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Supplementary Materials for

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

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The PDF file includes:

SUPPLEMENTARY MATERIALS

Supplementary Methods

Animals

All experiments were performed on adult (2-3 months old) wildtype 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 12/12hr light/dark cycle, with all behavioral procedures being performed during the light cycle. All procedures used were approved by the Institutional Animal Care and Use Committee of Emory University and in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Immobilization to wooden board (IMO)

Immobilization procedure was conducted in a different room separated from the fear conditioning (FC) and Fear expression (EXP) test apparatus. Each mouse was immobilized by gently restraining their four limbs in a prone position to metal arms attached to a wooden board for 2 hours. All animals of the same cage received the same treatment, IMO or compensatory handling (Control group, Ctrl) the same day of the stress (*9*). After stress exposure or compensatory handling, animals where returned to their home-cage where remained undisturbed until fear training. The fear conditioning started 6 days after the immobilization stress or the compensatory handling.

RNA extraction and Microarray hybridization

Mice were sacrificed under basal conditions (Home Cage group, HC), 2 hours after FC or 2 hours after EXP test. Brains were immediately fresh frozen on dry ice and stored at -80ºC. Amygdala and striatal tissue from both hemispheres was extracted by 1mm micropunch (Fig. S1A) and each structure from each mouse was individually stored. Total RNA was isolated and purified from the tissue with the RNeasy Mini Kit catalog # 74106 (Qiagen) following the manufacturer's instructions and previous studies (*38*). We obtained ~2 ug RNA per side for a total of ~4 ug per brain. For the microarray, only amygdala tissue was used with 4 animals per condition (HC, Ctrl-EXP and IMO-EXP groups). Electrophoresis assay and electropherogram to ensure the RNA quality was performed with Agilent 2100 BioAnalyzer PicoChip (Agilent Technologies) before the microarray. Illumina Mouse WG-6 v2 Expression BeadChip microarray (Illumina, Inc.) was assayed for 45,281 transcripts. RNA quality control, hybridizations and preliminary data analysis were conducted at the Cancer Genomics shared resource, Winship Cancer Institute (Emory University). Data retuned were Pair-wise two-condition comparisons including relative levels of RNA, fold change and p value. Heat maps were created with Genesis 1.4.0 (Institute for Genomics and Bioinformatics, Graz University of Technology, Austria) (*41*).

cDNA synthesis and quantitative PCR (q-PCR)

RNA isolation for q-PCR was performed as described above in a different cohort of animals than the microarray. Total RNA was reverse transcribed using the RT2 First Strand Kit catalog # 330401 (Qiagen) according to the manufacturer's instructions. The primer used for the q-PCR was the TaqMan *Oprl1* Mm00440563_m1 from Applied Biosystems (Carlsbad, CA). q-PCR

thermal cycling parameters were 10 minutes at 95°C, followed by 40 cycles of amplifications for 15 seconds at 95°C, 1 minute at 60°C. A dissociation stage, consisting of 15 seconds at 95°C, 1 minute at 60°C, and 15 seconds at 95°C, was added at the end. Quantification of mRNA was performed using the Applied Biosystems 7500 Real-Time PCR System. Relative levels of mRNA expression were normalized in all the samples with expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*38*). Graphics are represented by fold change obtained with the 2^-ddCt method (*42*).

Experimental procedures for the synthesis of SR-8993

The overall process to prepare SR-8993 is a 7-step route summarized below, completed in three separate stages as shown in the Fig. S3A.

(a) Steps 1 & 2: Synthesis of 1-(cyclooctylmethyl)piperidin-4-amine (Fig. S3B). Sodium triacetoxyborohydride (2.60 g, 12.3 mmol) was added to a solution of cyclooctanecarbaldehyde (1.55 g, 11.1 mmol), 4-Boc-aminopiperidine (2.00 g, 10.0 mmol), and acetic acid (1.5 mL) in methylene chloride (75 mL). The resulting mixture was stirred at room temperature overnight. The solvent was removed and the residue was treated with an aqueous solution of $NaHCO_3$ and extracted with methylene chloride. The organic layers were combined, washed with brine, dried over Na_2SO_4, and concentrated to dryness. Without further purification, the residue was dissolved in methanol (10 mL) and to this solution was added 4 M HCl in dioxane (6 mL). The mixture was stirred at room temperature overnight. The solvent was removed and dried under vacuum overnight to afford 1-(cyclooctylmethyl)piperidin-4-amine as a hydrochloride salt (2.60 g, 88%): LRMS (ES+) *m/z* for $C_{14}H_{29}N_2$ $[M+H]^+$calc'd 225, found 225.

