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

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SUMMARY

Maladaptive responses to stress adversely affect human behavior, yet the signaling mechanisms underlying stress-responsive behaviors remain poorly understood. Using a conditional gene knockout approach, the α isoform of p38 mitogen-activated protein kinase (MAPK) was selectively inactivated by AAV1-Cre-recombinase infection in specific brain regions or by promoter-driven excision of p38α MAPK in serotonergic neurons (by Slc6a4-Cre or ePet1-Cre) or astrocytes (by Gfap-CreERT2). Social defeat stress produced social avoidance (a model of depression-like behaviors) and reinstatement of cocaine preference (a measure of addiction risk) in wild-type mice, but not in mice having p38α MAPK selectively deleted in serotonin-producing neurons of the dorsal raphe nucleus. Stress-induced activation of p38α MAPK translocated the serotonin transporter to the plasma membrane and increased the rate of transmitter uptake at serotonergic nerve terminals. These findings suggest that stress initiates a cascade of molecular and cellular events in which p38α MAPK induces a hypo-serotonergic state underlying depression-like and drug-seeking behaviors.

INTRODUCTION

Stress has significant effects on mood and can act as a motivational force for decisive action, seeking food or reward, and coping with novel environmental conditions. However, sustained stress exposure can lead to maladaptive responses including clinical depression, anxiety, and increased risk for drug addiction (Bale and Vale, 2004; Krishnan and Nestler, 2008; Bruchas et al., 2010; Koob, 2008). Recent studies have proposed that the dysphoric components of stress are coded in brain by corticotropin releasing factor (CRF) and subsequent release of the endogenous dynorphin opioid peptides in brain (Land et al., 2008; Bruchas et al., 2010; Koob 2008). Systemic blockade of these neural pathways prevents the aversive and proaddictive effects of stress, but how these systems orchestrate affective responses at the molecular and cellular levels remain unresolved.

One group of signaling pathways involved in the cellular stress response includes the family of mitogen-activated protein kinases (MAPK). Using pharmacological approaches, p38 MAPK (also called SAPK, for stress-activated protein kinase) activity has been identified as a critical mediator of stroke-induced apoptosis, osmotic shock response, and in the regulation of transcriptional pathways responsible for cell death and differentiation (Raman et al., 2007; Coulthard et al., 2009). Recently however, inhibition of p38 MAPK was also found to block stress-induced behavioral responses including aversion (Land et al., 2009; Bruchas et al., 2007) and to prevent reflex-conditioned responses (Zhen et al., 2001). Although the cellular and molecular bases for these behavioral actions are not known, one possible site of action is the serotonergic nuclei because this transmitter has an established role in the regulation of mood (Roche et al., 2003; Paul et al., 2011; Richardson-Jones et al., 2010). The dorsal raphe nucleus (DRN) is the primary neuronal source of serotonin, and DRN neurons send diffuse projections to multiple forebrain and hindbrain structures that are critical for regulating affective state (Land et al., 2009; Hensler 2006; Zhao et al., 2007). The DRN is modulated by several afferent systems (Wylie et al., 2010; Land et al., 2009; Scott et al., 2005; Kirby et al., 2008), but how these inputs regulate serotonin neurotransmission remains unclear, and little is known about the essential signal transduction kinase cascades in the DRN that regulate serotonergic output to ultimately control behavior.

In the DRN, we found that p38α MAPK expression was widely distributed in tryptophan hydroxylase 2 (TPH) expressing cells,
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non-TPH cells, and astrocytes (Land et al., 2009). Several reports have demonstrated that there is a high degree of coexpression between the serotonin transporter (Slc6a4, SERT) and TPH positive neurons (MacGillivray et al., 2010; Lowry et al., 2008). Recent studies have also determined that expression of the transcription factor Pet1 is largely restricted to serotonergic (TPH-immunoreactive, ir) neurons (Scott et al., 2005; Liu et al., 2010). Thus, SERT and Pet1 represent potentially useful markers for the discrimination of serotonergic neurons within the brain. Here, we used a combination of conditional p38α MAPK null alleles generated in serotonergic neurons or astrocytes to determine the effects of p38α MAPK deletion in models of depression behaviors including place aversion and social avoidance and of drug addiction behaviors modeled by reinstatement of extinguished cocaine place preference.

RESULTS

p38α MAPK in DRN Is Required for Behavioral Responses to Stress

Since prior reports suggested that p38 MAPK is activated during the stress response, we first determined if social defeat stress (SDS) induces phosphorylation of p38 MAPK in the DRN. Following a single, 20 min session of SDS, mice showed an increase in phospho-p38 immunoreactivity (p38-ir) in the DRN (Figures 1A and 1A'). G protein coupled receptor activation can lead to p38 MAPK phosphorylation via recruitment of arrestin-dependent pathways (Tan et al., 2009; Gong et al., 2008), and activation of the dynorphin/kappa opioid receptor (KOR) system was shown to increase pp38-ir by this mechanism (Bruchas et al., 2006, 2007). Consistent with this concept, the increase in pp38-ir caused by SDS was prevented by blocking endogenous dynorphin activation of KOR with the selective antagonist norbinaltorphimine (norBNI) (Figures 1A and 1A').

