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Supplemental Information

Selective p38α MAPK Deletion in Serotonergic Neurons Produces Stress Resilience in Models of Depression and Addiction

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Inventory of Supplemental Information

Supplemental Figure 1 provides additional characterization of the specificity of the p38 MAPK phosphorylation response following kappa opioid receptor stimulation.

Supplemental Figure 2 provides a diagram describing the relationship between the DRN cell types specifically targeted in the CKO strategy and provides an explicit outline of the mouse breeding scheme required to generate the CKO and control mice used in the study. It supports the summary provided in Table 1.

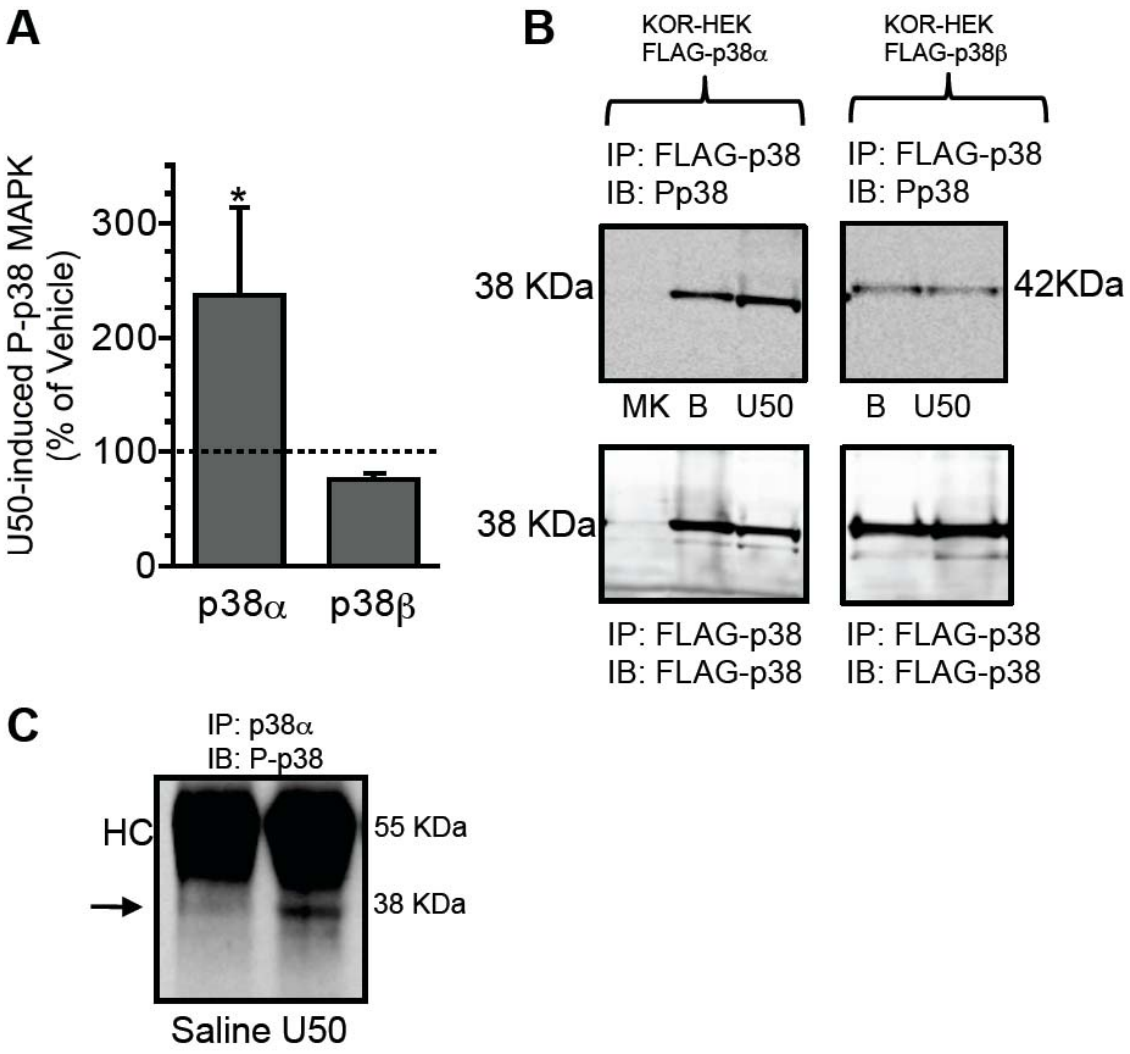
Supplemental Figure 3 provides additional characterization of the regional specificity of the Cre excision showing the effects of SERT-Cre and ePet-Cre on reporter expression in cingulate cortex, hippocampus, thalamus and caudal cortical regions to complement the DRN images shown in Figure 2. Also shown are image quantitation results to support the interpretation of the representative DRN image results shown in figure 2.