(b) Steps 3 & 4: Synthesis of N^1-(1-(cyclooctylmethyl)piperidin-4-yl)-4-fluorobenzene-1,2-diamine (Fig. S3C). To a solution of 1-(cyclooctylmethyl)piperidin-4-amine (1.10 g, 3.70 mmol) in DMF (10 mL) was added 2,5-difluoro-1-nitrobenzene (0.62 g, 3.9 mmol) and K_2CO_3 (1.80 g, 13.0 mmol) and the resulting suspension was stirred at 70 ˚C overnight. After removal of solvent by rotary evaporation, the residue was partitioned between methylene chloride (50 mL) and water (30 mL). The aqueous layer was extracted with three additional portions of methylene chloride (30 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4, and concentrated to dryness. Without further purification, the residue was dissolved in ethanol (50 mL), treated with stannous chloride (4.40 g, 19.4 mmol), and the resulting mixture was stirred at 70 ˚C for 3 h. The solvent was removed by rotary evaporation and the residue was dissolved in methylene chloride (50 mL) and an aqueous solution of 10% NaOH (40 mL). The layers were separated and the aqueous phase was washed with two additional portions of methylene chloride (40 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4, and concentrated to dryness. The residue was purified by flash column chromatography (1-9% methanol in methylene chloride with 0.1% NH_4OH) to afford the title compound as a purple oil, 0.97 g, 79%: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.61 (dd, J = 8.4, 5.2 Hz, 1H), 6.43 (m, 2 H), 3.61 (br s, 2 H), 3.10 (m, 1 H), 2.80 (m, 2 H), 2.02 (m, 6 H), 1.68 (6 H), 1.59-1.43 (m, 10 H), 1.21 (m, 2 H). LRMS (ES+) *m/z* for $C_{20}H_{33}FN_3$ $[M+H]^+$calc'd 334, found 334.

(c) Steps 5- 7: Synthesis of (*R*)-1-(1-(cyclooctylmethyl)piperidin-4-yl)-5-fluoro-2-(pyrrolidin-3-yl)-1H-benzo[d]imidazole (SR-8993) (Fig. S3D). To a solution of 1-(cyclooctylmethyl)piperidin-4-amine (273 mg, 0.817 mmol) in methylene chloride (10 mL) was added (*R*)-*N*-Boc-β-proline(180 mg, 0.836 mmol), *N*-hydroxybenzotriazole (HOBT, 140 mg, 0.899 mmol), *N*-methylmorpholine (0.2 mL, 1.82 mmol), and 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 200 mg, 1.05 mmol). The resulting mixture was stirred at room

temperature for 2 days. After removal of solvent by rotary evaporation, the residue was dissolved in methylene chloride (30 mL) and washed with a saturated aqueous solution of $NaHCO_3$ (30 mL). The aqueous layer was extracted twice with additional methylene chloride (10 mL) and the combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Without further purification, the brown residue was dissolved in toluene (6.0 mL) and acetic acid (2 mL) and stirred at 90 ˚C overnight. The solution was cooled, concentrated, and partitioned between a saturated aqueous solution of $NaHCO_3$ (30 mL) and methylene chloride (30 mL). The layers were separated, and the aqueous layer was extracted with two additional portions of methylene chloride (10 mL each). The organic layers were combined, dried over Na_2SO_4, filtered, concentrated *in vacuo*, and purified by flash column chromatography (10 to 50% ethyl acetate in hexanes). The obtained oil was dissolved in methanol (4.5 mL) and to this solution was added 4 M HCl in dioxane (1.5 mL). After stirring at room temperature overnight, the solvent was removed and the residue was dissolved in methylene chloride (30 mL). This solution was extracted with a saturated aqueous solution of $NaHCO_3$ (30 mL). The aqueous layer was extracted two times with methylene chloride (15 mL each time). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by flash column chromatography (3-16% methanol in CH_2Cl_2 with 0.1% NH_4OH) to provide the title compound as a colorless oil. This free amine was again converted to a salt using 4N HCl in dioxane, giving 260 mg of the bis-HCl salt of SR-8993, 0.536 mmol, 66%). LRMS (ES+) *m/z* for parent $C_{25}H_{38}FN_4$ $[M+H]^+$calc'd 413, found 413. ^{1}H NMR for salt (400 MHz, d6-dmso): δ10.94 (br. s, 1H), δ9.89 (br. s, 1H), δ9.53 (br. s, 1H), δ8.50 (dd, J=8.8, 4.4 Hz, 1H), δ7.50 (dd, J=9.2, 2.4Hz, 1H), δ7.20 (td, J=9.2, 2.4Hz, 1H), δ4.98 (m, 1H), δ1.1-3.4 (overlapping multiplets, 39H).