There are four isoforms of p38 MAPK: α, β, δ, and γ. p38α and p38β are both expressed in neurons and glial cells, whereas p38δ and p38γ are exclusively expressed in immune cell types (Zhang et al., 2007; Zarubin and Han, 2005). Since the p38 isoforms share consensus phosphorylation sites and there are no known isoform-selective phospho-antibodies, we used non-phospho-selective, but isoform-selective antibodies in immunoprecipitation approaches to determine the phosphorylation state of each isoform. agonist stimulation of KOR resulted in significant (p < 0.05, t test) phosphorylation of the p38α, but not p38β isoform (see Figure S1A available online) in HEK293 cells expressing KOR-GFP and either FLAG-tagged p38α or p38β isoforms. No difference in immunoprecipitation efficiency or isoform expression was observed (Figure S1B) as evidenced by equal FLAG staining. Finally, using nucleus accumbens cell lysates, we found that in vivo treatment with KOR agonist increased pp38α-ir (Figure S1C). Together these data suggest that KOR activation during stress exposure selectively increased the phosphorylation of the α isoform of p38 MAPK.

To determine if p38α activation in DRN was required for stress-induced behavioral responses, we used a genetic approach to selectively inactivate p38α MAPK in DRN cells. Using mice with a floxed gene (Mapk14lox/lox) encoding p38α MAPK (Nishida et al., 2004), local inactivation of p38α MAPK in the DRN was achieved by stereotoxic injection of adeno-associated virus serotype 1 vector encoding Cre recombinase (AAV1-CreGFP) (Ahmed et al., 2004). These mice were also bred to carry a Gr(ROSA)26Sor-YFP (R26-YFP) reporter cassette in which Cre-mediated recombination of a transcriptional STOP promotes YFP expression as a marker of Cre activity (Figure 1B). p38α-ir was absent in AAV1-CreGFP transduced cells that coexpressed the YFP reporter (Figure 1C). In contrast, injection of AAV1-CreGFP vector expressing an inactive, mutated form of the recombine (Creδ) did not affect p38α MAPK expression in DRN (Figure 1C).

Prior reports established that stress causes relapse to drug seeking (Nestler and Hyman, 2010; Krishnan et al., 2007), and in particular, social defeat stress (SDS) represents an ethologically relevant stressor for evoking dysphoria-like behavioral states (Miczek et al., 2008). The Mapk14lox/lox mice were injected in the DRN with AAV1-CreGFP to determine whether p38α MAPK was required for SDS induced reinstatement. We followed this injection with a conditioning paradigm for cocaine place preference (Figure 1D). Both AAV1-CreGFP and AAV1-CreGFP injected mice developed normal place preference to cocaine (Figure 1E), suggesting that deletion of p38α in DRN cells does not disrupt associative learning components required for initial acquisition of cocaine place preference. We then extinguished the conditioned preference by substituting saline for cocaine in the drug-paired chamber (Figure 1D). After mice met extinction criteria (≤15% of their initial preference score; Figure 1E), mice were exposed to social defeat stress (20 min session) and then place preference was reassessed. Importantly, AAV1-CreGFP-induced deletion of p38α in the DRN completely blocked SDS-induced reinstatement of cocaine CPP, whereas floxed p38α mice injected with virus expressing the inactive form of Cre recombinase still showed robust SDS-induced reinstatement of cocaine CPP (Figure 1E). These data suggest that expression of p38α in the DRN is required for stress-induced reinstatement of reward seeking behavior.

To expand on this concept and to parallel other studies showing that stress negatively modulates reward to initiate the drive for reward seeking (Koob, 2008), we injected Mapk14lox/lox (floxed p38α) mice with either AAV1-CreGFP or AAV1-CreGFP in either the DRN or nucleus accumbens (NAc), and then assessed conditioned avoidance of a context paired with an aversive stimulus. Since KOR activation results from stress and is known to produce aversive behavioral responses in stress-paired contexts (Land et al., 2008, 2009; Bruchas et al., 2010; Carlezon et al., 1998; Shippenberg et al., 1986) we conditioned mice with the KOR agonist U50,488 (2.5 mg/kg, i.p.) over 2 days and then assessed their avoidance of the drug-paired context. AAV1-CreGFP injection in the DRN of Mapk14lox/lox mice, but not the NAc, blocked conditioned place aversion (Figure 1F). This result suggests that p38α MAPK in the DRN is also required for stress-induced dysphoria-like avoidance behavior.

Selective Disruption of p38α in 5HT Neurons

p38α MAPK is ubiquitously expressed in cells of DRN including serotonergic and nonserotonergic neurons, as well as astrocytes.
Figure 1. p38α Expression in the Dorsal Raphe Nucleus Is Required for Stress Behavior

(A) Representative low-power immunofluorescence images of social defeat stress induced pp38-ir (green) in TPH-ir cells (red) of the DRN. (A1) Quantification ± SEM of pp38-ir in DRN from unstressed (naive), social defeat stress (SDS), and social defeat stress exposed norBNI (10 mg/kg, i.p.) injected mice (**p < 0.01, SDS versus naive). Inset, representative black and white low power immunofluorescence images of social defeat stress induced pp38-ir, scale bar = 200 μm.

