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### ► To cite this version:

Charlène Faye, René Hen, Bruno P. Guiard, Christine A. Denny, Alain M. Gardier, et al.. 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. *Biological Psychiatry*, 2020, 87, pp.514 - 525. 10.1016/j.biopsych.2019.08.009 . hal-03489347

HAL Id: hal-03489347

<https://hal.science/hal-03489347v1>

Submitted on 7 Mar 2022

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**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**

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**Running title:** 5-HT<sub>4</sub> receptor contribution to fast anxiolytic response

**Keywords:** anxiety, 5-HT<sub>4</sub> receptor agonist, benzodiazepine, optogenetic, fast onset, prefrontal cortex, dorsal raphe nucleus

**Number of:**

**Word in abstract:** 242

**Words in main text:** 3,982

**Words in figure legends:** 1,116

**Figures in Main Text:** 5

**Tables in Main Text:** 0

**Words in Supplementary Text:** 2,255

**Supplemental figures:** 4

**Supplementary tables:** 4

## ABSTRACT

**Background:** Activating the serotonin (5-HT) 4 receptors (5-HT<sub>4</sub>Rs) 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<sub>4</sub>R 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<sub>4</sub>R agonist, RS67333 (0.5 µg/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 µg/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<sub>4</sub>R acutely in the mPFC or targeting mPFC pyramidal cell terminals in the DRN, may constitute a strategy to produce a fast-anxiolytic response.

## INTRODUCTION

Anxiety disorders are among the most common psychiatric disorders, with a lifetime prevalence of over 25 % (1) and an annual financial burden of more than \$40 billion (2). 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 (3). 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 key 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 (4), there is a need to develop novel fast-acting anxiolytics.

Findings have indicated that the serotonin 4 receptor (5-HT<sub>4</sub>R) may represent a new target for treating both depression and anxiety (5-9). The 5-HT<sub>4</sub>R is a G-protein coupled receptor (GPCR) that activates G<sub>s</sub> 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 (10). The majority of 5-HT<sub>4</sub>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 (11). In addition, 5-HT<sub>4</sub>Rs are also found in hypothalamus, ventral pallidum, olfactory bulbs, septal area, and substantia nigra. Mice lacking the 5-HT<sub>4</sub>R display anhedonia and a context-dependent anxiety-like behavior (12) and various 5-HT<sub>4</sub>R agonists can exert an antidepressant and anxiolytic-like activity (6). For instance, unlike SSRIs, treatment with RS67333, a 5-HT<sub>4</sub>R agonist, (13), induced fast

anxiolytic/antidepressant-like effects through a neurogenesis-independent mechanism (5). Although a number of studies have assessed the anxiolytic/antidepressant-like activity of 5-HT<sub>4</sub>R modulation after subchronic or chronic treatment, few have evaluated their anxiolytic-like profile acutely. Conflicting evidence suggests that 5-HT<sub>4</sub>R antagonists have acute anxiolytic-like effects (14, 15). Two reports showed an anxiolytic effect of 5-HT<sub>4</sub>R antagonists SB 204070, GR 113808 (15) and SB 207266A (14, 15) 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 (15). Similarly, a direct effect of the 5-HT<sub>4</sub>R antagonists on anxiety-like behavior in the light/dark choice test was not detected (16). The reasons for these discrepancies are unclear. In contrast, acute 5-HT<sub>4</sub>R 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 (17) and reversed the anxiogenic effects of chronic exposure to cannabinoids during adolescence (18).

Interestingly, approximately 60 % of pyramidal neurons recorded in the medial prefrontal cortex (mPFC) contain both the 5-HT<sub>4</sub>R transcript and protein. Activation of these somatodendritic 5-HT<sub>4</sub>R in the mPFC results in glutamate release in the DRN to stimulate the firing of 5-HT neurons (19). A large body of evidence also suggests that mPFC projections to the DRN modulate anxiety and depression-related behaviors (20-22). 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 (23), and inhibition of mPFC terminals targeting to the DRN induces a long-lasting suppression of anxiety-like behavior in socially stressed mice (24). Lastly, a recent study revealed a key role for DRN circuits in environment-specific adaptive behaviors (25). As a result, it is possible that

projection from the mPFC to DRN may mediate the anxiolytic effects of 5-HT<sub>4</sub>R 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<sub>A</sub> modulator diazepam in male BALB/cJrj anxious mice (26). 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**

Additional experimental procedures are available online in Supplemental Data

### **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).

## **RESULTS**



### **Acute systemic 5-HT<sub>4</sub>R stimulation induced fast anxiolytic-like effect.**

To assess putative fast anxiolytic 5-HT<sub>4</sub>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 1A). 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 1D and inset).

To assess the selectivity of RS67333-induced anxiolytic-like effects, we also tested whether the 5-HT<sub>4</sub>R 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 S1A). 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 S1B-S1C 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 1E-1F 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, Supplemental Figures 1D-1E 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) (Supplemental Figures 2A-C). We found that, unlike fluoxetine, acute RS67333 and diazepam increase the percent time spent in Center (one-way ANOVA, \* $p < 0.05$ , \*\* $p < 0.01$ , Supplemental Figures 2B) 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, Supplemental Figures 2C and inset). In summary, these data indicate that RS67333-induced fast anxiolytic-like effects through 5-HT<sub>4</sub>R activation.

