Activation of the kappa opioid receptor in the dorsal raphe nucleus mediates the aversive effects of stress and reinstates drug seeking

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Although stress has profound effects on motivated behavior, the underlying mechanisms responsible are incompletely understood. In this study we elucidate a functional pathway in mouse brain that encodes the aversive effects of stress and mediates stress-induced reinstatement of cocaine place preference (CPP). Activation of the dynorphin/kappa opioid receptor (KOR) system by either repeated stress or agonist produces conditioned place aversion (CPA). Because KOR inhibition of dopamine release in the mesolimbic pathway has been proposed to mediate the dysphoria underlying this response, we tested dopamine-deficient mice in this study and found that KOR agonist in these mice still produced CPA. However, inactivation of serotonergic KORs by injection of the KOR antagonist norBNI into the dorsal raphe nucleus (DRN), blocked aversive responses to the KOR agonist U50,488 and blocked stress-induced reinstatement of CPP. KOR knockout (KO) mice did not develop CPA to U50,488; however, lentiviral re-expression of KOR in the DRN of KOR KO mice restored place aversion. In contrast, lentiviral expression in DRN of a mutated form of KOR that fails to activate p38 MAPK required for KOR-dependent aversion, did not restore place aversion. DRN serotonergic neurons project broadly throughout the brain, but the inactivation of KOR in the nucleus accumbens (NAc) coupled with viral re-expression in the DRN of KOR KO mice demonstrated that aversion was encoded by a DRN to NAc projection. These results suggest that the adverse effects of stress may converge on the serotonergic system and offers an approach to controlling stress-induced dysphoria and relapse.

depression | drug addiction | dynorphin | serotonin

Stress has profound effects on human health and can lead to mood disorders including clinical depression, anxiety, and can increase comorbid drug addiction risk (1–3). Corticotropin releasing factor (CRF) orchestrates the complex endocrine and neuronal responses to behavioral stress exposure (4), and recent studies have suggested that the dysphoric properties of stress are encoded by CRF-induced activation of the endogenous dynorphin opioid system (5). Systemic administration of kappa opioid receptor (KOR) antagonists block the aversive (5) and pro-addictive effects of stress (6–9), and dynorphin activation of KOR is thought to mediate opponent processes evoked by addictive drugs (10), yet the key sites of dynorphin/KOR action mediating these behavioral responses are not resolved.

Mice subjected to behavioral stress show dynorphin release and robust KOR activation in both dopaminergic and serotonergic nuclei (5), implying that these neurotransmitters could be important for KOR-dependent stress-induced behavioral responses. Ventral tegmental area (VTA) dopaminergic projections to the nucleus accumbens (NAc) have been linked to addiction (11), making this an obvious target for the regulation of appetitive and aversive behaviors. However, prior studies have shown that mice lacking dopamine can still develop place preference for drugs of abuse (12, 13). These data implicate other monoamine systems such as serotonin as mediators of hedonic responses. The dorsal raphe nucleus (DRN) is a primary source of serotonin in the mammalian brain, projecting to multiple hindbrain and forebrain structures important for the regulation of stress, affective state, and analgesic responses (14, 15). We used a combination of receptor inactivation and lentiviral gene expression methods to show that activation of KOR in the serotonergic DRN was both necessary and sufficient to encode the dysphoric effects of stress.

Results

Dopamine Is Not Necessary for Kappa Opioid-Mediated Place Aversion. Activation of KOR in the VTA reduces dopamine release and has been postulated to mediate aversion (16, 17). Dopamine-deficient ($Th^{fs/fs}$, $Dbh^{Th/+}$) mice cannot produce dopamine due to a selective knockout (KO) of the tyrosine hydroxylase gene in dopaminergic cells, yet produce norepinephrine normally through selective restoration of tyrosine hydroxylase under the dopamine-β-hydroxylase promotor (18). We found that dopamine-deficient mice were still able to show significant conditioned place aversion (CPA) (mean: -449 ± 165 s; one-sample t-test, $P < 0.05$) to the KOR agonist U50,488. The degree of aversion was slightly greater than that produced in littermate control ($Th^{+/+}$, $Dbh^{Th/+}$) animals (mean: -196 ± 75 s; one-sample t-test, $P < 0.05$) (Fig. 1*B*). Dopamine-deficient mice were hypoactive during the conditioning sessions when they completely lacked dopamine (Fig. S1), but received caffeine (15 mg/kg) 5 min before pre test and test on day 4 to facilitate exploration of the apparatus (13). Dopamine-deficient mice given saline in both AM and PM conditioning sessions did not develop significant conditioned place preference or aversion (mean: -96 ± 106 s). Normal U50,488-induced aversion in dopamine-deficient mice was surprising, and these results suggest that while dopamine may contribute to KOR-mediated place aversion (16, 17), other systems are also likely to participate.