Supplemental Figure 4 provides additional behavioral characterization data for the p38alpha CKO mice supporting the interpretation of results shown in Figure 4.

Supplemental Figure 5 provides additional quantitation of the effects of genotype on SERT expression supporting the interpretation of results shown in Figure 6.

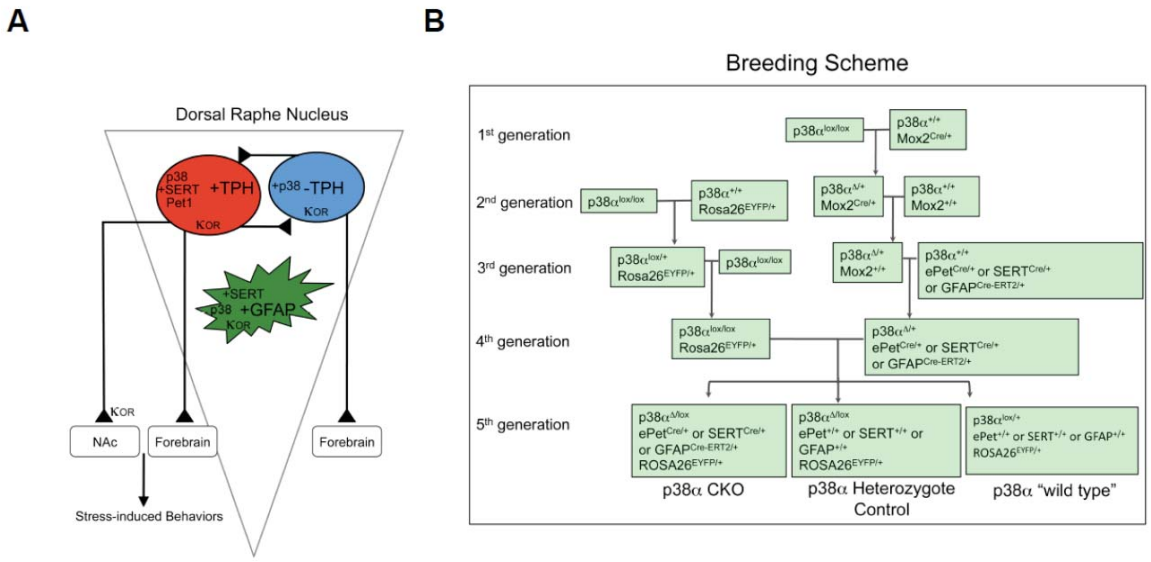
The **Supplemental Experimental Procedures** section provides more complete descriptions of the specific experimental methods used in this study than can fit in the main text of the manuscript.

Supplemental Figures



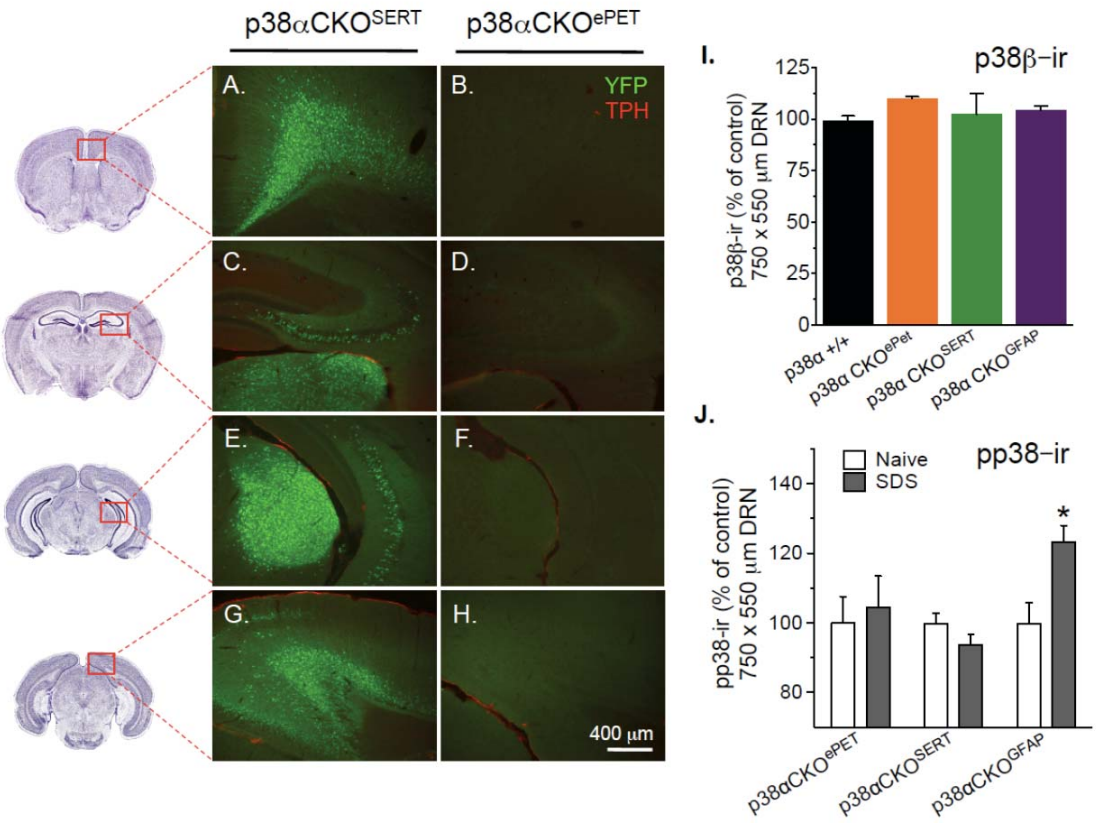
Supplemental Figure 1 (related to Figure 1): KOR stimulation increases p38α but not p38β MAPK (A) Quantification of phosphorylated p38 isoforms ± SEM following U50,488 (10 μM, 15 min) treatment in KOR expressing HEK293 cells. (n = 3, P < 0.05, t-test). (B) Representative Western blots of immunoprecipitation pull downs of FLAG-tagged p38 isoforms followed by immunoblots with phospho-p38 MAPK antibodies. Top Panels show