cAMP Functional Assay

Unless otherwise stated, reagents were purchased from commercial sources. The cAMP biosensor assay, ACTOne Membrane Potential assay kit, was purchased from Codex Biosolutions. Puromycin and Ro 20-1724 were from Sigma-Aldrich (St. Louis, MO). NECA, nociceptin, DAMGO and U-50488 were from Tocris Bioscience (Ellisville, MO). Human liver microsomes were from Xenotech (Lenexa, KS). Black, clear-bottomed 384-well tissue culture-treated microtiter plates were from Greiner Bio-One (Longwood, FL). The cAMP biosensor assay cell lines were purchased from BD Biosciences (now Codex Biosolutions) as human embryonic kidney (HEK) 293 cells stably expressing a cyclic nucleotide-gated (CNG) channel and either the nociceptin, μ opioid or κ opioid receptors. Cells were cultured in T-175-cm2 flasks at 37°C and 95% relative humidity. Cells were plated and maintained in growth medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 25 mM HEPES, 5 mM L-glutamine, 250 ug/ml G418 (Geneticin), 1 ug/ml puromycin, and 1% antibiotic mix containing penicillin and streptomycin. HEK293-CNG cells were diluted in growth medium and dispensed into 384-well black-walled, clear-bottomed plates (final concentration, 14,000 cells/well) and allowed to incubate for 24 h at 37°C. Next, 20 μl concentrated membrane potential dye, prepared according to manufacturer's instructions, was dispensed into each well. An initial fluorescent measurement (T0) was performed (510–545 nm excitation and 565–625 nm emission), with the use of a EnVisionfluorescence plate reader (Perkin Elmer), after incubating of the cells for 3 h at room temperature. The potency of SR-8993 was determined using dose-response assays, and EC50 values were calculated from the resultant data. A 10-point dose-response curve with a customized dilution series was used for each receptor. SR-8993,

DMSO alone or control agonists (nociceptin, DAMGO or U-50488) was added to sample or appropriate control wells, respectively in the presence of phosphodiesterase inhibitor (Ro 20-1724) and NECA (to stimulate Gs mediated cAMP production). The plates were incubated for 30 min at room temperature before the final fluorescence measurement (T30) was taken. The data were normalized with the T0 measurement by dividing the fluorescence at T30 by the initial basal fluorescence at T0 to normalize for well-to-well variation, then by dividing by the fractional fluorescence in vehicle (DMSO, Ro 20-1724 and NECA) treated cells to normalize to 100% activity. Data were analyzed Prism (version 5.01; GraphPad Software Inc., San Diego, CA). Curvefitting and EC50 determinations were performed using the variable slope sigmoidal dose-response analysis tool in Prism.

Hepatic Microsomal Stability Assays
Microsome stability was evaluated by incubating 1 μM SR-8993 with 1 mg/mL hepatic microsomes from either human or mouse in 100 mM potassium phosphate buffer, pH 7.4 as previously described (*37*). The reactions were held at 37ºC with continuous shaking. The reaction was initiated by adding NADPH (1 mM final concentration) and the final incubation volume was 300 μL. Aliquots (40 μL) were removed and added to 160 μL acetonitrile containing an internal standard (propranolol) to stop the reaction and precipitate the protein. At the end of the assay, the samples were centrifuged through a 0.45 micron filter plate (Millipore Solventer low binding hydrophilic plates, catalog # MSRLN0450) and analyzed by LC-MS/MS.

SR-8993 administration
SR-8993 was dissolved in physiological saline. Systemic intraperitoneally (i.p.) 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 100ng dose per side. Vehicle was physiological saline at the same volume of injection.

Brain Penetration Assays
Plasma and brain levels were assessed after dosing SR-8993 i.p. as indicated (*37*). Samples were formulated at 2 mg/mL in 10/10/80 DMSO/tween/water. Blood was collected into EDTA containing tubes at 30 min and plasma was generated using standard centrifugation techniques. Brain samples were frozen upon collection and all samples were stored at -80ºC until analyzed. Brain tissue was not perfused prior to freezing to prevent diffusion of the compound out of the tissue during the process. Plasma samples were analyzed by treating 25 μL of plasma with 125 μL of acetonitrile containing an internal standard (propranolol) and filtering through a Millipore Multiscreen Solvinter 0,45 μm low binding PTFE hydrophilic filter. The filtrate was analyzed by LC-MS/MS using an API Sciex 4000. MRM methods were developed in positive ion mode and concentrations were determined using a standard curve between 2 to 2,000 ng/mL. Samples with concentrations outside of the curve were diluted with blank plasma and reanalyzed. Similar conditions were used to determine brain levels except the samples were weighed and acetonitrile was added (10x, weight by volume). The samples were sonicated to extract the compound from the brain matrix and then filtered as described above. A density of 1 g/mL was used to convert compound per mg tissue into molar equivalents.

P450 inhibition
P450 inhibition for the four major human isoforms are evaluated by following the metabolism of

specific marker substrates (CYP1A2 phenaceten demethylation to acetaminophen; CYP2C9, tolbutamide hydroxylation to hydroxytolbutamide; CYP2D6, bufuralol hydroxylation to 4′-hydroxybufuralol; and CYP3A4, midazolam hydroxylation to 1′-hydroxymidazolam) in the presence or absence of 10 μM SR-8993 (*35*). Reactions are then analyzed by LC-MS/MS for remaining SR-8993.