Kappa-Opioid Receptors in the DRN Mediate Aversion and Drug Reinstatement. We suspected that the serotonergic system might be involved in mediating aversive effects of kappa agonists because of the known effects of this system in regulating mood. The principal source of serotonin in the mammalian brain is the DRN (14, 15), and to assess its role, we first used local injection of the long-acting antagonist norBNI that stably inactivates KOR signaling; the antagonist effects of norBNI were previously shown to last more than 3 weeks in mice (19, 20), a duration sufficient for these behavioral experiments. Local norBNI injection into the DRN

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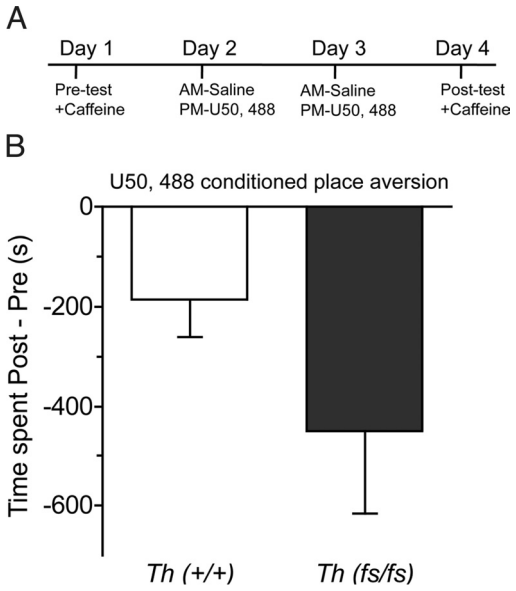

Fig. 1. Dopamine-deficient mice show U50,488-induced place aversion. (*A*) Timeline of the experiment (caffeine was injected before pre tests and post tests to induce locomotor activation). (*B*) U50,488-induced CPA scores, calculated as post test minus pre test times in the U50,488-paired side, for dopamine-replete [control, Th (+/+)] and dopamine-deficient [Th (fs/fs)] mice (n = 5–6).

significantly blocked CPA caused by administration of U50,488 (Fig. 2*A*). Mice injected with norBNI in an adjacent brain structure, the subpenduncular tegmental nucleus (SPTg), developed normal place aversion to U50,488. These results suggest that local injection of norBNI had a restricted sphere of effect, and this conclusion was confirmed anatomically using a phospho-selective antibody (KORp) that recognizes ser369 in the carboxyterminal domain of KOR that has been phosphorylated by G protein receptor kinase 3 (GRK3) (21). Following completion of the behavioral study, mice were systemically injected with U50,488, and sites of opioid action were visualized immunohistochemically by an increase in KORp-immunoreactivity (ir) (Fig. 2*B*). The U50,488-induced increase in KORp-ir was selectively reduced in the DRN (and inactivated KOR as far as the rostral DRN) of norBNI-injected mice, but not the saline-injected controls. In addition, local DRN injection of norBNI did not affect the increase in KORp-ir in the adjacent SPTg (Fig. 2*B*). These anatomical results support the conclusion that local injection of norBNI caused a persistent and spatially restricted inactivation of KOR signaling in the DRN. Interestingly, norBNI injection into DRN also blocked stress-induced analgesia (Fig. 2*C*), confirming local KOR antagonism. This result corroborates reports of KOR-dependent serotonergic control of the analgesic response (15), and it further establishes a supraspinal mechanism for KOR in the regulation of pain responses.

Recent evidence supports a critical role of KOR in stress-induced reinstatement to drug seeking (6, 7), but the sites of action in brain responsible and the relationship between KOR-dependent aversion and reinstatement are not clear. Animals injected with norBNI or saline in DRN showed equivalent cocaine place preference (CPP) after three training sessions and showed no differences in cocaine-induced locomotor activity during the training sessions (Fig. S2*A*). Subsequent extinction training also produced equivalent rates of place preference extinction, suggesting that the motivational and learning responses to cocaine were equivalent in both groups of animals (Fig. S2*B*). Consistent with prior results (6), a single social defeat stress session, previously shown to activate the dynorphin/KOR system (8), effectively reinstated CPP in saline-injected, control mice (Fig. 2*D*). However, mice receiving local norBNI in DRN did not show stress-induced reinstatement of CPP (Fig. 2*D*). In contrast, reinstatement of CPP induced by a cocaine priming injection (6) was unaffected by local injection of norBNI in DRN (Fig. S2*C*). Importantly, both DRN-saline and norBNI groups displayed equal stress-induced defeat behaviors and received an equal number of attacks from aggressor mice (Fig. S3); these results demonstrate that the block of stress-induced reinstatement in norBNI-DRN-injected mice is not due to a reduction in the stress experience of that group. These results further highlight a specific role KOR in the DRN in mediating stress-induced behaviors, including relapse to drug seeking as assessed in this paradigm.