antiphospho-p38 immunoblots. Bottom panels show anti-FLAG immunoblots. (C) Representative western blot of p38 immunoprecipitations in NAc cell lysates. Lysates were immunoprecipitated with anti-p38α antibody and blotted with anti-pp38. (n = 4, each taken from a different animal).



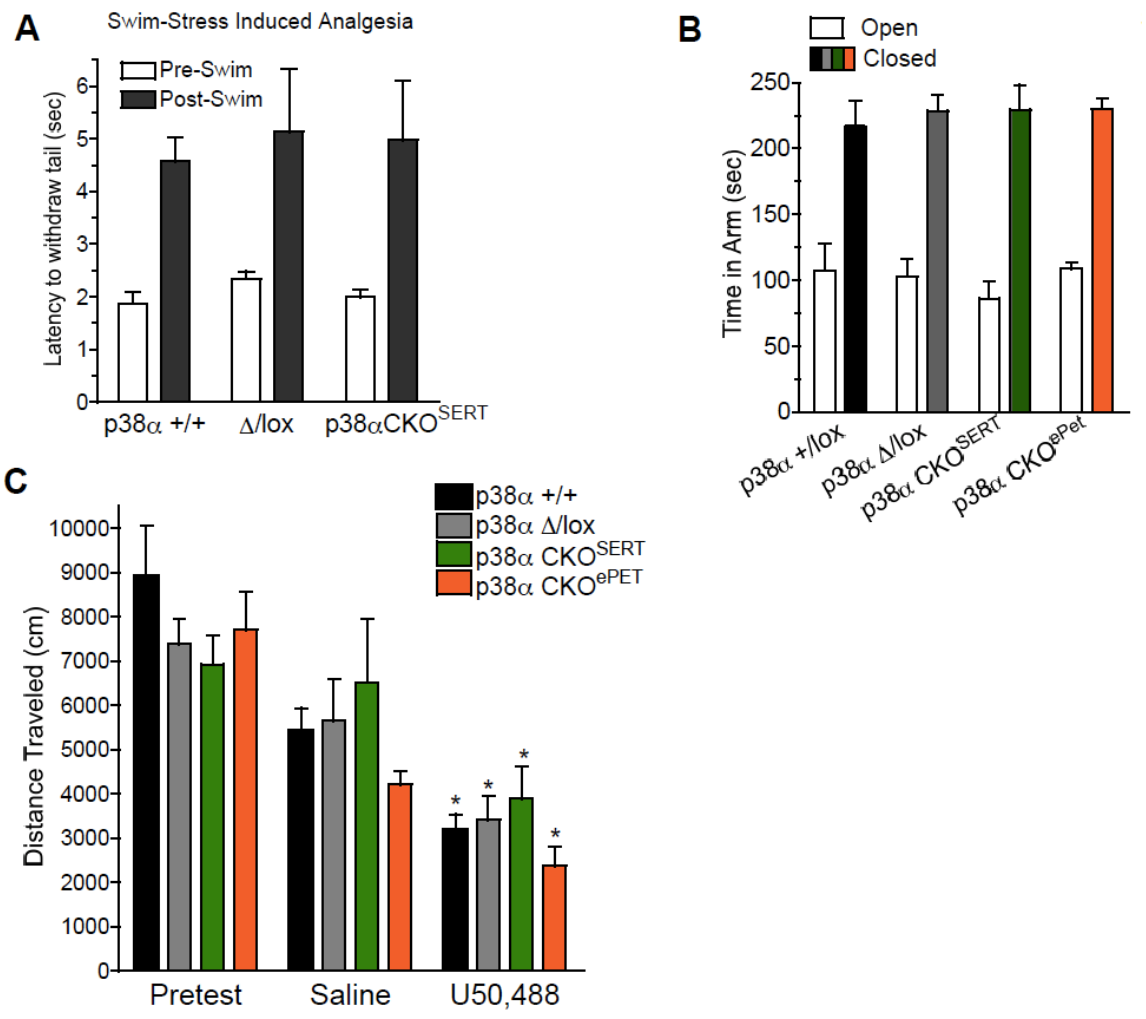
Supplemental Figure 2 (related to Table 1): Conditional Deletion of p38α MAPK in DRN cells (A) Cartoon depicting the cellular and molecular contexts of the dorsal raphe nucleus. This diagram depicts p38 expressing cells, Kappa opioid expressing cells (KOR), and astrocytes (GFAP-ir cells). The model highlights the diversity of cells within the DRN, and the relevant neuronal and non-neuronal markers used in this study. (B) Flowchart breeding scheme for mouse conditional knockout lines used in the present study. Details highlight the use of the Mox2-Cre deleter line to avoid transient expression during

