Neuropsychopharmacology (2018) 43, 362–372

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κ-Opioid Receptor Activation in Dopamine Neurons Disrupts Behavioral Inhibition

Antony D Abraham[1], Harrison M Fontaine[1], Allisa J Song[1], Mackenzie M Andrews[1], Madison A Baird[1], Brigitte L Kieffer[2], Benjamin B Land[1] and Charles Chavkin*[,1]

[1]Department of Pharmacology, University of Washington School of Medicine, Seattle, WA, USA; [2]Research Center of the Douglas Mental Health University Institute, Department of Psychiatry, McGill University, Montreal, Quebec, Canada

The dynorphin/κ-opioid receptor (KOR) system has been previously implicated in the regulation of cognition, but the neural circuitry and molecular mechanisms underlying KOR-mediated cognitive disruption are unknown. Here, we used an operational test of cognition involving timing and behavioral inhibition and found that systemic KOR activation impairs performance of male and female C57BL/6 mice in the differential reinforcement of low response rate (DRL) task. Systemic KOR antagonism also blocked stress-induced disruptions of DRL performance. KOR activation increased 'bursts' of incorrect responses in the DRL task and increased marble burying, suggesting that the observed disruptions in DRL performance may be attributed to KOR-induced increases in compulsive behavior. Local inactivation of KOR by injection of the long-acting antagonist nor-BNI in the ventral tegmental area (VTA), but not the infralimbic prefrontal cortex (PFC) or dorsal raphe nucleus (DRN), prevented disruption of DRL performance caused by systemic KOR activation. Cre-dependent genetic excision of KOR from dopaminergic, but not serotonergic neurons, also blocked KOR-mediated disruption of DRL performance. At the molecular level, we found that these disruptive effects did not require arrestin-dependent signaling, because neither global deletion of G-protein receptor kinase 3 (GRK3) nor cell-specific deletion of GRK3/arrestin-dependent p38α MAPK from dopamine neurons blocked KOR-mediated DRL disruptions. We then showed that nalfurafine, a clinically available G-biased KOR agonist, could also produce DRL disruptions. Together, these studies demonstrate that KOR activation in VTA dopamine neurons disrupts behavioral inhibition in a GRK3/arrestin-independent manner and suggests that KOR antagonists could be beneficial for decreasing stress-induced compulsive behaviors.
Neuropsychopharmacology (2018) **43,** 362–372; doi:10.1038/npp.2017.133; published online 23 August 2017

INTRODUCTION

Dynorphin, an endogenous opioid peptide released following stress, activates the κ-opioid receptor (KOR) to produce depression-like behaviors (Mague *et al*, 2003; Knoll and Carlezon, 2010) and aversion (Shippenberg and Herz, 1986). In humans, acute KOR activation with selective and highly efficacious KOR agonists or the natural products from the *Salvia divinorum* plant (Salvinorin A; a selective KOR agonist) produces potent psychotomimetic and hallucinogenic effects (Johnson *et al*, 2011), suggesting that KOR activation may also contribute to cognitive disruptions following a behavioral stress experience.

Although it is challenging to measure psychotomimetic drug effects using animal models, KOR activation has been shown to alter multiple domains of cognition in rodents, including attention, memory, and impulsivity (Nemeth *et al*, 2010; Cole *et al*, 2013). KOR activation has no effect on impulse control (Paine *et al*, 2007) or impulsive choice, but does disrupt behavioral performance in a response inhibition task (Walker and Kissler, 2013). Degradation of response inhibition may be indicative of a loss of inhibitory control, a cognitive feature that is disrupted in psychiatric illnesses such as substance use disorder (Jentsch and Taylor, 1999), affective disorders (Murphy *et al*, 1999), and compulsive disorders (Chamberlain *et al*, 2005). KOR activation following stress may exacerbate behavioral symptoms or contribute to the etiology of these diseases. Although KOR antagonists are in development for the therapeutic goal of decreasing stress-induced mood disorders and relapse of substance abuse (Carroll and Carlezon, 2013), it is unknown whether KOR antagonists may also be useful for decreasing stress-induced cognitive disruptions or compulsive behaviors.

Behavioral and cognitive disruptions in patients with obsessive–compulsive disorder (OCD) can worsen following stressful events (Fornaro *et al*, 2009). Many of the symptoms observed in OCD may be a consequence of loss of inhibitory control (Chamberlain *et al*, 2005) leading to obsessive or compulsive thoughts and behaviors. One method to investigate disruption of inhibitory control in rodents is through the use of the differential reinforcement of low response rate (DRL) task. The DRL task requires an animal to withhold responding for a set wait period before making a reinforced response (Sidman, 1955). Responses occurring

*Correspondence: Dr C Chavkin, Department of Pharmacology, University of Washington School of Medicine, Box 357280, Seattle, WA 98195-7280, USA, Tel: 206 543 4266, Fax: 206 685 3822, E-mail: cchavkin@u.washington.edu
Received 1 November 2016; revised 15 June 2017; accepted 19 June 2017; accepted article preview online 26 June 2017

before the end of the wait period (nonreinforced responses) reset the wait period. This task has previously been used to measure temporal discrimination (Sidman, 1956), antidepressant efficacy (O'Donnell *et al*, 2005), and impulsive action (Selleck *et al*, 2015). Nonspecific opioid receptor activation in the prefrontal cortex disrupts performance in the DRL task (Selleck *et al*, 2015), demonstrating that opioid receptor activation decreases inhibitory control. Stress-induced release of dynorphin would be expected to disrupt cognitive performance, but the specific nature of these disruptions, the sites of action in brain, and the cellular mechanisms underlying these disruptions are not yet known. Here, we find that KOR activation in dopamine neurons of the ventral tegmental area (VTA) disrupts inhibitory control to increase compulsive responses. We also determine that KOR-mediated DRL disruptions are likely to use G-protein receptor kinase 3 (GRK3)/arrestin-independent intracellular signaling pathways, indicating that some G-biased KOR agonists may produce compulsive responses.

MATERIALS AND METHODS

Subjects

Male and female C57BL/6 mice ($n=174$) ranging from 3 to 10 months of age were used in these experiments.