### **Acute cortical 5-HT<sub>4</sub>R activation induces fast anxiolytic-like effects.**

Since 5-HT<sub>4</sub>R<sub>s</sub> are expressed in the mPFC (27), a brain region involved in the pathophysiology of mood disorders related to central 5-HT dysfunction (19, 28, 29), we examined the contribution of 5-HT<sub>4</sub>R activation in the mPFC to fast anxiolytic-like activity (Figure 1B). 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 1G 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 1H). 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 1I-1J and inset) confirming the anxiolytic-like effects of 5-HT<sub>4</sub>R 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 2A). 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 RS67333, Figures 2B-2C).

To further confirm that anxiolytic-like effects of mPFC 5-HT<sub>4</sub>R 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 2D). 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 2E). 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 groupe, Figures 2F-2G). p-CPA –induced 5-HT depletion did not affect locomotor activity (Inset Figure 2G). These results point out the critical role of the 5-HT neurotransmission in fast anxiolytic-like effects. In summary, RS67333, as suggested previously (29), and diazepam act on 5-HT function through a modulation of the mPFC.

## **Fast anxiolytic-like effects of 5-HT<sub>4</sub>R agonist recruit medial prefrontal cortex-brainstem neural circuit.**

While emotional behaviors are mediated by mPFC pyramidal neurons projecting to the DRN (22), 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 a AAV5-CaMKII $\alpha$ -virus, that specifically expresses ChR2 in mPFC pyramidal cell terminals in the DRN (Figures 3A-3B). AAV5-CaMKII $\alpha$ -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 ## $p < 0.01$  vs. CaMKII $\alpha$ -ChR2-eYFP or CaMKII-eYFP respectively during light ON, Figures 3C 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 ## $p < 0.01$  vs. CaMKII $\alpha$ -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<sub>4</sub>R 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

(Supplemental Figures 3A-C). 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, Supplemental Figures 3B 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  $##p < 0.01$  vs. CaMKII $\alpha$ -ChR2-eYFP or CaMKII-eYFP during light ON, Supplemental Figure 3C and inset).

Next, we probed the effects of optogenetic inhibition of mPFC projections to the DRN after cortical infusion of RS67333 or diazepam (Figure 4A). 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 4B). In the EPM, RS67333 (0.5  $\mu$ g/side) and diazepam (1.5  $\mu$ g/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  $\pm$  8 % and 85  $\pm$  5 % of inhibition for RS67333 and diazepam respectively, two-way ANOVA,  $**p < 0.01$  or  $##p < 0.01$  vs. CaMKII $\alpha$ -ArchT or CaMKII-GFP respectively during light ON for appropriate treatment Figures 4C 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. CaMKII $\alpha$ -ArchT or  $\#p < 0.05$  CaMKII-GFP respectively during light OFF for appropriate treatment, Figure 4D). No changes in ambulatory distance were observed during light OFF or light ON (Figures 4D

and inset). These results were also confirmed in the OF (Supplemental Figure 4A-C). 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. CaMKII $\alpha$ -ArchT or CaMKII-GFP during light ON, Supplemental Figures 4B-4C 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 4A-4B). 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 4E-4F 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 4E-4F 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 4E-4F and inset). Overall, these data suggest that the mPFC terminals in the DRN are recruited for fast anxiolytic effects of RS67333 and diazepam.

## **DISCUSSION**

### **Acute 5-HT<sub>4</sub>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<sub>4</sub>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 (30). These data also suggest that rapid anxiolytic-like activity requires selective activation of some key postsynaptic receptors such as the 5-HT<sub>4</sub>R or the 5-HT<sub>1A</sub>R (31) more than a global increase in 5-HT neurotransmission.

### **Involvement of mPFC-DRN circuit for the 5-HT<sub>4</sub>R activation-mediated rapid anxiolytic-like activity.**

In both humans and also in rodents, 5-HT<sub>4</sub>R are mainly localized in limbic areas involved in psychiatric disorders, such as anxiety (27, 32). We explored the role of the 5-HT<sub>4</sub>R activation expressed in mPFC in fast anxiolytic-like effects. Indeed, 5-HT<sub>4</sub>R is expressed in excitatory pyramidal neurons of the mPFC, a region showing glutamate

dysregulation in patients with generalized anxiety disorders (33, 34). Interestingly, fast anxiolytic-like effects observed after acute systemic administration of RS67333 were reproduced by an acute infusion of this 5-HT<sub>4</sub>R 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<sub>4</sub>R overexpression in the mPFC yields a robust anxiolytic-like behavioral phenotype (7, 19).

Anatomical studies have shown that the prelimbic/cingulate cortices also project abundantly to DRN 5-HT neurons (35). This connectivity has attracted great interest as a potential circuit involved in modulating stress and depressive behaviors (22). 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 (36). There is also evidence that 5-HT<sub>4</sub>R activation in the mPFC controls the firing rate of midbrain serotonergic neurons via descending inputs (19, 29, 37). A reduction in the spontaneous activity of 5-HT neurons and a decrease in 5-HT content in the DRN of 5-HT<sub>4</sub>R-null mice were observed (38). Conversely, administration of RS67333 in rat, at different time points, drives effects on DRN 5-HT neuronal activity (19, 29, 39), and increase 5-HT release at projection sites (40). 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<sub>4</sub>R agonist is dependent on activation of this neuronal population, despite the fact that DRN does not express 5-HT<sub>4</sub>R (27). In fact, the fast onset of action of the 5-HT<sub>4</sub>R agonist could be a consequence of an increase in serotonergic output to projection areas including the mPFC (19, 41). 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 (42).