(B) Schematic of AAV1 induced cre-recombination of the floxed p38α MAPK allele and STOP sequence controlling Rosa26YFP gene expression.

(C) Representative images of pp38-ir (red) and YFP (green) fluorescence following AAV1-Cre-GFP or AAV1-DCre-GFP injection into the DRN. Mice were pre-treated with KOR agonist (U50,488, 20 mg/kg, i.p, 20 min prior to perfusion). Images show that AAV-cre expressing cells lack pp38-ir, confirming effective localized DRN p38α deletion in cells where Cre activity also promoted YFP expression by the Rosa reporter.

(D) Conditioning procedure for SDS induced reinstatement of cocaine seeking.

(E) Cocaine place preference scores, calculated as post-test minus pre-test on the cocaine-paired side, and SDS-induced reinstatement scores of extinguished place preference in DRN-injected animals (n = 5–8; *p < 0.05 t test compared to AAV1-DCre). Bars represent means ± SEM.

(F) Preference scores (mean ± SEM) for conditioned place aversion to kappa opioid agonist U50,488 (2.5 mg/kg, i.p.) from mice injected with either AAV1cre-GFP or AAV1Δcre-GFP into their DRN or nucleus accumbens (NAc) (*p < 0.05, AAV1cre-GFP versus AAV1Δcre-GFP; n = 8).

See also Figure S1.
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Serotonergic p38α Is Required for Stress-Induced Avoidance Behavior

Previous reports have demonstrated that mice subjected to defeat by an aggressor mouse show subsequent decreases in motivation for social interaction that can be prevented by clinically effective antidepressants (Nestler and Hyman, 2010; Cao et al., 2010; Berton et al., 2006; Avgustinovich and Kovalenko, 2005; Siegfried, 1985). Using this approach, we assessed the role of p38α MAPK in stress-induced social avoidance. Previously untraversed mice readily explore and interact with a novel male mouse in the social interaction chamber (Figures 3A and 3B). However, socially defeated mice showed a significant social avoidance (ANOVA, F(3,30) = 2.51, p < 0.05, Bonferroni; Figure 3A). Pretreatment with the KOR antagonist norBNI (24 hr prior to SDS, 10 mg/kg, i.p.) significantly blocked the SDS-induced avoidance behavior (ANOVA, F(3,30) = 2.843, p < 0.05, Bonferroni). As expected, littermate control mice (Mapk14+/lox: ePet1-Cre) showed avoidance behavior following SDS, whereas p38α CKOePet mice were resilient to the effects of social defeat and showed significant reduction in the SDS-induced interaction deficit (t test, p < 0.05; Figure 3B). Because social avoidance behavior may also be considered to be an anxiety-like response, we determined if behavior in the elevated plus maze was also affected by disruption of p38α MAPK in serotonergic neurons (Figure S4B). Unexpectedly, there were no significant differences in the time spent in the open arms of the maze by the p38α CKOePet, p38α CKOSERT and littermate control groups (Figure S4B), suggesting that the blockade of SDS-induced social avoidance caused by serotonergic p38α MAPK deletion was not a consequence of a generalized decrease in anxiety-like responses.

Avoidance behavior is a complex response known to be regulated by serotonergic systems as well as other hormones and neuropeptides (Bari et al., 2010; Eriksson et al., 2011; Cao et al., 2010; Bromberg-Martin et al., 2010; Pamplona et al., 2011). To determine if context-dependent avoidance requires serotonergic p38α MAPK expression, we assayed conditioned place aversion (CPA) to U50,488, a KOR agonist that acts as a pharmacological stressor. KOR activation causes aversion behavior in rodents in Pavlovian conditioning paradigms (Shippenberg et al., 1986; Land et al., 2009). We conditioned mice with U50,488 (2.5 mg/kg, i.p.) over 2 days and then assessed their preference for the drug-paired context. As expected, wild-type and Mapk14+/lox mice showed significant CPA to the drug-paired context (Figures 3C and 3D). In contrast, mice lacking p38α MAPK in either their ePet1- or SERT-expressing cells (p38α CKOePet or p38α CKOSERT, respectively) failed to show significant place aversion (for p38α CKOePet, ANOVA, F(3,30) = 5.626, p < 0.05 Bonferroni; for p38α CKOSERT, ANOVA, F(3,32) = 4.193, p < 0.05 Bonferroni; Figures 3C and 3D). Since previous studies have shown SERT is also expressed in astrocytes (Hirst et al., 1998; Bal et al., 1997; Pickel and Chan, 1999) and to further confirm 5HT neuronal selectivity of the behavioral effects, we induced Cre activity by tamoxifen in p38α CKOSERT (Mapk14+/lox:Gfap-CreERT2) then assayed their behavioral responses to KOR agonist. Although Cre activity was confirmed in astrocytes of tamoxifen-treated p38α CKOGfap mice (Figure 2D), they still developed significant CPA (Figure 3E), suggesting that aversion does not require p38α MAPK.
Figure 2. Cell Type Selective Deletion of p38α MAPK