Viral Expression of KOR Only in the DRN of KOR KO Mice Recovers Aversion. Because we found that activation of KOR in the DRN was necessary for place aversion, we next assessed whether KOR

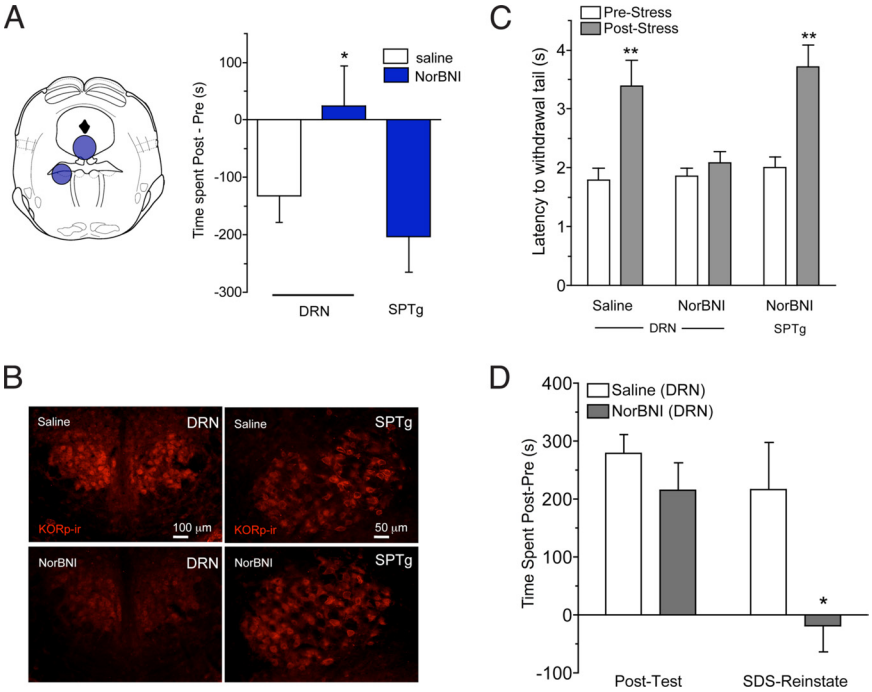

Fig. 2. NorBNI in the DRN blocks behavioral responses to KOR activation. (*A*, left) Image of a coronal section (plane −4.65 mm from bregma) highlighting the sites of saline and norBNI injection in the DRN (upper circle) or SPTg (lower circle); (right) U50,488-induced CPA scores, calculated as post test minus pre test times in the U50,488-paired side, for mice microinjected 1 week previously with either saline (1 μL) or norBNI (2.5 μg/μL) in DRN or SPTg (n = 7; ANOVA $F_{2,20}$ = 3.92; *, $P < 0.05$ Bonferroni posthoc compared to saline-DRN and norBNI-SPTg). (*B*) Representative photomicrographs of KORp-ir in the DRN and SPTg of saline or norBNI DRN-injected animals, perfused 30 min after U50,488 injection. (*C*) Tail withdrawal latency for DRN- and SPTg-injected animals before and after a 20-min SDS (n = 4–8; **, $P < 0.01$ t-test compared to pre stress). (*D*) Cocaine CPP 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 = 6–7; *, $P < 0.05$ t-test compared to saline). Bars represent means ± SEM.