Interestingly, despite different pharmacological targets, the 5-HT<sub>4</sub>R 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 (35), 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 (43). 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 (43). In SD mice, chronic photoactivation of mPFC pyramidal cells increased the time spent in the open arms in the EPM (23), whereas stimulation had no effect on anxiety-related behavior in non-stressed animals (23, 44). Conversely, photosilencing mPFC terminals in the DRN prevented a

decrease in social interaction in SD mice, suggesting a contributing role in anxiety-like behavior (24).

To ensure that the behavioral response to local 5-HT<sub>4</sub>R agonist infusion is in line with the idea that glutamatergic mPFC pyramidal neurons are mediators of 5-HT<sub>4</sub>R 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 (7). Interestingly, in line with our results, a recent study show that rescuing the mPFC-5-HT<sub>4</sub>R expression in 5-HT<sub>4</sub>R KO mice partly reduced stress levels (28). Although these results were not surprising for the 5-HT<sub>4</sub>R agonist, they were unexpected for diazepam. However, cortical GABA<sub>A</sub> receptor activation through intra-mPFC muscimol infusion (a direct agonist of GABA<sub>A</sub> receptor) has been proposed to attenuate anxiety-related behavior in adult Wistar rats (45). These findings indicate that despite different pharmacological targets, 5-HT<sub>4</sub>R 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 (35) suggesting that GABA interneurons influence may be secondary (37).

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<sub>4</sub>R 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<sub>4</sub>R 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 (47), basolateral amygdala to ventral hippocampus (48) or DRN to bed nucleus of the stria terminalis (31) inputs (Figure 5). For instance, RS67333 administration into the basolateral amygdala, hippocampus, or nucleus basalis magnocellularis modulated also emotional memory formation and consolidation (49-51), suggesting that the anxiolytic-like effect of 5-HT<sub>4</sub>R 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<sub>A</sub> receptor and the 5-HT<sub>4</sub>R, 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<sub>4</sub>R 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<sub>4</sub>R (16).

Taken together, our study reveals the importance the mPFC-DRN circuit in mediating the fast anxiolytic-like effects of both 5-HT<sub>4</sub>R agonists and BZD. Stimulating the 5-HT<sub>4</sub>R 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<sub>4</sub>R agonists as a fast-acting anxiolytics may be hampered by the fact that the 5-HT<sub>4</sub>R are expressed outside the central nervous system in the heart, gastrointestinal tract, adrenal gland, and urinary bladder (52). It may be worth identifying other components of the mPFC-DRN circuits that are more specific and amenable to drug development.

## **Acknowledgements**

We thank K. Deisseroth and E. Boyden for AAV5-CaMKII-hChR2(H134R)-EYFP, AAV5-CaMKII-ArchT-GFP or AAV5-CaMKII-eYFP, M. Kheirbeck and J. McGowan for comments on previous drafts. We also thank L. Tritschler, C. Defaix, T.H. Pham and V. Domergue and the staff of the animal care facility of the SFR-UMS Institut Paris-Saclay Innovation Thérapeutique for their technical support. This work was supported by a National Alliance for Research on Schizophrenia and Depression (NARSAD) 2017 Young Investigator award from Brain & Behavior Research Foundation and Deniker Foundation (I.M.D).

## **Contribution**

C.F, C.A.D, B.P.G, R.H, A.M.G, I.M.D, D.J.D conceived, planned, oversaw the study. C.F, B.P.G, I.M.D, D.J.D performed the experiments. C.F, D.J.D analyzed the data. R.H, I.M.D, D.J.D wrote the paper. CAD, B.P.G, R.H, A.M.G assisted in revising the manuscript. I.M.D and DJD obtained funding and resource used in the experiments.

## **Disclosures**

DJD serves as a consultant for Lundbeck Inc. DJD receives compensation from Lundbeck and Janssen. DJD, IMD, CF, AMG, CAD are named on non-provisional patent applications for the prophylactic use of RS67333 against stress-related psychiatric disorders. RH receives compensation as a consultant for Roche, Lundbeck and Servier in relation to the generation of novel antidepressants. CAD is named on non-provisional

patent applications for the prophylactic use of ketamine against stress-related psychiatric disorders. BPG reports no biomedical financial interests or potential conflicts of interest.

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## Figure Legends

### **Figure 1. Acute 5-HT<sub>4</sub> receptor stimulation induces fast anxiolytic-like effects in an anxious BALB/cJRj mouse strain.**

(A, B) Experimental design. 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 i.p., 45 minutes before behavioral testing (A). 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 i.p., 45 minutes before the start of behavioral paradigms (B). (C-D and G-H) Anxiolytic-like effect was measured in the elevated plus maze (EPM) as mean time or percent time spent in open arms (C, G and inset), or as mean ratio ambulatory distance in open arms/total distance (D, H) 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). (E-F and I-J) In the Novelty Suppressed Feeding (NSF) paradigm, anxiolytic-like effect is expressed as fraction of animals that have not eaten over 10 minutes (E, I), as mean of the food consumption (inset) or as mean of latency to feed (F, J) (n = 10 mice per group, n = 4-8 mice per group). Values plotted are mean ± SEM. \*p<0.05, \*\*p<0.01 vs. vehicle group.

