

# Somatostatin Receptor 4 Agonism Normalizes Stress-Related Excessive Amygdala Glutamate Release and Pavlovian Aversion Learning and Memory in Rodents

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## ABSTRACT

**BACKGROUND:** Excessive processing of aversive life events is a major pathology in stress-related anxiety and depressive disorders. Current pharmacological treatments have rather nonspecific mechanisms of action. Somatostatin is synthesized and released as an inhibitory co-neurotransmitter by specific GABA (gamma-aminobutyric acid) interneurons, and one of its receptors, SSTR4 (somatostatin receptor 4), is localized in brain regions involved in adaptive aversion processing and implicated in negative valence neuropathology, including the amygdala.

**METHODS:** Rat and mouse experiments were conducted to investigate effects of specific SSTR4 agonism on neurobehavioral aversion processing, including any normalization of stress-related hyperresponsiveness. A mouse experiment to investigate stress and SSTR4 agonism effects on reward processing was also conducted.

**RESULTS:** In male rats ( $n = 5\text{--}10/\text{group}$ ) fitted with glutamate biosensors in basolateral amygdala, SSTR4 agonism attenuated glutamate release to restraint stress in control rats and particularly in rats previously exposed to chronic corticosterone. In male mice ( $n = 10\text{--}18/\text{group}$ ), SSTR4 agonism dose-dependently attenuated Pavlovian tone/footshock learning and memory measured as freezing behavior, in both control mice and mice exposed to chronic social stress, which induces excessive Pavlovian aversion learning and memory. Specificity of SSTR4 agonism effects to aversion learning/memory was demonstrated by absence of effects on discriminative reward (sucrose) learning/memory in both control mice and mice exposed to chronic social stress; SSTR4 agonism did increase reward-to-effort valuation in a dose-dependent manner and in both control mice and mice exposed to chronic social stress, which attenuates reward motivation.

**CONCLUSIONS:** These neuropsychopharmacological findings add substantially to the preclinical proof-of-concept evidence for SSTR4 agonism as a treatment in anxiety and depressive disorders.

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GABA (gamma-aminobutyric acid) (inter)neurons are essential for adaptive processing of aversion and reward stimuli in corticolimbic circuits. Different subclasses of GABA interneurons can be identified with respect to the marker proteins they synthesize, one of which is somatostatin (SST). SST GABA interneurons release GABA and SST as inhibitory neurotransmitters. The latter has 5 postsynaptic G protein-coupled receptors, SST receptors 1 through 5 (SSTR1-5). SST receptor binding results in signal transduction via the  $G_{\alpha_i}$  or  $G_{\alpha_o}$  proteins, inhibition of adenylate cyclase, and modulation of mitogen-activated protein kinases (1). SST GABA interneurons are major mediators of aversion processing. In mice, knockout of the *Sst* gene led to increases in novelty suppression of feeding and basal levels of plasma corticosterone (CORT) (2), consistent with involvement of SST GABA interneurons in adaptive inhibition of glutamate neurons in the

neural (micro)circuitry of aversion processing (3). SST GABA interneuron involvement in the pathophysiology of stress-related neuropsychiatric disorders has also been proposed (1); for example, there is human postmortem evidence for decreased *SST* messenger RNA expression in corticolimbic regions in major depressive disorder (4–6).

Concerning individual postsynaptic SST receptors, mouse *Sstr4*/SSTR4 expression is high in regions integral to corticolimbic circuitry of aversion processing, most notably in glutamatergic neurons in prelimbic cortex, hippocampus, habenula, somatosensory cortex, and amygdala; human *SSTR4* is expressed in cortical regions and, in particular, amygdala (7,8). Relative to wild-type (WT) mice, *Sstr4* knockout mice displayed decreased open-arm time in an elevated plus maze anxiety test and increased time immobile in a forced swim test (FST). While *Sstr4*<sup>-/-</sup> mice did not display altered immobility in a tail

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suspension test (TST) (the FST and TST are screening tests for certain antidepressants), the TST did lead to more Fos-expressing cells in amygdala in knockout mice relative to wild-type mice (8). Also, *Sstr4*<sup>-/-</sup> mice were more sensitive than wild-type mice to chronic variable stress in terms of lower body weight and increased immobility in the TST, although chronic variable stress normalized immobility in the FST in *Sstr4*<sup>-/-</sup> mice (9). Systemic administration of an SSTR4 agonist (J-2156) reduced immobility in the TST and increased time spent on open arms in the elevated plus maze (8). SSTR4 agonism also increased the number of TST-stimulated Fos-expressing cells in various brain regions, including the basolateral and central amygdala (8). Intrahippocampal injection of an SSTR4 agonist (L-803,087) led to reduced immobility in the FST and attenuated footshock-induced increases in CORT levels in plasma and hippocampus (10).

In the present study, our overall aim was to investigate the effects of a selective SSTR4 agonist on neurobiological and behavioral measures of aversion processing in the basal state and, particularly, states related to or of chronic stress. In rats, using glutamate biosensors, we investigated whether SSTR4 agonism attenuates the increase in basolateral amygdala glutamate release induced by restraint stress (RS) in control rats and in rats after chronic CORT exposure. In mice, we investigated whether SSTR4 agonism attenuates Pavlovian aversion learning and memory (PALM), measured as freezing in control mice and, furthermore, normalizes excessive PALM in mice after exposure to chronic social stress (CSS).

## METHODS AND MATERIALS

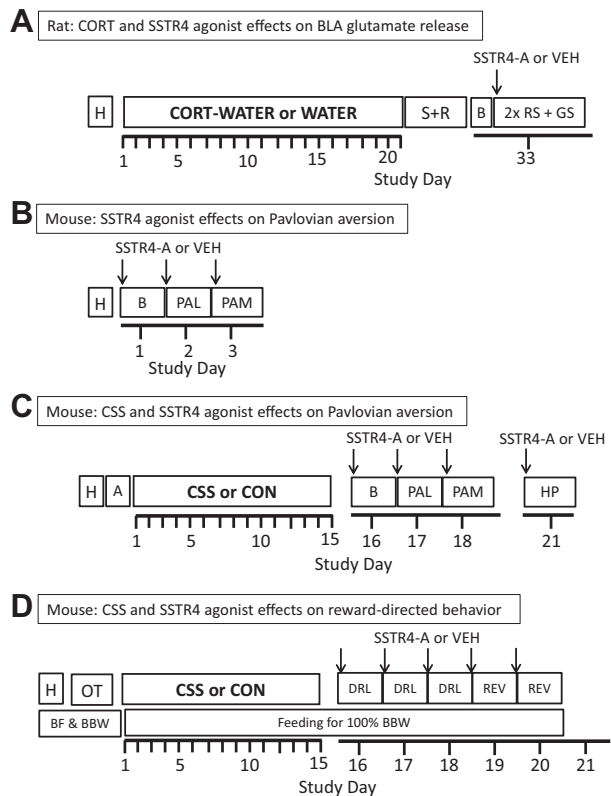
For a complete description of the animals and methods, see [Supplemental Methods and Materials](#).