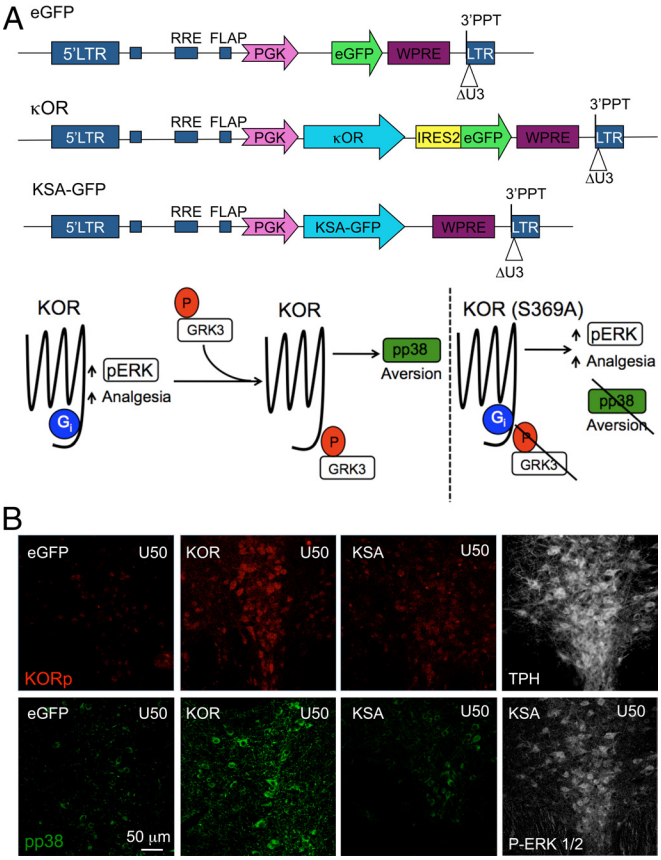

Fig. 3. Viral injection results in functional expression of KORs. (*A*, *Upper*) Schematic of the three lentiviral constructs used [adapted from (14); see for abbreviations, KSA represents KOR(S369A)]; (*Lower*) schematic of KOR signaling through Gαi and GRK/β-arrestin and the behaviors each is thought to control. KOR(S369A) can only signal through the Gαi upon activation. (*B*) Representative photomicrographs of KORp-ir (red) and phospho-p38 (pp38-ir) (false-green) in the DRN of KOR KO animals injected with the three lentiviral constructs. Photomicrographs of cells expressing TPH (serotonin cells) and *p*-ERK1/2 in KOR(S369A) (KSA) are also shown in white.

activation in the DRN was also sufficient for this behavior. We generated a bicistronic lentiviral vector based on a construct developed by the Changeux group (22) to express KOR-GFP under a PGK promoter (lenti-KOR) (Fig. 3*A*, *Upper*) in KO mice lacking KOR (KOR KO) (23). As a control, we also engineered a lentivector to express KOR(S369A), a serine to alanine point mutation at position 369 in the C-terminal domain that allows the receptor to activate Gαi-mediated responses, but is not phosphorylated by GRK3 (schematically diagramed in Fig. 3*A*, *Lower*) (21). GRK3 phosphorylation of KOR is necessary for β-arrestin-dependent activation of p38 mitogen-activated protein kinase (MAPK) (24), and p38 MAPK activation is required for KOR-dependent aversion (25). A second control lentiviral vector containing only eGFP was generated to assess nonspecific effects of viral-mediated gene transfer on behavior (Fig. 3*A*).

Three weeks after injection of lenti-KOR into the DRN of mice lacking the endogenous receptor (KOR KO), systemic U50,488 administration increased KORp-ir and phospho-p38-ir in the DRN, whereas mice injected with either lenti-KOR(S369A) or lenti-eGFP vectors showed no such increase (Fig. 3*B*). These results are consistent with previous in vitro results using these KOR constructs in heterologous expression systems (22). This enhanced KORp-ir staining was visualized only in cells that also expressed eGFP (Fig. 4*A*) and strongly co-localized with serotonergic DRN cells expressing tryptophan hydroxylase (TPH) (Fig. 4*B*). Greater than 90% (± 3%) are both KOR and TPH positive inside the "fountain" of the DRN, whereas 75% (± 5%) are both KOR and TPH positive throughout the DRN. Although unable to increase KOR-P-ir or phospho-p38 MAPK-ir, lentiviral KOR(S369A) expression restored robust ERK activation (*p*-ERK) in DRN neurons following U50,488 administration (Fig. 3*B*). These results confirm that the Gαi functionality of KOR was intact (25), in concordance with reports showing that KOR-mediated ERK activation does not require GRK3 or β-arrestin expression (24, 26).

KOR KO mice did not develop place aversion after systemic U50,488 injection; whereas KOR wild-type (WT) littermate mice developed aversion (Fig. 5*A*). Lenti-KOR injection into the DRN of KOR KO mice completely rescued the KOR-agonist-induced CPA (Fig. 5*A*), whereas neither lenti-KOR(S369A)- nor the lenti-eGFP-injected mice developed U50,488-induced place aversion (Fig. 5*A*). As a behavioral control for receptor functionality, lenti-KOR(S369A)-injected mice showed analgesic responses comparable to lenti-KOR-injected mice following U50,488 (Fig. 5*B*). In contrast, control lenti-eGFP injection in DRN of KOR KO mice did not restore the antinociceptive response to U50,488 (Fig. 5*B*). The selective rescue of the antinociceptive response by KOR(S369A) further supports the conclusion that KOR activation in the DRN regulates pain responses. The ability of the KOR(S369A) receptor to restore the analgesic, but not aversive response is consistent with a GRK3/phospho-p38-dependent role of KOR in mediating the aversive response to U50,488 (25). These data also suggest that KOR activation of p38 MAPK in the DRN is sufficient to produce CPA. The combination of local lentiviral expression in KO mice represents an effective technique for determining G-protein-coupled receptor-dependent behaviors.

