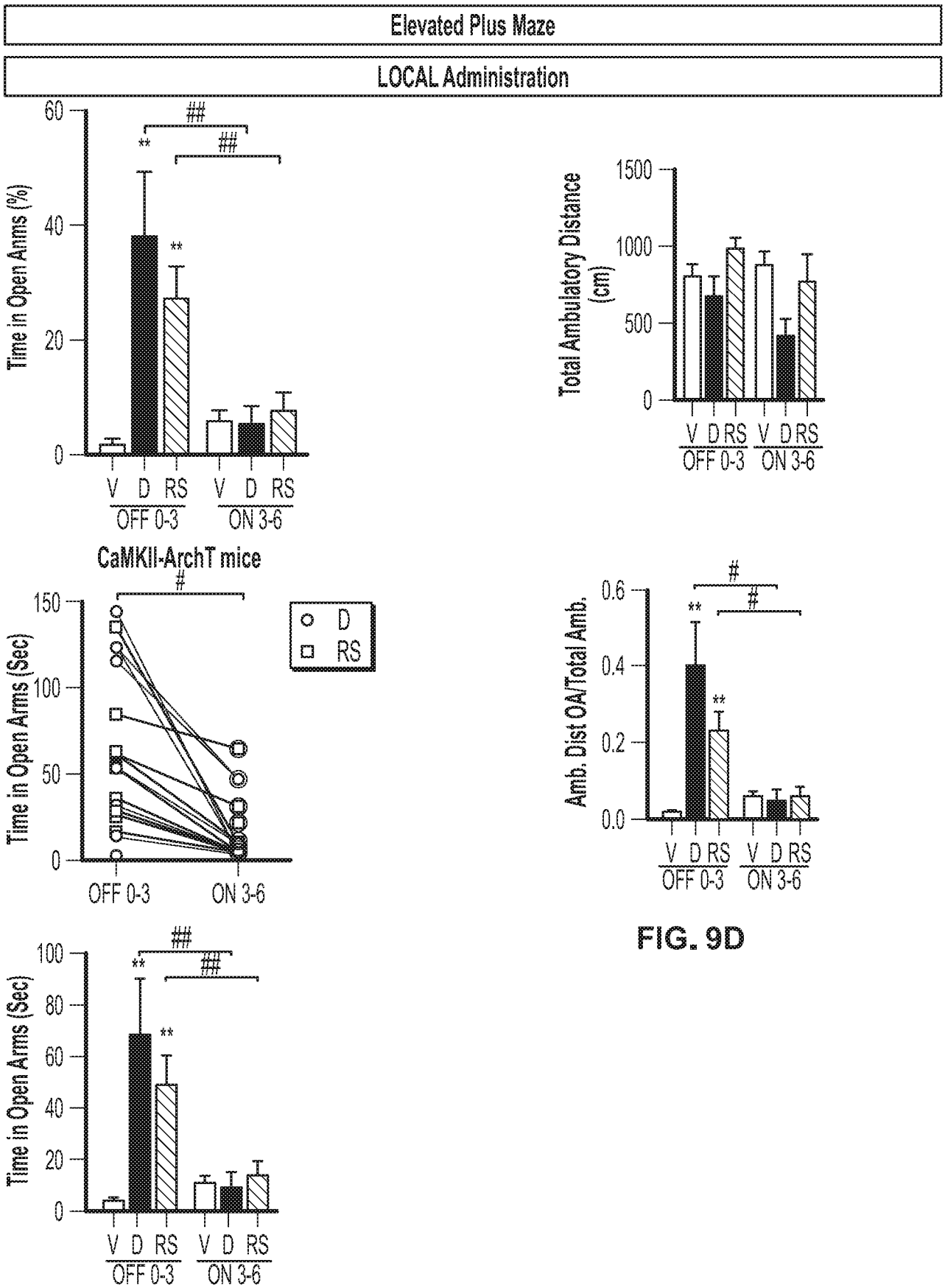

Elevated Plus Maze
LOCAL Administration
Time in Open Arms (%)
60
40
20
0
##
**
##
**
V D RS V D RS
OFF 0-3 ON 3-6
Total Ambulatory Distance (cm)
1500
1000
500
0
V D RS V D RS
OFF 0-3 ON 3-6
CaMKII-ArchT mice
Time in Open Arms (Sec)
150
100
50
0
#
D
RS
OFF 0-3 ON 3-6
Amb. Dist OA/Total Amb.
0.6
0.4
0.2
0.0
#
**
#
**
V D RS V D RS
OFF 0-3 ON 3-6
Time in Open Arms (Sec)
100
80
60
40
20
0
##
**
##
**
V D RS V D RS
OFF 0-3 ON 3-6