### Compound

In vitro, the novel SSTR4 agonist used had efficacy at SSTR4 in the nanomolar range and was highly selective in terms of binding to and efficacy at SSTR4 compared with the other SST receptor subtypes and a panel of 44 central nervous system ion channels, receptors, and enzymes.

### Rat Chronic CORT Administration and Basolateral Amygdala Glutamate Release

Chronic CORT exposure in rodents produces long-term neurobehavioral changes relevant to stress-related neuropsychiatric disorders (11,12). Adult male Wistar Han rats were administered CORT via drinking water for 21 days or received normal water (Figure 1A). They were then implanted with a guide cannula over the basolateral amygdala (BLA) for subsequent insertion of a glutamate biosensor. There was a 12-day interval between cessation of CORT exposure and testing BLA glutamate release. The baseline glutamate signal was collected for 30 minutes followed by administration of vehicle (VEH) (2 mL/kg subcutaneous [sc]) or SSTR4 agonist (3 or 10 mg/2 mL/kg sc). Starting 30 minutes later, two episodes of RS (RS I, RS II) were conducted, separated by 120 minutes. Glutamate biosensor recording was conducted during RS and in the home cage between and after RS.



**Figure 1.** Overview of the designs used in the experiments with in vivo readouts. In all experiments, the first procedure was several days of handling. **(A)** Effects of CORT and SSTR4 agonist on BLA glutamate release in rats. Rats received CORT in their drinking water or drinking water only for 21 days, followed by surgical implantation of a guide cannula above the basolateral amygdala to accommodate a glutamate biosensor and 1-week recovery. One day before testing, the glutamate biosensor was inserted. On the test day, baseline recording of GS was followed by SSTR4 agonist injection (VEH, 3, 10 mg/kg subcutaneous), a 5-minute period of RS (RS I), GS recording (RS + GS), another 5-minute period of RS (RS II), and further GS recording. **(B)** Effects of SSTR4 agonist on Pavlovian aversion learning and memory in otherwise naïve mice. Mice received daily SSTR4 agonist (VEH, 10, 30 mg/kg) followed by baseline testing in the context without stimuli (baseline, day 1), PAL of the association between tone and footshock (0.25 mA) (PAL, day 2), and successive PAM tests of context and tone (PAM, day 3). Freezing was the main dependent variable. **(C)** Effects of CSS and SSTR4 agonist on Pavlovian aversion learning and memory in mice. Mice were placed in the Pavlovian aversion learning and memory arena for an activity test, and activity was used to counterbalance mouse group × dose allocation. Mice underwent CSS or CON on days 1–15 and then received daily SSTR4 agonist (CON: VEH, 10 mg/kg; CSS: VEH, 10, 30 mg/kg) followed by baseline testing in the context without stimuli (baseline, day 16), PAL of the association between tone and footshock (0.15 mA) (PAL, day 17), and successive PAM tests of context and tone (PAM, day 18). On day 21, mice received SSTR4 agonist/VEH followed by HP test. **(D)** Effects of CSS and SSTR4 agonist on reward-directed behavior in mice. Daily BF consumption and BBW were measured across 1 week. Mice were then food restricted for OT with sucrose pellet reinforcement. Mice underwent CSS or CON on days 1–15 and then received daily SSTR4 agonist (VEH, 10, 30 mg/kg) followed by DRL on days 16–18 and REV on days 19–20, with chocolate-flavored sucrose pellets as reward. A, activity; B, baseline; BBW, baseline body weight; BF, baseline food; BLA, basolateral amygdala; CON, control holding; CORT, corticosterone; CSS, chronic social stress; DRL, discrimination reward learning; GS, glutamate signal; H,

### Mouse CSS and Aversion- and Reward-Directed Behavior

In otherwise naïve C57BL/6 adult male mice, effects of SSTR4 agonist (VEH, 10 or 30 mg/10 mL/kg sc) were investigated in PALM, measured as percentage of time spent in freezing behavior (Figure 1B) (13). Mice were assigned at random to dose and received this dose each test day at 30 minutes before testing, as follows: day 1, baseline freezing; day 2, acquisition of tone/footshock (conditioned stimulus [CS]/unconditioned stimulus [US]) freezing; day 3, expression of freezing to context and to tone CS. Footshock intensity of 0.25 mA was selected to achieve freezing scores of 50% to 60% time in the CS-memory expression test in VEH mice [e.g. (13)] and thereby provide a sufficient window for detection of any anxiolytic effect of SSTR4 agonist. In subsequent experiments, naïve adult males underwent either 15-day control handling (CON) or CSS. In one experiment, SSTR4 agonist effects on CSS-induced excessive PALM were investigated (Figure 1C) (14,15). After CSS/CON, mice were assigned to VEH (10 mL/kg sc) or SSTR4 agonist (10 or 30 mg/10 mL/kg sc) and received the same dose on each test day at 30 minutes before testing: day 16, baseline freezing; day 17, acquisition of CS/US freezing; day 18, expression of freezing to context and to tone CS. In the CSS/PALM model [e.g. (15,16)], footshock intensity of 0.15 mA is used because it maximizes the absolute difference in percentage of time spent freezing in the CS expression test between CSS (50%–60%) and CON (30%) in mice. It thereby provided an optimal window to detect potential SSTR4 agonist reversal of CSS-induced excessive PALM. Using higher-intensity footshock, e.g., 0.25 mA, reduces CSS/CON absolute difference and therefore the sensitivity of the model for detecting CSS reversal effects. On day 21, a hot plate test was conducted to assess effects on pain sensitivity.

In an experiment with different mice, SSTR4 agonist effects on CSS-induced attenuated discriminative reward learning and memory (DRLM) and reward-to-effort valuation (REV) were investigated using sucrose-pellet reward (17–19) (Figure 1D). Mice were assigned to VEH or SSTR4 agonist (10, 30 mg/kg sc) and received the same dose on each test day (days 16–20) at 30 minutes before testing. Mice were tested at 100% baseline body weight, maintained under mild food restriction, and normal diet was available in the test chamber as a low-effort/low-reward choice. In the DRLM test, conducted on days 16–18, a tone discriminative stimulus (DS) indicated that a nosepoke response into the feeder port led to delivery of sucrose, whereas nosepoking in inter-DS intervals did not. Reduction in DS response time relative to responding during inter-DS intervals indicates DS-reward learning and memory. In the REV test, an operant nosepoke stimulus was introduced, and mice could respond on a progressive ratio reinforcement schedule to obtain sucrose. Mice underwent a training REV test on day 19 and the actual REV test on day 20.