Drugs

The (±)U50488H (U50488), nor-binaltorphimine (nor-BNI), and nalfurafine were provided by the National Institute of Drug Abuse Drug Supply Program (Bethesda, MD). U50488 (5 and 10 mg/kg), nor-BNI (10 mg/kg), or nalfurafine (50 μg/kg) was dissolved in saline and administered intraperitoneally (i.p.) in a volume of 10 ml/kg. The nor-BNI (2.5 μg/μl) dissolved in sterile artificial cerebrospinal fluid (ACSF) was intracranially microinjected.

Procedures

Differential reinforcement of low rates of responding (DRL). Following training to discriminate between active and inactive nose poke holes, mice were trained in a 60-min DRL procedure (Horwood *et al*, 2001). During a DRL session, a single nose poke led to food reward delivery. Following the first reinforced nose poke, subjects were required to withhold responding for a specified wait period. Nose pokes that occurred before the end of the wait period reset the wait period and were nonreinforced. Once trained for stable performance on the DRL task (below 40% error over at least 4 days; 15–25 training sessions), animals underwent sessions where they received saline treatment on a baseline DRL day, and a U50488 treatment the following day immediately before a DRL session. For microinjection studies, male mice were used that had previously been tested for the systemic effect of KOR activation in the DRL task. Male mice were trained in the DRL task, and then received a single microinjection of nor-BNI (2.5 μg/μl; long-acting κ-antagonism by JNK activation; Bruchas *et al*, 2007; Land *et al*, 2009) into the infralimbic prefrontal cortex (PFC; $n=4$), dorsal raphe nucleus (DRN; $n=4$), or VTA ($n=10$). Controls received an injection of ACSF into the VTA ($n=8$). Mice recovered from surgery for 3 days before retraining for stable performance in the DRL task (3–5 days). Following retraining, mice received a saline baseline test day and U50488 the following day. Conditional and global knockout mice and their littermate controls received two DRL sessions with 5 mg/kg U50488 and one DRL session with 10 mg/kg U50488. Each test was separated by at least 5–10 daily sessions of DRL training between the drug test days to ensure that there were no persistent effects of drug administration on DRL performance. Each drug test day was preceded by a saline baseline DRL test day.

Forced-swim stress. To induce stress, C57BL/6 male mice ($n=8$) were exposed to a modified forced-swim test as previously described (McLaughlin *et al*, 2003). Briefly, the modified-Porsolt forced-swim paradigm used was a 2-day procedure in which mice swam in 30 °C water for 15 min the first day following a baseline DRL session, and then 6 min during each of four trials on the second day without the opportunity to escape. Mice were tested for DRL performance within 10 min following the second day of stress (stress 1). After 1 week, mice received the 2-day FSS procedure (stress 2) with nor-BNI (10 mg/kg; i.p.) or saline 24 h pretreatment ($n=4$ per group) and DRL performance was tested the following day.

Marble burying. C57BL/6 male mice were placed in a novel rectangular context (50.8 × 25.4 × 25.4 cm; corn cob bedding packed to 5 cm depth) housed in a sound- and light-attenuating cabinet for a 30 min habituation period. Mice then received an injection of U50488 ($n=10$) or saline ($n=9$) and were immediately returned to the context for another 30 min habituation period. Mice were removed from the context and 18 marbles (16 mm diameter) were placed on the bedding. Mice were immediately returned for a 30 min marble burying test. Total number of marbles buried (at least 2/3 covered) at the end of the 30 min session were counted by an experimenter blinded to drug treatment (Deacon, 2006).

Data Analysis

Error percentage was calculated by the number of responses that led to a reset of the wait time (nonreinforced responses) divided by total number of responses (reinforced+nonreinforced responses). Stable performance in the DRL task was defined as at least 4 days with $<40\%$ error (Sinden *et al*, 1986). After mice showed stable performance, they received baseline (saline) and U50488 test days. Mice that did not show stable performance also received baseline and test days to match drug treatment between cage-mates. Thus, mice that showed $>40\%$ error during the DRL baseline day were removed from analysis. Experimenters were blind to genotype (Figures 4e–h and 5) and group assignment (Figures 4a–d). Interresponse time (IRT) was the time between each nose poke on the active port. MATLAB software (version R2016a) was used to extract burst response data. Data were analyzed with *t*-tests or two-way ANOVAs as required by experimental designs for DRL, fixed ratio 1 (FR1), fixed ratio 5 (FR5), and marble burying experiments. For experiments in Figure 4, data from two baseline days and two U50488 days were averaged for analysis. The *post hoc* comparisons were