Stereotaxic surgery and infusion of SR-8993

Mice were anesthetized by i.p. injections of a Ketamine – Domitor (medetomidine) mixture and placed in a stereotaxic apparatus. Small holes were drilled into the skull and 6 mm stainless-steel guide cannulas (Plastics One) were lowered bilaterally in to the Central Amygdala (CeA). CeA coordinates were as follows: anteroposterior, -1.34mm; dorsoventral, -4.4mm; mediolateral, -2.4mm relative to bregma. Dorsoventral coordinates were measured from the skull surface with the internal cannula extending 2 mm beyond the end of the guide cannula. Coordinates were based on the mouse brain atlas of Paxinos and Franklin (*43*). The guide cannula was fixed to the skull using dental acrylic and jeweler's screws and dummy cannulas (Plastics One) were inserted into each guide cannula to prevent clogging. All animals were allowed to recover for 7 days before testing. During this time, mice were handled daily for acclimation and inspection of cannula fixture. Intracerebral Infusions of 0.5 μl of drug or vehicle were made using an injection cannula (33 gauge cannula, Plastics One), which extended 2.0 mm beyond the tip of the guide cannula. SR-8993 was delivered manually with a 5 μl Hamilton syringe attached to the injection cannula via polyethylene tubing (PE-10). Administration of a volume of 0.5 μl/side was delivered over a period of 60 seconds by slowly turning the microsyringe plunger. After each infusion, the injection cannula was allowed to remain for 2 minutes. After finishing the behavioral studies mice where perfused with 4% paraformaldehyde. After fixation, brains were equilibrated in 30% sucrose, sectioned on a cryostat and stained with cresyl violet. Visualization of the cannula placement was performed with a 4X objective on a light microscope to verify its location. Dots indicate the lowest point of the injector tip for each mouse for each group (Fig. 6B). Black dots represents SR-8993 group whereas white dots represents vehicle.

Water Maze

The apparatus was a 122-cm diameter pool (San Diego Instruments). Water was at room temperature between 19-20ºC and made opaque by adding non-toxic latex. Swim paths were monitored and stored using the video-tracking system SMART 2.5.19 (Panlab, Harvard Apparatus) for later analysis. Animals were gently released to the water with head facing the pool wall from a peripheral pseudorandom starting point (N, S, E or W). When a mouse did not reach the platform within 90 seconds, it was guided by hand to the platform. The water maze procedure lasted 5 days. On day 1, six trials of visible platform training were done to assess sensorimotor skills and motivation. The platform was at 2 cm above water level, and a pole holding a flag was mounted on it. Platform's location was changed on each trial to avoid spatial-learning strategies. On days 2–4, the invisible platform training involved four daily trials with the platform that remained always in the same position, between S and E, with the center at 25 cm from the wall and 2 cm underwater level. The intertrial interval (ITI) was 5–7 min for all days. On days 2 and 3, after training, the atlantis platform remained 30 seconds at the bottom of the pool. After 30 seconds it was raised to its original position (but hidden at 2 cm below underwater level) and the trial ended when the mouse found it. When a mouse did not reach the platform within 60 seconds after the end of a probe trial, it was gently guided by hand to the platform.

These probe trial were performed to assess short-term memory and atlantis platform was used to avoid extinction. On day 5, only a 60 seconds probe trial was performed to assess long-term memory. Heat maps were created with jTracks as previously described (*44*).

Open Field
The open field was an open box (27,9cm x 27,9cm) made of Plexiglas. The mice were placed in the apparatus to explore for 30 min, and then returned to home cages. Locomotor and center/periphery activity data was obtained by a video camera placed over the apparatus and analyzed using the SMART 2.5.19 video-tracking system (Panlab, Harvard Apparatus).

Elevated Plus Maze
The elevated plus maze consisted of two open arms (50 × 6.5 cm) and two closed arms with a wall (50 × 6,5 × 15 cm) attached to a common central platform (6,5 × 6,5 cm) to form a cross. The maze was elevated 65 cm above the floor. Test sessions lasted 5 minutes and behaviors were continuously recorded using a video camera placed over the apparatus. Activity was analyzed with stopwatch by a researcher blind to the each mouse treatment. Arm entry was considered complete if all four paws entered a closed or open arm from the central platform.

Cued-Fear Conditioning and Fear Expression test
Mice were given fear conditioning and fear expression in standard rodent modular test chambers (ENV-008-VP; Med Associates Inc) with an inside area of 30,5cm (L) x 24,1cm (W) x 21,0cm (H). Mice were given a 10-minutes chamber exposure session to habituate mice to handling and the training context. Mice that had immobilization stress were habituated to the test chambers before the stress session. The 2 habituation days were carried out the same days for all mice, independently if they were going to be submitted to the stress procedure or not. The tone conditioned stimulus was generated by a Tektronix function generator audio oscillator delivered through a high-frequency speaker (Motorola, Model 948) attached to side of each chamber. Mice received 5 trials of a conditioned stimulus (CS) tone (30 seconds, 6 kHz, 70 db) co-terminating with a US footshock 500ms, 1mA. The expression of fear was assessed 48 hours after fear conditioning and consisted of 15 CS tone trials (30 s each) with a 1.5 minutes inter-trial interval (ITI). Tone presentation and freezing data were controlled, stored, and analyzed with FreezeView software (Coulbourn Instruments) (*9*).