← handling; HP, hotplate; OT, operant training; PAL, Pavlovian aversion learning; PAM, Pavlovian aversion memory; R, recovery; REV, reward effort valuation; RS, restraint stress; S, surgical implantation; SSTR4, somatostatin receptor 4; VEH, vehicle.

### Mouse CSS and Amygdala Expression of *Sst* and *Sstr4* Genes and SST

In a separate cohort, mice underwent CSS/CON, and on day 16 brains were collected, amygdala total RNA was extracted, and reverse transcription was conducted. With polymerase chain reaction primer pairs for *Sst* and *Sstr4* complementary DNAs, quantitative reverse transcription polymerase chain reaction was conducted together with *Actb* as endogenous control. In a further cohort, mice underwent CSS/CON, with perfusion fixation of brains done on day 16 for immunofluorescence staining of SST in sections including lateral amygdala and BLA, followed by confocal imaging and quantification of SST-positive cell density.

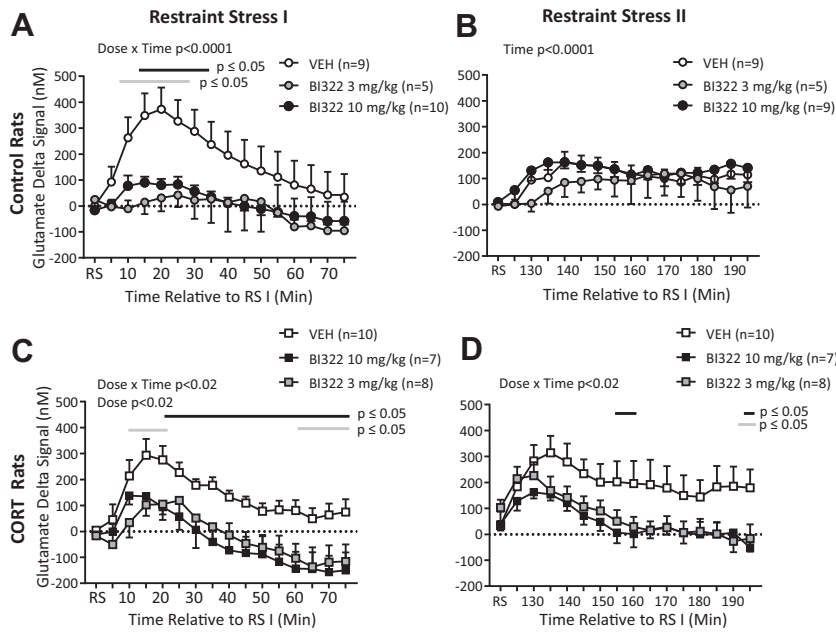
## RESULTS

### SSTR4 Agonist Normalizes CORT-Induced Excessive BLA Glutamate Release in Rats

Rats that underwent chronic CORT exposure had higher fecal CORT levels than control rats, followed by immediate return to basal levels (Figure S1A). To investigate effects of group (control, CORT) and restraint stressor (RS I, RS II) on BLA extracellular glutamate levels relative to baseline during the 75-minute period after each RS episode, a first analysis was conducted with VEH rats (Figure S1B). There was a main effect of time (5-minute bins) ( $F_{15,527} = 11.56, p < .0001$ ), with post hoc testing identifying that glutamate was relatively high at 15 to 30 minutes after RS I and RS II. There was an RS  $\times$  time interaction effect ( $F_{15,527} = 2.12, p < .008$ ), with the increase in glutamate being less at 15 to 30 minutes after RS II than after RS I. Furthermore, the effect of RS repetition was dependent on group (group  $\times$  RS interaction effect:  $F_{1,527} = 27.29, p < .0001$ ): while control rats responded less to RS II than RS I ( $p < .001$ ), mean glutamate release in CORT rats was higher at RS II than RS I ( $p < .001$ ).

SSTR4 agonist effects on BLA glutamate were analyzed separately for each group  $\times$  RS (Figure 2). In control rats at RS I (Figure 2A), there was a dose  $\times$  time interaction effect ( $F_{30,315} = 2.16, p < .0001$ ): relative to VEH rats, rats that received SSTR4 agonist at 3 mg/kg had lower glutamate at 10 to 30 minutes after RS I ( $p \leq .05$ ), and rats that received 10 mg/kg had lower glutamate at 15 to 35 minutes after RS I ( $p \leq .05$ ). At RS II (Figure 2B), control rats in each dose group displayed a modest and similar increase in glutamate after RS (time main effect:  $F_{15,300} = 4.08, p < .0001$ ) without any SSTR4 agonist effect ( $p \geq .55$ ). In CORT rats at RS I (Figure 2C), there was a dose  $\times$  time interaction effect ( $F_{30,330} = 1.65, p < .02$ ) and a main effect of dose ( $F_{2,22} = 5.28, p < .02$ ): relative to VEH rats, rats at 3 mg/kg had lower glutamate at 10 to 20 minutes and 60 to 75 minutes after RS I ( $p \leq .05$ ), and rats at 10 mg/kg had lower glutamate at 20 to 75 minutes after RS I ( $p \leq .05$ ); both doses resulted in lower glutamate than at pre-RS baseline. At RS II (Figure 2D), there was again a dose  $\times$  time interaction effect ( $F_{30,330} = 1.67, p < .02$ ): relative to VEH rats, rats at 3 mg/kg had lower glutamate at 70 to 75 minutes after RS II ( $p \leq .05$ ), and rats at 10 mg/kg had lower glutamate at 35 to 40 minutes and 75 minutes after RS II ( $p \leq .05$ ). Therefore, acute SSTR4 agonist attenuated BLA glutamate release in response to RS I in both control rats and chronic CORT rats; the latter displayed high BLA glutamate release to RS I and