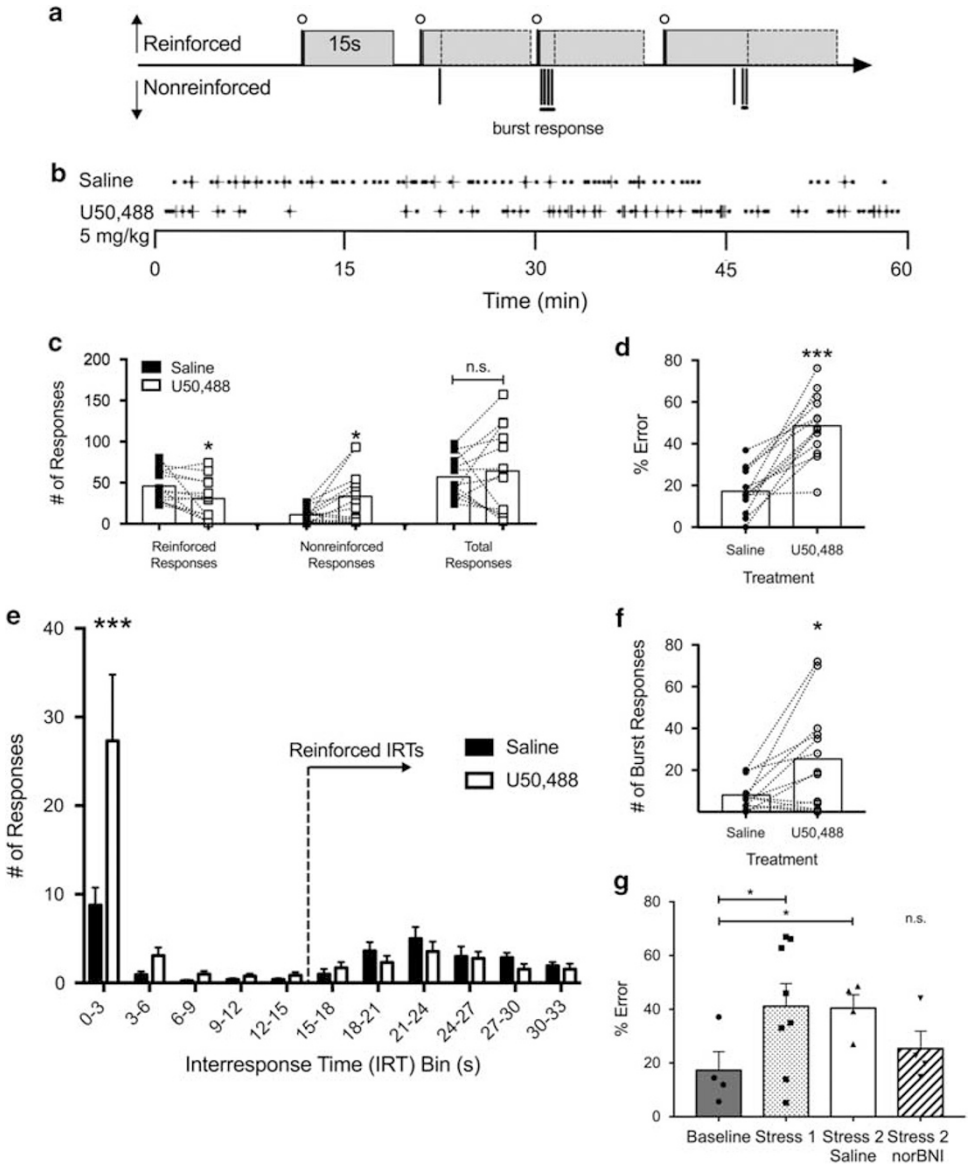


Figure 1 In male C57BL/6 mice, systemic KOR activation disrupted DRL performance. (a) Schematic of the DRL task. Reinforced responses (circle above vertical line) are shown as a positive deflection above the center time line and nonreinforced responses are shown as a vertical line below the center time line. Both reinforced and nonreinforced responses reset the 15 s DRL wait period (gray box), leading to longer wait periods (dashed box) if animals responded before the end of the wait period. Burst responses (horizontal line) were nonreinforced responses that occurred within 1 s of the previous response. (b) To illustrate a typical data set, responses from a single male animal are shown during 60 min DRL sessions with both saline or U50488 pretreatment. Closed circles represent reinforced responses and '+' symbols represent nonreinforced responses. (c) KOR activation by U50488 decreased total number of reinforced responses and increased nonreinforced responses without altering total number of responses. The number of responses made by an individual mouse during a 60 min period following saline pretreatment (■) were compared with responses following U50488 pretreatment (□) by a paired *t*-test (*$p<0.05$). (d) KOR activation significantly increased percent error during the DRL session. (e) A histogram of interresponse times showing the number of responses per 3 s bin. U50488 increased responses occurring within 0–3 s of the previous response. (f) KOR activation significantly increased the number of burst responses (an additional response <1 s after the previous response). (g) Repeated forced-swim stress increased percent error. Pretreatment with nor-BNI (KOR antagonist), but not saline, blocked increases in percent error following a second exposure to repeated forced-swim stress compared with baseline. Error bars indicate SEM. *$P<0.05$; ***$p<0.0001$; NS, not significant.

conducted with Sidak's test. For all statistical tests, the α was set to 0.05.

RESULTS

Systemic KOR Activation Disrupts DRL Performance in Male Mice

Following training for stable reinforcement under a DRL 15 s schedule, we tested the effect of systemic KOR activation on DRL performance in male mice. The experimental paradigm is diagrammed (Figure 1a). Responses during a DRL session could be either reinforced or nonreinforced, and either response resets the 15 s wait period. A subset of nonreinforced responses occurred with RT intervals of ‹1 s. These 'burst responses' have been described as a consequence of reward omission and a disruption in positive feedback for reinforced responses (Kramer and Rilling, 1970). Mice ($n=13$) were administered saline before a DRL session on a

baseline day, and 1 day later received a KOR agonist (U50488) immediately before a DRL session. A representative raster plot of responses in the DRL task is shown from a male mouse following saline and then U50488 pretreatment (Figure 1b).

Administration of U50488 (5 mg/kg) significantly increased the number of nonreinforced responses ($t_{12} = 2.78$, $p = 0.016$) and significantly decreased the number of reinforced responses ($t_{12} = 2.54$, $p = 0.026$) during the DRL test session compared with the saline test session, but there was no significant difference between saline and U50488 treatment in total response number (Figure 1c). Treatment with U50488 significantly increased percent error during the DRL session ($t_{12} = 6.59$, $p < 0.0001$) compared with the baseline saline day (Figure 1d). To investigate the temporal patterns of responding in the DRL task following KOR activation, we compared IRTs during U50488 and saline treatment days (Figure 1e). There was a significant main effect on IRT ($F(10, 120) = 39.86$, $p < 0.0001$), a main effect of Treatment ($F(1, 12) = 16.67$, $p = 0.002$), and an interaction between IRT and Treatment ($F(10, 120) = 24.4$, $p < 0.0001$). There was a significant difference between saline and U50488 treatment in the 0–3 s IRT bin ($p < 0.0001$), indicating a loss of inhibitory control of behavior (Selleck *et al*, 2015). The loss of inhibitory control caused by KOR activation was evident as a significant increase in burst responding ($t_{12} = 2.85$, $p = 0.015$) (Figure 1f).

Stress-induced release of dynorphin generated similar disruptions to those observed with systemic U50488 treatment in a separate cohort of mice. Following a repeated forced-swim stress procedure (stress 1), mice ($n = 8$) showed a significant increase in percent error ($t_8 = 2.93$, $p = 0.022$). These mice were then retrained and retested following a second repeated forced-swim stress (stress 2) with saline ($n = 4$) or nor-BNI ($n = 4$) pretreatment. Mice that received saline before stress 2 showed a significant increase in percent error ($t_4 = 3.59$, $p = 0.037$), but mice that received nor-BNI before stress 2 did not show a significant increase in percent error (Figure 1g). Together, these results demonstrate that pharmacological or stress-induced KOR activation impaired performance in the DRL task by disrupting behavioral inhibition and indicate that KOR activation increased compulsive responses to unexpected reward omissions.