Shock reactivity
Shock reactivity was assessed in startle-footshock chambers (SRLAB, San Diego Instruments) consisting of a nonrestrictive acrylic plastic cylinder, 5,5 cm in diameter and 13 cm long, mounted on a Plexiglas platform which was located in a ventilated, sound-attenuated chamber. The footshock, US, was delivered through a removable stainless steel grid floor using one of four constant current shock generators (SDI, San Diego, CA) located outside the isolation chambers. A piezoelectric accelerometer mounted under each platform detected cylinder movements that were digitized and stored by an interfacing computer assembly. Shock reactivity was defined as the peak activity/accelerometer voltage that occurred during the 200 ms after the onset of the US. Response sensitivities were calibrated (SR-LAB Startle Calibration System) to be nearly identical in all startle cylinders. The tone CS was generated by a Tektronix function generator audio oscillator (Model CFG253, Beaverton, OR) and delivered through a high-frequency speaker (Motorola, Model 948) located 13 cm from the rear of each sound intensities were

measured by an audiometer (Radio Shack, Ft. Worth, TX, #33-2055). Stimuli presentation and data acquisition were controlled, digitized and stored by an interfacing IBM PC compatible computer using SRLAB software. On each of 2 days prior to training, mice were given a 10-minutes startle chamber exposure session to habituate mice to handling and the training context. During cued fear training used to measure shock reactivity, mice received 5 trials of a conditioned stimulus tone (30 s, 6 kHz, 70 db) co-terminating with a US footshock 500ms, 1mA (*9*). The training inter-trial interval was 5-min.

Human subjects

Detailed trauma interviews using the PTSD symptom scale (PSS) were collected on approximately 2,000 highly traumatized males and females and the Clinician Administered PTSD scale (CAPS) on a smaller subset following informed consent. As noted in some of our prior work (*18,19*), these subjects are adult (average age ~40 years old), 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 Childhood Trauma Questionnaire (CTQ) as our primary child abuse measure, and current substance abuse.

GWAS

All samples were tracked using a Laboratory Information Management System (LIMS; ThermoLab systems). DNA from saliva was collected in Oragene vials (DNA Genotek Inc., Ontario Canada) and extracted using the DNAdvance extraction kit (Beckman Coulter Genomics, Danvers MA). DNA from whole blood was collected in EDTA tubes and extracted using the E.Z.N.A. Blood DNA Midi Kit (Omega Bio-tek, Norcross, GA). All DNA for genotyping was quantified by gel electrophoresis using Quantity One (BioRad, Hercules, CA) and then normalized to 400 ng. Using the Illumina Human Omni1-Quad BeadChip (Illumina Inc.), SNP genotyping was performed according to instructions by the manufacturer.

We used PLINK for data cleaning and generating summary statistics (*45*). The five SNPs in the OPRL1 locus had a genotyping rate of 0,998, minor allele frequencies that were comparable to expected frequencies in African Americans (rs6089789, 0.0618; rs6011291, 0.09104; rs2229205, 0.09972; rs6010719, 0.208; rs6090043, 0.4507), and Hardy-Weinberg equilibrium (HWE) p-values that were greater than 0,05 (range 0.35-1.0). To control for population stratification, we conducted principal components analysis. Using the principal components, we extracted a cluster of unrelated African Americans as subjects for the association analyses (N=1793).

Human Fear Potentiated Startle

Startle response data were acquired at a 1000 Hz sampling frequency using the electromyography (EMG) module of the BIOPAC MP150 for Windows (Biopac Systems, Inc., Aero Camino, CA). The eyeblink component of the acoustic startle response was measured by EMG recordings of the right *orbicularis oculi* muscle using two 5-mm Ag/AgCl electrodes filled with electrolyte gel. One electrode was positioned 1 cm below the pupil of the right eye and the other was placed 1 cm below the lateral canthus. Impedance levels were less than 6 kilo-ohms for each participant. The acquired data were filtered, rectified, and smoothed using the MindWare software suite (MindWare Technologies, Ltd., Gahanna, OH) and exported for statistical analyses. The EMG signal was filtered with low- and high-frequency cutoffs at 28 and

500 Hz, respectively. The maximum amplitude of the eyeblink muscle contraction 20-200ms after presentation of the startle probe was used as a measure of the acoustic startle response. The startle probe was a 106-dB (A) SPL, 40-ms burst of broadband noise with near instantaneous rise time, delivered binaurally through headphones. The startle session began with a habituation phase to reduce startle reactivity and familiarize the subjects to the CSs. The fear conditioning phase immediately followed habituation and consisted of three blocks with four trials of each type (a reinforced conditioned stimulus, CS+; a nonreinforced conditioned stimulus, CS-; and the 40 ms, 108 dB noise probe alone, (noise alone, NA), for a total of 36 trials. Both CSs were colored shapes presented on a computer monitor for 6sec. The US was a 250ms air blast with an intensity of 140 p.s.i. directed at the larynx. The inter-trial intervals were randomized to be 9-22 sec in duration. The data shown in Fig. 7D corresponds to the average of block 2 and 3 (late acquisition).