development, as well as, the use of Rosa-YFP reporter lines for confirmation of successful Cre-mediated recombination.



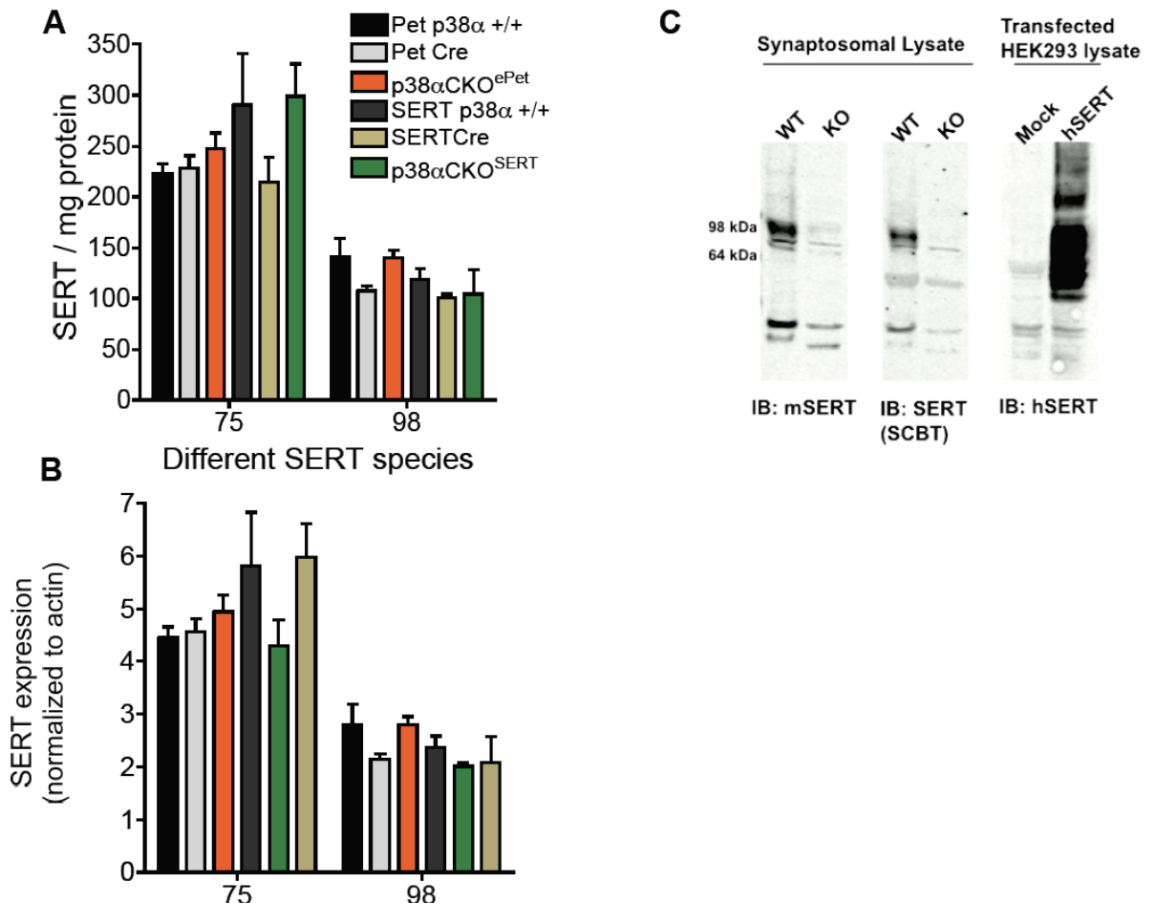
Supplemental Figure 3 (related to Figure 2): Anatomical specificity of SERT-Cre and ePet-Cre driver lines (A-H). Representative images of YFP and TPH-ir colocalization in p38αCKO^{SERT} and p38αCKO^{ePet} (n=3-4 independent experiments). Panels A and B are representative 10x magnification micrographs of the cingulate cortex. Panels C and D are 10X magnification images of the hippocampus, and Panels E-F are of the thalamus imaged at 10X magnification. More caudal regions of the cortex are shown in Panels G-H. (I) Quantification of