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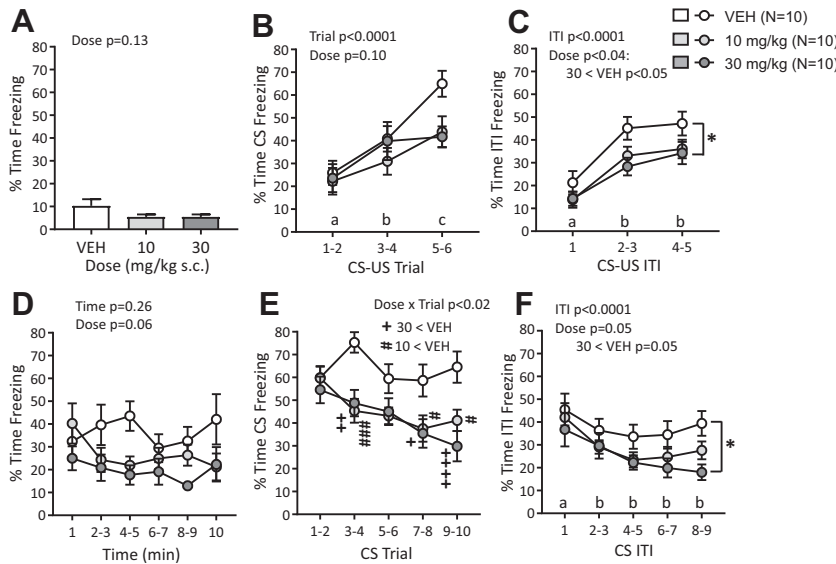
**Figure 2.** Effects of acute somatostatin receptor 4 agonist on post-RS basolateral amygdala glutamate levels in control rats and rats administered chronic CORT. Control rats at (A) RS I and (B) RS 2. Chronic CORT rats at (C) RS I and (D) RS 2. Somatostatin receptor 4 agonist or VEH was administered 30 minutes before RS I, and RS II was conducted 120 minutes after RS I. Group mean  $\pm$  SEM values for the glutamate delta signal normalized to the mean baseline are shown for each 5-minute time bin from 1–5 minutes of RS to 70–75 minutes after each RS. For the data in each figure separately, a mixed-model two-way analysis of variance was conducted with between-subject factor of somatostatin receptor 4 agonist dose (VEH, 3, 10 mg/kg) and within-subject factor of time. In cases where there was a dose  $\times$  time interaction effect, time-specific dose effects were analyzed with post hoc Sidak’s test; the horizontal bars denote time bins at which the dose indicated was significantly different from VEH. CORT, corticosterone; RS, restraint stress; VEH, vehicle.

RS II, and the SSTR4 agonist was effective at each of these stress responses.

**SSTR4 Agonist Reduces PALM in Mice**

Effects of SSTR4 agonism on PALM freezing behavior were investigated first in otherwise naïve mice using 20-second tone

CS and 2 second  $\times$  0.25 mA footshock US (Figure 3). Baseline freezing in the novel arena/context without conditioning stimuli was unaffected by SSTR4 agonist (dose main effect:  $p = .13$ ) (Figure 3A). In CS/US trials (Figure 3B), freezing increased across trials (CS/US trial main effect:  $F_{2,54} = 15.49, p < .0001$ ) and in the absence of a significant dose effect, i.e., neither a



**Figure 3.** Effects of repeated somatostatin receptor 4 agonist on mouse Pavlovian aversion learning and memory measured as time spent in conditioned freezing behavior. The compound was administered at 30 minutes before each test stage, at 0 (VEH), 10, or 30 mg/kg sc. (A) Day 1 baseline freezing during placement in the novel context without CS or US presentation. (B) Day 2 acquisition of aversive conditioning to a 20-second tone CS paired with a 2 second  $\times$  0.25 mA footshock US for 6 CS/US trials. (C) Day 2 acquisition of aversive conditioning in the 5 120-second intervals between CS/US trials. (D) Day 3 expression of aversive context memory during a 10-minute test. (E) Day 3 expression of aversive CS memory during 10 30-second CS trials delivered with intervals of 90 seconds. (F) Day 3 expression of aversive memory during 9 90-second intervals between CS trials. Group mean  $\pm$  SEM values for percentage of test time spent freezing for each time bin are shown. For each test/measure, linear mixed model analysis was conducted with fixed effects of dose and trials and random effect of mouse identification. For measures where there was a trial main effect, pairwise comparison of trials was conducted using Sidak’s test;

trials denoted by different letters (a, b, or c) were significantly different from each other. For measures where there was a dose main effect, pairwise comparison of groups was conducted using Sidak’s test; groups indicated with an asterisk were significantly different from each other:  $*p \leq .05$ . For measures where there was a dose  $\times$  trials interaction effect, trial-specific group effects were analyzed with post hoc Sidak’s test; significant 10 mg/kg vs. VEH differences are indicated with # and significant 30 mg/kg vs. VEH differences are indicated with +:  $+/#p \leq .05$ ,  $+/##p \leq .01$ ,  $+/###p \leq .001$ ,  $+/####p \leq .0001$ . CS, conditioned stimulus; ITI, intertrial interval; sc, subcutaneous; US, unconditioned stimulus; VEH, vehicle.

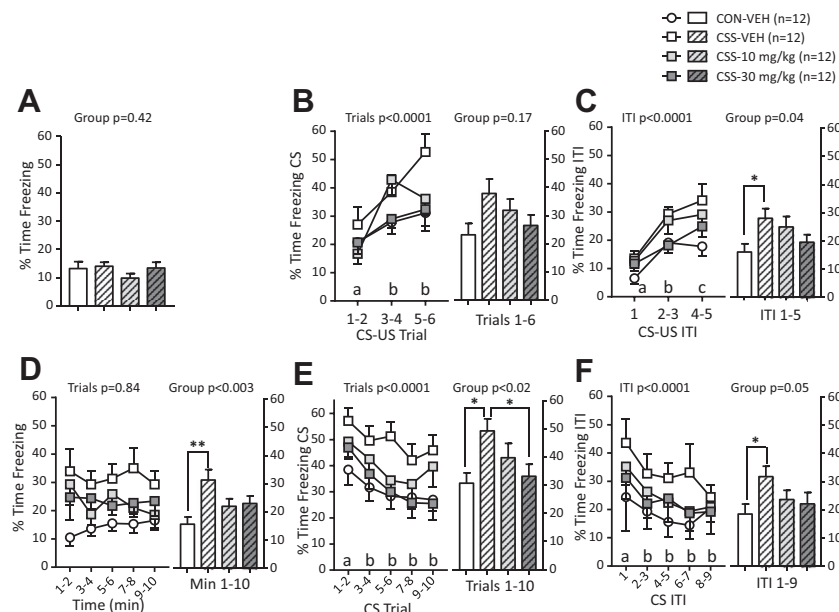


dose main effect nor an interaction with CS/US trial ( $p \geq .10$ ). In the intertrial intervals (ITIs) (Figure 3C), freezing increased between the initial and subsequent ITIs (ITI main effect:  $p < .0001$ ), and there was a dose main effect ( $F_{2,27} = 3.80, p < .04$ ): mice at 30 mg/kg spent less time freezing than VEH mice ( $p < .05$ ). In the context memory test (Figure 3D), there was neither a time effect ( $p \geq .12$ ) nor a dose effect ( $p = .06$ ). In the CS test, for CS trials (Figure 3E) there was a dose  $\times$  trial interaction effect ( $F_{8,108} = 2.44, p < .02$ ) as well as a dose main effect ( $F_{2,27} = 7.67, p < .02$ ) and a trial main effect ( $F_{4,108} = 6.33, p < .0001$ ): dose groups displayed a similar, high amount of freezing at CS trials 1 and 2; whereas VEH mice then remained at these levels across trials, mice at 10 or 30 mg/kg displayed a consistent decrease in freezing such that freezing time was less than that of VEH mice at trials 3 and 4, 7 and 8, and 9 and 10 at each dose ( $p < .05-.0001$ ). For ITIs of the CS memory test (Figure 3F), freezing was reduced after ITI 1 (ITI main effect:  $p < .0001$ ) and was lower in mice at 30 mg/kg than VEH mice (dose main effect:  $F_{2,27} = 3.34, p = .05$ ).