FIG. 9C

FIG. 9D

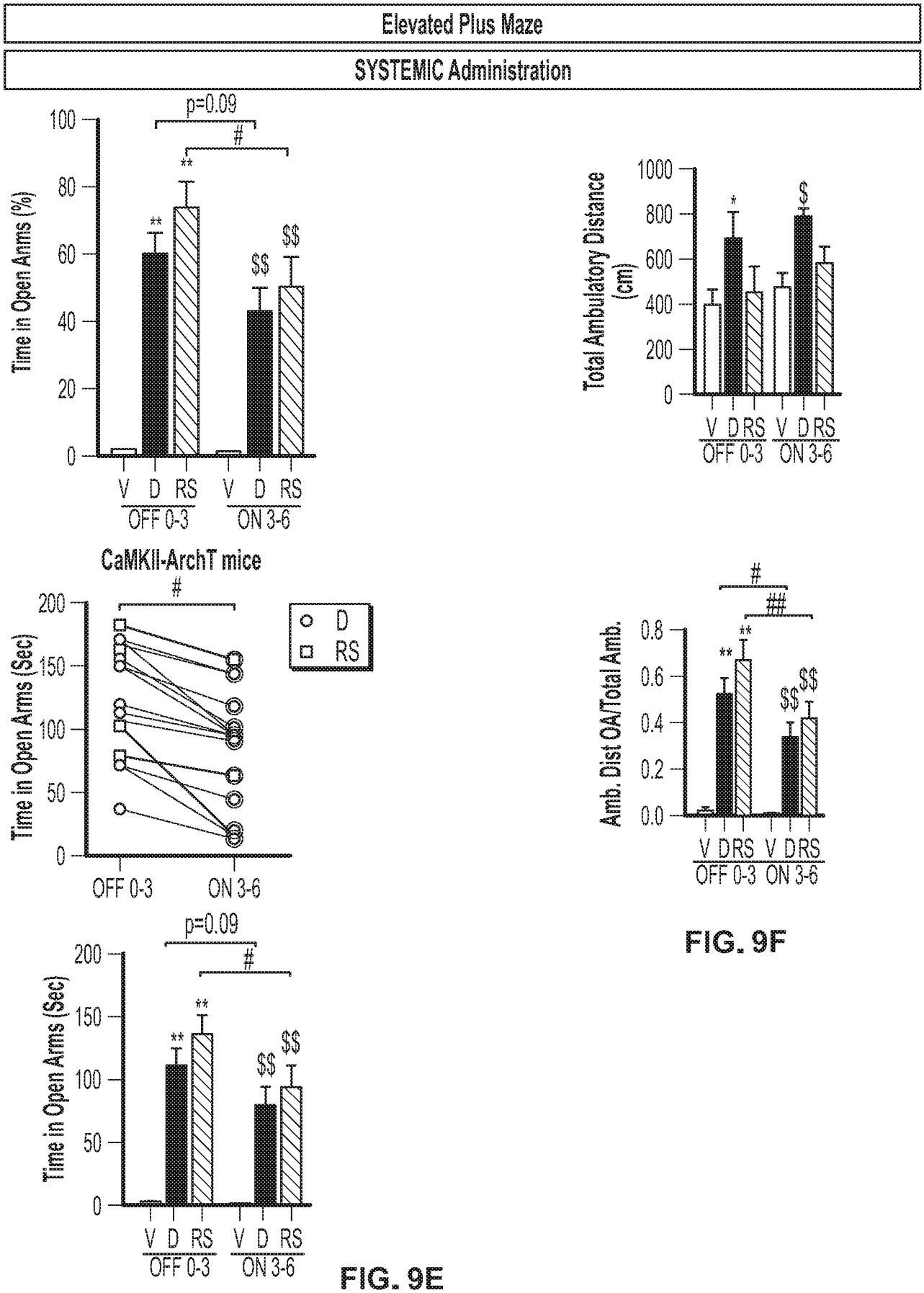

Elevated Plus Maze
SYSTEMIC Administration
p=0.09
#
**
**
$$
$$
Time in Open Anms (%)
100
80
60
40
20
0
V D RS V D RS
OFF 0-3
ON 3-6
Total Ambulatory Distance (cm)
1000
800
600
400
200
0
*
$
V D RS V D RS
OFF 0-3 ON 3-6
CaMKII-ArchT mice
#
D
RS
Time in Open Arms (Sec)
200
150
100
50
0
OFF 0-3
ON 3-6
#
##
Amb. Dist OA/Total Amb.
0.8
0.6
0.4
0.2
0.0
**
**
$$
$$
V D RS V D RS
OFF 0-3 ON 3-6
p=0.09
#
Time in Open Arms (Sec)
200
150
100
50
0
**
**
$$
$$
V D RS V D RS
OFF 0-3
ON 3-6