Systemic KOR Activation Disrupts DRL Performance in Female Mice

KOR activation has sex-dependent effects on depressive behaviors (Russell *et al*, 2014; Chartoff and Mavrikaki, 2015), but it is unknown whether there are sex differences in KOR-mediated cognitive disruption. Female mice ($n = 10$) showed lower error rates during initial training in the 15 s DRL task compared with male mice (data not shown). Female mice were instead trained with a 25 s DRL schedule to ensure that DRL performance was learned, rather than reflecting a baseline rate of performance. Figure 2a shows a raster plot of responses in the DRL task from a representative female mouse with saline then U50488 pretreatment. Following training for stable performance in the 25 s DRL task, KOR activation (U50488; 5 mg/kg) significantly increased nonreinforced responses ($t_9 = 4.38$, $p = 0.002$) and total responses ($t_9 = 2.70$, $p = 0.024$). There was no significant effect of U50488 on reinforced responses compared with saline treatment (Figure 2b). However, there was a significant increase in percent error ($t_9 = 11.48$, $p < 0.0001$) during the DRL task in female mice (Figure 2c). A two-way ANOVA (IRT and Treatment as factors; Figure 2d) showed a significant main effect on IRT ($F(15, 135) = 27.9$, $p < 0.0001$), a main effect of Treatment ($F(1, 9) = 20.9$, $p = 0.001$), and an interaction between IRT and Treatment ($F(15, 135) = 16.21$, $p < 0.0001$). There was a significant difference between saline and U50488 treatment in the 0–3 s ($p < 0.0001$) and 3–6 s time bins ($p = 0.031$). KOR activation also significantly increased ($t_9 = 4.19$, $p = 0.002$) burst responding (Figure 2e). These results show that despite procedural differences in the DRL paradigm (ie, wait times), male and female mice display qualitatively similar disruptions in DRL performance efficiency following KOR activation and KOR-mediated disruptions of cognition may not be sex dependent.

KOR Activation Does Not Affect FR Responding and Increases Marble Burying

In the DRL task, burst responses have been hypothesized to occur when the delivery of reward is ambiguous or omitted (Sidman, 1956). To determine whether the burst responding caused by KOR activation could be attributed to a nonspecific change in operant performance or compulsive increases in responding, we tested the effect of KOR activation in two fixed ratio tasks. Male mice ($n = 7$) were trained in the FR1 procedure, where one nose poke led to the delivery of one food pellet. Following stable performance in the FR1 task, mice were given saline on one day and U50488 (5 mg/kg) the following day. There was no significant difference in the total number of reinforced responses following U50488 treatment (Figure 3a), and unlike mice trained in the DRL task, all responses were separated by at least 1 s, showing that FR1-trained animals did not produce burst responses with saline or U50488 treatment. We trained a separate cohort of male mice in an FR5 task ($n = 6$), where five nose pokes led to the delivery of one food pellet. KOR activation did not significantly alter the number of total responses during an FR5 session (Figure 3b) and produced no burst responses following a reinforced response. These experiments demonstrated that when reward delivery was predictable, KOR activation did not promote compulsive burst responses.

However, when tested in a task producing mild anxiety, such as in the marble burying test (Deacon, 2006), KOR activation with U50488 significantly ($t_{17} = 2.71$, $p = 0.015$) increased the number of marbles buried (Figure 3c). These results suggest that KOR-mediated increases in burst responses may be specific to anxiogenic or ambiguous reward contexts.

KORs in the VTA and in Dopamine Neurons Are Required for KOR-Mediated DRL Disruptions

The infralimbic PFC, DRN, and VTA contain KORs (Mansour *et al*, 1987) and have been implicated in response inhibition (Dalley *et al*, 2011). To identify the brain regions involved in KOR-mediated DRL disruptions, male mice were trained in the DRL task, and then received either ACSF into the VTA (Control; $n = 8$) or a microinjection of nor-BNI