DRN Neurons Projecting to the NAc Mediate Aversion. Serotonergic projections from the DRN innervate nearly all forebrain regions, including the NAc. The NAc has been widely associated with the regulation of hedonic state (27) and was therefore a plausible projection site for KOR-containing neurons originating in the DRN to affect mood. Consistent with this concept, local injection of norBNI into the rostral NAc (Fig. 6*B*) also significantly attenuated U50,488-induced CPA of WT mice (Fig. 6*A*). Injection of NorBNI in the NAc also decreased swim immobility, an effect previously shown to depend on dynorphin-KOR activation (28) (Fig. S4). This finding is consistent with a role of the NAc in KOR-mediated depression-like behaviors (28). To assess whether the site of norBNI action in NAc was pre- or postsynaptic, we injected lenti-KOR into the DRN of KOR KO mice, then injected norBNI or saline into the NAc 3 weeks later (Fig. 6*C*). Mice receiving lenti-KOR in the DRN and saline in the NAc showed CPA to U50,488; whereas KOR KO mice receiving lenti-KOR in the DRN and subsequent norBNI in the NAc did not develop aversion (Fig. 6*D*). This experiment suggests that norBNI injection in the NAc blocked the functioning of KOR expressed on nerve terminals of DRN cells, because only axons from the lenti-KOR-injected DRN cells express KOR in the KO mice.

Discussion

The principal findings of this study are that mice lacking dopamine still show a place aversion to U50,488. Second, by selective inactivation of KOR signaling and lentiviral rescue of KOR function in KO mice, we establish a DRN to NAc serotonergic projection as being both necessary and sufficient to mediate aversive behavioral responses. Third, KOR signaling in the DRN is necessary for stress-induced reinstatement of CPP. Our results demonstrate that activation of KOR either pharmacologically or by stress-evoked dynorphin release may regulate a serotonergic projection from the DRN to the NAc to regulate drug seeking behaviors.

Although serotonin has been implicated in the modulation of drug reward (29), identifying the sources of the serotonergic

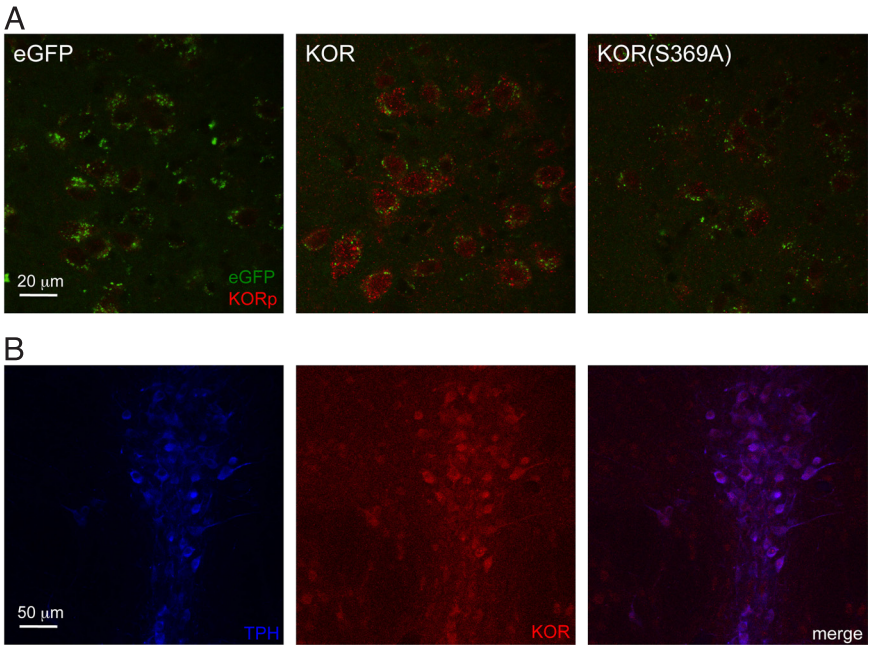

Fig. 4. Functional KOR is expressed only in neurons containing eGFP and on serotonin neurons. (*A*) Confocal photomicrographs showing eGFP (green) and KORp (red) in the DRN of animals injected with one of the three lentiviral constructs 3 weeks previously. (*B*) Confocal photomicrographs of DRN in a lenti-KOR-injected KOR KO animal, showing overlap of TPH (blue) and KOR (red).

projections and the regulation of this connection by stress-mediators has not previously been investigated. Instead, prior studies have focused on the contributions of various serotonin receptor types in classic drug target regions including the NAc (30) or have used various KO strategies to implicate serotonin (13). Indeed, only one other study has looked at the contribution of a median raphe nucleus to drug seeking (31).