FIG. 9E

FIG. 9F

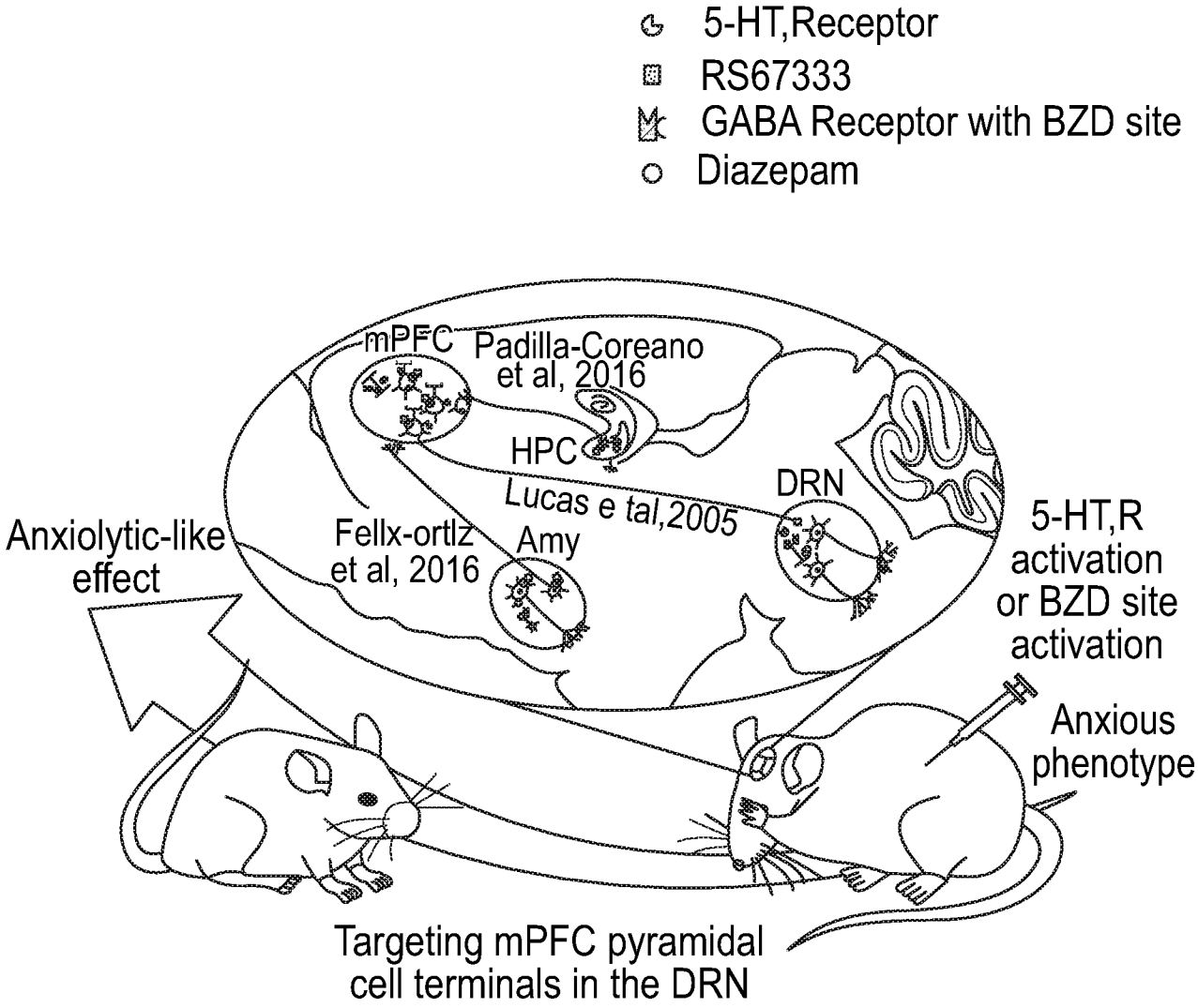

5-HT,Receptor
RS67333
GABA Receptor with BZD site
Diazepam
mPFC
Padilla-Coreano et al, 2016
HPC
Lucas e tal,2005
DRN
Anxiolytic-like effect
Fellx-ortlz et al, 2016
Amy
5-HT,R activation or BZD site activation
Anxious phenotype
Targeting mPFC pyramidal cell terminals in the DRN

FIG. 10

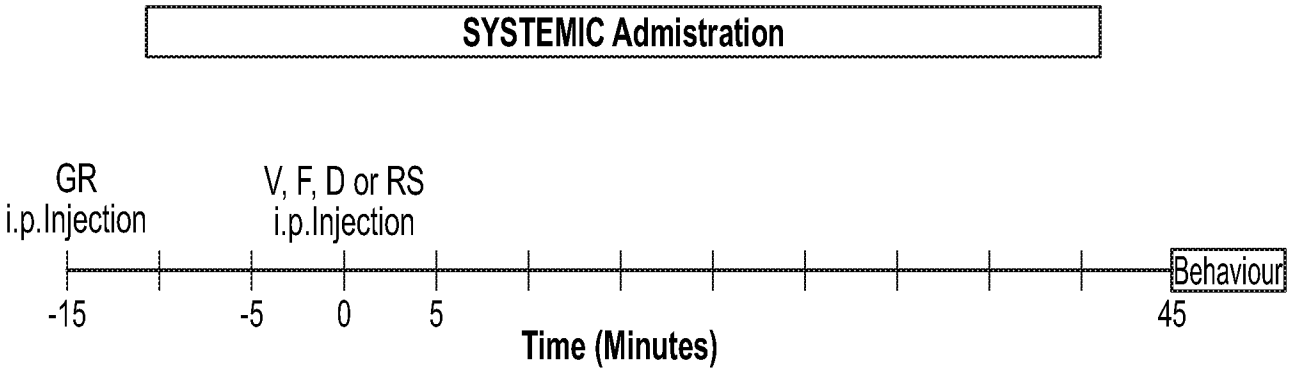
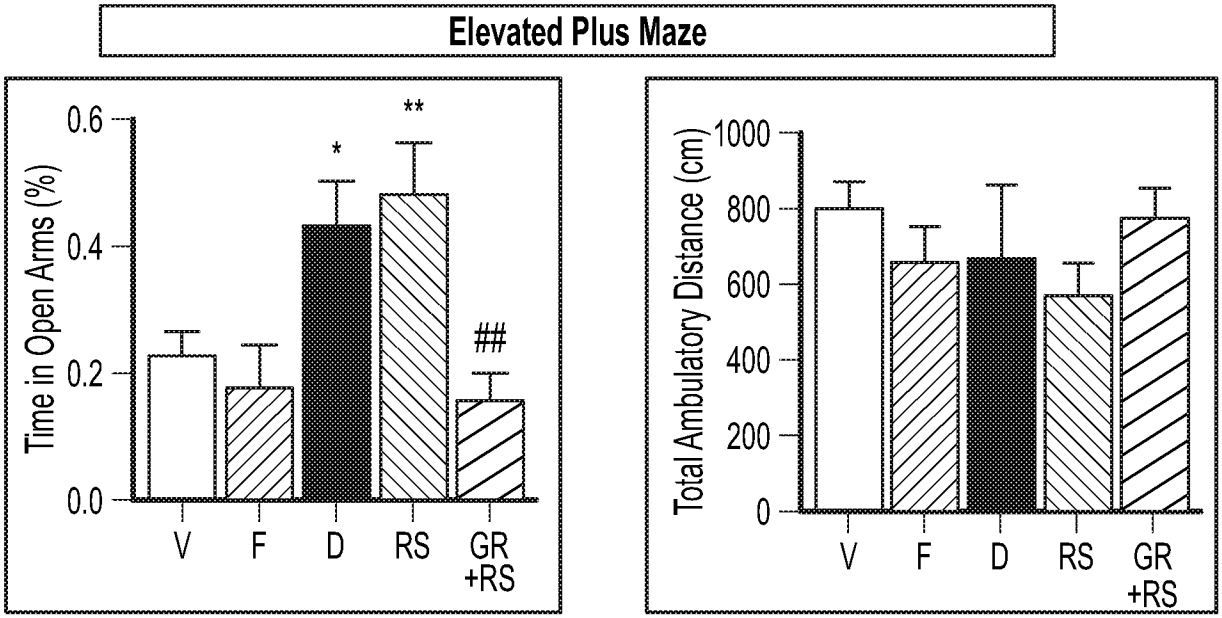