### SSTR4 Agonist Normalizes Excessive PALM in CSS Mice

Effects of SSTR4 agonism were investigated in a mouse model in which CSS leads to increased PALM relative to CON mice, with footshock delivered at 0.15 mA. CON mice received either VEH or 10 mg/kg, and CSS mice received VEH or 10 or 30 mg/kg. First analysis was conducted with CON mice only: in line with the lower US intensity, CON/VEH mice acquired/expressed less freezing than naïve/VEH mice in the previous experiment; consequently, the window to detect a dose effect

at any test stage was insufficient ( $p \geq .17$ , data not shown). In line with the experimental aim, to investigate whether SSTR4 agonism reverses CSS-induced excessive PALM, the main analysis was conducted with CON/VEH, CSS/VEH, CSS/10 mg/kg, and CSS/30 mg/kg mice. In the baseline freezing test, there was no group effect ( $p = .42$ ) (Figure 4A). At conditioning, in CS/US trials (Figure 4B), freezing increased from trials 1 and 2 to trials 3 and 4 (CS/US trial main effect:  $F_{2,88} = 14.16, p < .0001$ ), and there was no group effect ( $p \geq .07$ ). In ITIs (Figure 4C), freezing increased across conditioning (ITI main effect:  $p < .0001$ ), and there was a group main effect ( $F_{3,44} = 3.16, p < .04$ ), with higher freezing in CSS/VEH than in CON/VEH mice ( $p = .04$ ) and CSS/10 mg/kg and CSS/30 mg/kg mice at intermediate levels. For the context test (Figure 4D), freezing remained consistent across time intervals ( $p \geq .84$ ). There was a group main effect ( $F_{3,44} = 5.30, p < .003$ ): freezing was higher in CSS/VEH mice than CON/VEH mice ( $p = .002$ ) and at intermediate levels in CSS/10 mg/kg and CSS/30 mg/kg mice. In the subsequent CS test, in CS trials (Figure 4E), freezing decreased after CS 1 and 2 (trial main effect:  $p < .0001$ ). There was a group main effect ( $F_{3,44} = 3.81, p < .02$ ) with higher freezing in CSS/VEH mice than CON/VEH mice ( $p = .03$ ) and lower freezing in CSS/30 mg/kg than CSS/VEH mice ( $p = .05$ ). In ITIs (Figure 4F), freezing decreased after ITI 1 (ITI main effect:  $p < .0001$ ). There was a group main effect ( $F_{3,44} = 2.75, p = .05$ ): freezing was higher in CSS/VEH than CON/VEH mice ( $p < .05$ ) and at intermediate level in CSS/10 mg/kg and CSS/30 mg/kg mice. On day 21, a hot plate test was conducted: there was no effect of CSS or SSTR4 agonist on the latency to display a pain response ( $F_{4,55} < 1, p = .97$ ) (Figure S2).



**Figure 4.** Effects of repeated somatostatin receptor 4 agonist in a mouse model of CSS-induced excessive Pavlovian aversion learning and memory measured as time spent in conditioned freezing behavior. CSS and CON were conducted on days 1–15. Compound (CSS mice: 10 or 30 mg/kg subcutaneous) or VEH (CON and CSS mice) was administered at 30 minutes before each test stage. (A) Day 16 baseline freezing during placement in the novel context without CS or US presentation. (B) Day 17 acquisition of aversive conditioning to a 20-second tone CS paired with a 2 second  $\times$  0.15 mA footshock US for 6 CS/US trials. (C) Day 17 acquisition of aversive conditioning in the 5 120-second intervals between successive CS/US trials. (D) Day 18 expression of aversive context memory during a 10-minute test. (E) Day 18 expression of aversive CS memory during 10 30-second CS trials delivered with intervals of 90 seconds. (F) Day 18 expression of aversive memory during 9 90-second intervals between successive CS trials. In panels (B–F), the left-side graph shows group mean  $\pm$  SEM values for percentage of time spent freezing for each time bin, and the right-side graph shows group overall mean  $\pm$  SEM values for all time bins. For each test/measure, linear mixed model analysis was conducted with fixed effects of group/dose (CON-VEH,

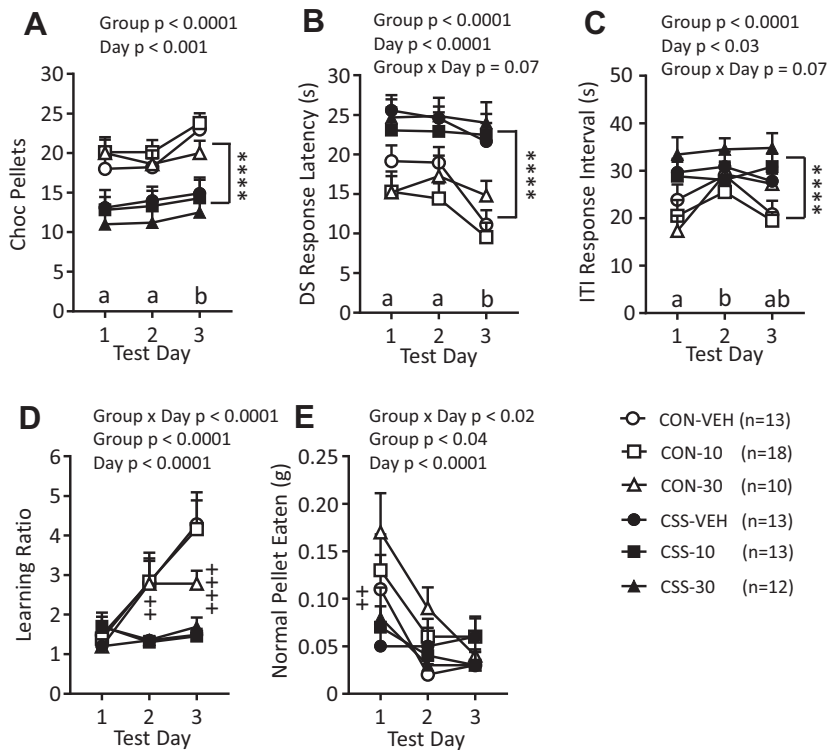
CSS-VEH, CSS-10, CSS-30) and trials, and a random effect of mouse identification. In cases where there was a trial main effect, pairwise comparison of trials was conducted using Sidak's test; trials denoted by different letters (a, b, or c) were significantly different from each other. For measures where there was a group main effect, pairwise comparison of groups was conducted using Sidak's test; groups indicated with an asterisk were significantly different from each other: \* $p \leq .05$ , \*\* $p \leq .01$ . CON, control handling; CS, conditioned stimulus; CSS, chronic social stress; CSS-10, CSS and 10 mg/kg; CSS-30, CSS and 30 mg/kg; ITI, intertrial interval; US, unconditioned stimulus; VEH, vehicle.