We have also resolved a previously unknown connection between the DRN and NAc in mediating aversive behaviors. The molecular aspects of this DRN projection were not defined, but re-expression of KOR in serotonergic neurons (marked by TPH-ir) and prior studies showing that KOR activation inhibits serotonin release (32), suggests the concept that dynorphin release induced by stress produces aversion and induces reinstatement by reducing serotonergic tone in the NAc. Local norBNI injection in DRN also blocked CPA, suggesting that both hyperpolarization of somatic membranes (33) and inhibition of transmitter release from nerve terminals (32) in NAc by KOR activation may be required to reduce serotonin release in NAc. Further molecular and biophysical characterizations of this circuit are necessary to understand the consequences of KOR-mediated DRN modulation, and further exploration is warranted to determine the contributions from other KOR containing brain areas, such as the VTA, that have been previously shown to modulate aversion (16).

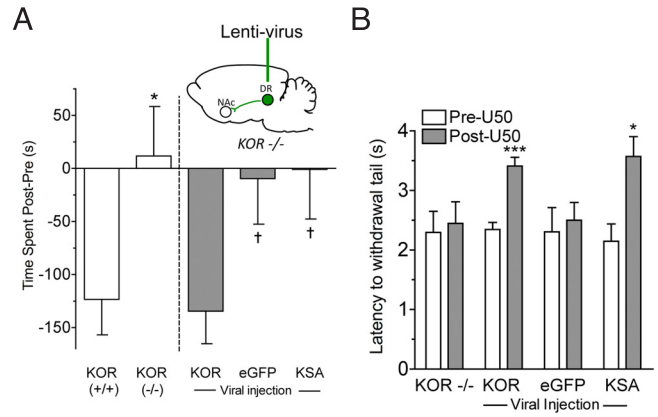

Fig. 5. Viral expression of KOR in the DRN of KOR KO mice rescues the behavioral response to agonist. (*A*) CPA scores induced by U50,488 (2.5 mg/kg) for KOR WT, KOR KO, and lentiviral-injected KOR KO animals. (Inset) Sagittal schematic illustrating the DRN injection site ($n = 9$–12; *, $P < 0.05$ *t*-test compared to KOR KO; ANOVA $F_{2,31} = 3.55$; † $P < 0.05$ Bonferroni posthoc compared to KOR). (*B*) Tail withdrawal latency before and after a 10 mg/kg U50,488 injection for KOR KO and lentiviral-injected KOR KO animals ($n = 4$–11; *, $P < 0.05$, ***, $P < 0.001$ *t*-test compared to pre-U50). Bars represent mean ± SEM.

Although we report that selective disruption of dopamine failed to blocked KOR-agonist-induced conditioned place aversion, it is still likely that dopamine plays a key role in the modulation of these negative affect processes because other reports have shown that infusion of KOR agonist in the VTA induces CPA in rodents (16) by inhibiting dopamine cell firing (17) and have demonstrated that dopamine D1-receptor antagonism blocks KOR agonist CPA (34, 35). However, results from the dopamine-deficient mice suggest the decrease in dopamine release caused by KOR agonists may not be necessary for place aversion. KO approaches come with caveats including compensatory changes during development, which can limit the interpretation of behavioral data. In any case, our observation that dopamine-deficient mice have an intact response to a KOR agonist led us to investigate other potential circuits and structures mediating the behavioral responses to dynorphin/KOR activation. Coupled with prior work showing that serotonin is critical for CPP in the absence of dopamine (13), our study suggests that both serotonergic and dopaminergic circuits can mediate hedonic processing in response to stress. Future study is required to understand the relationship between the convergence of dopaminergic and serotonergic projections in the NAc.

We also demonstrate that p38 MAPK activation in the DRN is necessary for the aversive properties of KOR activation. Injection of a lentiviral-KOR construct into the DRN was sufficient to restore aversion to a KOR agonist, whereas injection of a construct encoding a mutant receptor (KOR369A) that is unable to activate p38 MAPK (24) did not restore aversive responses. This finding corroborates our report showing that p38 MAPK activation is critical for KOR-dependent CPA (25) and highlights the importance of intracellular MAPK signaling cascades in mediating mood-associated behaviors. The mechanisms by which p38 MAPK activation evokes aversive responses is unknown, however some possibilities are suggested by data

