Editor's Summary

Blocking PTSD with an Opioid Receptor Agonist

There are currently no approved pharmacological preventive treatments for posttraumatic stress disorder (PTSD), an anxiety disorder with altered fear learning that occurs in some individuals after exposure to a highly traumatic event. Andero *et al.* now demonstrate that the opioid receptor–like 1 gene (*Oprl1*), encoding the nociceptin (NOP)/orphanin FQ receptor, is involved in stress-mediated enhancement of amygdala-dependent fear in mouse models and humans with PTSD. These findings build on published data showing that opioid activity appears to be involved in fear inhibition and may block the consolidation of fear memories. Recent clinical observations suggest that opioid analgesia, provided shortly after trauma, may decrease the development of PTSD. Here, Andero *et al.* use a gene discovery approach to identify a specific member of the opioid receptor family, Oprl1, which may be involved in this process. Using a mouse model of dysregulated fear, the authors found altered expression of *Oprl1* in the amygdala, the part of the brain where fear memories are consolidated. Systemic infusion of SR-8993, a new highly selective NOP receptor agonist, or injection into the central amygdala of mice impaired the consolidation of fear memories. Additionally, in humans, a single-nucleotide polymorphism (SNP) was found within *Oprl1* and may be associated with PTSD symptoms. The authors show that this SNP is associated with physiological startle measures of fear discrimination and correlates with functional connectivity between the amygdala and insula. Together, these data suggest that *Oprl1* is associated with amygdala function, fear processing, and PTSD symptoms. Furthermore, activation of the NOP receptor encoded by *Oprl1* may block fear memory consolidation, with implications for preventing PTSD after traumatic events.

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POST-TRAUMATIC STRESS DISORDER

Amygdala-Dependent Fear Is Regulated by *Oprl1* in Mice and Humans with PTSD

Raül Andero,[1,2] Shaun P. Brothers,[3] Tanja Jovanovic,[2] Yen T. Chen,[4]* Hasib Salah-Uddin,[3] Michael Cameron,[5] Thomas D. Bannister,[4] Lynn Almli,[2] Jennifer S. Stevens,[2] Bekh Bradley,[2] Elisabeth B. Binder,[2,6] Claes Wahlestedt,[3] Kerry J. Ressler[1,2,7]†

The amygdala-dependent molecular mechanisms driving the onset and persistence of posttraumatic stress disorder (PTSD) are poorly understood. Recent observational studies have suggested that opioid analgesia in the aftermath of trauma may decrease the development of PTSD. Using a mouse model of dysregulated fear, we found altered expression within the amygdala of the *Oprl1* gene (opioid receptor–like 1), which encodes the amygdala nociceptin (NOP)/orphanin FQ receptor (NOP-R). Systemic and central amygdala infusion of SR-8993, a new highly selective NOP-R agonist, impaired fear memory consolidation. In humans, a single-nucleotide polymorphism (SNP) within *OPRL1* is associated with a self-reported history of childhood trauma and PTSD symptoms (*n* = 1847) after a traumatic event. This SNP is also associated with physiological startle measures of fear discrimination and magnetic resonance imaging analysis of amygdala-insula functional connectivity. Together, these data suggest that *Oprl1* is associated with amygdala function, fear processing, and PTSD symptoms. Further, our data suggest that activation of the Oprl1/NOP receptor may interfere with fear memory consolidation, with implications for prevention of PTSD after a traumatic event.

INTRODUCTION

Posttraumatic stress disorder (PTSD) is an anxiety disorder that is composed, in part, of altered fear learning that develops after exposure to a highly traumatic event. Current therapeutic approaches are used only after PTSD is already present and debilitating symptoms have appeared, but prevention of PTSD development is an important unmet medical need. Within the neural circuitry of fear formation, the amygdala is a critical site for development and storage of fear memory (*1*). At a molecular level within the amygdala, gene regulation and protein synthesis are needed for the consolidation of fear memory formation (*2*, *3*). A new and more cost-effective therapeutic approach, although not yet a reality, would comprise early treatments that could prevent PTSD development by impairing fear memory consolidation (*4*). This early intervention would be especially appropriate for individuals at higher risk for PTSD after trauma, such as those with a history of previous trauma or those carrying genetic polymorphisms that have been associated with PTSD risk (*4*, *5*).

There is little consensus as to what constitutes a PTSD-like rodent model (*6*). However, most models have in common the study of emotional memories according to a Pavlovian learning paradigm. This associative learning process consists of the pairing of a neutral conditioned stimulus with an aversive unconditioned stimulus eliciting a conditioned fear response. The conditioned stimulus can be a cue (for example, a tone) or a context (for example, a room). Examples of conditioned fear responses are increased freezing and heart rate. Fear extinction consists of new inhibitory learning after repeated or prolonged conditioned stimulus presentations, without the unconditioned stimulus, which causes a gradual decrease in the magnitude and frequency of the conditioned response (*5*). Here, we use a single severe stress exposure, immobilization to a wooden board (IMO), to model PTSD-like behavior in mice and to study resultant differential gene regulation in fear learning. We use this model for several reasons: A valid PTSD-like model requires a highly traumatic stress exposure, much more stressful than other mild stressors such as fear conditioning (*6*, *7*). IMO is one of the most stressful among the emotional models used in the laboratory (*7*). Moreover, the severity of the stressor during trauma exposure is related to the likelihood of PTSD development (*8*). Previous IMO causes PTSD-like impaired fear extinction (*9*), whereas fear conditioning alone is not resistant to extinction (*6*). IMO is one of the few acute stressors that result in a wide range of long-term (more than 24 to 48 hours) PTSD-like symptoms after a single exposure in rodents (*7*). Among those symptoms, IMO elicits long-term impaired declarative memory and enhanced anxiety similar to what occurs in PTSD patients (*4*, *7–10*).

RESULTS

Immobilization of mice causes long-term impaired declarative memory and enhanced anxiety

Mice were initially exposed for 2 hours using the IMO paradigm, followed 6 days later by a water maze task (Fig. 1A). There were differences in long-term, but not short-term, spatial memory on this spatial task after IMO (Fig. 1B). However, there was no difference in time to reach the platform between the groups during visible platform training, which indicated intact motivation and sensorimotor skills after IMO. Results from the long-term memory test showed that IMO animals spend less time than control animals in the target area (**P* < 0.05). These data suggest that a single exposure to IMO elicits