(A) Schematic of cell type specific p38α deletion. Floxed p38α and ROSA<sup>YFP</sup> reporter mice were crossed to mice expressing Cre-recombinase under the control of Pet1, serotonin transporter, or the tamoxifen inducible glial fibrillary acidic protein (GFAP) Cre<sub>ERT2</sub> transgene. Representative images showing TPH-ir and YFP in p38α CKO<sub>Pet1</sub> (B), p38α CKO<sub>SERT</sub> (C), and p38α CKO<sub>GFAP</sub> (D) mice. Insets show higher-power images with arrows directed toward yellow cells indicating overlap of TPH/YFP expression. Representative images showing TPH and p38α-ir in wild-type (E), p38α CKO<sub>Pet1</sub> (F), and p38α CKO<sub>SERT</sub> (G). Representative images from wild-type mice showing the absence of phosphorylated p38 MAPK (pp38-ir) following saline treatment (H) and increased pp38-ir following treatment with U50,488 20 mg/kg, i.p., 20 min prior (I). Insets show intact TPH labeling in the same fields. (J) Quantitation of p38α-ir in TPH positive cells in the dorsal raphe nucleus. Data show a significant reduction in p38α expression in both p38α CKO<sup>Pet1</sup> and p38α CKO<sup>SERT</sup> mice (**p < 0.001, ANOVA, Bonferroni). (K) Representative images from in p38α CKO<sup>Pet1</sup> mice showing the absence of pp38-ir following saline treatment (K) and following treatment with U50,488 (L). Insets show intact TPH staining. (M) Quantitation of p38α-ir expressed in TPH-negative cells in the DRN. Data are representative of 4–8 animals per group. See also Figure S3.
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Table 1. Mouse Cell Lines Generated

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<th>Reference</th>
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<th>Genotype</th>
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<td>Nishida et al., 2004</td>
<td>floxed p38α</td>
<td>Mapk14lox/lox</td>
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<tr>
<td>B6.129-Tg(Slc6a4-cre)1Xz</td>
<td>Zhuang et al., 2005</td>
<td>p38α CKO</td>
<td>Mapk14lox/lox</td>
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<tr>
<td></td>
<td></td>
<td>Slc6a4Cre/4</td>
<td>Mapk14lox/lox</td>
</tr>
<tr>
<td>B6.129-Tg(Slt/Cg)26Sertm1(EYFP)Cre/J</td>
<td>Scott et al., 2005</td>
<td>p38α SERT</td>
<td>Mapk14lox/lox</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ePetCre only</td>
<td>Mapk14lox/lox</td>
</tr>
<tr>
<td>B6.129X1-Gt(Rosa)26Sertm1(EYFP)Cre/J</td>
<td>Hirrlinger et al., 2006</td>
<td>p38α SERT</td>
<td>Mapk14lox/lox</td>
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<tr>
<td></td>
<td></td>
<td>ePet-Cre only</td>
<td>Mapk14lox/lox</td>
</tr>
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<td>Tallquist and Soriano, 2000</td>
<td>Mox2-Cre</td>
<td>As heterozygote to produce null p38α allele</td>
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<td>B6.129(Cg)-Slc6a4tm1Cre/J</td>
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<td>Conventional SERT KO</td>
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expression in astrocytes. Furthermore, since place conditioning requires locomotor activity for normal exploratory behavior and aversive compounds such as KOR agonists can reduce locomotion, we also measured locomotor activity in p38α CKOs and controls. We did not observe any effect of genotype on basal or U50,488-induced locomotor scores before or during conditioning (Figure S4C), suggesting that the lack of context dependent place aversion to a pharmacological stressor is not attributable to a deficit in locomotor activity or lack of pharmacological activation of KOR.

Serotonergic systems have been widely studied in models of depression and many groups use forced swim stress (FSS) as an animal model of stress-induced affect and for measuring behavioral efficacy of anti-depressant-like compounds (Porsolt et al., 1977). To determine if p38α MAPK deletion in SERT-expressing cells prevents swim-stress-immobility, we exposed mice to FSS and then measured their immobility during the first trial and again 24 hr later. p38α CKO mice showed significantly less immobility compared to control groups (Figures 3F; ANOVA, p < 0.05 versus matched control; Figure 4C). Finally, since cocaine injection (i.e., priming) is known to initiate reinstatement to drug seeking by distinct mechanisms (Thomas et al., 2008; Shaham and Hope, 2005), on the following day mice that did not reinstate to stress were injected with 15 mg/kg of cocaine and retested for place preference. All four groups of mice reinstated following a cocaine priming injection (Figure 4D), suggesting that serotonergic p38α MAPK deletion selectively alters only stress-induced modulation of drug-seeking. In conclusion, these results implicate serotonergic p38α MAPK in the regulation of affective state and show that selective deletion of p38α MAPK in serotonergic neurons protects mice from stress-induced relapse of cocaine-seeking behaviors.
Figure 3. Negative Affective Behavior Requires Expression of p38α in Serotonergic Neurons

(A) Representative traces of mouse locomotion (red lines) in unstressed and social defeat stressed wild-type or mice lacking p38α in serotonergic neurons (p38α CKO<sup>ser</sup>). Data show that SDS caused mice to retreat to zone 2 or 3 (Z2, 3, far corners). Mice pretreated with norBNI (10 mg/kg, i.p., 24 hr prior) or with serotonergic p38α deletion (p38α CKO<sup>ser</sup>) show normal exploration of the interaction zone (IZ).