DRN p38β MAPK expression levels in a 750 x 550 μm field (p38β-ir, Mean pixel intensity ± SEM) in p38 wild type, p38αCKO^{ePet}, p38αCKO^{SERT}. Data show no evidence of compensatory changes in p38β in conditionally deleted p38α mouse lines (n =4-5). (J) Quantification of DRN pp38α MAPK expression levels in a 750 x 550 μm field (pp38-ir, Mean pixel intensity ± SEM) in naïve vs social defeat stress (SDS) exposed p38αCKO^{ePet}, p38αCKO^{SERT}, and p38αCKO^{GFAP}. Data show no evidence of pp38-ir in serotonergic selective knockouts, but as expected p38aCKO^{GFAP} mice show a significant increase (*$p<0.05$, SDS vs naïve, n=6-9) in DRN pp38-ir.



Supplemental Figure 4 (related to Figure 4): Additional behavioral Characterization Data for p38α CKO mice (A) Tail withdrawal latency before and after swim stress for wild type, Δ/lox, p38αCKO^{SERT} (*n* = 4–6; all groups show significant $P < 0.05$ *t*-test compared to pre-swim values). Bars represent mean ± SEM swim-stress induced analgesia. (B) Mean elevated plus maze (EPM) data for time spent in the open (white bars) or closed (colored bars) in each mouse genotype under brighter light, stressful conditions (120 lux). Data are expressed as ± SEM and show no significant difference between each

conditional p38α mouse line in terms of reduction in open-arm time (n = 6-8 per group) (C). Locomotor activity expressed as distance traveled during conditioning for conditioned place aversion experiments. As expected, U50,488 (2.5mg/kg i.p.) produced a significant ($p < 0.05$) reduction in locomotor activity in each genotype as compared to saline treated conditioning during the morning session.



Supplemental Figure 5 (related to Figure 6): SERT expression in CKOs (A) Mean ± SEM of Total SERT-ir expression (normalized to total mg protein) in mouse lines used in this study. Data were obtained using Odyssey imaging system (LiCOR) and quantitation of pixel intensity of each fluorescent band.

Data show that both species of SERT-ir (75 and 98) are expressed at equal levels (n= 4-5, where each n represents a separate animal). (B) Mean ± SEM of total SERT-ir expression (normalized to total actin) in mouse lines used in this study. (C) Representative western blots showing SERT antibody specificity. Data show loss of 98 kDa band using Santa Cruz Biotech antibody in SERT-KO mice. Furthermore, in untransfected HEK293 cells, neither of the 75k Da and 98k Da SERT-ir bands were evident. However HEK293 cells transfected with hSERT show robust SERT-ir as well as oligomeric complex formation using the Santa Cruz Biotechnology antibody (n=2).

Supplemental Experimental Procedures

Animals

Male C57BL/6 mice (20-30 gm) were group-housed, four to a cage, in ventilated mouse cages (Thoren Caging Systems, Hazelton, PA) within the Animal Core Facility at the University of Washington, given access to food pellets and water *ad libitum,* and maintained in specific pathogen-free housing. Mice were transferred at least 1 wk before testing into a colony room adjacent to the behavioral testing room to acclimate to the study environment. Housing rooms were illuminated on a 12-hr light/dark cycle with lights on at 7 A.M. All procedures with mice were approved by the University of Washington Institutional Animal Care and Use Committee in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Generation of serotonin-specific conditional knockout mice

Mice (Nishida et al., 2004) with loxP sites flanking the third exon of p38α MAPK (*Mapk14*$^{lox/+}$) were obtained from the RIKEN Bioresearch Center. *Mapk14*$^{lox/+}$ mice were crossed to *Mox2-Cre* mice (Tallquist and Soriano, 2000) to generate *Mapk*$^{\Delta/+}$ mice so that the null allele would not be susceptible to Cre recombination and thereby guard against ectopic germline Cre-mediated excision of the *Mapk14*^{lox} allele by Cre recombinase. Backcrossing with C57BL/6 wild-type mice allowed the *Mapk*$^{\Delta}$ allele to be segregated away from the *Mox2-Cre* allele.