[1]Yerkes National Primate Research Center, Atlanta, GA 30329, USA. [2]Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, Atlanta, GA 30329, USA. [3]Center for Therapeutic Innovation and Department of Psychiatry and Behavioral Sciences, University of Miami Miller School of Medicine, Miami, FL 33136, USA. [4]Department of Chemistry, The Scripps Research Institute—Scripps Florida, Jupiter, FL 33458, USA. [5]Molecular Therapeutics, The Scripps Research Institute—Scripps Florida, Jupiter, FL 33458, USA. [6]Max Planck Institute of Psychiatry, Munich 80804, Germany. [7]Howard Hughes Medical Institute, Chevy Chase, MD 20815, USA.
*Present address: The Boston Consulting Group, Tokyo 102-0094, Japan.
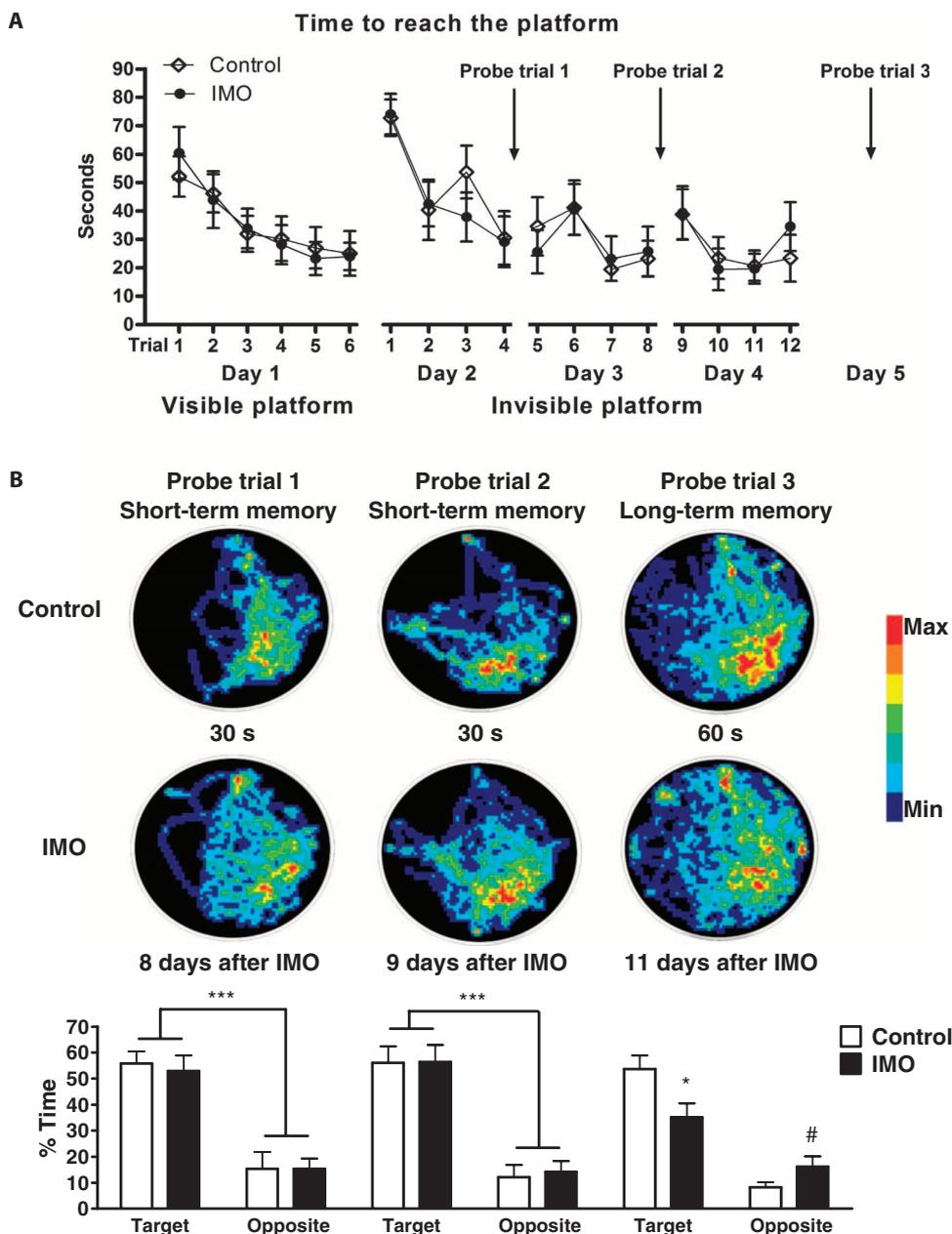†Corresponding author. E-mail: kressle@emory.edu

Fig. 1. Immobilization of mice causes long-term impaired declarative memory. (**A** and **B**) Mice were exposed for 2 hours using the immobilization to a wooden board (IMO) procedure, 6 days before testing in the water maze test, which is used to evaluate spatial declarative memory. The water maze procedure lasted for 5 days. (A) Training started with six trials in 1 day of the visible platform test. Results showed no difference in the time to reach the platform between groups, which indicated intact motivation and sensorimotor skills. The day after, the invisible platform training started and consisted of four trials a day for three consecutive days, showing no differences in learning evaluated by time to reach the platform. (B) On days 2 (8 days after IMO) and 3 (9 days after IMO) after training, a probe trial was performed to assess short-term memory. Results showed no differences between IMO and control group in the short-term memory tests [***$P < 0.001$, Target versus Opposite, repeated-measures analysis of variance (ANOVA)]. On day 5 (11 days after IMO), only a probe trial was performed to assess long-term memory. Results showed that IMO animals spent less time than control animals in the target area (*$P < 0.05$ versus control in target area, repeated-measures ANOVA followed by Bonferroni post-test). IMO mice spent more time than the control mice in the opposite area of the maze (#$P < 0.05$ versus control in opposite area, repeated-measures ANOVA followed by Bonferroni post-test). These data replicate previous findings in rats (10).

long-term declarative memory impairment. A separate group of mice was tested for anxiety-like measures 6 days after they were exposed for 2 hours to the IMO procedure. Data showed that IMO-treated mice exhibited enhanced anxiety in both the elevated plus maze (Fig. 2A) and the open-field maze (Fig. 2B). Overall, these findings suggest that mice, like rats, exhibit prolonged enhanced anxiety-like behavior and deficits in spatial learning after a single immobilization stress. We next examined whether previous IMO also altered fear extinction, another deficit observed in PTSD patients.

Amygdala *Oprl1* gene regulation is altered in a PTSD-like mouse model during fear consolidation

We then studied cued-fear expression in mice with a single previous history of IMO versus compensatory handling (control) 6 days before fear conditioning. Here, we used auditory-fear conditioning where the conditioned stimulus was a tone (30 s, 6 kHz) that coterminated with an unconditioned stimulus, which was a mild electric footshock (0.5 s, 1-mA intensity). Mice that received fear conditioning showed significantly more freezing during this test, independently of having received IMO or not, than animals exposed to the fear conditioning box without receiving stimuli (box-only exposure) (main effect of fear conditioning: $F_{1,36} = 42.5$; $P \leq 0.0001$, repeated-measures ANOVA followed by Bonferroni post-test; Fig. 3A). During the cued-fear expression test, the IMO-fear conditioning group presented more enhanced freezing than did the control-fear conditioning group (fear conditioning main effect: $F_{1,36} = 508$; $P \leq 0.0001$; IMO main effect: $F_{1,36} = 12$; $P \leq 0.001$; Fear conditioning × IMO interaction: $F_{1,36} = 10.8$; $P \leq 0.001$, repeated-measures ANOVA followed by breakdown of the interaction; Fig. 3A). In the fear conditioning test, control-fear conditioned mice showed more freezing during the final conditioned stimulus than during the intertrial interval (Trial × Cue × Group interaction: $F_{1,20} = 10.3$; $P \leq 0.01$, repeated-measures ANOVA; conditioned stimulus and intertrial interval 5 breakdown of the interaction: $P \leq 0.01$, repeated-measures ANOVA), whereas the IMO-fear conditioning group had similar freezing during all of the conditioned stimulus and intertrial interval periods
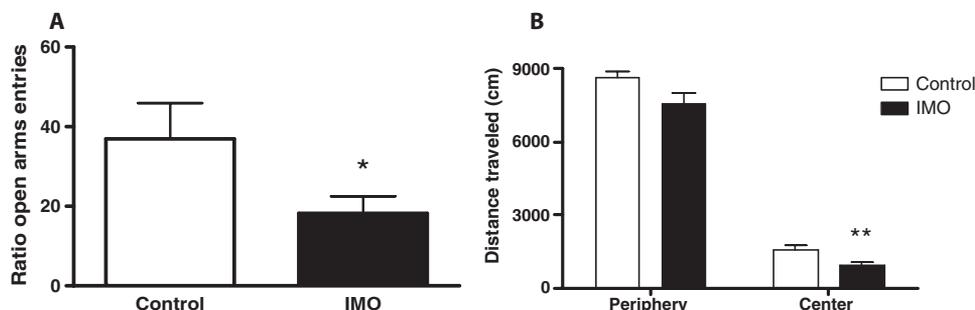
Fig. 2. Immobilization causes long-term enhanced anxiety-like symptoms in mice. (**A** and **B**) Mice were exposed for 2 hours to immobilization to a wooden board (IMO) 6 days before testing in (A) an elevated plus maze or (B) an open-field test. (A) Five-minute exposure to elevated plus maze revealed that IMO mice exhibited long-term enhanced anxiety according to the ratio of the time spent in the open arms (*$P < 0.05$, two-tailed Student's t test). (B) Concordantly, 30-min exposure to an open field resulted in mice showing enhanced anxiety when the time that control or IMO mice spent in the center of the apparatus was calculated (**$P < 0.01$, two-tailed Student's t test). These data replicate previous findings in rats (*49*).