### **Figure 2. Acute 5-HT<sub>4</sub> receptor stimulation induces fast anxiolytic-like effects on 5-HT function through a modulation of the mPFC in an anxious BALB/cJRj mouse strain.**

(A) 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. (B) 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  $\pm$  frequency (Hz) of DRN 5-HT neurons determined before the administration of RS67333. (C) 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. (D) p-CPA was injected i.p. twice a day during 3 days and treatments [vehicle (V), diazepam (D,  $1.5 \mu\text{g}/\text{side}$ ) and RS ( $0.5 \mu\text{g}/\text{side}$ )] 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. (E) Cortical 5-HT depletion by p-CPA pre-treatment is measured as mean 5-HT levels ( $n = 6-8$  mice per group). (F and G) 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 (G) and as mean total ambulatory distance (inset) ( $n = 8-11$  mice per group). Values plotted are mean  $\pm$  SEM.  $*p < 0.05$ ,  $**p < 0.01$  vs. vehicle group,  $\#p < 0.05$ ,  $\#\#p < 0.01$  vs. appropriate group.

**Figure 3. Effects of cortical terminals stimulation in the dorsal raphe nucleus of anxious BALB/cJRj mouse strain.**

(A) Timeline regarding the behavioral consequences after stimulation of glutamatergic terminals in the DRN. AAV5-CamKII $\alpha$ -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). (B) Expression of virus was confirmed in the mPFC (left) and in the DRN (right). (C-E) 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 (C 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 (D) and as mean total ambulatory distance (inset) or (eYFP: n = 12 mice per group; Chr2: n = 19 mice per group). Values plotted are mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  between laser ON and laser OFF; # $p < 0.05$ , ## $p < 0.01$  between CHR2 and eYFP group.

**Figure 4. Modulation of anxiolytic-like activity following optogenetic inhibition of glutamatergic terminals in the DRN of anxious BALB/cJRj mouse strain.**

(A) Timeline regarding the behavioral consequences after inhibition of glutamatergic terminals in the dorsal raphe nucleus (DRN) after medial prefrontal cortex (mPFC) infusion [Diazepam (D, 1.5  $\mu$ g/side) or RS67333 (RS, 0.5  $\mu$ g/side)] or systemic administration of diazepam (D, 1.5 mg/kg, i.p), RS67333 (RS, 1.5 mg/kg, i.p) 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, one week before testing. For the local injection protocol, two injection cannulae were also implanted in the mPFC. Drug treatment were infused in mPFC or injected i.p, 45 min before testing. (B) Expression of control CaMKII-ArchT-GFP virus was confirmed in the mPFC (left) and in the DRN (right). (C-J) For the behavioral consequences of a local infusion or systemic administration with vehicle, diazepam or