(B) Quantification of social interaction scores in mice following SDS. Dashed line represents the social interaction scores for unstressed mice (n = 8, *p < 0.05 versus control saline or p38α<sup>D</sup>/lox, t test).

(C) Place Preference scores following conditioning with U50,488 (2.5 mg/kg) in wild-type, p38α<sup>D</sup>/lox and p38α CKO<sup>ser</sup> mice (n = 8–10, ANOVA, p < 0.05 versus control).

(D) Place Preference scores (means ± SEM following conditioning with U50,488 (2.5 mg/kg) in p38α wild-type versus p38α<sup>D</sup>/lox and p38α CKO<sup>ser</sup> mice (n = 8–10, ANOVA, p < 0.05 versus control).

(E) Place Preference scores ± SEM following conditioning with U50,488 (2.5 mg/kg) in wild-type or p38α CKO<sup>ser</sup> mice (n = 6–8).

(F) Swim-stress induced immobility scores for wild-type mice, p38α<sup>D</sup>/lox, or p38α CKO<sup>ser</sup> mice (data are means ± SEM; ANOVA, *p < 0.01, n = 6–8).

See also Figure S3.
To define the mechanism for the effects of p38α MAPK, we looked to studies in heterologous gene expression systems that previously suggested the plasma membrane serotonin transporter could be a p38 MAPK substrate (Zhu et al., 2005; Samuvel et al., 2005). Building on in vitro data showing that p38 MAPK increases SERT activity, we first asked whether the serotonergic p38α-dependent CPA response was sensitive to the selective SERT reuptake inhibitor citalopram (Ravna et al., 2003). Mice were conditioned as previously described with a KOR agonist and then assayed for preference to the stressor-paired context. Control mice showed normal place aversion to the U50,488-paired compartment, whereas citalopram-pre-treated mice (15 mg/kg, s.c.) showed significantly less U50,488 place aversion (Figure 5B; ANOVA, F(2,15) = 4.082, Bonferroni, p < 0.05 versus saline). These behavioral data strongly implicate the regulation of extracellular serotonin as a plausible mechanism for p38α-dependent effects.

To determine if p38α MAPK activation actually modulates SERT function in vivo, we used rotating disk electrovoltammetry (RDEV), a validated measure of monoamine transport kinetics (McElvain and Schenk, 1992; Burnette et al., 1996; Earles and Schenk, 1998; Hagan et al., 2010), to measure 5HT uptake rates in synaptosomes isolated from stressed or unstressed mice. To isolate G protein-coupled receptor-mediated p38α MAPK activation and to mimic the conditioned aversion paradigm described above, mice received either saline or U50,488 (2.5 mg/kg, i.p.) 24 hr prior to and again 30 min prior to preparation of whole-brain synaptosomes. Synaptosomes isolated from mice injected with KOR agonist (Figure 5C) showed a marked increase rate of SERT specific 5HT clearance compared with synaptosomes from control, saline-injected mice (Figures 5B and 5D). This increase in uptake rate was blocked by in vivo pretreatment with norBNI (2 × 2 ANOVA, significant effect of pretreatment, p < 0.05; Figure 5D). We then determined whether deletion of p38α in serotonergic cells blocked the KOR induced...
increase in SERT uptake. Both wild-type (p38α+/+) (t test versus saline control, p < 0.05) and control Mapk14Δ/lox mice (t test versus saline control, p < 0.001) showed a significant U50,488-mediated increases in SERT uptake as compared to saline treated controls (n = 10–16, "p < 0.01). This effect of U50,488 was blocked by pretreatment of the mice with norBNI (10 mg/kg).

(E) Administration of U50,488 (2.5 mg/kg, i.p.) 30 min prior to preparation of synaptosomes did not significantly increase serotonin uptake by the low-affinity transporters (n = 10–16).

Figure 5. Investigation of 5HT Uptake by SERT

(A) Place preference scores (±SEM) following conditioning of wild-type mice treated either with U50,488 (2.5 mg/kg) (U50/Saline), with the selective SERT re-uptake inhibitor citalopram (CPM) (15 mg/kg, i.p., 30 min prior to U50,488) (U50/CPM), or with citalopram alone (Saline/CPM). Citalopram prior to KOR agonist significantly blocked U50,488 CPA (ANOVA, p < 0.05, n = 8–10).