(Fig. 3B). This suggests that IMO mice have difficulties discriminating between nonthreat periods (intertrial interval) and danger signals (conditioned stimulus). This is concordant with a recent report that shows that PTSD patients present altered discrimination of safety and danger signals (*11*).

Two hours after the cued-fear expression test, animals were sacrificed and amygdala tissue was collected to perform mRNA microarray analyses (Fig. 3, C to E, and fig. S1A). To focus on differential gene expression as a function of previous IMO history, and not fear conditioning per se, we then selected probes that were statistically different between control-fear expression versus IMO-fear expression groups. About 4.5% of the examined genes (45,281) were differentially expressed on the basis of previous IMO exposure (Fig. 3D). From those genes, we then selected those that presented >1.3- and <0.7-fold changes in both groups versus the home cage group (fig. S1B). Of those probes, the only one that represented a gene with highly specific expression in the amygdala compared to other areas of the brain was the opioid receptor–like 1 (*Oprl1*) gene (Fig. 3F).

Oprl1 is found in mice in the central amygdala, whereas its expression in other amygdala regions is relatively low (*12*). The *Oprl1* gene encodes the nociceptin receptor (NOP-R), also known as the orphanin FQ receptor, which is activated by the nociceptin peptide (NOP) (or orphanin FQ) (*13*, *14*). On the microarray, *Oprl1* mRNA was significantly down-regulated in the control-fear expression group when compared to IMO-fear expression group (group effect: $F_{2,11} = 30.7$; $P < 0.05$, ANOVA followed by Bonferroni post-test; Fig. 3E). Additionally, quantitative polymerase chain reaction (qPCR) analysis confirmed the microarray finding: *Oprl1* mRNA was down-regulated after fear conditioning and fear expression tests in control animals but not in animals with previous IMO exposure [Stress × Fear conditioning interaction: $F_{1,29} = 5$; $P < 0.05$ (Fig. 3G); Stress × Fear expression interaction: $F_{1,29} = 5.5$; $P < 0.05$, ANOVA followed by Bonferroni post-test (Fig. 3H)]. Moreover, *Oprl1* mRNA down-regulation only occurred when the conditioned and unconditioned stimuli are paired but not when they are unpaired (fig. S1C). *Oprl1* mRNA concentrations in the striatum do not change after fear conditioning or fear expression test in control or IMO groups (fig. S1, D and E). We hypothesize that amygdala-specific dysregulation of *Oprl1* gene expression after fear conditioning and fear expression in IMO mice may partial-

ly explain the mechanisms of altered fear learning in this PTSD-like animal model.

SR-8993 is a new specific NOP-R agonist

Using peptides and nonselective compounds, it has been suggested that NOP-R agonism impairs fear learning, whereas antagonism enhances fear (*15*, *16*). We wished to examine a more selective NOP-R agonist, with >100-fold selectivity over the μ opioid receptor and essentially no κ or δ opioid receptor activity, in our dysregulated fear model. We thus used a newly developed and selective NOP agonist, SR-8993 (Fig. 4A and fig. S2), which potently activates NOP-R [median effective concentration (EC_{50}) = $8.8 ± 1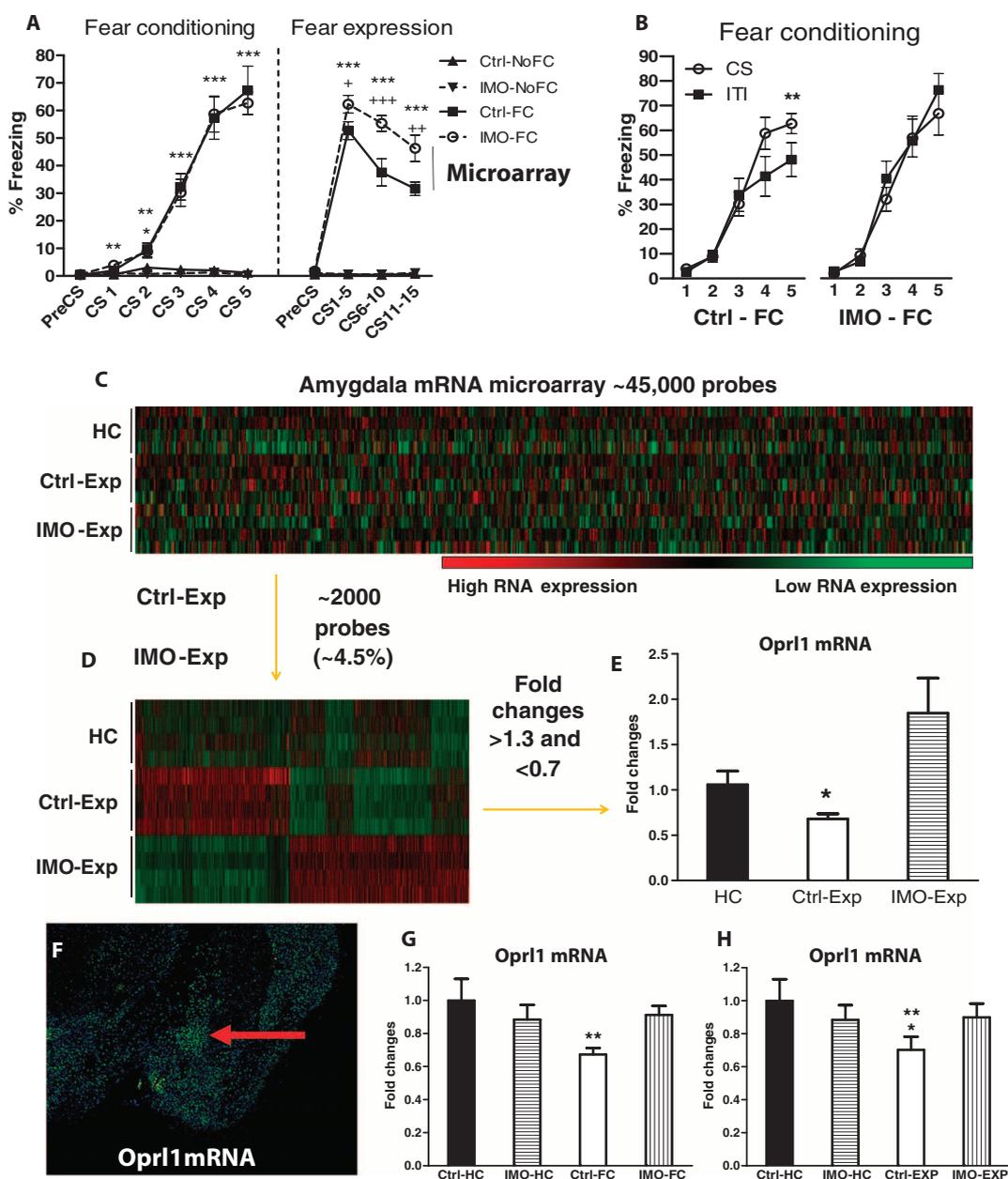.38$ nM; Fig. 4B]. SR-8993 is also characterized by unusually high selectivity for NOP-R over the closely related opioid receptors μ ($EC_{50} = 4800 ± 3300$ nM; Fig. 4C), κ ($EC_{50} > 10,000$ nM, estimated; Fig. 4D), and δ (no activity). SR-8993 passes common drug-likeness criteria and is not predicted to be associated with toxicity (*17*). Drug metabolism and pharmacokinetic studies in mice showed an in vivo half-life of $4.8 ± 0.6$ hours and a brain/plasma ratio of 0.55, measured 2 hours after intravenous dosing. It is stable in human and mouse liver microsome preparations and does not show inhibition of most major CYP450 isoforms, with the exception of 2D6 (Fig. 4A).