Neuroimaging

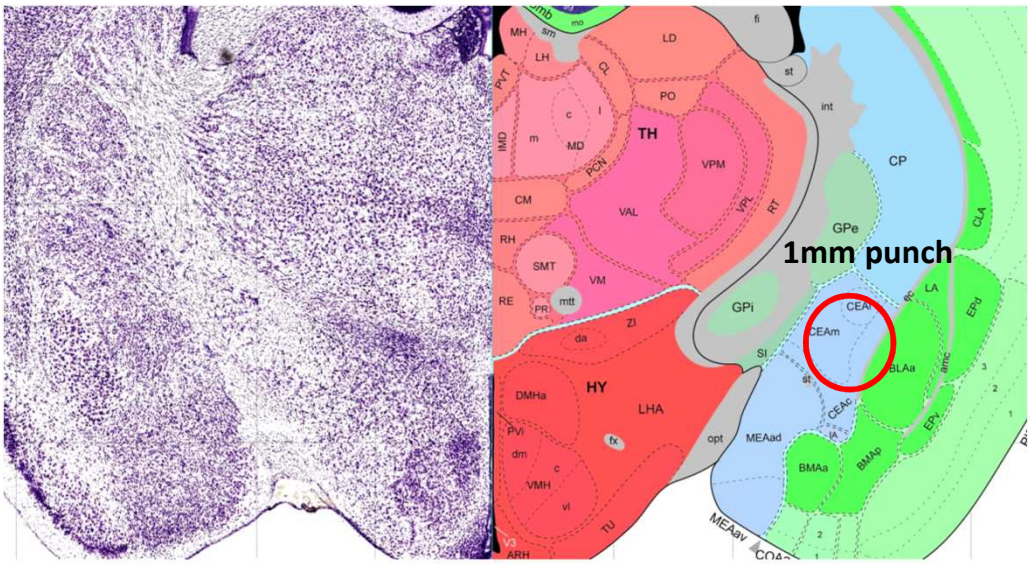
Eight fearful and eight neutral (4 male and 4 female) faces were selected from the stimulus set of Ekman and Friesen (*39*). Stimuli were projected onto a 24 inch screen at a resolution of 1280 x 1024 using EPrime 2.0 software (Psychology Software Tools, Pittsburgh, PA). Blocks of neutral and fearful stimuli (15 blocks each) were presented in a pseudorandom order. Each block was composed of all eight faces presented in a random order for 500 ms with a 500 ms presentation of a fixation cross separating each face stimulus. After every 10th block, a 10 sec rest period with the instruction "relax and look at the screen" was presented. Brain imaging data were acquired on a Siemens 3.0-Tesla Magnetom Trio TIM whole-body MR scanner (Siemens, Malvern, PA) using a standard head coil. Functional images were acquired using the Z-SAGA pulse sequence (*46*) to minimize signal loss due to susceptibility artifacts. Each scan volume contained 30 axially acquired 4mm thick images with an in-plane resolution of 3.44 x 3.44 mm2 utilizing the parameters: pulse repetition time 3000 ms, echo time 1= 30 ms, echo time 2 = 67 ms, at a flip angle of 90 deg. Structural images were acquired using a gradient-echo, T1-weighted pulse sequence (TR=2600ms, TE=3.02ms; 1mm×1mm×1mm voxel size). The EPI data were slice-timed and realigned using AFNI and the matrix to coregister the EPI images to the anatomical image was calculated using FSL. In FSL, the anatomical image was registered and normalized into standard MNI space using the ICBM152 template, and the resulting matrix was combined with the coregistration matrix and applied to the EPI images. The functional images were then smoothed with an 8mm Gaussian kernel. Individual participants' imaging data were analyzed using first level general linear models implemented in SPM5 (*47*). For each subject, evoked responses for each block were modeled with a boxcar function representing the onset and duration of the block, convolved with a canonical hemodynamic response function, as implemented in SPM5. Subject-specific motion parameters were included as regressors of non-interest. Statistical contrasts between conditions (e.g., fearful vs. neutral) were assessed using linear contrasts. Contrast images representing the linear comparison of beta values for the fearful versus neutral conditions were constructed for each participant, and were entered into group-level random effects analysis to identify clusters of significant activation. Task-based functional connectivity analyses were conducted using the CONN toolbox (http://web.mit.edu/swg/software.htm). Seed regions for the right and left amygdala were defined anatomically using the SPM Anatomy Toolbox (*47*). The mean timecourse across voxels within each seed region was extracted, and convolved with a regressor for fearful face blocks, and separately for neutral face blocks. For each voxel within the whole-brain mask, a contrast was

performed between covariance with the seed timecourses for fearful relative to neutral face stimuli. Individual participants' motion parameters and main effects of task condition were modeled as nuisance covariates. The resulting contrast images for individual participants then entered group-level analyses comparing participants carrying the G allele, and participants with the CC allele. Whole-brain results for the analyses of regional activation and functional connectivity were examined using a combined height-extent threshold to correct for multiple comparisons. Monte carlo simulation was implemented using Alphasim within the REST toolbox for SPM5 (*48*) for voxels within a gray-matter mask based on the ICBM 152-subject atlas.