(B and C) Representative RDEV traces of 5-HT uptake from paroxetine (red traces) and nonparoxetine (black traces) treated synaptosomes isolated from control (B) or U50,488 (2.5 mg/kg, i.p. x2)-treated animals (C). Note the larger difference in slope for U50,488 treated than control animals.

(D) Administration of U50,488 (2.5 mg/kg, i.p. x2, 24 hr apart) to mice, 30 min prior to synaptosomal isolation, increased 5-HT uptake by SERT compared to saline treated controls (n = 10–16, "p < 0.01). This effect of U50,488 was blocked by pretreatment of the mice with norBNI (10 mg/kg).

(F) Administration of U50,488 (2.5 mg/kg, i.p. x2) 30 min prior to preparation of synaptosomes did not significantly increase serotonin uptake by the low-affinity transporters (n = 10–16).

*p38α MAPK Regulates SERT Cell-Surface Trafficking*

To determine if the increase in uptake rate was caused by increased SERT expression, we isolated synaptosomes and immunoblotted for SERT in each mouse genotype. Consistent with previous reports (Samuvel et al., 2005; Zhu et al., 2005), we found that SERT-ir migrates at both 75 and 98 KDa (Figure 6A). We confirmed the selectivity of the two different SERT antibodies by showing an absence of staining in synaptosomes isolated from SERT knockout mice (Figure 6A) and absence of SERT-ir in untransfected HEK293 cells, but presence in cells transfected with cDNA encoding SERT (Figure S5). Total SERT expression in p38αCKO(SERT) or p38αCKO(Δ/Δ) mice was not significantly different from wild-type mice (Figures 6A and S5).

Using a membrane impermeant biotinylation procedure to label cell-surface proteins (Samuvel et al., 2005), we next assessed changes in SERT-ir expression on the synaptosomal surface. SDS (20 min exposure) of wild-type mice significantly
Figure 6. p38α MAPK Is Required for Social Defeat Stress-Induced Cell Surface SERT Trafficking

(A) Representative immunoblot of total SERT levels in the different mouse lines used in this study. Data show both species of SERT (75 and 98 kDa) are present in these strains and the absence of SERT-ir in the Slc6a4 knockout (SERT-KO) mouse. Actin-ir was used to control for protein loading.

(B) Representative immunoblot of surface SERT expression in biotinylated synaptosomes isolated from unstressed mice (no stress), from mice after SDS, and from mice pretreated with norBNI (10 mg/kg) 24 hr prior to SDS. (Anti-streptavidin-ir confirms equal protein loading after biotinylation and pull-down.

(C) Quantification of SERT-ir surface expression following SDS of saline-treated wild-type, norBNI-treated wild-type, and p38α CKO ePET mice (**p < 0.01, ***p < 0.001, ANOVA, Bonferroni post-hoc).

(D) Quantification of SERT-ir surface expression following U50,488 treatment of wild-type and p38α CKO ePET mice. (*p < 0.05, t test). n = 8–10 in replicate, and each was taken from a separate animal.

(E) Cartoon model depicting p38α MAPK-dependent SERT translocation and decreased extracellular 5HT.

See also Figure S5.
increased (ANOVA, F(2,24) = 4.7122, p < 0.05) synaptosomal surface SERT expression (Figure 6), and this increase was blocked by pretreatment with norBNI (10 mg/kg, i.p.) 1 hr prior to SDS (Figures 6B and 6C). Furthermore, socially defeated (20 min exposure) or KOR agonist treated (2.5 mg/kg, 2 × 24 hr, i.p.) p38α CKOpet1 mice did not show stress-induced increases in surface SERT expression, defining a critical role for p38α MAPK in SERT surface trafficking following stress and KOR activation (Figures 6C and 6D). The proposed mechanism of p38α MAPK-SERT interaction is illustrated in Figure 6E.

DISCUSSION

In this study, we present evidence that p38α MAPK is an essential mediator of stress-induced adverse behavioral responses through regulation of serotonergic neuronal functioning. Our data demonstrate that p38α expression in 5HT neural circuits is required for local regulatory control of serotonin transport that ultimately controls behavioral responses including social avoidance, relapse of drug seeking, and the dysphoria-like responses underlying aversion. These results are important because they implicate a critical requirement for p38α MAPK signaling in 5HT neuronal function during stress, and demonstrate that p38α MAPK, in spite of its ubiquitous expression profile, has the ability to specifically regulate selected downstream targets to shape behavioral output. The evidence presented here strongly links molecular events, physiological responses and behavioral output through p38α MAPK signaling actions in serotonergic neurons.