FIG. 11A



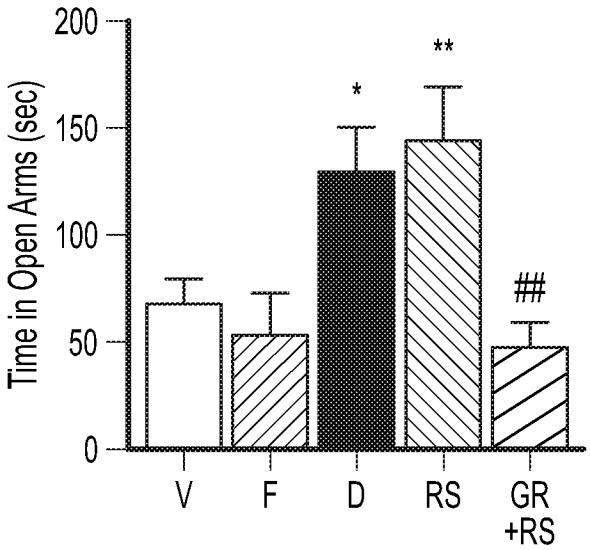


FIG. 11B

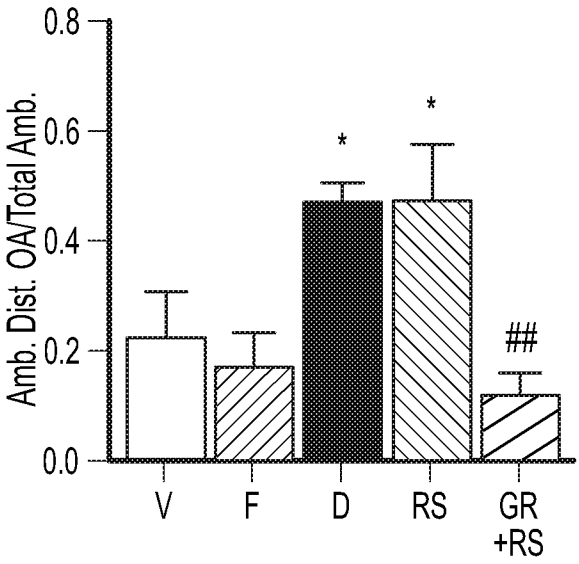


FIG. 11C

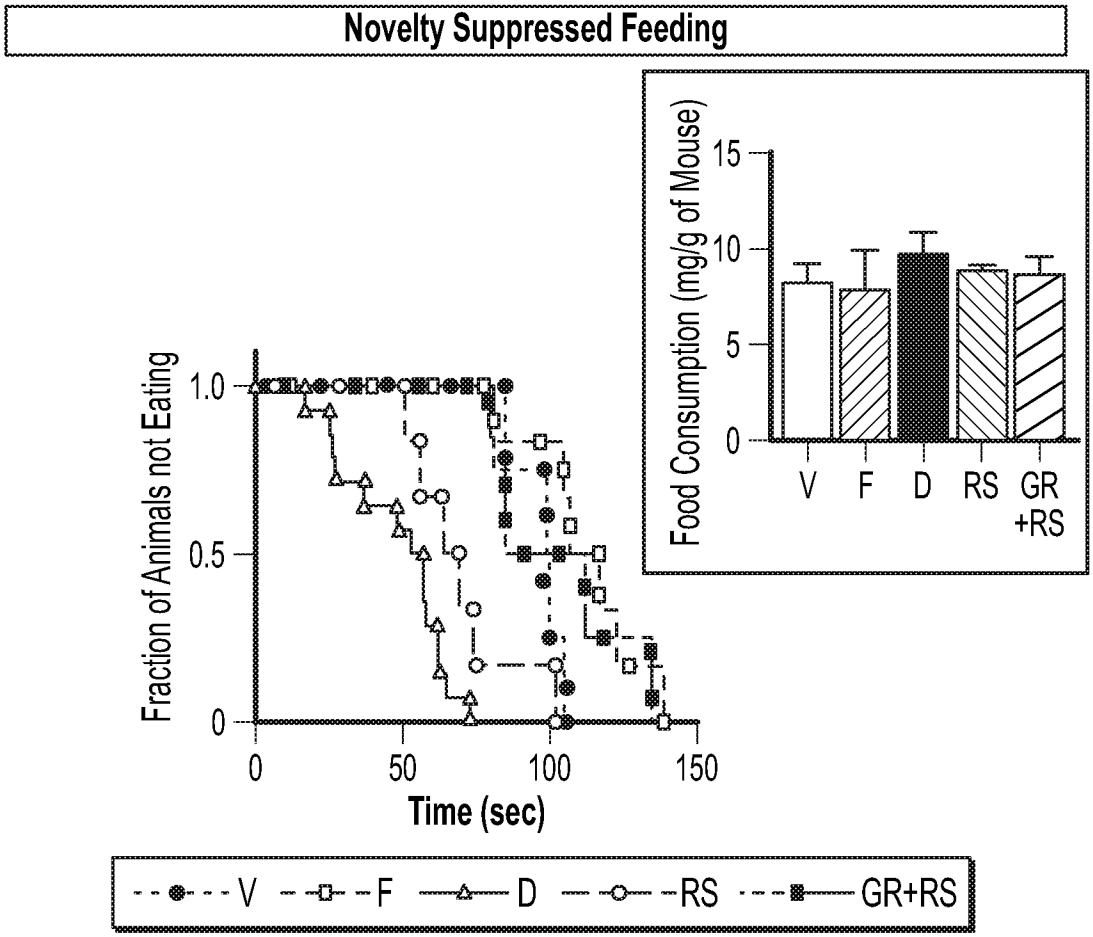


FIG. 11D

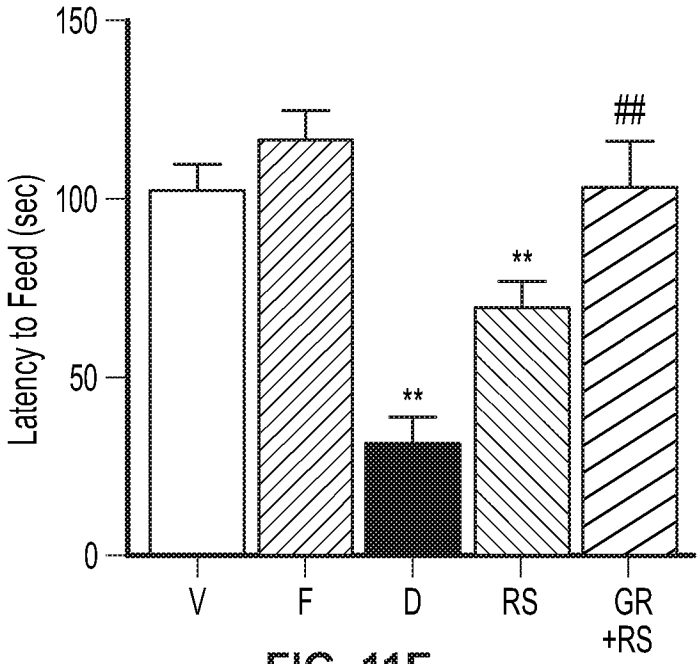


FIG. 11E

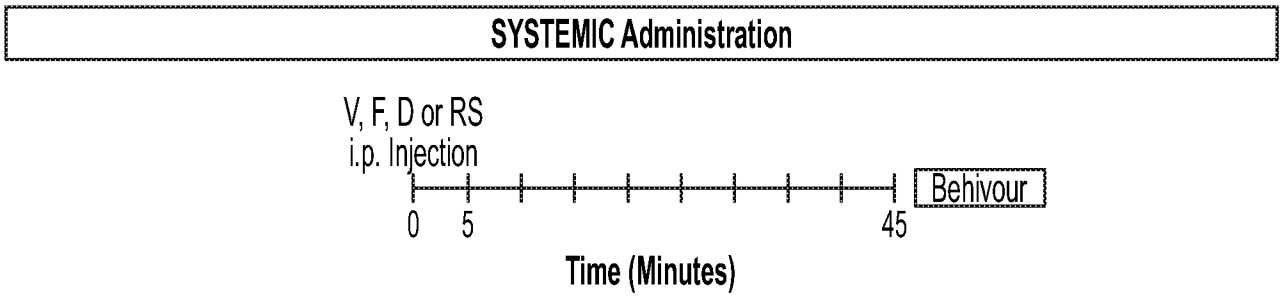


FIG. 12A

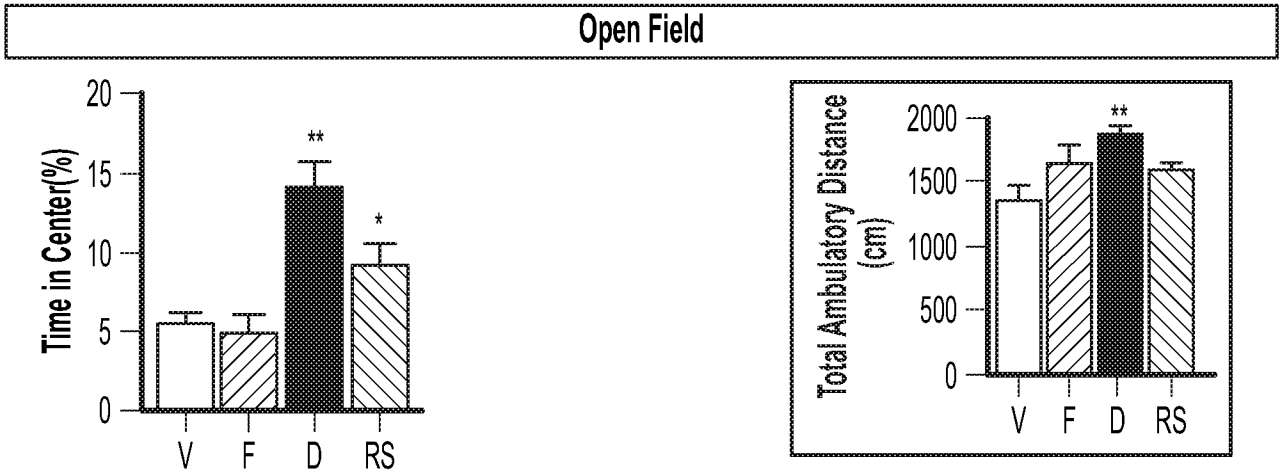


FIG. 12B

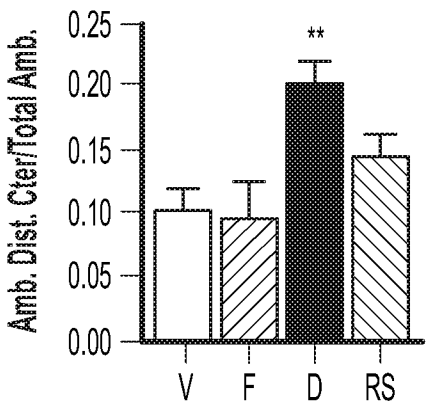


FIG. 12C

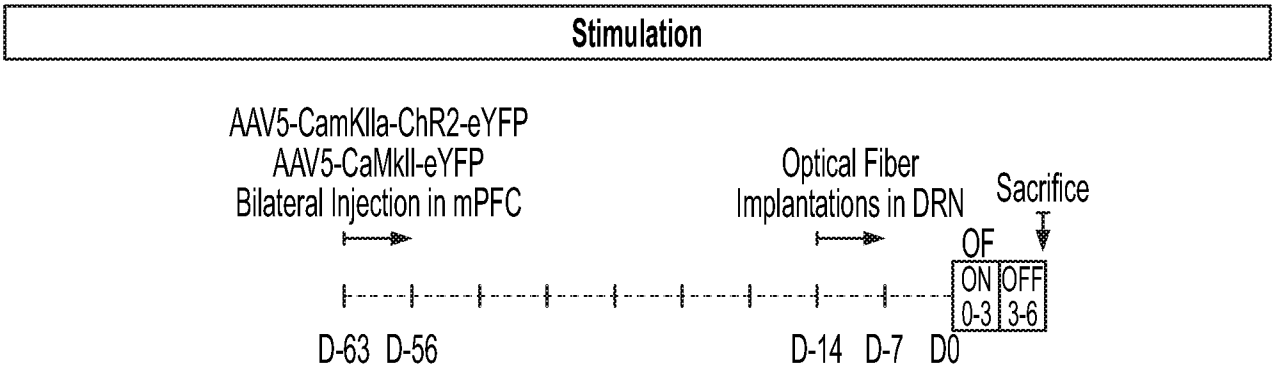


FIG. 13A