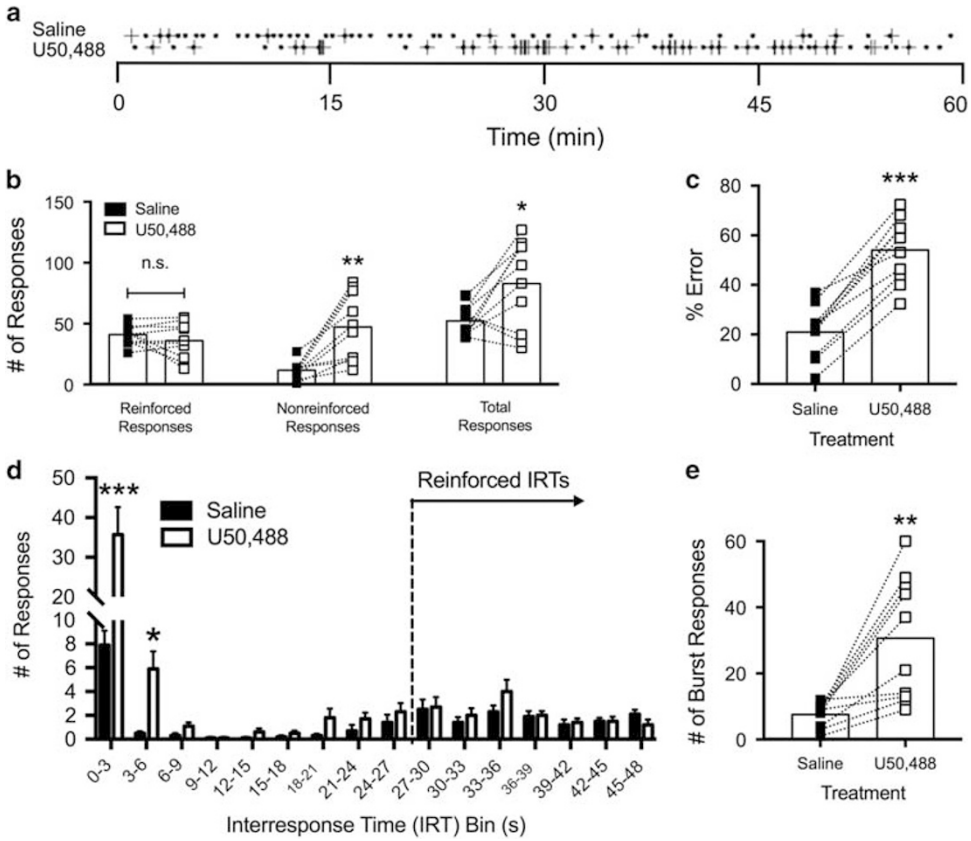


Figure 2 In female C57BL/6 mice, systemic KOR activation disrupted DRL performance. (a) To illustrate a typical data set for female mice, a raster display of responses from a representative animal are shown during 60 min DRL sessions after saline and then U50488 pretreatment. Closed circles represent reinforced responses and '+' symbols represent nonreinforced responses. (b) KOR activation did not affect reinforced responses, but increased nonreinforced and total responses during the DRL session. U50488 significantly increased (c) percent error, (d) the number of responses occurring within 0–3 s and 3–6 s of the previous response, and (e) the number of burst responses (< 1 s of the previous response). Error bars indicate SEM. Data were analyzed with paired *t*-tests; $^{*}P<0.05$; $^{**}p<0.01$; $^{***}p<0.0001$; NS, not significant.

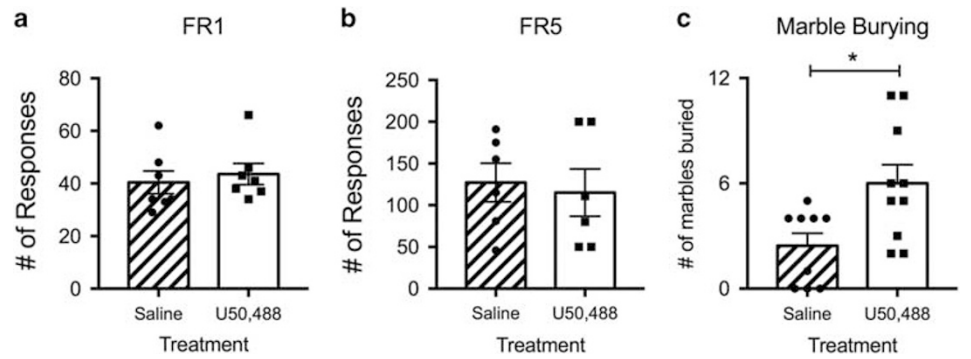


Figure 3 Systemic KOR activation increased marble burying. U50488 pretreatment did not affect the total number of responses during an (a) FR1 or (b) FR5 session compared with saline pretreatment. (c) There was a significant increase in marble burying following U50488 treatment. Error bars indicate SEM. $^{*}P<0.05$.

(2.5 μg/μl) bilaterally into the PFC ($n=4$) or VTA ($n=10$) or unilaterally into the DRN ($n=4$) (Figure 4a). For non-reinforced responses, a two-way ANOVA with Treatment (Saline; U50488) and Brain Region (PFC; DRN; VTA; Control) as factors showed that there was a significant effect of Treatment ($F(1, 22)=36.71$, $p<0.0001$) and a significant interaction between Treatment and Brain Region ($F(3, 22)=10.16$, $p=0.0002$). Nonreinforced responses were different between saline and U50488 treatment days in Control ($p=0.0003$) and DRN ($p=0.0001$) groups, but not in VTA or PFC (Figure 4b). There was a significant effect of U50488 treatment ($F(1, 22)=28.02$, $p<0.0001$) and Brain Region ($F(3, 22)=4.22$, $p=0.017$) on percent error, and a significant interaction between Treatment and Brain Region ($F(3, 22)=7.25$, $p=0.002$). U50488 treatment significantly increased percent error in Control ($p=0.003$), DRN ($p=0.024$), and

PFC ($p=0.004$) groups, but not in the VTA group (Figure 4c). This suggests that KOR activation in the VTA was required for disruptions in DRL performance. For burst responses (Figure 4d), there was a significant effect of Treatment ($F(1,23)=5.34$, $p=0.03$). There was no significant effect of U50488 on reinforced responses, total responses, or IRTs in VTA injected mice (Supplementary Figure 1). Histological confirmation for VTA targeting is shown in Supplementary Figure 2.

These results suggested that KOR activation in the VTA was required for the U50488-mediated effects on DRL performance. Although the VTA primarily comprises dopaminergic neurons, it contains other cell types and is innervated by a broad variety of neurons (eg, serotonergic or GABAergic) that would be affected by KOR inactivation (Polter and Kauer, 2014). Dopaminergic and serotonergic neuron activity is important for impulsivity (Dalley and Roiser, 2012) and KOR-mediated aversion (Ehrich *et al*, 2015), but cognitive disruptions may occur through distinct cellular mechanisms. We tested whether KOR-mediated disruptions in DRL performance occurred in male mice having either global KOR knockout (KOR KO; $n=13$), or KOR conditionally removed from ePet1-Cre-expressing serotonergic neurons (KOR CKO^{PET}; $n=4$), or KOR conditionally removed from DAT-Cre-expressing dopaminergic neurons (KOR CKO^{DAT}; $n=11$). Control mice ($n=18$) were $KOR^{lox/lox}$ or $KOX^{lox/+}$ littermates from CKO colonies (Figure 4e).

For nonreinforced responses, a two-way ANOVA with Treatment (Saline; U50488) and Genotype (Control; KOR CKO^{PET}; KOR CKO^{DAT}; KOR KO) as factors showed a main effect of Treatment ($F(1,42)=16.63$, $p<0.001$) and a nonsignificant trend toward an interaction between Treatment and Genotype ($F(3,42)=2.59$, $p=0.065$; Figure 4f). For percent error, there was a significant effect of Treatment ($F(1,42)=36.4$, $p<0.001$), Genotype ($F(1,42)=3.034$, $p=0.04$), and an interaction between Treatment and Genotype ($F(3,42)=7.187$, $p<0.001$). U50488 treatment significantly increased percent error in Control ($p<0.0001$) and KOR CKO^{PET} ($p=0.0001$) groups, but not KOR CKO^{DAT} or KOR KO groups (Figure 4g). For burst responses, there was a significant effect of Treatment ($F(1,42)=26.1$, $p<0.0001$) and a significant interaction between Treatment and Genotype ($F(3,42)=3.192$, $p=0.033$). KOR activation significantly increased burst responses in Control ($p=0.0007$) and KOR CKO^{PET} groups ($p=0.0035$), but not KOR CKO^{DAT} or KOR KO groups (Figure 4h). Analyses of reinforced responses, total responses, and IRTs for this experiment are shown in Supplementary Figure 3. Similar effects were observed when mice were given a 10 mg/kg dose of U50488 (Supplementary Figure 4). Similar to pharmacological blockade of VTA KORs, genetic excision of KORs from dopaminergic neurons prevents KOR-mediated increases in percent error.