The drug metabolism, pharmacokinetic, and biochemical selectivity data were used in the design of experiments. An intraperitoneal injection of SR-8993 was used to obtain high brain concentrations of SR-8993 that activated NOP-R with minimal activation of μ or κ opioid receptors. A dose of 10 mg/kg achieved a $660 ± 51$ nM brain concentration 120 min after intraperitoneal administration, which is 75-fold above the NOP-R EC_{50} and 7-fold below the μ EC_{50}. We chose a somewhat lower injection dose (3 mg/kg) to allow targeting of NOP-R with a low probability of affecting other brain receptors.

SR-8993 prevents fear memory consolidation when injected into the central amygdala or systemically

SR-8993, given intraperitoneally, does not induce differences in locomotor activity, anxiety-like behavior, footshock reactivity, or cued-fear acquisition (Fig. 5, A to D). In contrast, it has clear on-target NOP-R activation effects. SR-8993 impaired cued-fear memory consolidation when injected 30 min before fear conditioning. Concordantly, SR-8993 dosing immediately after fear conditioning caused impaired cued-fear memory consolidation ($t_{11} = 2.3$; $P < 0.05$, two-tailed Student's t test; Fig. 5E). Similarly, SR-8993 injected into the central amygdala immediately after fear conditioning also impaired cued-fear memory consolidation ($t_{10} = 2.4$; $P < 0.05$, two-tailed Student's t test; Fig. 6, A and B). Moreover, SR-8993 impaired cued-fear memory consolidation when mice had a previous IMO exposure before fear conditioning ($t_{14} = 2.5$; $P < 0.05$, two-tailed Student's t test; Fig. 6C). SR-8993, which targets NOP-R, is a potent, selective, and effective agent to block cued-fear memory consolidation in control animals and a PTSD-like model. This suggests that activation of *Oprl1* expression in the amygdala with an NOP-R agonist is sufficient to impair fear consolidation even in a robust model of fear dysregulation.

Fig. 3. Differential regulation of *Oprl1* in the amygdala during cued-fear conditioning and cued-fear expression in a PTSD-like mouse model. (**A**) Immobilization-fear conditioning (IMO-FC) and control-fear conditioning (Ctrl-FC) groups equally acquired cued-fear conditioning. IMO in the absence of fear conditioning did not elicit freezing (IMO-NoFC). During the cued-fear expression (Exp) test, the IMO-FC group presented enhanced freezing relative to the nonstressed fear conditioned group (***$P \leq 0.001$, IMO-FC versus IMO-NoFC, repeated-measures ANOVA followed by Bonferroni post-test; +$P < 0.05$, ++$P \leq 0.01$, +++$P \leq 0.001$, IMO-FC versus Ctrl-FC, repeated-measures ANOVA followed by Bonferroni post-test) ($n = 10$ mice per group). IMO alone did not induce freezing in the fear expression test. The term "microarray" and the line (under fear expression) denote that at 2 hours after fear expression, microarray analysis was performed to identify differential gene expression in the IMO-FC and Ctrl-FC groups. (**B**) During fear conditioning, the control-fear conditioning group (Ctrl-FC) presents discrimination of the conditioned stimulus 5 (CS5) presentation versus the intertrial interval (ITI) (period between stimulus) (**$P \leq 0.01$, repeated-measures ANOVA followed by Bonferroni post-test), whereas the IMO-fear conditioning group (IMO-FC) does not. (**C**) mRNA microarray analysis of amygdala tissue showing 45,281 transcripts of amygdala mRNA, obtained 2 hours after fear expression ($n = 4$ mice per group). (**D**) Selection of the 1963 probes (4.34%) that present statistically significant changes in control-fear expression (Ctrl-Exp) group versus the IMO-fear expression (IMO-Exp) group. (**E**) *Oprl1* mRNA is down-regulated in the control-fear expression (Ctrl-Exp) group versus the IMO-fear expression (IMO-Exp) group (*$P < 0.05$, ANOVA followed by Bonferroni post-test). (**F**) *Oprl1* mRNA is highly expressed in the mouse central amygdala (red arrow) (12). (**G**) In replication studies, fear conditioning induces down-regulation of *Oprl1* mRNA in the amygdala of control-fear conditioned (Ctrl-FC) mice [*$P < 0.05$ versus home cage group (HC), ANOVA followed by Bonferroni post-test, $n = 8$ mice per group]. The mice in the home cage group were undisturbed in the vivarium and had compensatory handling the same days that the IMO mice were exposed to stress. (**H**) The cued-fear expression test also induced down-regulation of *Oprl1* mRNA in the amygdala of the control-fear conditioning group (Crl-FC) (*$P < 0.05$ versus HC, **$P \leq 0.01$ versus IMO-FC, ANOVA followed by Bonferroni post-test; $n = 8$ mice per group).

The *OPRL1* gene is associated with altered fear learning and amygdala-insula functional connectivity in PTSD patients