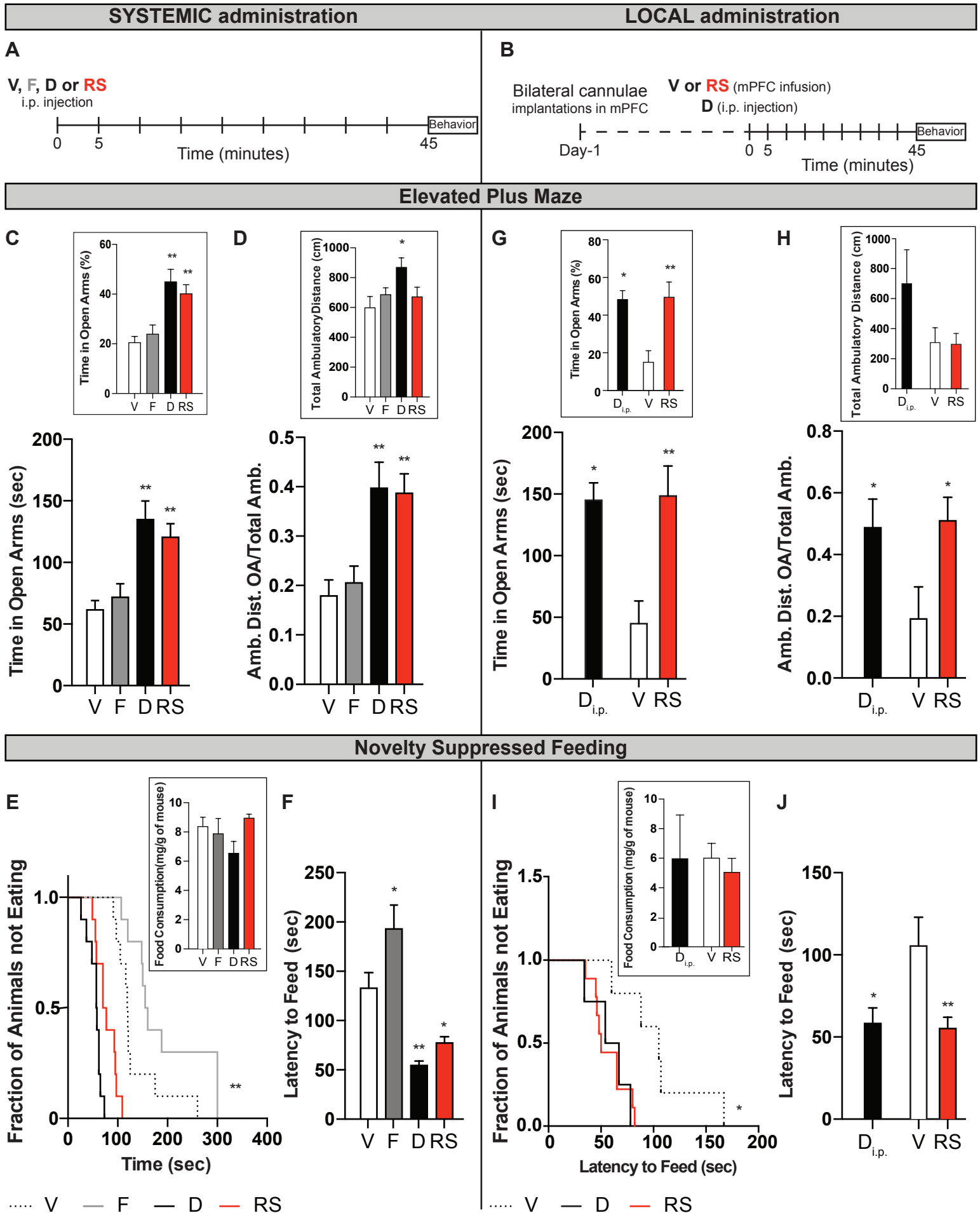


RS67333, anxiolytic-like effect is measured in the elevated plus maze (EPM) as time or percent time spent in the open arms across 2 epoch stimulation (3-min OFF and 3-min ON) (C or G and inset), the distribution of time spent in open arms prior to and following cortical terminals inhibition in the dorsal raphe nucleus (inset), as mean time in open arms (OA)/total time (inset), as mean time spent in the open arms (D or H), as mean total ambulatory distance (E-I) or as mean ratio of ambulatory distance in the open arms divided by total distance (F or J) (n = 7-11 mice per group and n = 7-9 mice per group for local and systemic administration respectively). Values plotted are mean  $\pm$  SEM. \*p<0.05, \*\*p<0.01 vs. vehicle group, #p<0.05, ##p<0.01 vs. appropriate group during light OFF.