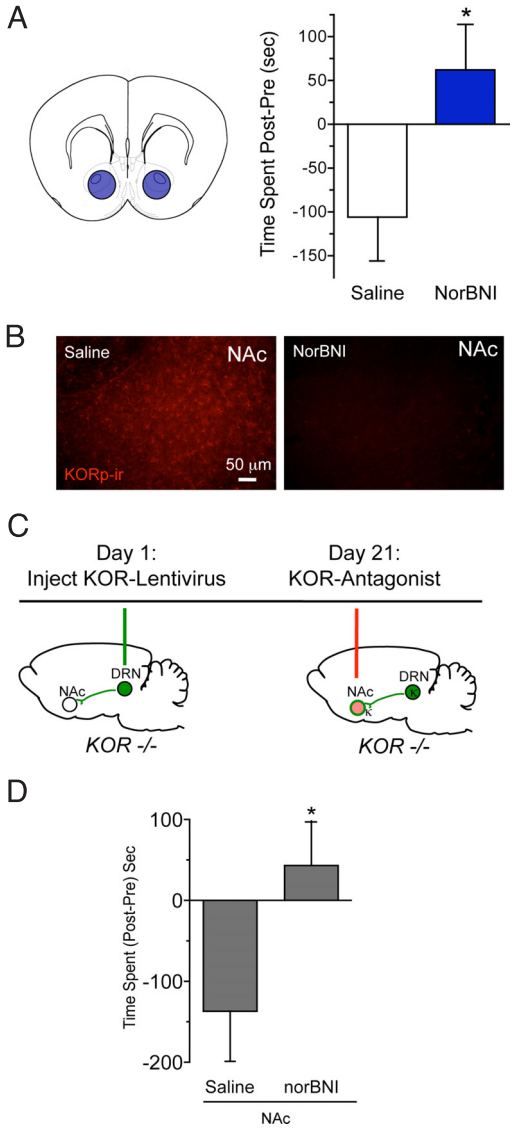

Fig. 6. A DRN-NAc circuit regulates KOR CPA. (*A*, left) Coronal section of the NAc highlighting saline and norBNI injection areas (+1.45 mm from bregma); (right) U50,488-induced CPA scores for NAc-injected animals ($n = 10$; *, $P < 0.05$ *t*-test compared to saline). (*B*) Representative photomicrographs of KORp-ir in the NAc after saline or norBNI injection, perfused 30 min after U50,488 injection. (*C*) Schematic of double-injection schedule for DRN and NAc place aversion study. (*D*) U50,488-induced CPA scores for lenti-KOR-DRN, saline/norBNI-NAc animals ($n = 7–11$; *, $P < 0.05$ *t*-test compared to saline). Bars represent mean ± SEM.

developed by the Blakely group (36), showing that p38 MAPK activation modulates function of the serotonin transporter. Other groups have demonstrated that p38 MAPK contributes to synaptic plasticity by affecting long-term depression (37), possibly through a phosphorylation of proteins including SynGAP and AMPA receptors (38). In any event, KOR-mediated p38-dependent behavioral responses require functional DRN-KOR expression, and future study of the p38 MAPK substrate is merited.

We used CPA as an operationally defined measure of dysphoria. Our previous behavioral characterization using odorant and contextual cues demonstrated that responses to stressful events requires dynorphin/KOR activation (5). Additionally, KOR agonists are known to produce depressed mood and dysphoria in humans (39); hence, we assume that the avoidance of contextual cues in our assay is due to their pairing with a qualitatively similar experience in the mouse. Although we do not directly measure dysphoria in mice, CPA acts as a well-validated proxy measure useful to dissect the brain circuitry and molecular mechanisms that mediate the negative affect associated with stress-induced behavior. The same caveat applies for the use of conditioned place preference as a proxy measure of "drug seeking" behavior. Although animals are not administering cocaine to themselves in this paradigm, mice are preferring to actively explore the chamber containing cocaine-associated contextual cues, which implies that the animal is engaged in reward-seeking activity.

The role of dynorphin as a key mediator of stress-induced dysphoria is gaining increasing appreciation. Kappa-selective opioid agonists produce dysphoric responses in humans (39) and aversive responses in rodents (5, 16, 40). Endogenous dynorphins are released during exposure to stressful events, and KOR receptor activation is required for stress-induced aversion (5) and anxiety responses (41). KOR antagonists have antidepressant-like effects in the Porsolt forced swim test and the Miczek social-defeat test (8, 42). These results support the concept that stress-induced depression may involve dynorphin-dependent effects. Similarly, stress activation of the dynorphin/KOR systems potentiates the rewarding properties of cocaine (8, 42) and induces reinstatement of extinguished drug seeking (6,7.9). The dynorphin/KOR system is thought to be activated during drug withdrawal (43) and may underlie the opponent process postulated to drive drug addiction (10). Understanding the pro-depressant and pro-addictive effects of the dynorphin/KOR system is providing insights to mechanisms linking stress, depression, and addiction.

Materials and Methods

Animals. Male C57BL/6 mice (Charles River) weighing 22–30 g were used. KOR (*Oprk1*) gene deletion on C57BL/6 background was generated as previously described (23), and homozygous (KOR KO) and WT (KOR WT) littermate controls were generated by heterozygote crosses. Dopamine-deficient mice ($Th^{fs/fs}$, $Dbh^{Th/+}$) carrying two conditionally inactive *Th* alleles, were generated as described (18). Dopamine-deficient mice are indistinguishable from littermates at birth and maintain normal growth when given daily L-dihydroxyphenylalanine (L-dopa) treatment (30 mg/kg) that restores locomotion for ≈8 h (18). Animal protocols were approved by the Institutional Animal Care and Use Committee.