We next examined if *OPRL1* is involved in PTSD and fear learning in a traumatized human population. For that purpose, we performed a tag single-nucleotide polymorphism (SNP) analysis (18, 19), spanning 20 kb of the *OPRL1* locus with a total of five SNPs obtained from an Illumina OmniQuad 1M array. We examined whether each SNP was associated with PTSD diagnosis in this cohort of highly traumatized urban civilian subjects ($n = 1847$). SNP rs6010719 was associated with increased PTSD symptoms in individuals exposed to moderate to severe child abuse [SNP × Child trauma interaction: $F_{2,1847} = 6.4$; $P < 0.005$, univariate ANOVA (UNIANOVA)]. Because *OPRL1* has also
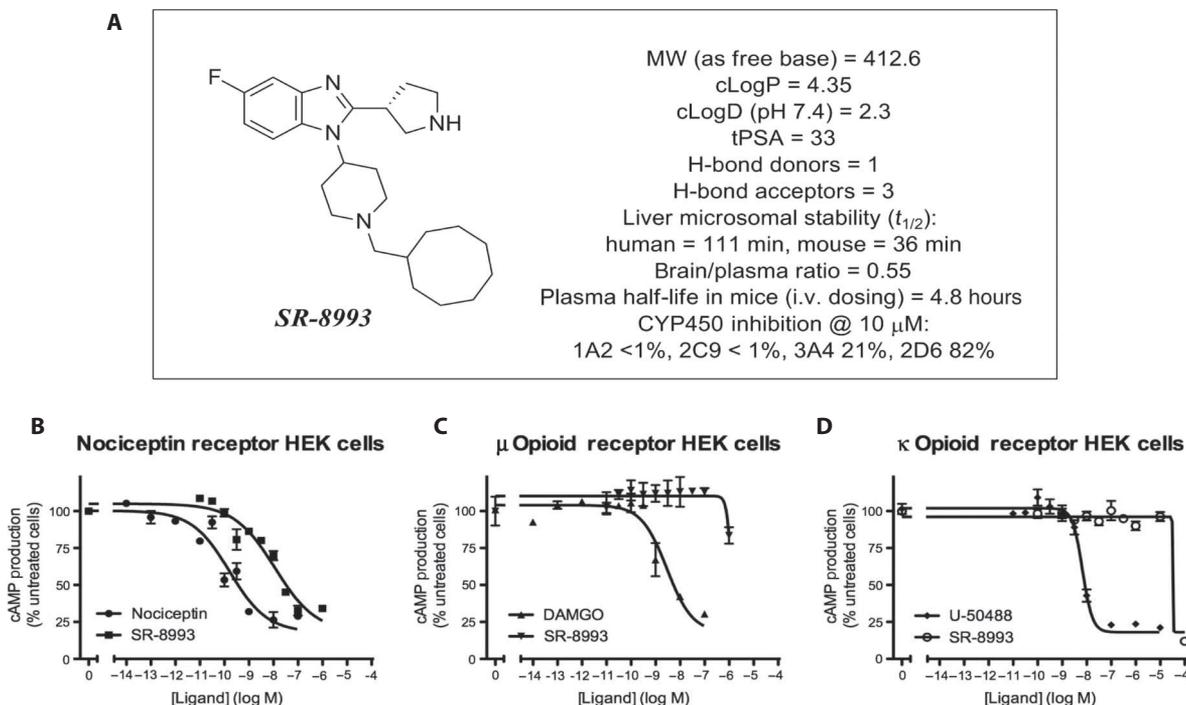
Fig. 4. SR-8993 is a new specific and potent NOP-R agonist. (**A**) SR-8993 is characterized by unusually high selectivity for the NOP-R over the closely related opioid receptors. Structure and physical and pharmacokinetic char- acteristics of SR-8993 are shown. (**B** to **D**) SR-8993 dose-response curves in human embryonic kidney (HEK) cells expressing the NOP/NOP-R (B), the μ opioid receptor (C), and the κ opioid receptor (D).

previously been associated with substance abuse (*20*) and PTSD is often comorbid with substance abuse (*21, 22*), and because PTSD is sometimes differentially associated with sex and age, we then reran the interaction, controlling for age, sex, and substance abuse. After these covariates were added, rs6010719 remained significant in the in- teraction (SNP × Child trauma interaction: $F_{2,1793} = 6.9$; $P \le 0.001$, ANOVA; Fig. 7, A and B). Additionally, we found no interaction with this SNP, and sex, age, or substance abuse was able to predict PTSD symptoms (all *P* values >0.1). Compared to CC carriers, the genetic association of G allele carriers for PTSD risk increased with the degree of trauma exposure (Fig. 7C).

To examine the effect of this *OPRL1* variant on human fear pro- cessing, we examined fear-potentiated startle (FPS) by measuring the acoustic startle reflex in a subset of this highly traumatized cohort. Given the above finding, we collapsed the GG and GC genotypes into G allele carriers versus the CC genotype during differential fear con- ditioning, in which one conditioned stimulus was paired with an aver- sive airblast (+), whereas a second conditioned stimulus was not (−) (*11, 23*). A significant interaction of genotype and type of conditioned stimulus ($F_{1,118} = 4.4$; $P < 0.05$, ANOVA) indicated that G allele car- riers did not discriminate on the FPS response between danger signals (+) and safety signals (−) during late fear conditioning. In contrast, those with the CC genotype showed an increase in the FPS response to the (+) conditioned stimulus versus the (−) conditioned stimulus ($F_{1,69} = 12.3$; $P \le 0.001$, ANOVA; Fig. 7D). Examining the difference score between the (+) and (−) conditioned stimuli, G allele carriers presented a significantly decreased discrimination rate even after tak- ing into account covariance for sex, age, and degree of childhood trau- ma and PTSD symptoms (SNP effect: $F_{1,113} = 5.7$; $P < 0.05$, ANOVA).

Notably, the *OPRL1* gene is highly expressed in the human amyg- dala in concordance with the mouse gene expression pattern [Figs. 3A and 7E and (*12*)]. To examine *OPRL1* association with fear proces- sing within the brain, traumatized women underwent functional magnetic resonance imaging (fMRI) as they viewed fearful and neutral face stimuli. We predicted that in G carriers, the amygdala would show increased reactivity and increased functional coupling with other fear-related regions such as the insula and anterior cingu- late (*24, 25*). Random-effects analysis of the main effect of fearful relative to neutral face stimuli showed robust bilateral amygdala ac- tivation within our sample with no differences between G and CC car- riers. However, for fearful relative to neutral faces, a two-group *t* test comparing task-based functional connectivity for G versus CC carriers showed greater functional connectivity between the amygdala and posterior insula in the GG/GC group (Fig. 7F; $P_{corr} < 0.05$).