**SSTR4 Agonist Has No Effect on Reward Learning and Memory and Increases Reward Motivation in CON and CSS Mice**

Effects of SSTR4 agonist were investigated in a mouse model in which CSS attenuates DRLM about and REV of sucrose. CSS and CON were conducted on days 1 to 15, the DRLM test was conducted on days 16 to 18, and the REV test was conducted on days 19 and 20. With regard to body weight, during testing (days 16–20), absolute body weight was higher in CSS mice ( $29.9 \pm 0.2$  g) than CON mice ( $29.0 \pm 0.3$  g) (group main effect:  $F_{1,73} = 5.20, p < .03$ ) and unaffected by SSTR4 agonist ( $p \geq .55$ ). Expressed as percentage of baseline body weight (determined at experiment onset), body weight was similar in CSS mice ( $100.9 \pm 0.5\%$ ) and CON mice ( $99.9 \pm 0.3\%$ ) ( $p \geq .12$ ), and there was no effect of SSTR4 agonist ( $p \geq .60$ ). As expected given the CSS effects on energy status (17,19,20), CSS mice required more home-cage food ( $117 \pm 3\%$  baseline food intake) than CON mice ( $81 \pm 1\%$  baseline food intake) to maintain baseline body weight during the test period (group main effect:  $F_{1,73} = 145.29, p < .0001$ ); there was no effect of SSTR4 agonist on this measure ( $p \geq .52$ ). All food in the home cage had been consumed at the latest 2 hours before behavioral testing.

In the DRLM test (Figure 5), relative to CON mice, CSS mice obtained fewer sucrose pellets (Figure 5A) and had longer DS response latencies (Figure 5B), longer ITI response intervals (Figure 5C), and lower ITI/DS learning ratios (Figure 4D). There was no significant effect of SSTR4 agonist on any of these measures. Statistical findings are exemplified using the ITI/DS learning ratio (Figure 5D): there was a significant group  $\times$  day interaction effect ( $F_{2,134} = 11.66, p < .0001$ ) as well as main effects of group ( $F_{1,66} = 14.59, p < .0001$ ) and day ( $F_{2,134} = 12.82, p < .0001$ ) and no effect of dose ( $p \geq .34$ ). This reflected that the ITI/DS learning ratio remained at about 1 in CSS mice, while it increased across test days in CON mice, and for both groups the ratio was independent of SSTR4 agonist dose. The amount of normal diet eaten (Figure 5E) was higher in CON mice than CSS mice at test day 1 specifically and similar thereafter, and amount of normal diet eaten was low compared with sucrose pellet, e.g., test day 2: normal diet: CON  $0.06 \pm 0.01$  g; CSS  $0.04 \pm 0.01$  g; sucrose: CON  $0.53 \pm 0.03$  g; CSS  $0.36 \pm 0.03$  g.

In the REV test (Figure S3), reward-to-effort valuation of sucrose was decreased in CSS mice compared with CON mice and increased at 10 mg/kg SSTR4 agonist. Thus, for number of operant responses (Figure S3A), there were main effects of group ( $F_{1,72} = 28.25, p < .0001$ ) and dose ( $F_{2,72} = 5.83,$



**Figure 5.** Absence of effects of repeated somatostatin receptor 4 agonist in a mouse model of CSS-induced attenuated discriminative reward learning and memory for sucrose. CSS and CON were conducted on days 1–15. Mice were tested on days 16–18. The discriminative reward learning and memory test comprises presentation of a tone DS, during which a single feeder-port response leads to chocolate pellet reinforcement and DS termination, and intertrial intervals, during which feeder-port responses are without consequence. Each test comprised 40 DS/reward trials, and the data were analyzed for trials 1–30 per test. A pellet of normal diet was placed in the test chamber to provide a low-effort/low-reward choice. Compound or VEH was administered 30 minutes before each each test, using a 2 group (CSS, CON)  $\times$  3 dose (VEH, 10, 30 mg/kg)  $\times$  3 test day (1, 2, 3) design. **(A)** Total number of chocolate pellets obtained: group:  $F_{1,73} = 29.87, p < .0001$ ; test day:  $F_{2,146} = 7.56, p < .001$ . **(B)** Median DS response latency: group:  $F_{1,73} = 30.74, p < .0001$ ; test day:  $F_{2,146} = 10.01, p < .001$ ; group  $\times$  test day:  $F_{2,146} = 2.75, p < .07$ . **(C)** Median interval between feeder responses during intertrial intervals: group:  $F_{1,73} = 19.81, p < .0001$ ; test day:  $F_{2,146} = 3.60, p < .03$ ; group  $\times$  test day:  $F_{2,146} = 2.91, p < .06$ . **(D)** Median ITI/DS learning ratio, calculated as intertrial interval response interval/DS response latency: group:  $F_{1,65.89} = 14.58, p < .0001$ ; test day:  $F_{2,134.39} = 12.82, p < .0001$ ; group  $\times$  test day:  $F_{2,134.39} = 11.66, p < .0001$ . **(E)** Mean weight of normal diet pellet eaten: group:  $F_{1,73} = 4.44, p < .04$ ; test day:  $F_{2,146} = 12.90, p < .0001$ ; group  $\times$  test

day:  $F_{2,146} = 4.26, p < .02$ . Group mean  $\pm$  SEM values for each test are given. Linear mixed model analysis was conducted with fixed effects of group, dose, and test and random effect of mouse identification. In cases where there was a trial main effect, pairwise comparison of trials was conducted using Sidak’s test; trials denoted by different letters (a, b, or ab) were significantly different from each other. Group main effects are indicated with asterisks: \*\*\*\* $p < .0001$ . For measures where there was a group  $\times$  test interaction effect, test-specific group effects were analyzed with post hoc Sidak’s test; significant differences are indicated with a plus: ++ $p \leq .01$ , +++ $p \leq .0001$ . CON, control handling; CON-10, CON and 10 mg/kg; CON-30, CON and 30 mg/kg; CSS, chronic social stress; CSS-10, CSS and 10 mg/kg; CSS-30, CSS and 30 mg/kg; DS, discriminative stimulus; ITI, intertrial interval; VEH, vehicle.