Slc6a4-Cre has the Cre gene knocked into the promoter region of the endogenous serotonin transporter gene locus. ePet^{Cre} is a transgene driven by an enhancer element for the Pet1 transcription factor that is uniquely expressed in serotonergic neurons (Scott et al., 2005). The tamoxifen-inducible *Gfap-Cre-ERT2* transgene utilizes the human promoter for glial fibrillary acidic protein (GFAP) (Hirrlinger et al, 2006). These mice were then mated with *Mapk14*$^{lox/lox}$ mice, which in the case of *Mapk*$^{\Delta/+}$:*Slc6a4-Cre* parent would yield *Mapk*$^{\Delta/lox}$ conditional knockout progeny as well as *Mapk*$^{\Delta/+}$ progeny with or without lacking *Slc6a4-Cre*, regarded as littermate controls carrying one or two functional p38α alleles, respectively. These were the three primary classes of mice used in behavioral and biochemical studies. All types of p38αCKO mice were produced in expected Mendelian frequency and showed no discernable differences in growth, lifespan or overt health from either their p38α$^{\Delta/lox}$ (heterozygote) or

functionally wild-type ($p38\alpha^{+/lox}$) littermates. As reported previously (Nishida et al, 2004), $p38\alpha^{lox/lox}$ mice are behaviorally indistinguishable from wild type $p38\alpha^{+/+}$. *Slc6a4 knockout mice were obtained from Taconic Farms.*

Genotyping of mouse lines:

DNA was isolated from tail tissue obtained from weanling mice (21-28 days of age), and PCR screening was performed using the following primers: A3 (5'-ATGAGATGCAGTACCCTTGGAGACCAGAAG-3') and A4 (5'-AGCCAGGGCTATACAGAGAAAAACCCTGTG-3') for the floxed and wild type (+) p38α alleles, giving bands of 230 and 180, respectively. Primers A1 (5'-CCACAGAAGAGATGGAGCTATATGGATCTC-3') and A4 were used to detect the null $p38\alpha^{\Delta}$ allele as a 420-bp PCR product. The $SERT^{Cre}$ allele (450-bp band) was differentiated from wild type (350-bp) using 5'-CATCCGACCACT GACTGACCA-3', 5'-GGCACTAACCTCCACCATTCTG-3' and 5'-GAACGAAC CTGGTCGAAATCAG-3', while the Mox2, $ePet1^{Cre}$ and $GFAP^{CreERT2}$ transgenes were detected using 5'-AGCGTTCGAACGCACTGATTTCG-3' and 5'-CGCCGTAAATCAATCGATGAGTTG-3', yielding a 330-bp band. The Rosa26-^{YFP} reporter gene was screened for with 5'-AAGACCGCGAAGAG TTTGTC-3', 5'-AAAGTCGCTCTGAGTTGTTAT-3', and 5'-GGAGCGGGAGA AATGGATATG-3', giving a 320-bp EYFP and 600-bp wild type band (Supplemental Figure 2C).

Behavioral Analysis

Conditioned Place Aversion: Mice were trained in an unbiased, balanced three-compartment conditioning apparatus as described (Land et al., 2009, Bruchas et al., 2007). Briefly, mice were pre tested by placing individual animals in the small central compartment and allowing them to explore the entire apparatus for 30 min. Time spent in each compartment was recorded with a video camera (ZR90; Canon) and analyzed using Ethovision software (Noldus). Mice were randomly assigned to saline and drug compartments and received saline in the morning (10 mL/kg, i.p.) and drug in the afternoon at least 4 h after the morning training on 2 consecutive days (3 for CPP reinstatement). CPA was assessed on day 4 by allowing the mice to roam freely in all three compartments and recording the time spent in each. Scores were calculated by subtracting the time spent in the drug paired compartment post-test minus the pre-test.

Stress-induced Social Avoidance.

On days 1-3, naïve untreated mice were habituated by having free access to explore the entirety of a white plexiglas chamber 42 cm x 42 cm x 20 cm tall containing a small wire mesh cash at each end center between each wall. Mice were then tested for 3 min to assess their interaction with small mesh wire area at the distal side of the apparatus. A novel mouse was introduced into the wire mesh compartment and social interaction time was recorded over a 3 min period. On day 4 animals were placed in the home cage of a dominant, resident male for 20 min and social defeat behaviors were recorded. After the SDS, animals were

placed into the social interaction chamber and allowed to freely explore the entire chamber for 3 min in the absence of a novel mouse. Next a novel mouse was introduced into the interaction chamber behind the mesh, and the stressed animal was allowed to explore for an additional 3 min. Interaction scores were calculated by dividing the time spent in the interaction zone in the presence of a novel mouse by the time spent in the absence of a mouse.