DISCUSSION

This study demonstrates translational convergence in a gene asso- ciated with PTSD in human patients and a PTSD-like mouse model. Moreover, the SNP rs6010719 of the *OPRL1* gene is associated with PTSD symptoms, and this may be explained by its effect on altered fear learning and fear discrimination mechanisms. Additionally, this SNP is associated with differential amygdala-insula functional con- nectivity, and the insula has been reported to be involved with PTSD (*26*). Given the potential role of the nociceptin peptide in pain percep- tion, the amygdala-insula functional connectivity is intriguing given this circuit's role in central pain processing (*27*).
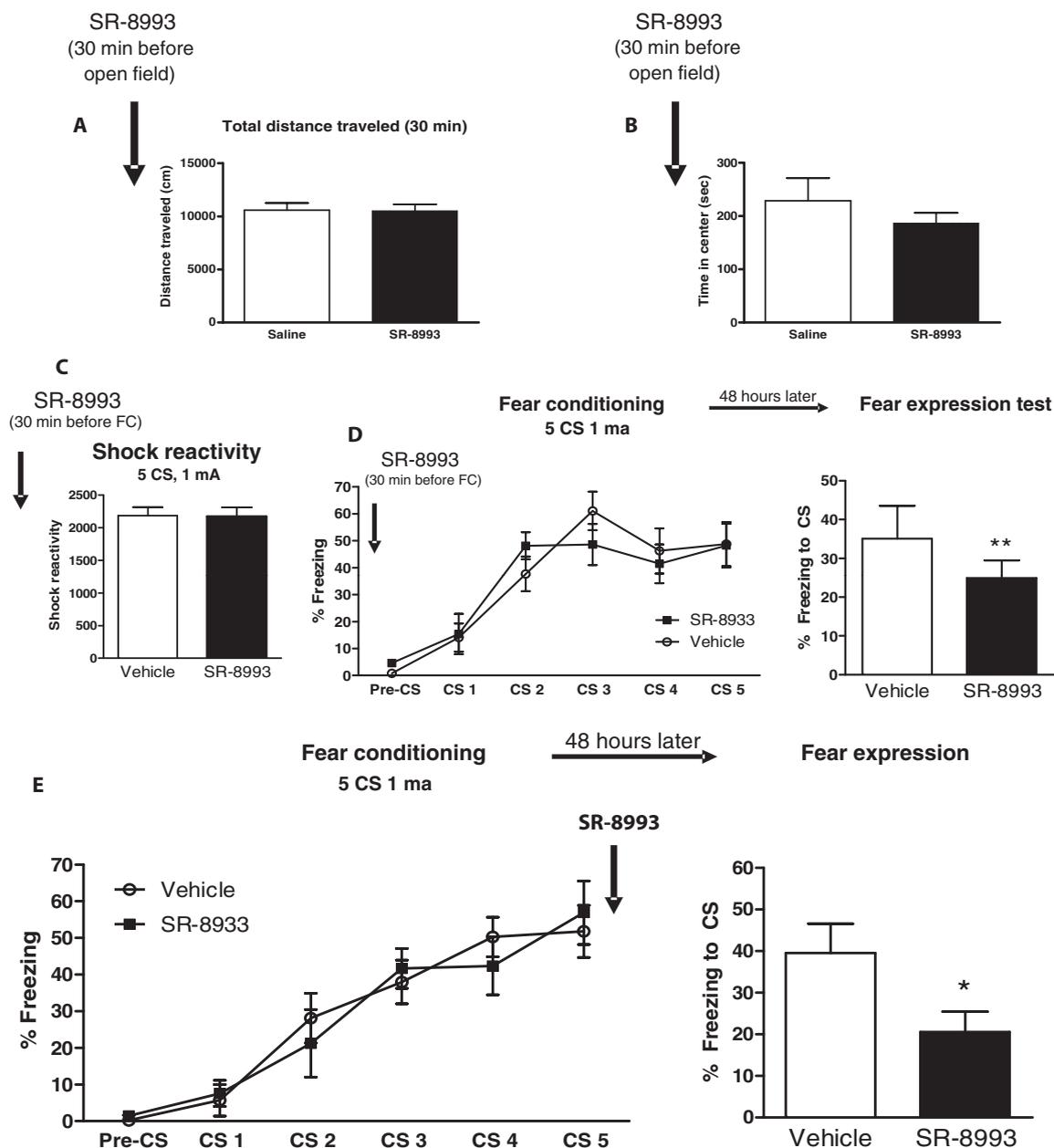
Fig. 5. The NOP-R agonist SR-8993 impairs cued-fear memory consolidation in mice. The NOP-R agonist SR-8993 (3 mg/kg), when systemically injected, impairs cued-fear memory consolidation but has no effects on anxiety, shock reactivity, or fear acquisition. (**A**) SR-8993 does not elicit locomotor changes evaluated with the open field ($n = 8$ mice per group). (**B**) SR-8993 has no effect on anxiety evaluated by the time spent in the center of the apparatus in the open field ($n = 8$ mice per group). (**C**) SR-8993 does not induce changes in pain sensitivity to mild electric footshock evaluated in the startle chamber ($n = 8$ mice per group). (**D**) SR-8993 does not alter freezing during cued-fear conditioning but impairs fear memory consolidation when evaluated 48 hours later in the fear expression test (**$P < 0.01$, two-tailed Student's t test; $n = 8$ mice per group). (**E**) Systemic injection of SR-8993 given immediately after cued-fear conditioning. Upon cue fear expression testing 48 hours later, the immediate post-training SR-8993 impaired fear memory consolidation, as shown by reduced freezing in the fear expression test (*$P < 0.05$, two-tailed Student's t test; $n = 6$ to 7 mice per group).

Concordantly, the amygdala *Oprl1* gene is dysregulated in a PTSD-like mouse model after fear learning. A previous study found that a single exposure to stress elicited *Oprl1* mRNA expression changes in amygdala (*28*). Thus, individual episodes of significant traumatic stress may alter amygdala *Oprl1* function, which affects later fear conditioning and cued-fear expression in our PTSD-like model. Additionally, using *Oprl1* knockout mice, *Oprl1* has been shown to be involved in contextual fear conditioning (*29*). That study did not find an effect on cued-fear learning, which might be explained by developmental compensation in a knockout mouse model. A recent report in rats
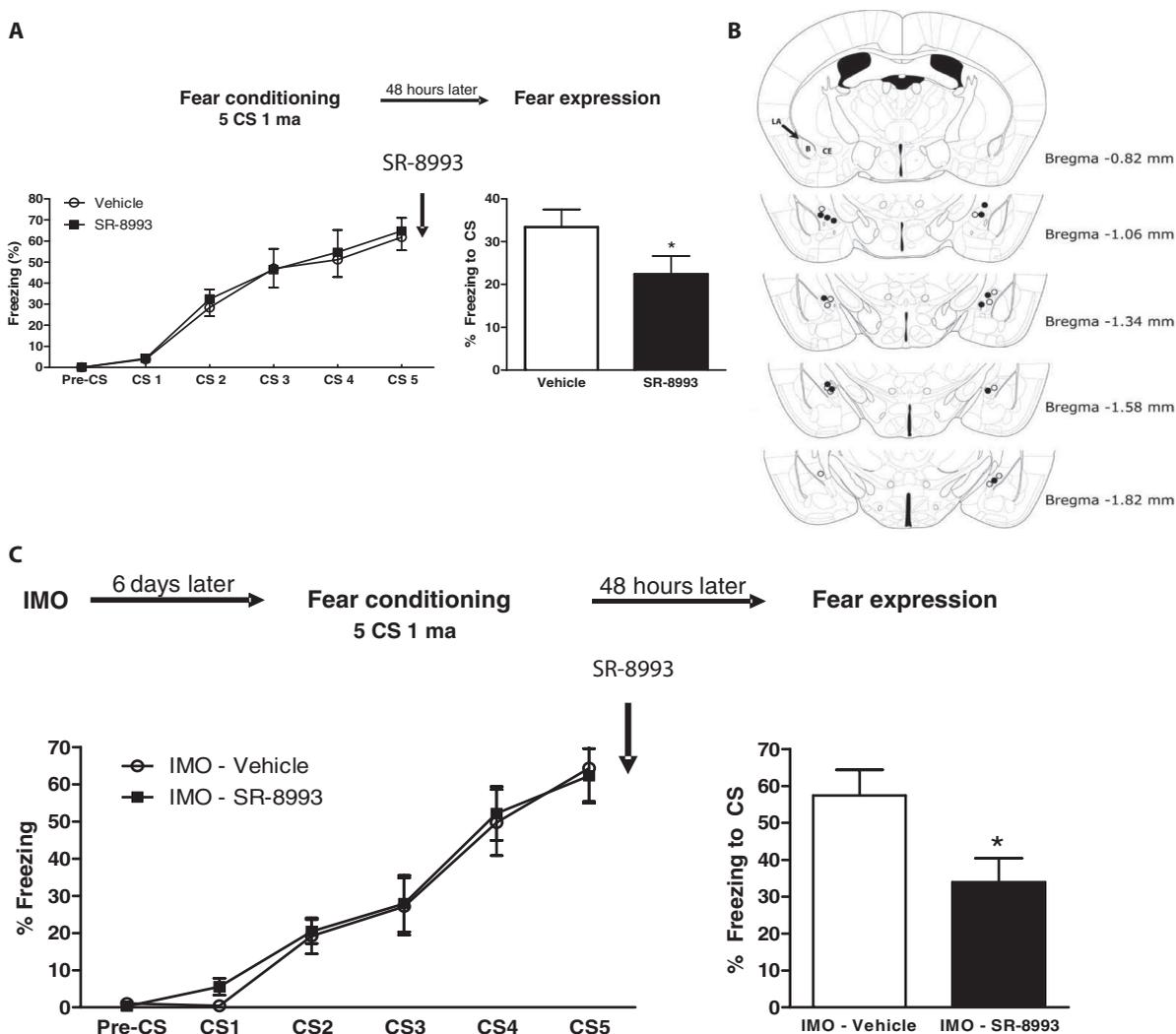
Fig. 6. SR-8993 impairs cued-fear memory when infused in the central amygdala and in a PTSD-like mouse model. (**A**) SR-8993 bilaterally injected into the central amygdala immediately after fear conditioning causes impaired fear memory consolidation as shown by the degree of freezing in the cued-fear expression test (*$P < 0.05$, two-tailed Student's t test; $n = 6$ mice per group). (**B**) Histological verification of SR-8993 infusion sites. The dots indicate the lowest point of the in-jector tip. Bregma is the anatomical point on the skull at which the coronal suture is intersected perpendicularly by the sagittal suture used as a reference point. (**C**) Systemic SR-8993 given immediately after fear conditioning in mice with a previous IMO exposure impaired fear memory consolidation as determined by reduced freezing in the fear expression test (*$P < 0.05$, two-tailed Student's t test; $n = 8$ mice per group).