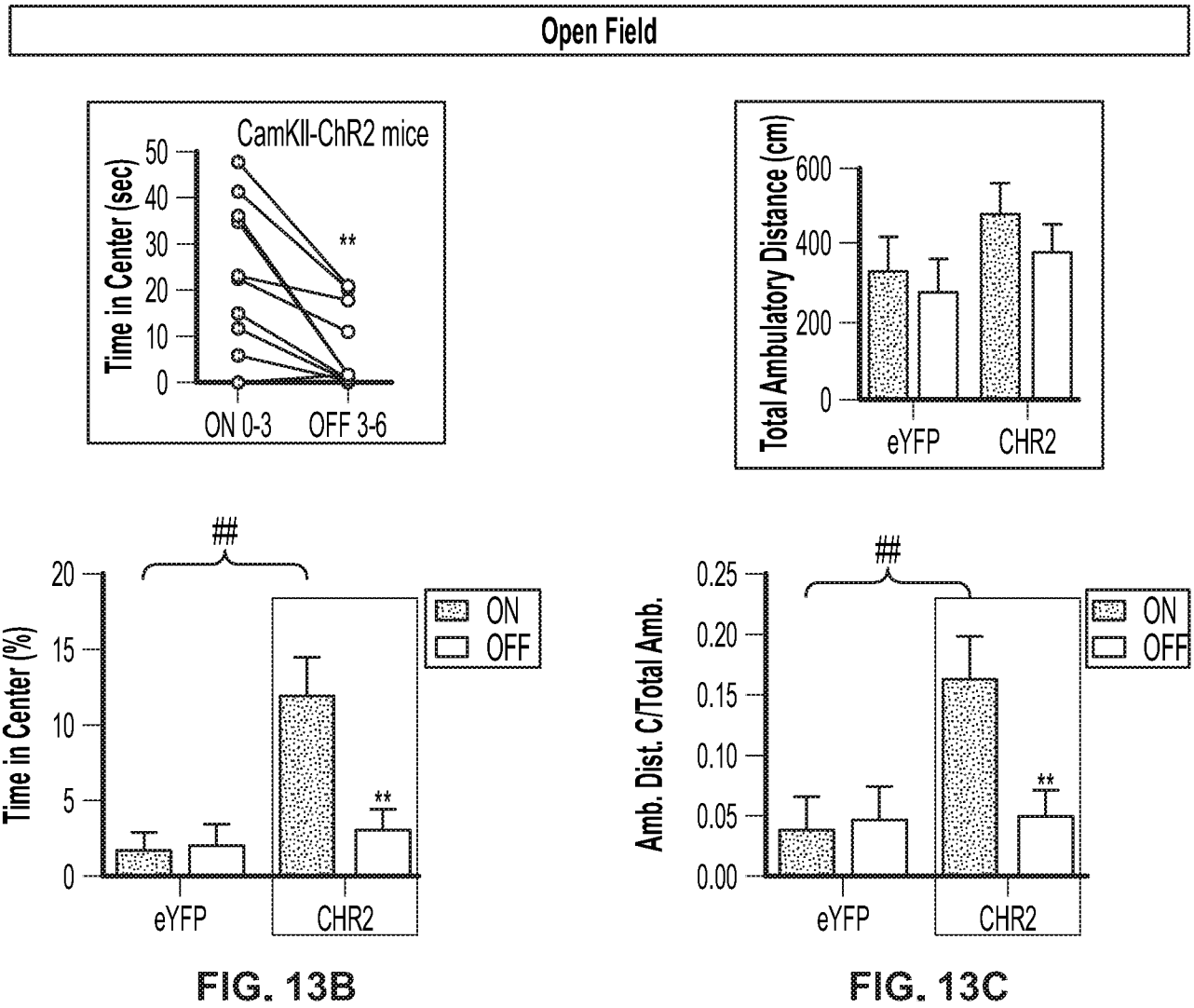


FIG. 13B

FIG. 13C

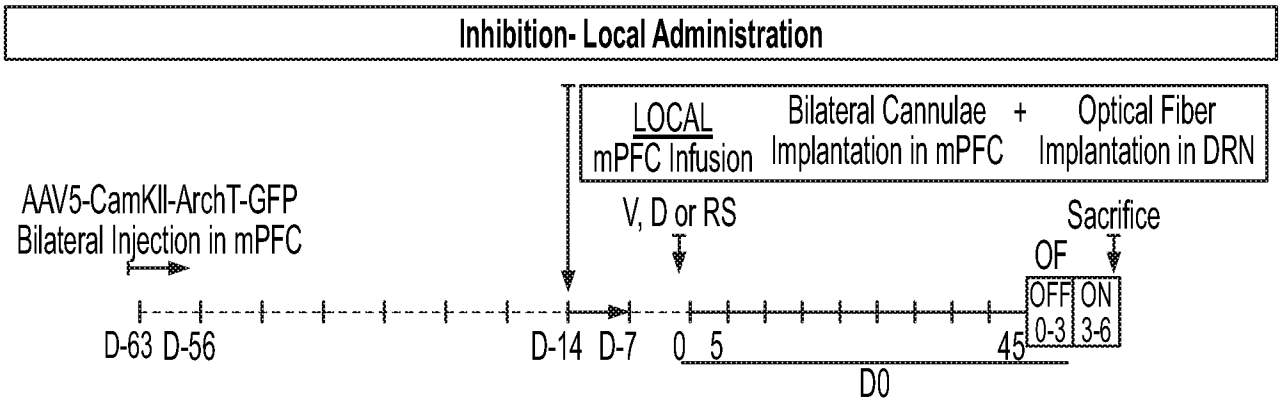


FIG. 14A

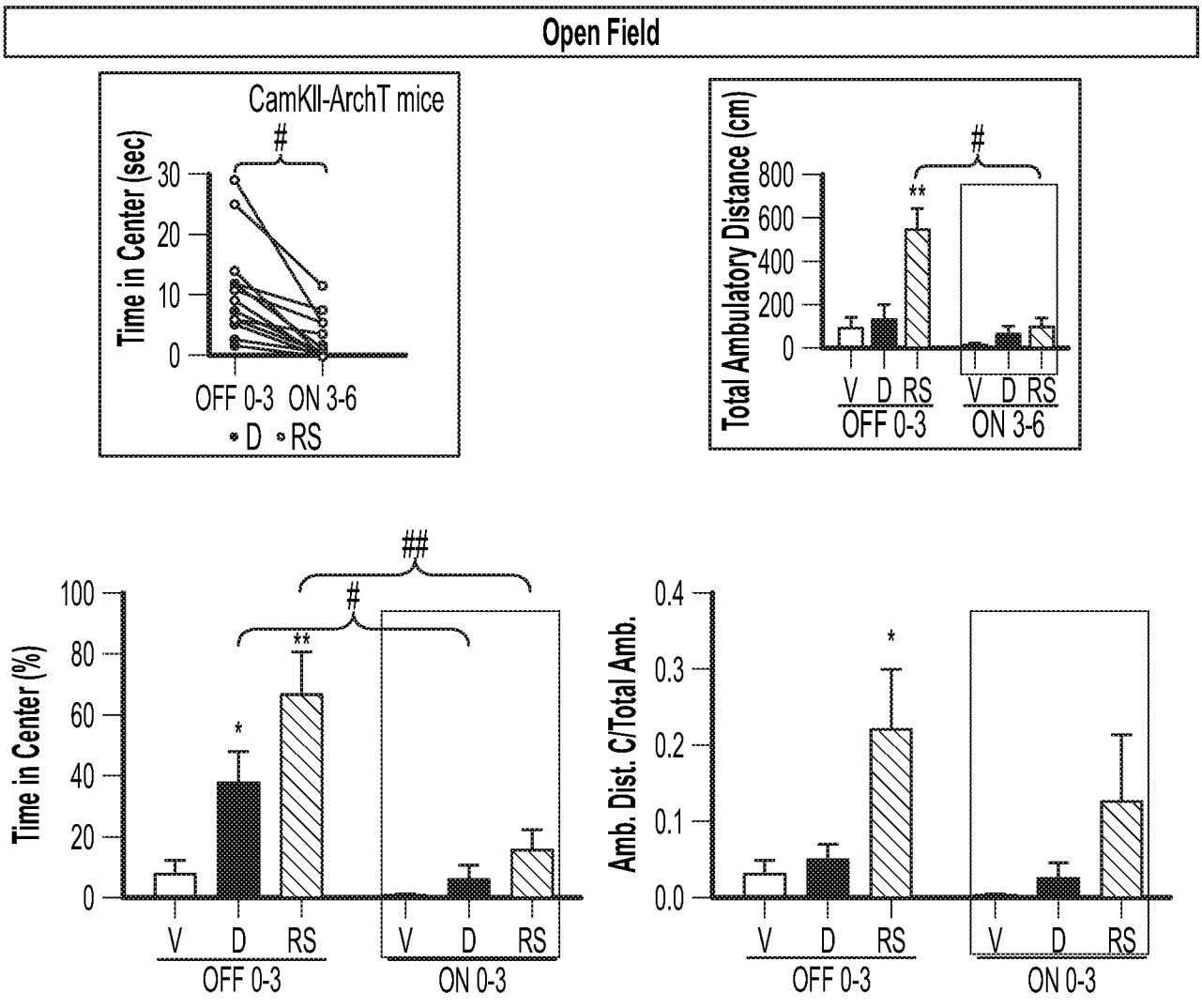


FIG. 14B

FIG. 14C

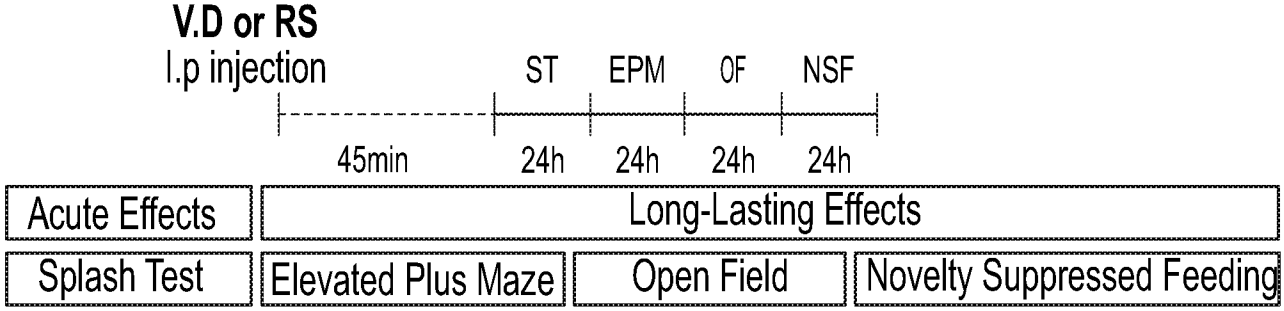


FIG. 15A

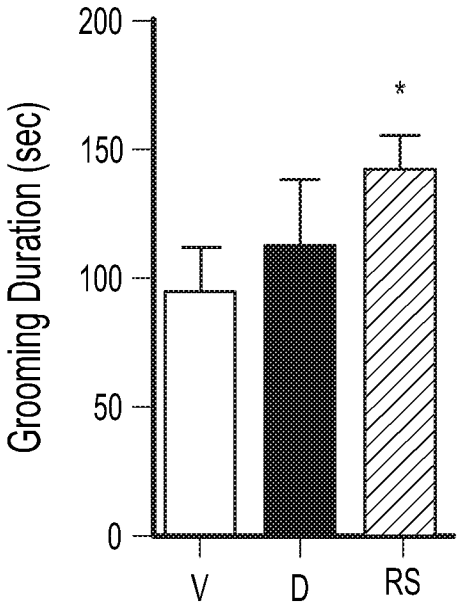


FIG. 15B

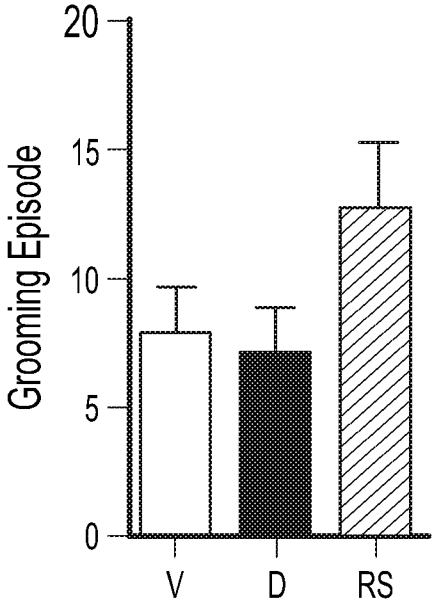


FIG. 15C

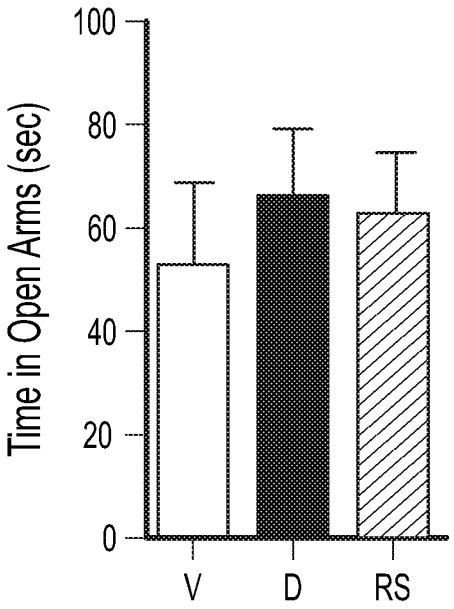


FIG. 15D

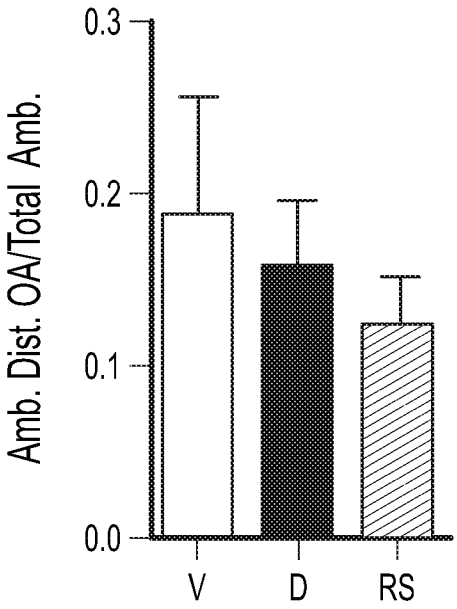


FIG. 15E