Stress-Induced Cocaine Reinstatement

Mice were trained in an unbiased, balanced three-compartment conditioning apparatus as described (Land et al., 2009, Bruchas et al., 2007). Mice were trained in the same conditioning apparatus as described above, and the pretesting phase (day 1) was identical. However, on days 2–4, mice received saline in the morning (10 ml/kg, s.c.) and cocaine (15 mg/kg, s.c.) in the afternoon (4 hr interval). On day 5, animals were allowed to explore all 3 chambers to determine cocaine place preference. Animals next had extinction training on days 6–8 by injection with saline in both the morning and afternoon prior being placed in the conditioning chambers. Animals were tested for place preference on day 9 with a second free exploration of all three chambers. Animals were judged to have extinguished cocaine preference if scores fell within 15% of their initial preference (~60% of animals). Animals that extinguished were put through stress-induced reinstatement. on day 11, cocaine trained and saline extinguished animals were exposed to a single 20-min social defeat stress (SDS)

session. Immediately after the SDS, place preference was again determined by allowing the mice to freely explore the 3 chambers. Animals that did not reinstate to stress were exposed on the following day to a priming injection of cocaine (15 mg/kg, s.c.) and placed in the testing apparatus to freely explore all chambers. Reinstatement scores were calculated by subtracting the time spent in the cocaine side post-test minus the extinction test.

Viral Procedures

***Preparation of AAV1-Cre$^{\Delta}$GFP*:** A plasmid with CMV and chicken β-actin promoter and first intron driving expression of a Cre-EGFP fusion protein with a myc-tag and nuclear localization signal at the N-terminus, that was followed by a WPRE sequence and a bovine growth hormone poly-adenylation site was digested with *Bst*B1 and *Eco*RV, treated with DNA polymerase in the presence of dCTP and then religated. The resulting plasmid precisely deleted 47 amino acids from the middle of Cre recombinase, while maintaining the reading frame. After transfection into HEK cells carrying a conditional, Cre-dependent DsRed2 reporter gene, there was no recombination (no red fluorescence), but EGFP expression from the fusion protein could still be detected, although it was less bright than that observed with the plasmid encoding intact Cre-EGFP recombinase. This virus was prepared as above.

Local intracranial injections: were performed as previously reported (Zweifel et al., 2008, Land et al., 2009). Briefly, AAV1*Cre-GFP* virus contains an opening

reading frame for Cre-EGFP fusion protein with a myc tag and nuclear location signal at the N-terminus. The promoter is a cytomegalovirus-chicken beta-actin promoter and it is followed by a woodchuck postregulatory element (WPRE) and bovine growth hormone polyadenylation signal. It was prepared by transfection of HEK293 cells with the helper plasmids and a plasmid containing AAV1 coat proteins. The virus was purified by iodixanol and Q column and tittered to a concentration of ~1.2 $x10^{12}$ particles/ml.

Mice were anesthetized with isoflurane and injected with 1ul of AAV1-Cre or AAV1-ΔCre-GFP unilaterally into the dorsal raphe (stereotaxic coordinates: x = 0.0, y = −4.65, z = −3.85 mm from bregma, or bilaterally into the Nucleus Accumbens: x = 0.75, y = +1.45, z = −5.00 mm from bregma (NAc). Mice were allowed to recover for 3 weeks prior to behavioral testing; this interval also permitted optimal AAV expression and Cre recombinase activity.

Drug Treatments

For $GFAP^{Cre\text{-}ERT2}$ mice inducible cre-driver mice, Tamoxifen (Sigma-Aldrich) was dissolved in autoclaved sunflower oil (Sigma-Aldrich) by rocking overnight before use. Tamoxifen was stored in the dark at 4°C for not more than 7 days before use, and given at a dose of 66 mg/kg once daily for 7 days. At least 5 days elapsed between the end of tamoxifen administration and any further testing to allow drug clearance, tamoxifen-dependent Cre-ERT2 mediated gene excision

and subsequent knockdown of p38α MAPK to occur. Norbinaltorphimine, (±)U50,488, and cocaine were provided by the National Institute on Drug Abuse drug supply program (Bethesda, MD), dissolved in sterile saline immediately before use. Sodium pentobarbital (Lundbeck USA, Deerfield, IL) and isoflurane (Hospira, Lake Forest, IL) were obtained from the University of Washington Drug Services.