$p < .004$ ); CSS mice made fewer operant responses than CON mice, and CON and CSS mice at 10 mg/kg made more operant responses than VEH mice ( $p = .03$ ) and 30 mg/kg mice ( $p = .008$ ). Test measures dependent on operant responding yielded similar findings: there were main effects of group and dose for number of sucrose pellets earned (group: CSS < CON, dose: 10 mg/kg > VEH and 30 mg/kg) (Figure S3B) and final ratio attained (group: CSS < CON, dose: Sidak's test  $p > .05$ ) (Figure S3C). The amount of normal pellets consumed (Figure S3D) was low and unaffected by group ( $p \geq .12$ ) or dose ( $p \geq .20$ ).

### Absence of Effect of CSS on *Sst*, *Sstr4*, and SST Expression in BLA Complex

CSS effects on BLA-complex expression of *Sst* and *Sstr4* were investigated in a separate cohort of mice. There was no effect of CSS on relative expression of either *Sst* ( $t_{23} = 1.27$ ,  $p = .22$ ) (Figure 6A) or *Sstr4* ( $t_{23} = -0.58$ ,  $p = .57$ ) (Figure 6B); there was also no effect of CSS on *Actb* expression ( $p \geq .85$ ). In a further separate mouse cohort, CSS effects on the density of SST-positive cells as well as of their SST-signal integrated density were investigated. In lateral amygdala, there was no CSS effect on SST<sup>+</sup> cell density ( $t_{19} = -0.70$ ,  $p = .49$ ) (Figure 6C, E) or mean integrated density of SST cell staining ( $t_{19} = -0.66$ ,  $p = .52$ ). Also in BLA there was no effect of CSS on SST<sup>+</sup> cell density ( $t_{19} = 0.56$ ,  $p = .58$ ) (Figure 6D, F) or mean integrated density of SST cell staining ( $t_{19} = -0.18$ ,  $p = .86$ ).

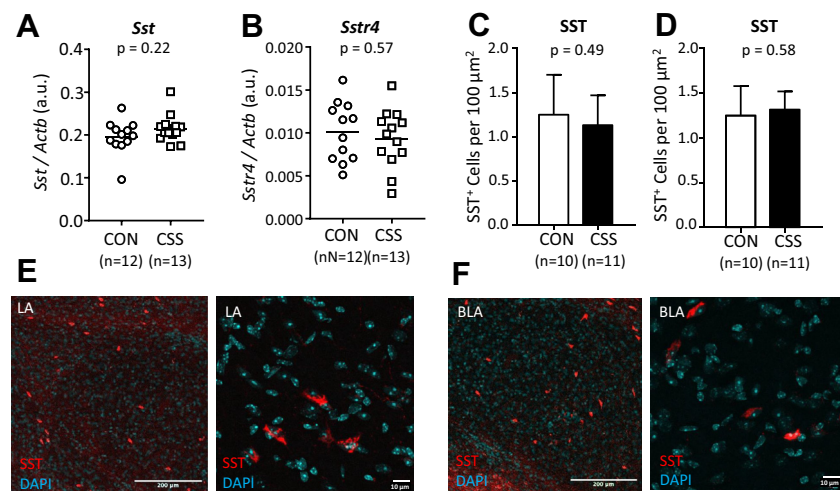
## DISCUSSION

This rodent study provides evidence that SSTR4 agonism exerts inhibitory effects on 1) RS-induced BLA glutamate release in control and CORT-exposed rats and 2) PALM in CON-exposed mice and excessive Pavlovian aversion memory in CSS-exposed mice, for which BLA GABA and glutamate neurons are of major importance. While it is a limitation of the study that both experimental models were not studied in both rats and mice, this evidence nonetheless constitutes

important preclinical validation of SSTR4 as a pharmacotherapy target in depressive and anxiety-related neuropsychiatric disorders.

In rats, chronic CORT exposure resulted in a precise period of elevated fecal CORT levels. Although there was a 12-day interval between cessation of CORT exposure and testing, rats with chronic CORT exposure showed an increase in BLA glutamate release to novel and repeated restraint, while control rats showed an increase in response to novel restraint specifically. Indeed, even acute CORT exposure results in dendritic atrophy in BLA glutamate neurons when assessed 12 days later, indicating the durability of CORT effects in BLA (11). Using microdialysis, rats exposed to 21-day daily RS displayed increased hippocampal glutamate release to an acute stressor (21). In transgenic mice expressing extracellular glutamate biosensors in cortical excitatory neurons, 10-day chronic social defeat exposure led to mesoscale corticolimbic glutamate functional hyperconnectivity (22). Here, in control rats, the SSTR4 agonist resulted in an almost complete block of BLA glutamate response to RS I, and in CORT rats, the SSTR4 agonist attenuated the BLA glutamate response to RS I and RS II. These effects were mildly more pronounced at 10 mg/kg than at 3 mg/kg.

Building on the efficacy of SSTR4 agonism in attenuating BLA glutamate release, we investigated its effects on PALM, neurobehavioral processes for which neurotransmission in the BLA-complex microcircuitry is of major importance (3,23). First, SSTR4 agonism effects were studied in naïve mice using 0.25 mA footshock. At conditioning, CS freezing was unaffected, while ITI freezing was reduced by SSTR4 agonist. At memory expression, extinction was accelerated markedly by SSTR4 agonist and independently of dose. Second, effects of SSTR4 agonism were studied in CSS mice using 0.15 mA footshock, which yields clear separation between CSS and CON mice allowing for the study of anxiolytic efficacy on excessive threat responsiveness [e.g. (15,16)]. At conditioning, CSS/VEH mice acquired more ITI freezing than CON/VEH mice, and there was no effect of SSTR4 agonism on aversion



**Figure 6.** Absence of effect of CSS on gene and protein expression in BLA-complex tissue. (A, B) CSS and CON were conducted on days 1–15, and fresh-frozen brains of these otherwise unmanipulated mice were collected on day 16. (A) Individual mouse values for *Sst* gene expression normalized with *Actb*. (B) Individual mouse values for *Sstr4* gene expression normalized with *Actb*. (C–F) CSS and CON were conducted on days 1–15, and brains following perfusion fixation of these otherwise unmanipulated mice were collected on day 16. (C) Lateral amygdala SST<sup>+</sup> cell densities (mean ± SD). (D) BLA SST<sup>+</sup> cell densities (mean ± SD). (E) Representative images for SST immunofluorescence staining in LA: left-side image taken at 20× (scale bar = 200 μm) and right-side image taken at 63× (scale bar = 10 μm). (F) Representative images for SST immunofluorescence staining in BLA: left-side image taken at 20× (scale bar = 200 μm) and right-side image taken at 63× (scale bar = 10 μm). All  $p$  values

were obtained with unpaired Student's  $t$  tests. a.u., arbitrary units; BLA, basolateral amygdala; CON, control handling; CSS, chronic social stress; LA, lateral amygdala; SST, somatostatin.