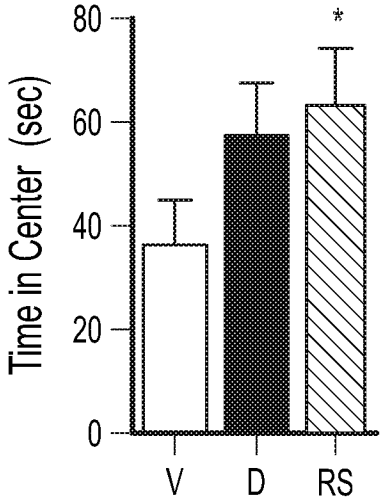


FIG. 15F

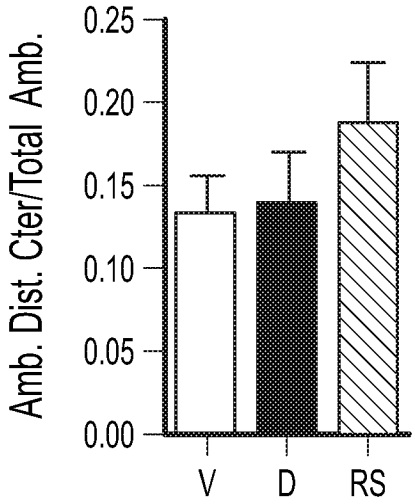


FIG. 15G

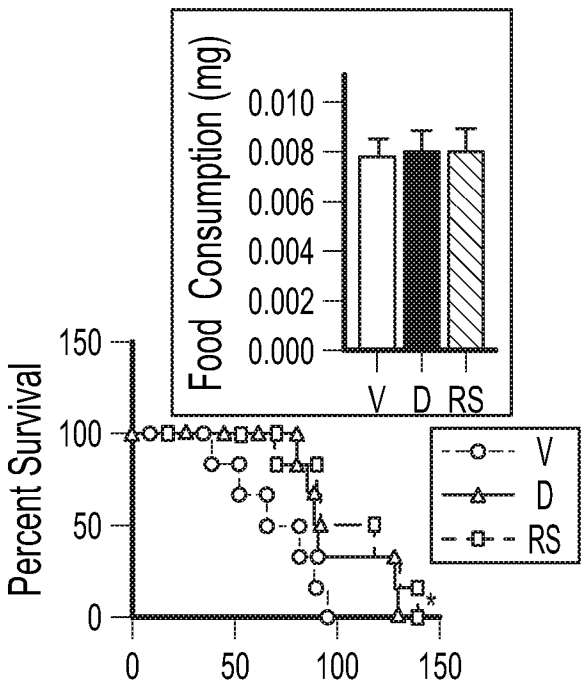


FIG. 15H

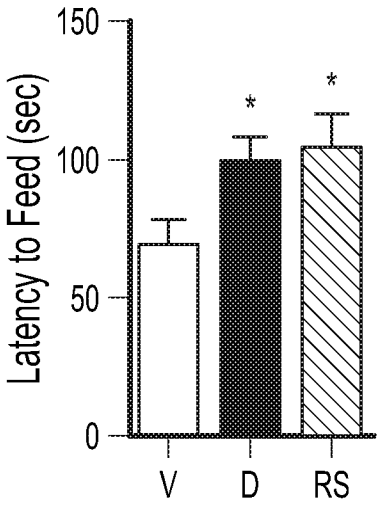


FIG. 15I

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/27321

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 13-21
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/27321

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 31/138; A61K 31/401; A61K 31/42 (2020.01)

CPC - A61K 31/445; A61K 31/428; A61K 2300/00; A61K 31/401

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2009/0170899 A1 (Debonnel et al.) 02 July 2009 (02.07.2009); abstract, para [0005], [0015]	1-12
Y	US 2018/0325844 A1 (The Trusttees of Columbia University in the City of New York) 15 November 2018 (15.11.2018); para [0002], [0012]-[0013], [0018]-[0019], [0021]-[0022], [0025]	1-12
A	US 2006/0116341 A1 (Compan) 01 June 2006 (01.06.2006); entire document	1-12
A	Mendez-David et al. "Rapid Anxiolytic Effects of a 5-HT4 Receptor Agonist Are Mediated by a Neurogenesis-Independent Mechanism" Neuropsychopharmacology. 24 June 2017 (24.06.2017) vol 39, pg. 1366-1378; entire document	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 June 2020	01 JUL 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300