KOR-Mediated DRL Disruptions Are GRK3/Arrestin Independent

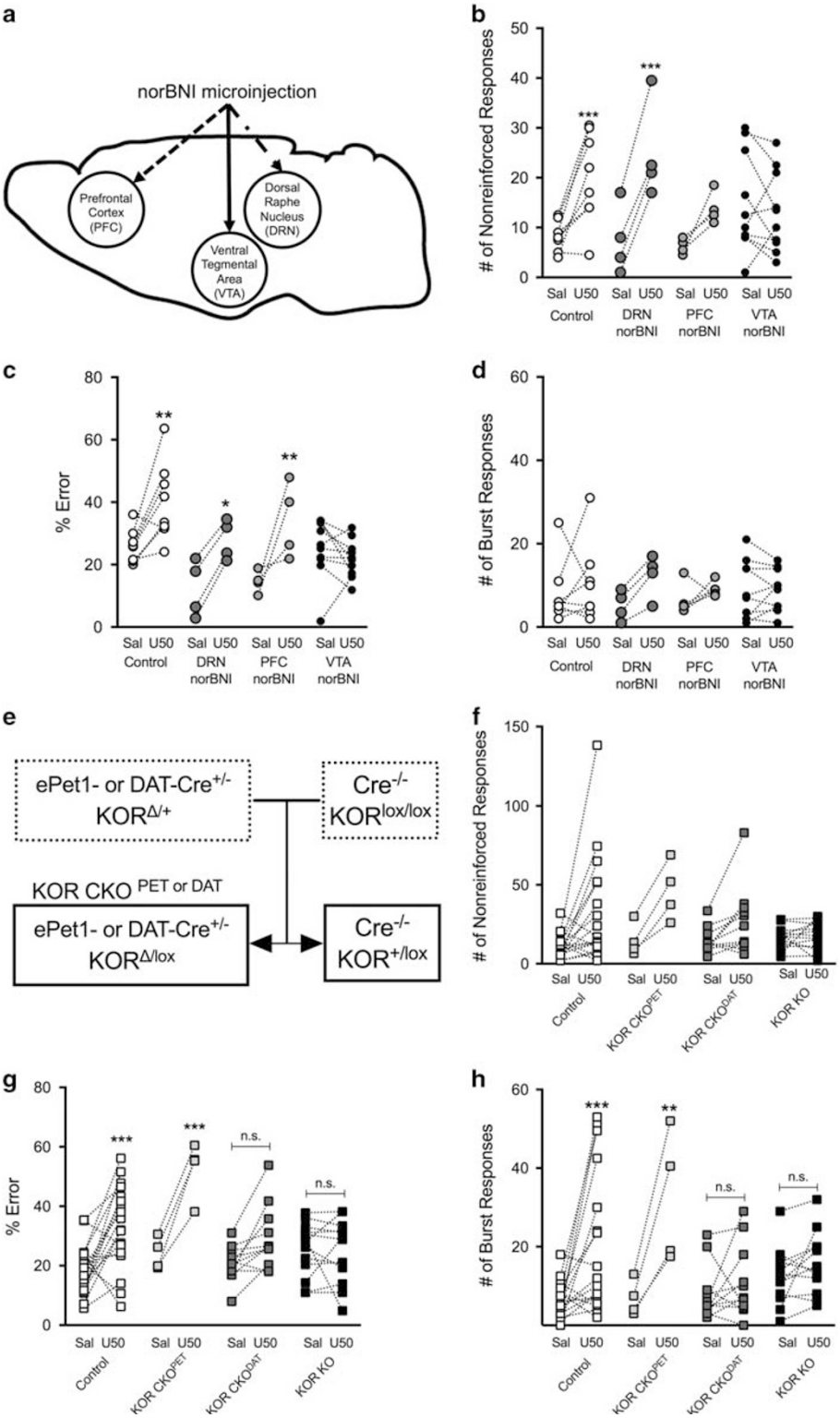
The aversive effects of KOR activation have been attributed to GRK3/arrestin-dependent activation of the p38α mitogen-activated protein kinase (p38 MAPK) in dopamine neurons (Bruchas *et al*, 2007; Ehrich *et al*, 2015). We tested whether KOR-mediated cognitive disruptions had similar molecular requirements to KOR-mediated aversion by measuring KOR effects on DRL performance in mice with conditional knockout of p38 MAPK from dopaminergic neurons ($p38\alpha CKO^{DAT}$). Male $p38\alpha CKO^{DAT}$ ($n=4$) and $p38\alpha^{lox/+}$ littermates ($n=5$) showed a main effect of Treatment in percent error ($F(1,7)=28.5$, $p=0.001$) but no effect of Genotype and no interaction between Genotype and Treatment (Figure 5a). Before p38 activation, GRK3 phosphorylates KOR and promotes arrestin binding to KOR to initiate MAPK signaling (Bruchas *et al*, 2006). To test whether the KOR-mediated DRL disruptions were arrestin dependent, we used male GRK3 knockout mice ($n=14$) and wild-type littermates ($n=9$). There was a significant effect of Treatment ($F(1,21)=45.2$, $p<0.001$), but no effect of Genotype and no interaction between Genotype and Treatment. We then tested whether a G-biased KOR agonist, nalfurafine (Schattauer *et al*, 2017), could produce deficits that were comparable to the unbiased KOR agonist U50488. Nalfurafine (50 µg/kg) pretreatment produced a significant increase in percent error ($t_{12}=9.125$, $p<0.0001$). Analyses of nonreinforced responses and burst responses for these experiments are shown in Supplementary Figure 5. Together, these results demonstrate that KOR-mediated disruptions of DRL performance are GRK3/arrestin independent.