suggests that nociceptive sensitivity and NOP concentrations are enhanced in a different PTSD-like model (single prolonged stress) than the one we have used in the present study (IMO) (30). Notably, the NOP agonist SR-8993 does not alter reactivity to footshock (Fig. 5C). This suggests that NOP-R regulation of nociception may be strongly influenced by the nature of the test, as previously reported (31).

This study does not support a model in which *Oprl1* within the amygdala is solely responsible for fear consolidation, but rather that it is required for normal fear memory formation to occur. Together, our data suggest that early intervention (shortly after fear learning) with a selective, centrally acting NOP agonist reduces fear memory consolidation. In humans, this may translate to the ability to prevent the likelihood of developing PTSD in the aftermath of trauma. Nota-

bly, the opiate agonist morphine, a primarily μ opioid receptor agonist given within 48 hours after trauma, reduces the risk of subsequent development of PTSD in observational studies (32, 33). The μ opioid receptor and NOP-R have a high level of homology but are involved in different biological functions (34). For example, the NOP agonist Ro 64-6198 has been shown to block the rewarding effects of morphine in mice (35) and does not induce rewarding effects per se in monkeys (36). Thus, the selective agonism of NOP-R in humans may mimic morphine's beneficial effect in reducing fear memory consolidation without the well-known undesired effects of opioids (for example, dependence, addiction, tolerance, gastrointestinal symptoms, and respiratory impairment). If this hypothesis is clinically validated and our studies are replicated, NOP-R agonists may be important candidates for

**Fig. 7. In humans, *OPRL1* is associated with PTSD and altered fear processing in PTSD. (A)** The significance level of each of the five *OPRL1* SNPs examined is shown as "log($P_{interaction}$)" for the interaction of each genotype and level of self-reported child abuse [using the Childhood Trauma Questionnaire (CTQ)]. For a Bonferroni-corrected P value of 0.01 (for five SNPs), the log(P) would be 2. We find that the rs6010719 SNP survives correction for the interaction test at $P \leq 0.005$. **(B)** Location of SNPs within the *OPRL1* gene (average 3.5-kb inter-SNP interval) and location of the gene on chromosome 20. **(C)** Specific interactions of the G allele carriers (GG, GC) versus CC allele carriers of the rs6010719 SNP in *OPRL1*, demonstrating that G allele carriers who have experienced greater trauma are at higher risk for PTSD symptoms ($F_{1,1847} = 10.5$; $P < 0.001$, UNIANOVA). Additionally, we found no interactions between this SNP and variables such as sex, age, or substance abuse that predicted PTSD symptoms (all P values >0.1). **(D)** G allele carriers of the rs6010719 SNP ($n = 49$) showed no discrimination between CS+ (danger signal) and CS− (safety signal) measured by the FPS response. In contrast, individuals of the CC genotype ($n = 70$) showed normal discrimination (interaction: *$P < 0.05$, ***$P \leq 0.001$, ANOVA). **(E)** *OPRL1* mRNA is highly expressed in the human central amygdala [picture modified from Allen Brain Atlas (12, 50)]. The arrow indicates the central amygdala. **(F)** (Left) Within-group random-effects analysis showed enhanced bilateral amygdala activation in response to fearful versus neutral face stimuli in all participants, irrespective of genotype (local maximum for left amygdala: $Z = 4.32$; $x, y, z = -32, -8, -16$; $P_{corr} < 0.05$; local maximum for right amygdala: $Z = 3.12$; $x, y, z = 28, -4, -28$; $P_{corr} < 0.05$). (Right) For fearful versus neutral face stimuli, G carriers ($n = 10$) have increased functional connectivity between amygdala (seed region) and right posterior insula, relative to CC allele carriers ($n = 19$) ($P_{corr} < 0.05$). Results are overlaid on a representative structural anatomical image in standard Montreal Neurological Institute space.

OPRL1 genotype x trauma interaction
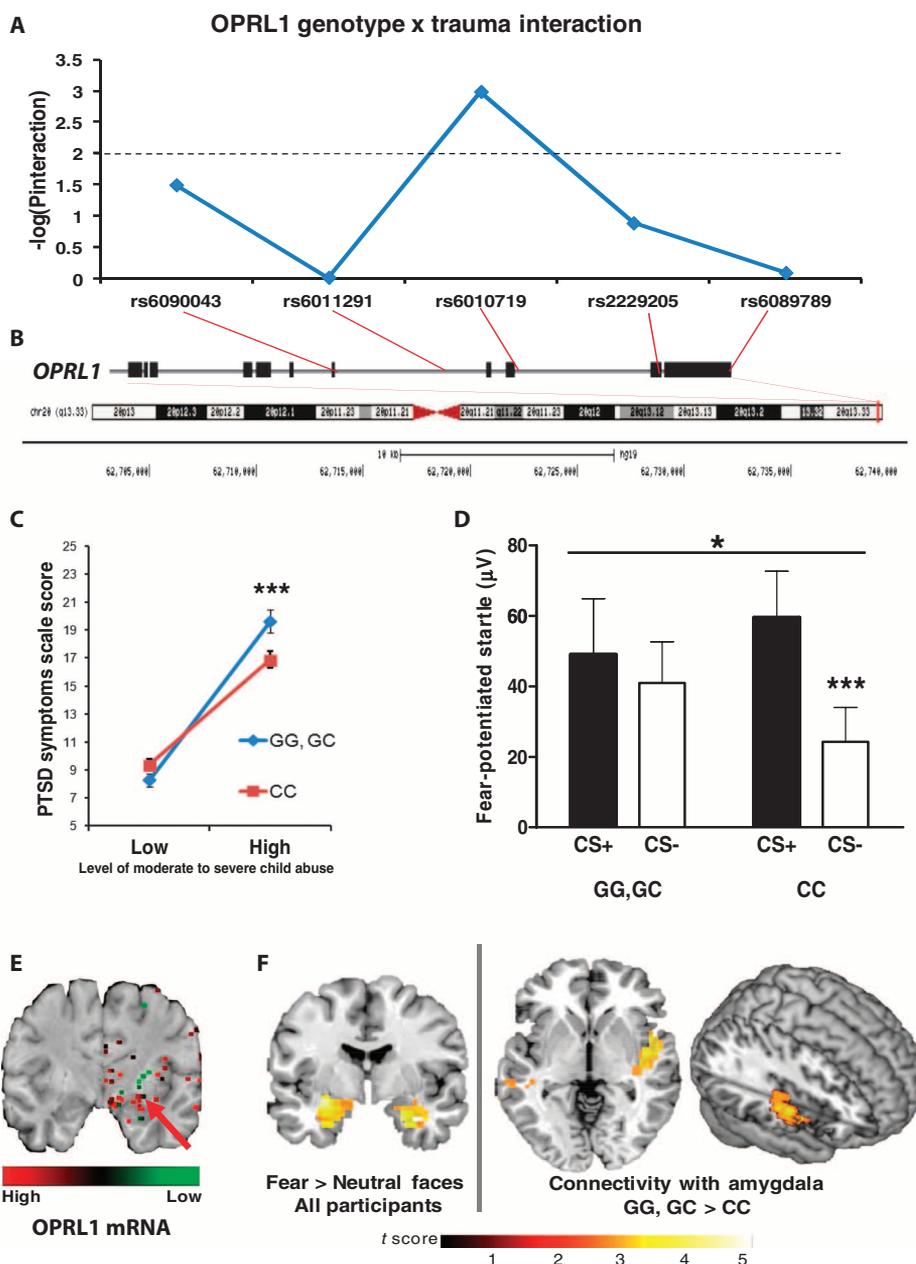
the prevention of PTSD, particularly in an early intervention setting, shortly after exposure to traumatic experiences.