Immunohistochemistry

Immunohistochemistry was performed as described in Land et al., 2007 and Bruchas et al., 2007. Briefly, mice were anesthetized with pentobarbital and intracardially perfused with ice-cold 4% paraformaldehyde in phosphate buffer (PB). Brains were dissected, post-fixed 2 hr at 4 ºC and cryoprotected with solution of 30% sucrose in 0.1M PB at 4ºC for at least 3 d, cut into 40 μm sections and processed for immunostaining. 40μm brain sections were washed three times in PBS and blocked in PBS containing 0.3% Triton X-100 and 5 % normal goat serum. Sections were then incubated for 36 hr at room temperature in rabbit anti phospho-p38 antibody (1:50, Cell Signaling 4511), the mouse phospho-p38 antibody (1:50, Santa Cruz sc-7973), chicken anti-GFAP (1:2000, Abcam 13970), and mouse anti-GFAP (1:1000, Sigma G3893), rabbit anti-p38α antibody (1:40, Santa Cruz sc-535) and mouse anti-TPH (1:1000, Sigma-Aldrich T0678). Following incubation, sections were washed six times in PBS and then incubated for 2 hr at room temperature in Alexa fluor 488 goat anti-mouse IgG

(1:500, Invitrogen-Molecular Probes A11001) and Alexa fluor 555 goat anti-rabbit IgG (1:500, Invitrogen-Molecular Probes A21428). Sections were then washed six times in PBS and followed by three 10-min rinses in PB and mounted on glass slides with Vectashield+DAPI (Vector Labs) and sealed with nail polish for microscopy. All sections were imaged on both epifluorescent and confocal microscopes. Gain and exposure time were constant throughout, and all image groups were processed at the same time.

Purification and biotinylation of synaptosomes

Synatosomes were prepared from whole brain according to published protocols (Hagan et al., 2010; Ramamoorthy, 2007). Briefly, brain was homogenized in 4 mL homogenizing buffer (300 mM Sucrose, 10 mM Hepes, with protease and phosphatase inhibitors from Calbiochem) using a Dounce homogenizer. The homogenates were transferred to polycarbonate tube (Beckman, Palo Alto, CA) and centrifuged at 1000xg (~3000 rpm) for 10 min at 4°C using a JA-21 rotor. The supernatants were transferred to fresh tubes and centrifuged at 15,900 x g. The crude synaptosomal pellets were then washed with 10-15 ml of Krebs-Ringer-Hepes buffer (KRH, 124 mM NaCl, 1.8 mM KCl, 1.3 mM $MgSO_4$, 1.24 mM KH_2PO_4, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose with protease and phosphatase inhibitors) spun at 15,900 x g. The crude synaptosomes were then purified by layering over a sucrose gradient- 2.6 ml each of 0.85 M, 1.0 M, 1.2 M sucrose (top to bottom) and were centrifuged at 85,000 X g for 2 hr at 4°C (L8-M

ultracentrifuge, Beckman) using the SW41 rotor. Purified synaptosomes, appearing as a creamy colored band at the interface 1.0 M and 1.2M sucrose, were collected. The purified synaptosomes were washed in 0.32 M sucrose and then once with KRH buffer. After protein concentrations were determined, the purified synaptosomes were biotinylated using the EZ-link-Sulfo-NHS-SS-Biotin (Pierce Biotechnology) to label cell surface proteins according to manufacturer's instructions. After quenching excess biotin with 100 mM glycine, synaptosomes were lysed in RIPA lysis buffer (10 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate) with 1% CHAPS detergent by passing the lysate through a 26.5 gauge needle 10x on ice. The lysates were solubilized further by gentle rocking for 45 min at 4°C and centrifuged at 15,000 rpm for 45 min. Supernatants were incubated with high capacity neutravidin beads (Pierce Biotechnology) to capture the biotinylated proteins. Beads were washed and bound proteins were eluted with Laemmli's buffer. Eluates were electrophoresed in a 10% tris-glycine gel and processed for western blotting with two different SERT antibodies, from Santa Cruz Biotechnology and from Millipore, respectively.

Rotating Disk Electrovoltammetry

Rotating disk electrode voltammetry (RDEV) was used to measure initial velocities of serotonin (5-HT) transport into mouse synaptosomal preparations as previously described (Hagan et al. 2010). Synaptosomes (480 ul) were placed in

the glass electrochemical well, a constant +550 mV potential (the previously defined optimum working potential for serotonin; Hagan et al, 2010) was applied to the carbon electrode, and the electrode was rotated at 3000 rpm. Each synaptosomal aliquot was allowed to stabilize for 10 min, and once a stable baseline was reached, 10 ul of 5 uM 5HT was added (100 nM final concentration) and uptake was recorded for 3 min. All experiments were performed in the presence of 1 uM GBR12935 (Sigma-Aldrich) and 100 nM nisoxetine (Sigma-Aldrich). SERT specific uptake was defined as the difference in the initial rates in the presence and absence of 1 uM paroxetine (Sigma-Aldrich).