DISCUSSION

The present study specifies the cellular and molecular pathways underlying KOR-mediated increases in compulsive responses. First, we found that systemic KOR activation disrupted inhibitory control and decreased response efficiency in the DRL task by increasing nonreinforced responses and burst responses in both male and female mice. Systemic KOR antagonism blocked disruptions of DRL performance caused by stress-induced release of dynorphin in male mice. The burst responses induced by pharmacological KOR activation could not be attributed to a simple disruption of operant responding, as KOR activation did not produce burst responding in an FR1 or FR5 task. Instead, U50488 administration promoted marble burying, suggesting that KOR activation increased compulsive behaviors in anxiogenic or uncertain environments. Second, we demonstrated that KOR-mediated disruptions in DRL performance were due to KOR activity in the VTA and KOR activation on dopamine neurons. Third, although arrestin-dependent signaling in dopaminergic neurons is required for KOR-mediated aversion (Ehrich *et al*, 2015), KOR-mediated cognitive disruptions can be generated via arrestin-independent intracellular signaling pathways. Together, these findings reveal a relationship between KOR activation and inhibitory control of behavior that may underlie interactions between stress and compulsivity.

Although KOR actions can have sex-dependent effects (Russell *et al*, 2014), we found that KOR agonism disrupted DRL performance in both male and female mice. Sex differences have been observed in stress circuitry (Goldstein *et al*, 2010) and impulsivity (Mitchell and Potenza, 2015), suggesting that there could be sex-specific effects of KOR

activation on DRL performance. One issue in comparing male and female mouse behavior in the DRL task is that female rats acquire DRL more efficiently than male rats, and we observed the same sex difference in mice. This effect on DRL performance has been suggested to result from an effect of ovarian steroids (Beatty, 1973) or baseline differences in locomotor activity in males and females (van Hest *et al*, 1987). To account for baseline differences in DRL acquisition in the present study, female mice were trained on a DRL 25 s protocol, rather than a DRL 15 s protocol. Despite the differences in DRL procedure, KOR activation induced qualitatively similar deficits in males and females in the DRL task, demonstrating that the cognition disrupting effects of KOR activation are likely to be sex independent.



Performance in the DRL task can be disrupted by several different factors, including dysregulation of temporal discrimination, general locomotor alteration, and the loss of inhibitory control (Kramer and Rilling, 1970). Alterations in temporal discrimination would shift the peak of observed IRTs (Cho and Jeantet, 2010), but in these experiments, there was no broad shift in IRTs observed following KOR activation. Instead, there were increases in responses occurring within the 0–3 s IRT bin, indicating deficits in inhibitory control (Selleck *et al*, 2015). We assessed how inhibitory control may be affected in the DRL task by analyzing burst responses (IRTs <1 s) and found that burst responses increased following U50488 administration. If burst responses increased in operant tasks where reinforcement probability is consistently predictable (eg, an FR1 schedule of reinforcement), it would suggest that KOR activation produced a broad increase in compulsive responding. However, we observed no increased burst responses and no differences in total responses in an FR1 or FR5 task following KOR activation. Many studies have reported hypolocomotion induced by KOR activation (Paris *et al*, 2011), suggesting that the KOR-mediated increase in burst responses is unlikely to reflect a simple motoric effect. We found that compulsive responses could be observed following KOR activation in the marble burying task, and corroborated Rose *et al* (2016), who demonstrated that KOR antagonism decreased drug-induced marble burying. Marble burying may reflect a perseverative or compulsive response that is increased in anxiogenic settings, but is not consistently correlated with other anxiety behaviors (Thomas *et al*, 2009). Compulsive responses have been hypothesized to arise as a reaction to environmental uncertainty to cope with ambiguous or threatening stimuli (Holaway *et al*, 2006). Novel objects in the marble burying task may provoke compulsive behavior, or uncertainty in the DRL task about the relationship between responses and outcomes following a nonreinforced response could produce compulsive responding. In contrast, during the FR tasks, there is a stable relationship between responses and outcomes, leading to a lack of burst responses following KOR activation. In addition to evidence suggesting that KOR activation can enhance dopamine D2 receptor-mediated compulsive responses (Perreault *et al*, 2007), our data indicate that KOR activity may have potent effects on compulsive behaviors via dopaminergic circuits.

Dopamine neurons in the VTA encode discrepancies between expected and actual outcomes (Schultz *et al*, 1997), as well as convey information about whether outcomes are better or worse than expected (Hart *et al*, 2014). Alterations in reinforcement probability can modify dopamine neuron responsivity to aversive events (Matsumoto *et al*, 2016). KOR activation on dopamine neurons in the VTA can produce aversion (Ehrich *et al*, 2015), and potentiate cocaine reward (Ehrich *et al*, 2014). We found that KOR activation in the VTA but not the DRN was necessary for KOR-mediated DRL disruptions. Mice with KOR blockade in the PFC increased percent error as a result of nonsignificant decreases in reinforced responses and increases in nonreinforced responses, rather than increases in burst responses. Local KOR activation in the PFC or in the VTA can decrease dopamine release in the PFC (Margolis *et al*, 2006; Tejeda *et al*, 2013) and dopamine depletion in the PFC has been shown to disrupt DRL performance by increasing burst

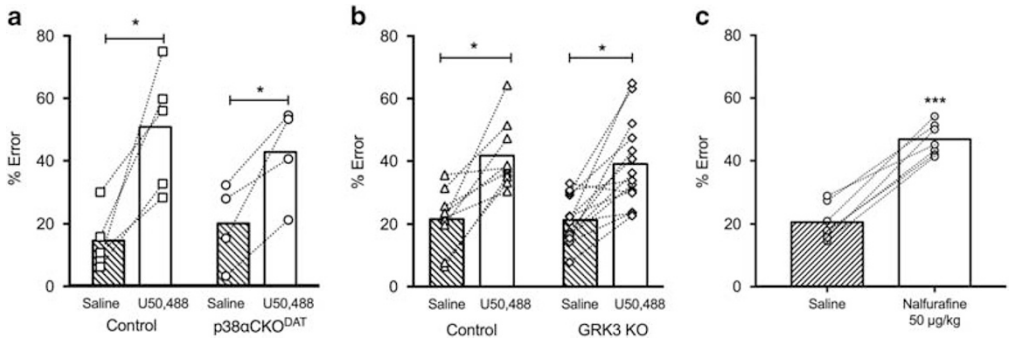


Figure 5 KOR-mediated DRL disruptions are arrestin independent. (a) Conditional deletion of the p38α MAPK in dopamine neurons does not prevent KOR-mediated increases in percent error in the DRL task. (b) Global deletion of the GRK3 does not prevent KOR-mediated increases in percent error in the DRL task. (c) The G-biased KOR agonist nalfurafine significantly increases percent error in the DRL task, indicating that DRL disruptions are arrestin independent. Error bars indicate SEM. $^{*}P<0.05$, $^{***}p<0.0001$.