Statistics

Statistics were performed with IBM SPSS Statistics 19.0. Detection of outliers was performed, and when necessary, removed from analyses. Repeated measures of ANOVA, one or two-way ANOVA and Student's ttest (two-tailed) for independent samples were tested. Bonferroni was the Post-hoc analysis. The results are presented as mean ± or + standard error of the mean (SEM) and statistical significance was set at $P < 0.05$.

A



B

PROBE_ID	SYMBOL	ACCESSION	PROTEIN PRODUCT	PROBE_SEQUENCE	CYTOBAND	TTEST Ctrl-Exp vs IMO-EXT
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ILMN_2469494	1700084C01Rik	NM_001033185.2	NP_001028357.1	GGCAAGAATGTGACAACATCTTTCGATCTCTTTTGAATTTCATGCCCCAC	1qH3	0.008
ILMN_1228406	Ugcgl2	NM_001081252.1	NP_001074721.1	CTGCTGAGTGGCCTTAAAAGTCTTGGGCTCAGTGAAGAAGAGAGGAACAG	14qE4	0.036
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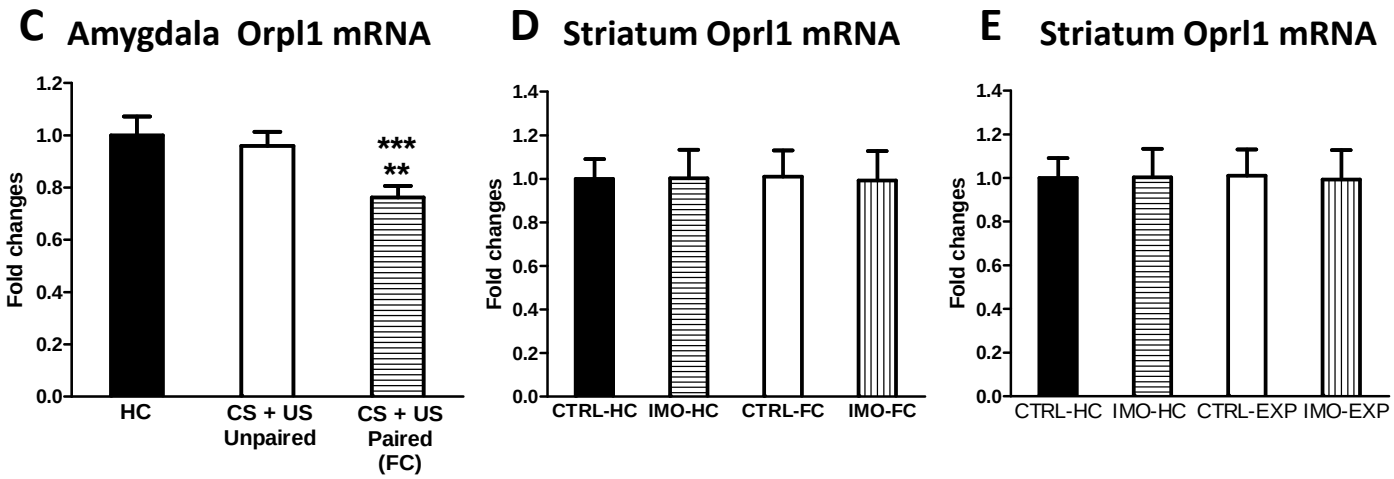
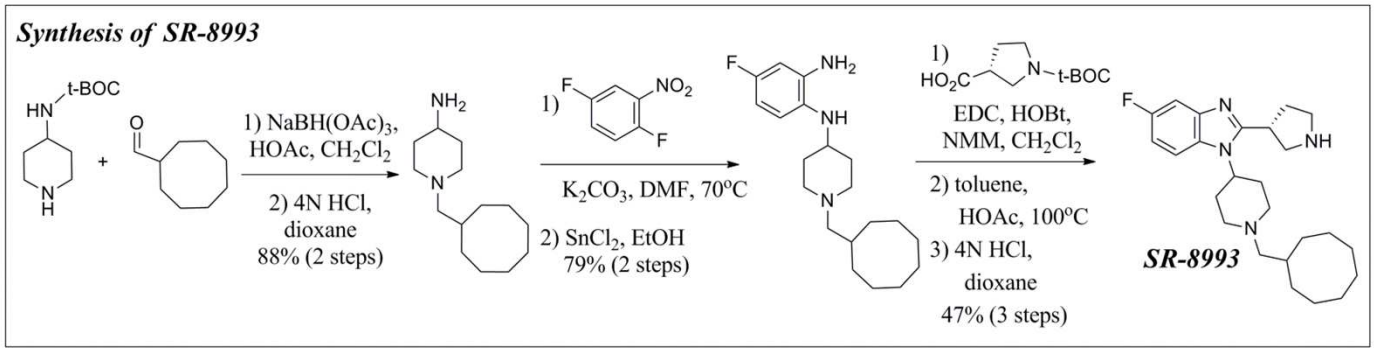
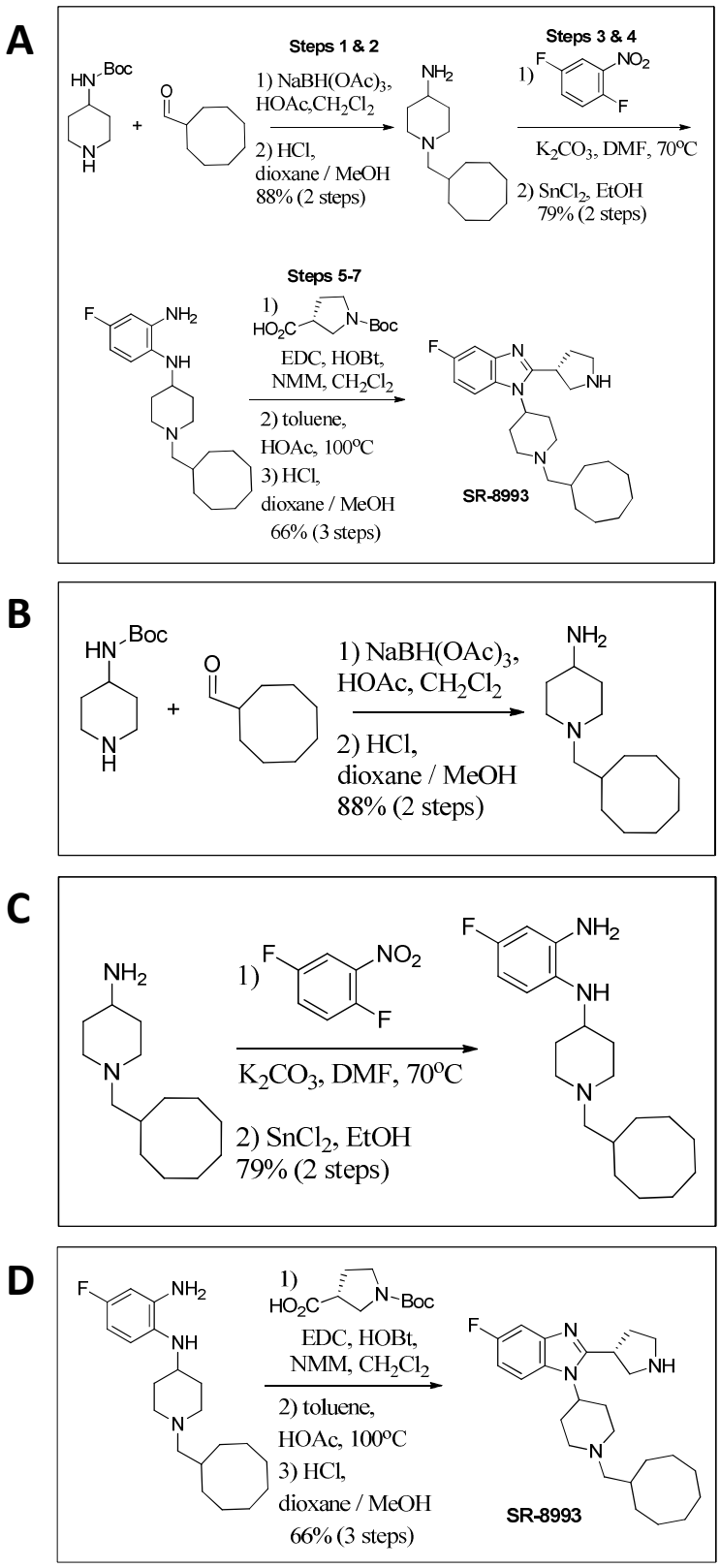