The dorsal raphe nucleus (DRN) contains a major cluster of serotonergic neurons that project broadly throughout the brain (Wylie et al., 2010). Its circuits have impact on mood regulation and nociception (Scott et al., 2005; Zhao et al., 2007). However, the DRN is not homogeneous and contains a diversity of cell types whose local circuit interactions and projections are not completely defined (Wylie et al., 2010). Expression of the transcription factor Pet1 during development is highly correlated with the production of TPH, the rate-limiting enzyme in 5HT synthesis (Liu et al., 2010; Scott et al., 2005). GABA and glutamatergic inputs are known to regulate tonic DRN neuronal activity (Lemos et al., 2011; Tao and Auerbach, 2000), although how these different systems are integrated remains an active area of study. All serotonergic cell bodies express SERT perisynaptically at their terminal regions to clear extracellular 5HT following transmitter release (Murphy and Lesch, 2008). Using the selective expression of Cre driven by SERT and Pet1 promoters, we found that the genetic inactivation of p38α MAPK in Pet1- and SERT-expressing cells caused a loss of p38α and pp38 staining selectively in TPH ir-positive cells of DRN. We were not surprised to find that expression of Cre driven by the SERT promoter was widespread (Figure S3) because transient SERT expression during brain development had previously been noted (Gaspar et al., 2003; Narboux-Nême et al., 2008). Nevertheless, the SERT-Cre mice provide important corroborative results consistent with the effects of two other tools we used to excise p38α in serotonergic neurons. The selectivity of Cre expression and subsequent p38α excision by AAV1-CreGFP, SERT-Cre or ePet1-Cre are demonstrably different. AAV1-CreGFP acts on all DRN cells at the site of injection; SERT-Cre expression was not restricted to DRN; and ePet1-Cre is expressed in TPH-ir neurons of the median raphe as well as DRN. Nevertheless, the consistent behavioral results suggest the p38α deletion in the common TPH-ir cells of DRN mediates these effects. In addition, although p38-dependent stress responses also include activation, hypertrophy, and proliferation of astrocytes (Xu et al., 2007), we found no evidence that activation of p38α in GFAP-ir astrocytes was involved in the behavioral responses assessed. The lack of effect of p38α deletion in astrocytes was surprising since other investigators have noted that many aspects of the brain’s response to stress resemble inflammation (Wager-Smith and Markou, 2011).

The conditional deletion of p38α and lack of compensation by p38β caused profound behavioral effects in models of stress-induced depression and addiction and establishes a distinct role of the p38α isoform over p38β isoforms in dorsal raphe function. The selective role for the p38α MAPK isoform was unexpected but is consistent with prior reports suggesting that the α and β isoforms may be expressed in different subcellular compartments (Lee et al., 2000). In addition, differences in functional roles are consistent with isoform differences in other signaling kinases including the various PKC isoforms (Hauben-Sak et al., 2010; Sajikumar and Korte, 2011).

The 5HT transmitter system in mammalian brain is known to be an essential modulator of homeostatic responses that control emotional behaviors and the interaction of animals with their environments (Holmes, 2008; Ansorge et al., 2004; Gingrich and Hen, 2001). It is widely accepted that 5HT function is necessary for the normal functioning of neural circuits required for adult emotional behaviors (Gaspar et al., 2003). However, few studies have identified the critical kinases involved in serotonergic function, and few have established how disruption of signal transduction in serotonergic neurons impacts emotional behaviors. Pharmacological blockade of p38 MAPK has been suggested to prevent conditioned place aversion and learned helplessness in animal models of depression (Bruchas et al., 2007). Furthermore, expression of mutant kappa opioid receptors that are ineffective at activating p38 MAPK prevents place aversion in behavioral assays (Land et al., 2009). However, a definitive role for p38 MAPK in behavioral regulation following stress had not previously been directly demonstrated.

Rodent models of social interaction have gained acceptance by neurobiologists as useful models of depression-like behavior since they respond to antidepressant compounds, and the DSM-IV criteria includes decreased motivation for social interaction as major component of human depression (Berton et al., 2006; Beidel et al., 2010). p38α MAPK may represent the first kinase mediator in a series of neurochemical events that underlie the chronic behavioral changes. The block of social avoidance by KOR antagonist further establishes the dynorphin system as a critical part of the stress response and strengthens the concept that this system may be a novel therapeutic target to promote stress resilience (Land et al., 2008, 2009; Bruchas et al., 2010).

The regulation of extracellular serotonin levels and subsequent postsynaptic effects have long been thought to be a primary component of depression and anhedonic behavioral responses in humans (Haenisch and Bönisch, 2011); however, few reports have demonstrated that interruption of the signal transduction
that controls SERT protects against the depressive-like effects of stress. Although regulation of SERT by p38 had been implicated based on in vitro studies (Zhu et al., 2005; Samuel et al., 2005), the demonstration that stress-induced p38α MAPK causes translocation of SERT to the plasma membrane in brain provides a clear molecular explanation for stress-induced dysphoria. The data presented here show that in serotonin neurons, p38α MAPK acts to directly influence SERT trafficking and ultimately to increase the rate of serotonin reuptake. In conclusion, understanding the molecular and cellular mechanisms that control stress-induced behaviors delineates the neurobiological mechanisms involved in depression and addiction-like behaviors, while also providing insight to potential therapeutic targets. Although prior studies have demonstrated a role for p38α MAPK in cellular development and apoptotic mechanisms, its role in the regulation of mood disorders and addiction risk was not previously appreciated. Furthermore, although antidepressant efficacies of drugs that inhibit the plasma membrane serotonin transporter are clear, the profound effects of stress on the serotonin system function defined by this study provide key molecular insight into the underlying mechanisms of stress-vulnerability and resilience.