Drugs. Cocaine-HCl, (±) U50,488 and norbinaltorphimine-HCl (norBNI) were provided by the National Institute on Drug Abuse Drug Supply Program.

Viral Vector Design and Production. Three constructs were developed based on the lentiviral construct expressing the β2 subunit of the nicotinic acetylcholine receptor under the mouse phosphoglycerol kinase (PGK) promoter (22). The lenti-KOR vector is a bicistronic construct expressing KOR and GFP. Lenti-KOR(S369A) is a monocistronic construct containing a mutant form of the KOR where serine 369 was mutated to alanine. A construct expressing only eGFP was used as a negative control. In each case, gene expression was under the control of the PGK promoter. The integrated virus was rendered replication incompetent by deletion of the U3 region of the 3′ long terminal repeat (44, 45). Sequences were incorporated to enhance RNA stability, transgene expression, and infection of nondividing cells (46), and the viral expression plasmid was inserted into the pUC18 plasmid. Viral particles were produced by the Fred Hutchinson Cancer Research Center, Seattle, WA. Briefly, viral particles were produced by cotransfection of the vector plasmid with a packaging plasmid and the VSV-G envelope plasmid; at 24–72 h following transfection, media were collected, and viral particles were isolated by filtration and ultracentrifugation. Virus was tested for replication competency by ELISA against the p24 capsid protein over a course of at least 3 weeks.

Microinjection Procedure (norBNI and Lentiviral Constructs). Isoflurane-anesthetized mice were mounted on a stereotaxic alignment system (David Kopf Instruments). A bevel-tipped Hamilton syringe was lowered into the dorsal raphe ($x = 0.0$, $y = -4.65$, $z = -3.85$ mm from bregma) or bilaterally into the NAc ($x = 0.75$, $y = +1.45$, $z = -5.00$ mm from bregma) and either norBNI (2.5 μg/side) or one of the three viral constructs (dorsal raphe only) were injected at a rate of 100 nL/min for 10 min (1 μL total). The syringe remained in place for at least 3 min after the infusion was finished and was then slowly removed. Animals were sutured with 5–0 polyviolene sutures (Sharpoint) and allowed to recover for at least 5–7

days before behavioral testing began. Virally injected animals recovered for at least 3 weeks, allowing adequate time for expression and distribution of protein.

Conditioned Place Aversion. Mice were trained in an unbiased, balanced three-compartment conditioning apparatus as described (5). 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). Dopamine-deficient mice were injected with caffeine (15 mg/kg) 5 min before pre test and test on day 4 to facilitate exploration of the apparatus (13). Mice were assigned to saline and drug compartments and received saline in the morning (10 mL/kg, i.p.) and U50,488 (2.5 mg/kg, i.p.) in the afternoon at least 4 h after the morning training on 3 consecutive days. 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 U50,488-paired compartment post test minus the pre test.

Stress-Induced Cocaine Reinstatement. Mice were trained in the same conditioning apparatus as described above, and the pre test (day 1) was identical. On days 2–5, mice received saline in the morning (10 mL/kg, s.c.) and cocaine (15 mg/kg, s.c.) in the afternoon, at least 4 h after the morning training. On day 5, animals were allowed to freely explore all three chambers to assess CPP. Animals then went through extinction training on days 6–8 by injection with saline in both the morning and afternoon. Animals were tested for extinction of CPP on day 9 with a second free exploration of all three chambers. Animals were judged to have extinguished CPP if scores fell within 15% of their initial preference (≈60% of animals). Animals that extinguished were put through stress-induced reinstatement. On day 10, animals were exposed to social defeat stress (SDS), a 20-min exposure to a dominant, resident male. Interactions were observed to ensure social defeat postures such as rearing with paws up took place, as well as to ensure serious damage (i.e., bleeding, paw injury) did not occur. Immediately after the SDS, animals were placed into the CPP apparatus and allowed to freely explore the three chambers. Scores were calculated by subtracting the time spent in the cocaine side post test minus the extinction test.

Tail-Flick Analgesia. The response latency for the mouse to withdraw its tail from immersion into 52.5 ± 1 °C water was measured using a stopwatch before and 30 min after stress or U50,488 administration (10 mg/kg, i.p.).

Immunohistochemistry. Standard immunohistochemical methods were used in this study. Details of antibody sources, dilutions, incubation conditions, buffers, and procedural and analytical methods are provided in the *SI Text*.

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 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). The number of mice (*n*) and specific statistical analyses used in each experiment are specified in the figure legend text.

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