MATERIALS AND METHODS

Animals

All experiments were performed on adult (2 to 3 months old) wild-type strain C57BL/6J from Jackson Labs, male mice that were group-housed in a temperature-controlled vivarium, with ad libitum access to food and water. They were maintained on a 12-hour/12-hour light/dark cycle, with all behavioral procedures being performed during the light cycle. All proce-

dures used were approved by the Institutional Animal Care and Use Committee of Emory University and in compliance with National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*.

Immobilization to wooden board (IMO)

Mice were exposed once for 2 hours to IMO, which was performed as previously described (9).

Water maze

The apparatus was a 122-cm-diameter pool (San Diego Instruments). Water was at room temperature between 19° and 20°C and made opaque by adding nontoxic latex. Swim paths were monitored and

stored with the video-tracking system SMART 2.5.19 (Panlab, Harvard Apparatus) for later analysis. Atlantis platform was used as previously described (10).

mRNA extraction and microarray hybridization

Total mRNA was isolated and purified from the tissue with the RNeasy Mini Kit (catalog 74106, Qiagen). Illumina Mouse WG-6 v2 Expression BeadChip microarray (Illumina Inc.) was assayed for 45,281 transcripts. We initially selected probes that were statistically different between control-fear expression (Ctrl-Exp) and IMO-fear expression (Exp) groups. From those genes, we initially selected for genes with >1.5- and <0.5-fold changes in both groups versus home cage (HC) group. However, no genes survived this combined stringent criteria. We then relaxed the criteria to select those that presented >1.3- and <0.7-fold change in both groups versus the home cage group, and those genes were prioritized on the basis of amygdala expression for further replication.

Complementary DNA synthesis and qPCR

Total mRNA was reverse-transcribed with the RT2 First Strand Kit (catalog 330401, Qiagen). The primer used for the qPCR was the TaqMan Oprl1 Mm00440563_m1 from Applied Biosystems.

SR-8993 synthesis and pharmacokinetics

Steps 1 and 2: Synthesis of 1-(cyclooctylmethyl)piperidin-4-amine. Steps 3 and 4: Synthesis of N^1-(1-(cyclooctylmethyl)piperidin-4-yl)-4-fluorobenzene-1,2-diamine. Steps 5 to 7: Synthesis of (R)-1-(1-(cyclooctylmethyl)piperidin-4-yl)-5-fluoro-2-(pyrrolidin-3-yl)-1H-benzo[d]imidazole (SR-8993). The cAMP (adenosine 3′,5′-monophosphate) biosensor assay, ACTOne Membrane Potential assay kit, was purchased from Codex BioSolutions. Hepatic microsomal stability assays and brain penetration assays were performed as previously described (37).

SR-8993 administration

SR-8993 was dissolved in physiological saline. Systemic intraperitoneal volume of injection was 8 μl/g, and the dose was 3 mg/kg for behavioral experiments and 10 mg/kg for brain penetration assays. Intramygdalar SR-8993 volume of injection was 0.5 μl with 100 ng of dose per side. Vehicle was physiological saline at the same volume of injection.

Stereotactic surgery and infusion of SR-8993

Stereotactic surgery was performed as previously described (38). Administration of a volume of 0.5 μl per side of SR-8993 was delivered over a period of 60 s.

Cued-fear conditioning and fear expression test

Cued-fear conditioning and fear expression tests were performed as previously described (9).

Human subjects

Detailed trauma interviews were performed using the PTSD Symptom Scale (PSS) on about 2000 highly traumatized males and females and the Clinician-Administered PTSD Scale (CAPS) on a smaller subset after informed consent were collected. As noted in some of our previous works (18, 19), these subjects are adult (average age, ~40 years), primarily female (60%), highly traumatized, impoverished, primarily African American, and with very large rates of current and lifetime PTSD. Other phenotype measurements included in the data collection were the CTQ as our primary child abuse measure, and current substance abuse.

Genome-wide association studies

All DNA for genotyping was quantified by gel electrophoresis with Quantity One (Bio-Rad) and then normalized to 400 ng. Using the Illumina Human Omni1-Quad BeadChip (Illumina Inc.), we performed SNP genotyping according to instructions by the manufacturer. Data were analyzed with PLINK.

Human FPS

Data were collected and analyzed as previously described (11, 23).

Neuroimaging

Eight fearful and eight neutral (four male and four female) faces were selected from the stimulus set of Ekman and Friesen (39). Faces were presented in a random order for 500 ms with a 500-ms presentation of a fixation cross separating each face stimulus. Brain imaging data were acquired on a Siemens 3.0-T Magnetom Trio TIM whole-body MR scanner (Siemens) with a standard head coil. Functional images were acquired with the Z-SAGA pulse sequence (40). Structural images were acquired with a gradient-echo, T1-weighted pulse sequence (repetition time, 2600 ms; echo time, 3.02 ms; voxel size, 1 mm × 1 mm × 1 mm).

Statistics

Statistics were performed with IBM SPSS Statistics 19.0. Detection of outliers was performed and, when necessary, removed from analyses. Repeated-measures ANOVA, one- or two-way ANOVA, or Student's t test (two-tailed) for independent samples was tested. Bonferroni was the post-hoc analysis. The results are presented as means ± or + SEM, and statistical significance was set at $P < 0.05$.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/5/188/188ra73/DC1
Methods
Fig. S1. Amygdala and striatal punches 2 hours after fear learning.
Fig. S2. SR-8993 was prepared in seven steps in 47% overall yield, as described in Materials and Methods.
Fig. S3. The overall process to prepare SR-8993 is a seven-step route completed in three separate stages.

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