EXPERIMENTAL PROCEDURES

For detailed Experimental Procedures, see Supplemental Information.

Animals

Experimental procedures were carried out in accordance with the USPHS Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Washington. Male C57BL/6 mice (20–30 g) 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.

Generation of Serotonin-Specific Conditional Knockout Mice

Breeding and genotyping procedures were described in the Supplemental Information.

Behavior

Conditioned Place Aversion

Mice were trained in an unbiased, balanced three-compartment conditioning apparatus as described (Land et al., 2009; Bruchas et al., 2007).

Stress-Induced Social Avoidance and Stress-Induced Cocaine Reinstatement

Stress-induced social avoidance and stress-induced cocaine reinstatement was performed as described in the Supplemental Information.

Viral Preparation and Local Intracranial Injections

Viral preparation and local intracranial injections were performed as previously reported (Zweifel et al., 2008; Land et al., 2009) and described more fully in the Supplemental Information.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Land et al., 2009; Bruchas et al., 2007) and described more fully in the Supplemental Information.

Synatosomes

Synatosomes were prepared from whole brain according to published protocols (Hagan et al., 2010; Ramamoorthy et al., 2007) and described more fully in the Supplemental Information.

Rotating Disk Electrovoltagmetry (RDEV)

RDEV was used to measure initial velocities of serotonin (5-HT) transport into mouse synaptosomal preparations as previously described (Hagan et al., 2010) and described more fully in the Supplemental Information.

Data Analysis/Statistics

Data are expressed as means ± SEM. Data were normally distributed, and differences between groups were determined using independent t tests or one-way ANOVA, or two-way ANOVAs followed by post hoc Bonferroni comparisons if the main effect was significant at p < 0.05. Statistical analyses were conducted using GraphPad Prism (version 4.0; GraphPad) or SPSS (version 11.0; SPSS).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2011.06.011.

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REFERENCES


Deletion of p38α Produces Stress Resilience


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


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 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 p38\(\alpha\)CKO\textsuperscript{SERT} and p38\(\alpha\)CKO\textsuperscript{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, p38αCKOεPet, 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 p38αCKOεPet, 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 (Mapk14lox/+\(\text{lox}^+\)) were obtained from the RIKEN Bioresearch Center. Mapk14lox/+\(\text{lox}^+\) mice were crossed to Mox2-Cre mice (Tallquist and Soriano, 2000) to generate Mapk\(^{-}\)+ 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\(^{\text{lox}}\) allele by Cre recombinase. Backcrossing with C57BL/6 wild-type mice allowed the Mapk\(^{-}\) 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\(^{\text{Cre}}\) 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\(^{\text{lox/lox}}\) mice, which in the case of Mapk\(^{\Delta^+}\):Slc6a4-Cre parent would yield Mapk\(^{\Delta^\text{lox}}\) 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\(^{\Delta^\text{lox}}\) (heterozygote) or...
functionally wild-type (p38α⁺/lox) littermates. As reported previously (Nishida et al., 2004), p38αlox/lox mice are behaviorally indistinguishable from wild type p38α⁺/+.

\textit{Slc6a4 knockout mice were obtained from Taconic Farms.}

\textbf{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'-ATGAGATGCGATACCTGGAGACCAGAAG-3') and A4 (5'-AGCCAGGGCTTACAGAGAAAACCCTGTG-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 p38αΔ allele as a 420-bp PCR product. The SERT\textsuperscript{Cre} allele (450-bp band) was differentiated from wild type (350-bp) using 5'-CATCCGACCACGTGACCA-3', 5'-GGCACTAACCTCCACCATTCTG-3' and 5'-GAACGAACCTGGTCGAAATCAG-3', while the Mox2, ePet1\textsuperscript{Cre} and GFAP\textsuperscript{CreERT2} transgenes were detected using 5'-AGCGTTTCGACCAGACCTGGATCT-3' and 5'-CGCCGTAAATCAATCGATGAGTT-3', yielding a 330-bp band. The Rosa26-YFP reporter gene was screened for with 5'-AAGACCGCAGAG AATGGATATG-3', 5'- AAAGTCGCTCTGAGTTTATG-3', and 5'-GGAGCGGGAGA AATGGATATG-3', giving a 320-bp EYFP and 600-bp wild type band (Supplemental Figure 2C).

\textbf{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 $\beta$-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, AAV1Cre-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 $\sim 1.2 \times 10^{12}$ 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$^{\text{Cre-ERT2}}$ 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 °C and cryoprotected with solution of 30% sucrose in 0.1M PB at 4°C for at least 3 d, cut into 40 μm sections and processed for immunostaining. 40 μm 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**

Synaptosomes 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 MgSO₄, 1.24 mM KH₂PO₄, 2.5 mM CaCl₂, 26 mM NaHCO₃, 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).