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conditioning in CSS mice. At memory expression, CSS/VEH mice displayed excessive freezing to context and CS relative to CON/VEH mice, as expected (14–16,24). The CSS effect on CS freezing expression was reversed by SSTR4 agonist at 30 mg/kg: in CSS/30 mg/kg mice, CS memory expression was reduced at test onset, and subsequent extinction was accelerated, relative to CSS/VEH mice. These same mice were also studied in a hot plate test to investigate nociception: as expected, there was no effect of CSS [e.g. (14)], and there was also no effect of SSTR4 agonist. These findings suggest that in PALM, CSS and SSTR4 agonism primarily affect the psychological aversive salience of footshock, and therefore of tone, and not nociception per se. Given the importance of CORT in Pavlovian aversion consolidation (25) and evidence that SSTR4 agonism attenuates the limbic-brain CORT response to footshock (10), it is possible that attenuation of CORT signaling in BLA and/or hippocampus contributed to SSTR4 agonism effects on Pavlovian aversion memory. In the mouse experiments, repeated SSTR4 agonist dosing was applied, and the findings do not allow for determination of whether the effects observed were due to acute or cumulative SSTR4 agonism.

The SSTR4-mediated attenuation of (excessive) CS/US aversion learning/consolidation and/or recall could involve increased signaling at GABA interneuron/glutamate projection neuron synapses within the BLA complex (3,26). Acceleration of extinction of CS aversion memory in CON/SSTR4 agonist and CSS/SSTR4 agonist mice could involve increased signaling at BLA GABA interneuron/glutamate neuron synapses in which the former receive excitatory input from infralimbic cortex long-range glutamate neurons during CS-only exposure (27). In CSS mice, this would necessitate that in these respective microcircuits, CSS either leads to deficient endogenous SST/SSTR4 synaptic signaling or to different (off-target) effects that can be compensated by additional SST/SSTR4 synapse signaling (see below). In both scenarios, SSTR4 agonism would be predicted to reduce aversion-induced BLA glutamate release, as observed in the rat glutamate biosensor experiment. Nonetheless, it is important to emphasize that in addition to the BLA, *Sstr4*/SSTR4 is expressed by glutamate neurons in basomedial and central nuclei of the amygdala, prelimbic cortex, hippocampus, habenula, and somatosensory cortex as well as by some GABA interneurons in these regions (7,8). Therefore, two or more of these regions could contribute to the effects of SSTR4 agonism observed.

It is important to consider the current findings relative to the evidence that mouse BLA SST GABA interneurons projecting onto dendrites of glutamate neurons are inhibited by parvalbumin GABA interneurons, resulting in disinhibition of glutamate principal neurons (parvalbumin/SST/pyramidal neuron disinhibitory microcircuits). In the context of Pavlovian aversion learning, in the BLA complex, parvalbumin interneurons are excited by the CS (28). Potentially, therefore, pharmacological SSTR4 agonism could function to override such disinhibitory microcircuits. In contrast to the mouse BLA complex, the mouse prelimbic cortex contains SST/parvalbumin/pyramidal neuron disinhibitory microcircuits (29). CS excitation of the prelimbic cortex SST GABA interneurons is

important for PALM, and a major afferent pathway comprises long-range glutamate neurons from the BLA complex, firing of which increases during CS/US processing (29). If SSTR4 agonism would have any effect in such SST/parvalbumin/pyramidal neuron microcircuits, it would be disinhibition of prelimbic cortex glutamate pyramidal neurons and increased PALM. However, given that *Sstr4*/SSTR4 is expressed by prelimbic cortex glutamate neurons, it is certainly possible that SSTR4 agonism has direct effects on aversion CS/US processing in the prelimbic cortex.

The therapeutic potential of SSTR4 agonism in attenuating association learning and memory depends on specificity to aversion processing. Accordingly, in the DRLM test, two findings are of major importance. First, CON mice acquired and consolidated the association between DS and sucrose availability, as indicated by the increased learning ratio across tests, and there was no effect—positive or negative—of SSTR4 agonism. Second, CSS mice displayed the expected deficit in DRLM (19), and this was also unaffected by SSTR4 agonism. Finally, here, although the SSTR4 agonist had no effect on DRLM, it did exert a dose-dependent, positive effect on REV and in both CON and CSS mice. Interestingly, this effect was specific to 10 mg/kg, while effects on PALM were obtained with 10 mg/kg and particularly 30 mg/kg, suggesting that attenuation effects on aversion processing and enhancing effects on reward processing can to some extent be separated by dose. The mouse REV test is sensitive to mesolimbic dopamine function (17,18), suggesting that SSTR4 agonism might act to disinhibit ventral tegmentum dopamine release. In this regard, it is relevant that the (medial) habenula is a major region of *Sstr4*/SSTR4 expression (7) and that injection of an SSTR4 agonist into the hippocampus, another major region of *Sstr4*/SSTR4 expression (7), led to increased operant responding for sweet reward (30). While SST is important in the regulation of feeding, including stimulation of nonhomeostatic food intake (31), this would appear to primarily involve the hypothalamus and its SSTR2 expression (32).

There was no effect of CSS on expression of the *Sst* or *Sstr4* genes in BLA-complex tissue and no effect on the density of SST<sup>+</sup> cells or the integrated density of SST staining in the lateral amygdala and BLA. If the absence of an effect of CSS on BLA complex *Sstr4* expression reflects protein level—we were unable to identify a suitable SSTR4 antibody (7)—it would indicate that in these mouse models of stress-induced disrupted aversion and reward processing, SSTR4 agonism is functioning via indirect compensation for circuitry dysregulation elsewhere, rather than acting to repair pathological SST-SSTR4 signaling.

In conclusion, the complementary rat and mouse experiments presented here provide neurochemical and behavioral proof-of-concept evidence for SSTR4 agonism as a treatment in depressive and anxiety-related neuropsychiatric disorders. The data are consistent with increased and compensatory signaling in disinhibitory microcircuits in which SST GABA interneurons synapse directly on glutamate projection neurons in BLA and other regions of *SSTR4* expression. Recent data indicate that the amygdala is a region of relatively high *SSTR4*



expression in the human brain (7), and a recently developed human SSTR4 transgenic mouse line (33) will be invaluable in translational studies aimed at optimizing SSTR4 agonist drug candidates.

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