**Figure 5. Cartoon summarizing the neuronal circuits involved in fast anxiolytic-like effects induced by acute systemic RS67333 and diazepam administration.**

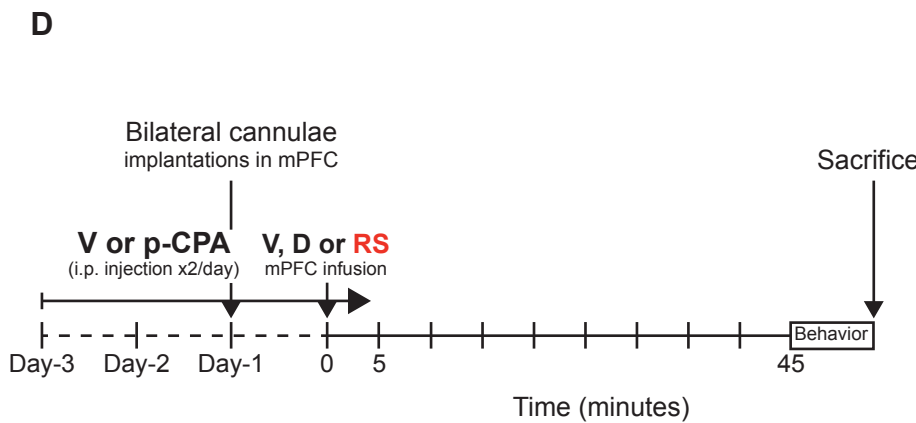
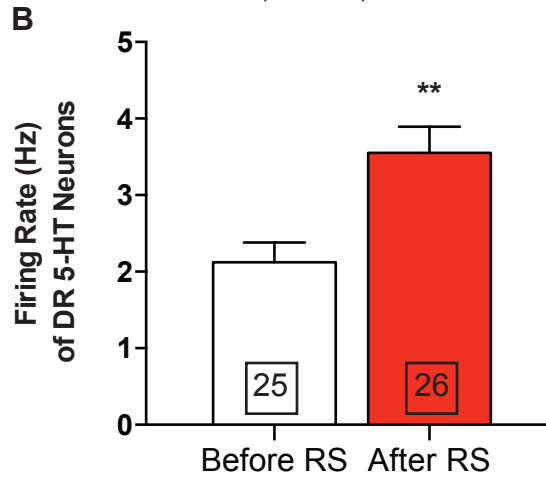
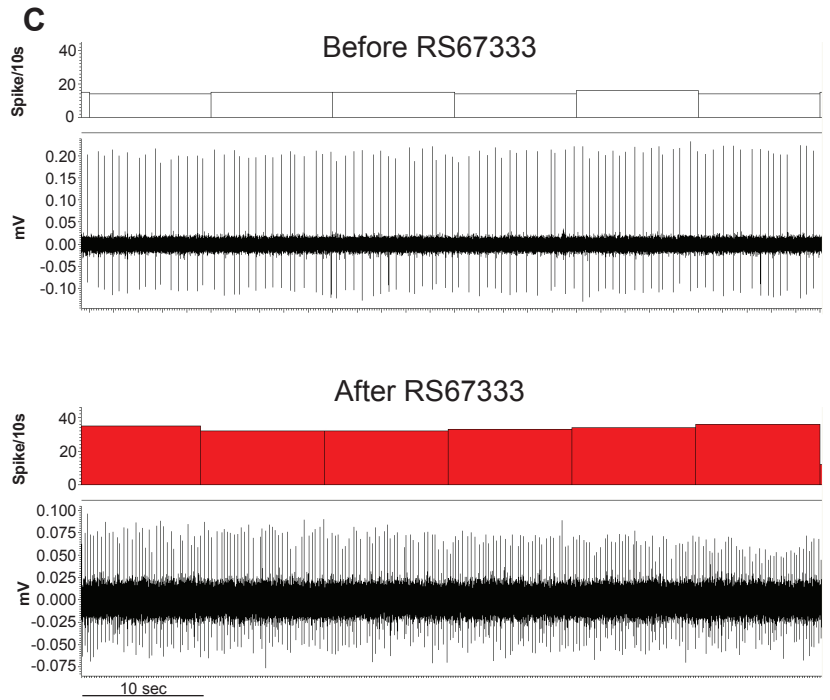
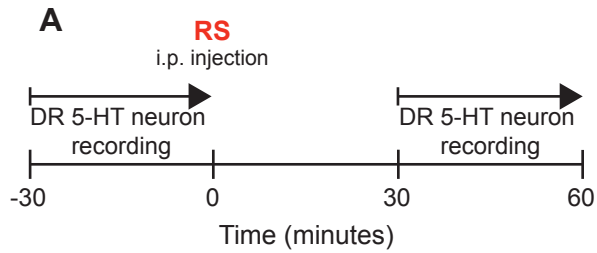
Acute systemic administration with RS67333, a serotonin type 4 receptor agonist, induces fast anxiolytic-like effects in BALB/cJRj mice through at least activation of the cortical glutamatergic terminals (Glu) in the dorsal raphe nucleus (DRN) confirming previous studies (19). Similarly, diazepam, a benzodiazepine, 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 5-HT<sub>4</sub>R agonist. Previous studies demonstrated that hippocampus (HPC)/medial prefrontal cortex (mPFC) (46) circuit and also the mPFC/amygdala (Amy) pathway (48) are also recruited to modulate anxiety-like phenotype suggesting that these circuit should be also investigated for 5-HT<sub>4</sub>R induced fast anxiolytic-like effects.