Supplementary Figure 1. Amygdala and striatum punches 2 hours after fear learning. (**A**) Graphical representation of the amygdala punches. Image modified from Allen Brain Atlas (*12*). (**B**) Microarray analysis of the amygdala punches lead to only 4 significant probes according to this criterion: 1) significantly differentially regulated in mice with previous IMO exposure 2) More than 1 ± 0.3 fold changes versus home cage (HC) group. (**C**) *Oprl1* mRNA downregulation occurs when the CS (tone) and US (electric footshocks) are paired but not unpaired (***P < 0.001 vs HC; **P < 0.01 vs unpaired, ANOVA followed by Bonferroni post-test). (**D**) Fear conditioning (FC), IMO nor IMO exposure in FC mice changes mRNA expression in the striatum. (**E**) Fear expression (EXP), IMO nor IMO exposure in EXP mice alters mRNA expression in the striatum.



Supplementary Figure 2. SR-8993 was prepared in seven steps in 47% overall yield, as described in Materials and Methods.



Supplementary Figure 3. The overall process to prepare SR-8993 is a seven-step route completed in three separate stages. (**A**) Summary of the three different stages to synthesize SR-8993. (**B**) Steps 1 & 2: Synthesis of 1-(cyclooctylmethyl)piperidin-4-amine. (**C**) Steps 3 & 4: Synthesis of N^1-(1-(cyclooctylmethyl)piperidin-4-yl)-4-fluorobenzene-1,2-diamine. (**D**) Steps 5- 7: Synthesis of (*R*)-1-(1-(cyclooctylmethyl)piperidin-4-yl)-5-fluoro-2-(pyrrolidin-3-yl)-1H-benzo[d]imidazole (SR-8993).