Figure 4 KORs in the VTA and in dopamine neurons are required for KOR-mediated DRL disruptions. (a) Schematic shows microinjection sites for nor-BNI, a long-lasting KOR antagonist. Mice received nor-BNI pretreatment 5 days before DRL test sessions. (b) There was a significant increase in the number of nonreinforced responses in Control and DRN/nor-BNI, but not VTA nor-BNI or PFC/nor-BNI, following U50488 treatment. (c) U50488 caused a significant increase in percent error in Control, DRN/nor-BNI, and PFC/nor-BNI mice, but not VTA/nor-BNI-injected mice. (d) There was a significant effect of U50488 treatment on the number of burst responses, but no significant interaction between treatment and brain region. (e) Schematic shows breeding strategy for generating conditional knockout mice. Dashed boxes indicate parental mice. Parental mice were heterozygous for Cre-recombinase within serotonergic (ePet1) or dopaminergic (DAT) neurons and were heterozygous for a null KOR allele. Cre-recombinase-negative mice with a floxed KOR gene were bred with heterozygous Cre-recombinase mice to produce conditional knockout mice with KOR specifically deleted from serotonergic or dopaminergic neurons or littermate controls. (f) U50488 treatment significantly increased the number of nonreinforced responses but there was no significant interaction between treatment and genotype. KOR activation significantly increased percent error (g) and burst responses (h) in Control and KOR CKO^{PET} mice, but not KOR CKO^{DAT} or KOR KO mice. Error bars indicate SEM. $^{*}P<0.05$; $^{**}p<0.01$, $^{***}p<0.0001$.

responding (Sokolowski and Salamone, 1994). KOR blockade on dopamine neurons in the PFC may block burst responses, but this may not be sufficient to overcome the percent error increasing effects of KOR activation in the VTA. It is also possible that KOR activation in the VTA could dysregulate the dopamine signals received in the corticostriatal network to disrupt normal feedback mechanisms that control behavioral inhibition (Jentsch and Taylor, 1999).

Cre-driven excision of KOR can simultaneously remove KOR activity from somatic and terminal regions of particular neuronal populations. We compared the effect of conditional knockout of KOR from dopaminergic or serotonergic neurons against littermate controls and mice with global deletion of KOR. KOR activation in dopaminergic, but not serotonergic neurons, was necessary for disruptions in DRL performance. KOR activation on serotonergic neurons is important for affective processing (Bruchas *et al*, 2011) and cocaine reward potentiation (Schindler *et al*, 2012), but may not contribute to this particular feature of cognition. Many serotonergic antidepressant drugs that are effective in decreasing compulsive behaviors (Kellner, 2010) are also effective in improving DRL performance (O'Donnell *et al*, 2005), and hence it is possible that chronic KOR activation could lead to serotonin-dependent disruptions in DRL performance. Conditional deletion of KOR from dopaminergic neurons prevented increases in burst responding, demonstrating that KOR activation on dopaminergic neurons may be important for generating responses to reward omissions. One challenge with a conditional deletion of KOR from dopamine neurons is that dopamine neurons in the substantia nigra (SN) also contain KORs (Tempel and Zukin, 1987). Although SN KORs do not contribute to aversion (Bals-Kubik *et al*, 1993), KORs in the SN could produce the observed cognitive disruptions or compulsive behaviors. However, in combination with the microinjection experiments targeting the VTA, we show that KOR activation on dopamine neurons of the VTA disrupts inhibitory control.

Ehrich *et al* (2015) demonstrated that KOR-mediated aversion requires GRK3/arrestin-dependent p38α MAPK activation in VTA dopamine neurons. However, we found no effect of global GRK3 deletion or p38α MAPK deletion from dopamine neurons on KOR-mediated DRL disruptions. These findings show that KOR effects on cognition are distinct from KOR-mediated effects on aversion and likely to be arrestin independent. We also found that nalfurafine, a G-biased KOR agonist (Schattauer *et al*, 2017), could produce the observed DRL disruptions. An important implication of this finding is that a highly G-biased KOR agonist used in the treatment of pain or itch without producing dysphoria might still be complicated by unwanted cognitive side effects at high doses (Chavkin, 2011).

In summary, our studies demonstrate that KOR activation in dopamine neurons in the VTA disrupts inhibitory control of behavior. Dopamine neurons have been shown to modulate compulsive behaviors in mice (Pascoli *et al*, 2015), and our results show that KOR activation in dopamine neurons may increase compulsive behaviors when reinforcement probability is ambiguous. In contrast, when reinforcement probability was well predicted, KOR activation had no effect or promoted inhibition of behavior. Together, these studies suggest that KOR activation disrupts behavioral inhibition in a reward context-dependent manner. Our results demonstrate that KOR antagonism during periods of chronic stress could decrease cognitive disruptions and may be beneficial for treating stress-mediated increases in compulsive responses. In agreement with our preclinical findings, there are some case studies reporting that opioid antagonists, such as naltrexone, can decrease compulsive behaviors (Kim, 1998) and buprenorphine, a μ-opioid receptor partial agonist and KOR antagonist, can decrease treatment resistant compulsive behaviors (Liddell *et al*, 2013). Future studies could identify the molecular mechanisms underlying KOR-mediated increases in compulsive behaviors to generate novel therapeutic interventions for stress-induced cognitive disruptions.

FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank Dr Scott B Evans for help with data acquisition and analysis. We also thank Salina D Johnson and Daniel I Messinger for genotyping and animal care. This work was supported by NIH grants T32DA07278 (to ADA), T32GM007750 (to HMF), RO1DA030074 (to CC), P50MH106428 (to CC), and the NARSAD Young Investigator Award (to BBL).

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)