Figure 1

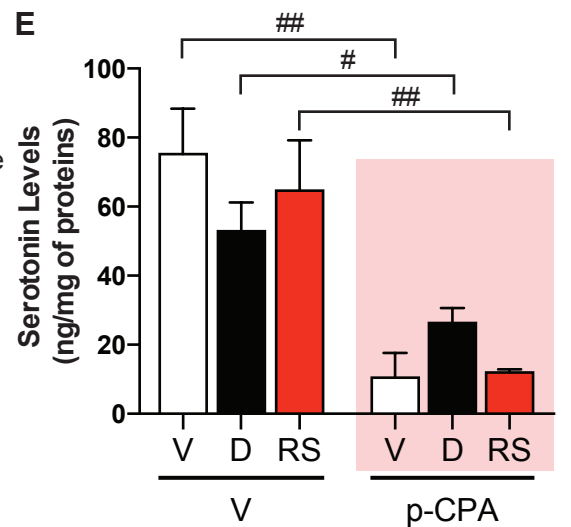


**Figure 2**

**Electrophysiological Recording**



**Cortical Serotonin Levels**



**Elevated Plus Maze**

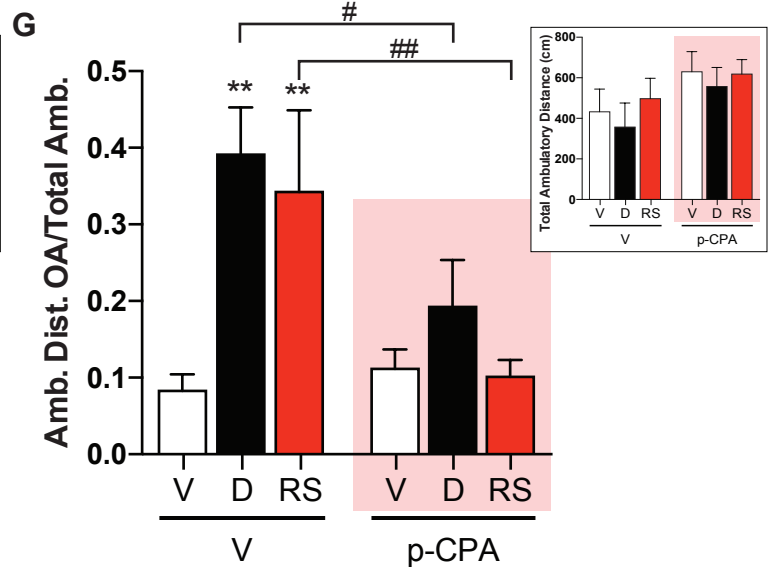
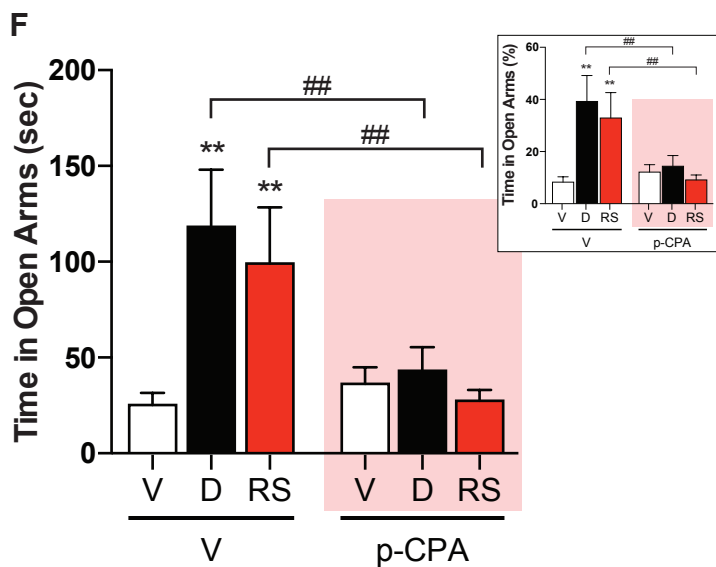
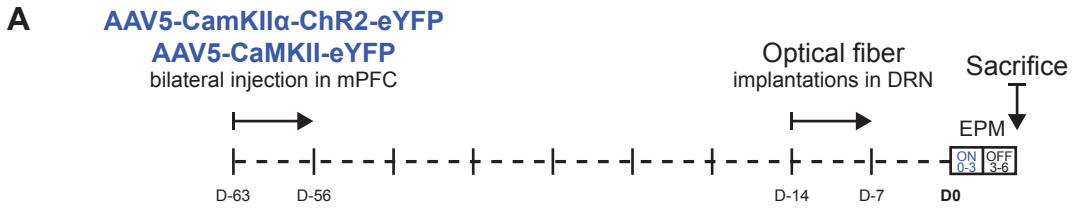
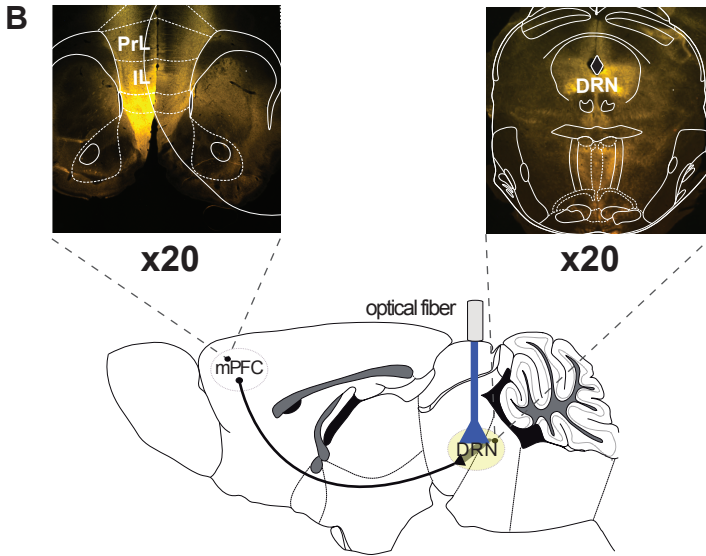


Figure 3

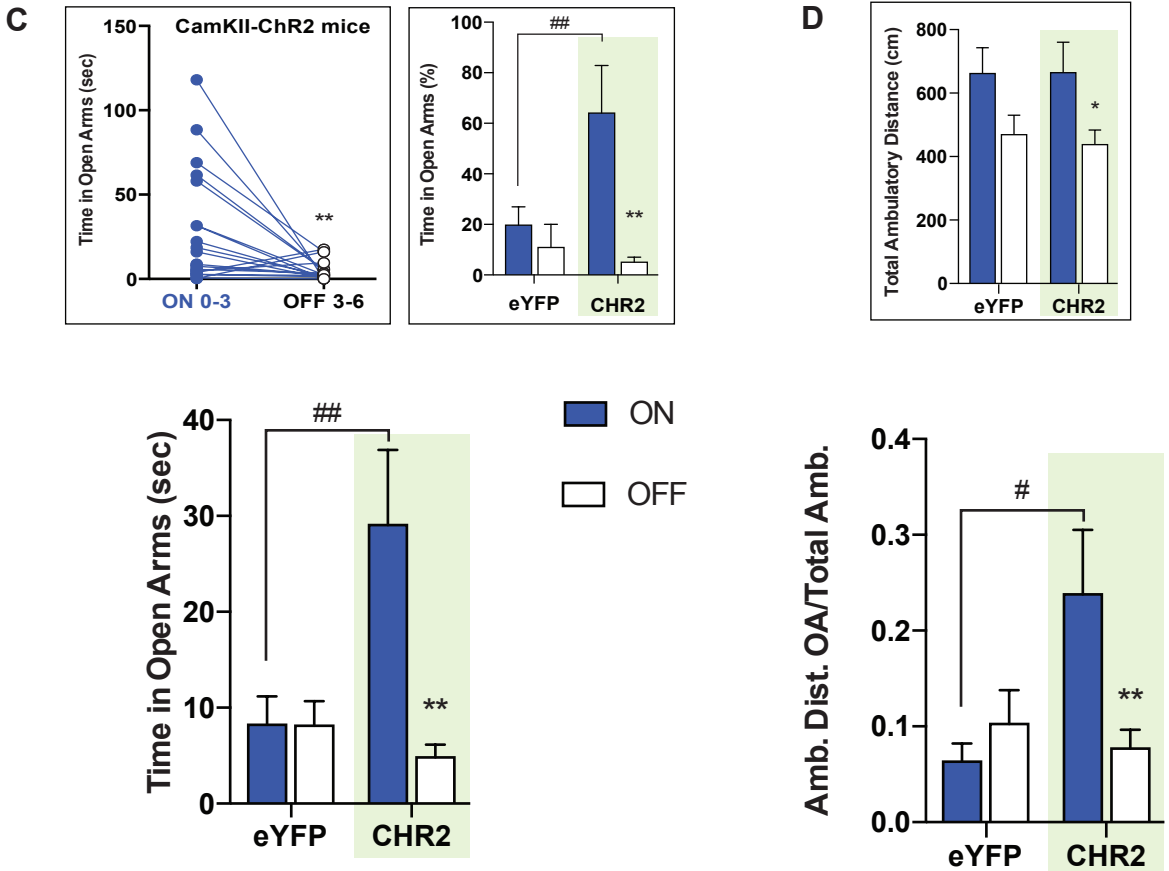
Stimulation



Immunohistochemistry



Elevated Plus Maze



**Figure 4**

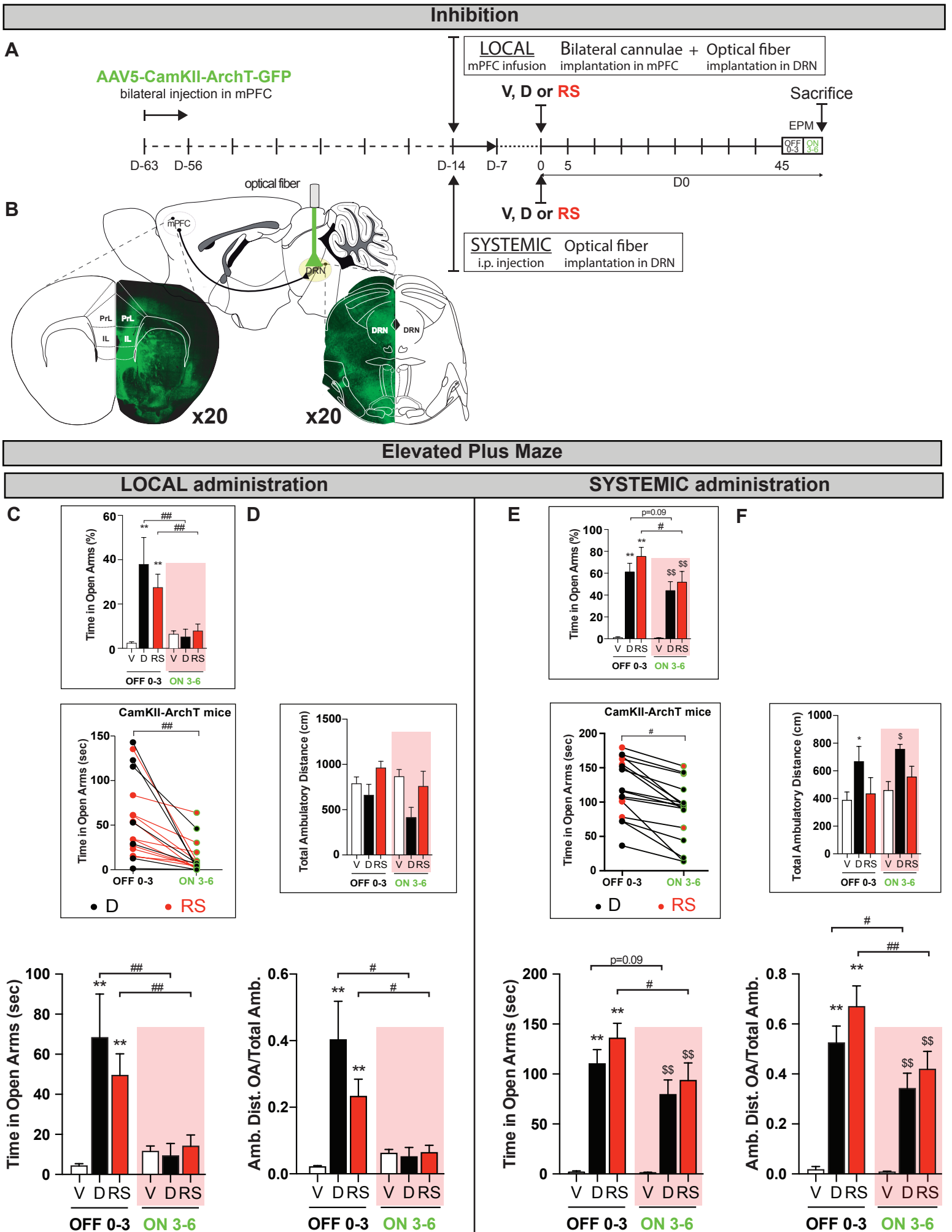


Figure 5

- 5-HT<sub>4</sub> Receptor
- RS67333
- ⌋ GABA Receptor with BZD site
- Diazepam

