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Optogenetic stimulation of dynorphinergic neurons within the dorsal raphe activate kappa opioid receptors in the ventral tegmental area and ablation of dorsal raphe prodynorphin or kappa receptors in dopamine neurons blocks stress potentiation of cocaine reward

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Abstract

Behavioral stress exposure increases the risk of drug-taking in individuals with substance use disorders by mechanisms involving the dynorphins, which are the endogenous neuropeptides for the kappa opioid receptor (KOR). KOR agonists have been shown to encode dysphoria, aversion, and changes in reward valuation, and kappa opioid antagonists are in clinical development for treating substance use disorders. In this study, we confirmed that KORs were expressed in dopaminergic neurons in the ventral tegmental area (VTA) of male C57BL6/J mice. Genetic ablation of KORs from dopamine neurons blocked the potentiating effects of repeated forced swim stress on cocaine conditioned place preference (CPP). KOR activation inhibited dopamine neuron GCaMP6m calcium activity in VTA during swim stress and caused a rebound enhancement during the period after stress exposure. Transient optogenetic inhibition of VTA dopamine neurons with AAV5-DIO-SwiChR was acutely aversive in a real time place preference assay and blunted cocaine CPP when inhibition was administered concurrently with cocaine conditioning. However, when inhibition preceded cocaine conditioning by 30 min, cocaine CPP was enhanced. Retrograde tracing with CAV2-DIO-ZsGreen identified a population of prodynorphin^{Cre} neurons in the dorsal raphe nucleus (DRN) projecting to the VTA. Optogenetic stimulation of dynorphinergic neurons within the DRN by Channelrhodopsin2 activated KOR in VTA and ablation of prodynorphin blocked stress potentiation of cocaine CPP. Together, these studies demonstrate the presence of a dynorphin/KOR midbrain circuit that projects from the DRN to VTA and is involved in altering the dynamic response of dopamine neuron activity to enhance drug reward learning.

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Declaration of Competing Interest

None.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.addicn.2022.100005.

Keywords

Stress; Dopamine; Cocaine; Kappa opioid receptors; Dynorphin; Dorsal raphe nucleus; Ventral tegmental area

1. Introduction

People with psychiatric illnesses are vulnerable to stress-induced disruptions in behavior [1]. Stress alters reward seeking behaviors and increases the likelihood of relapsing drug-taking behaviors in individuals with substance use disorder [2]. Alterations in dopaminergic signaling in the brain is a commonly reported feature of substance use disorders [3], and aversive events can both increase and decrease dopamine neuron activity and dopamine release [4–8]. These stress-induced changes in dopamine neuron activity often contribute to relapsing drug-taking behaviors [9,10], suggesting that targeting the specific elements controlling stress reactivity in dopamine neurons would enable better therapeutic interventions for substance use disorders.

Kappa opioid receptor (KOR) antagonists have been in clinical development for decreasing stress-induced relapse of psychostimulants [11–14]. Activation of KORs in dopamine neurons is known to produce aversive effects in mice [15,16] and ablation of KORs from dopamine neurons increases locomotor sensitization to cocaine [17]. Previous studies have shown that there is a complex temporal relationship between KOR activation and cocaine reward. When KOR activation is paired with cocaine administration, the rewarding properties of cocaine are blunted, leading to decreased cocaine conditioned place preference (CPP) and decreases in dopamine release in the nucleus accumbens [18]. KOR activation can also enhance the extinction of cocaine self-administration [19]. However, when stress or KOR agonist administration occurs 30 to 60 min prior to the administration of a drug reward, it can enhance the reinforcing properties of that drug [20–25] and increase stimulated dopamine release [18]. Prior studies have aimed to understand these effects through measuring agonist-mediated changes in electrophysiological activity in the ventral tegmental area [16,26–29] and voltammetric and microdialysis measurements of dopamine release in terminal regions [16,18,30–33]. However, the effect of stress-induced dynorphin release on cell body activity *in vivo* has been challenging to measure. KOR is thought to be present in a significant proportion of dopamine neurons in mice [34,35], and our goal was to determine the cellular and circuit mechanisms that underlie stress-induced potentiation of cocaine reward.

Here using immunohistochemistry, we confirm that KOR colocalizes with a majority of VTA dopamine neurons. We found that stress-induced activation of KORs in dopamine neurons increases preference for cocaine reward, and we determined that stress relief, or removal of an aversive stimulus, increases post-inhibitory rebound of dopamine neuron excitability to drive increased reward seeking behaviors through a KOR-dependent mechanism. We then tested whether optogenetic inhibition of dopamine neurons could recapitulate the effects observed with physiological stress and found that the potentiation effect was present when optical inhibition of dopamine neurons preceded cocaine

conditioning. Lastly, we identified a dynorphinergic projection from the dorsal raphe nucleus (DRN) to the VTA that is involved in these KOR-mediated effects, further demonstrating how coordination between the DRN and VTA guides reward learning during stress.

2. Methods

2.1. Subjects

Adult male C57BL/6 mice ranging from 2–6 months of age were used in these experiments. We have previously reported that estrogen-regulated sex differences in intracellular signaling pathways alter female responses to KOR agonists and antagonists [28,36,37]. In Abraham et al. [36], we found that stress-induced potentiation of cocaine conditioned place preference required a different drug dosing procedure than males, preventing pooling of data across sexes. However, qualitatively both males and females showed KOR-mediated potentiation of cocaine reward. Due to the variability produced by differing responses to KOR activation caused by estrogen, stress, and cocaine between sexes [36], we focused our initial studies of this circuit in male mice. All experimental procedures were approved by the University of Washington Institutional Animal Use and Care Committee and were conducted in accordance with National Institutes of Health (NIH) “Principles of Laboratory Animal Care” (NIH Publication No. 86–23, revised 1985). Mice were group housed (2–5 mice per cage). Food and water were available ad libitum in their home cages. All testing was conducted during the light phase of the 12 h light/dark cycle. Floxed KOR (KOR^{lox}) mice were generated by Dr. Brigitte Kieffer (Institut Clinique de la Souris), in which exon 1 of KOR was flanked by loxP sites [16]. Floxed prodynorphin mice were generated by Dr. Richard Palmiter (University of Washington), in which exon 3 of the prodynorphin gene was flanked by loxP sites [38,39]. DAT^{Cre} mice were bred with floxed KOR mice to generate DAT floxed KOR mice [15–17,40].

2.2. Genotyping

Transgenic mice were genotyped using Transnetyx (Cordova, TN, USA) genotyping services. Prodynorphin^{Cre}, DAT^{Cre} and DAT^{IRES-Cre} mice were genotyped by DNA isolated from tail tissue obtained from weanling mice (21–28 days of age), and PCR screening was performed for the presence of Cre recombinase. For KOR^{lox} mice, the following primers were used for PCR screening: Forward Primer: CACTTTTAAACATGGAGTAGGGTGATG; Reverse Primer: GGCCGCATAACTTCGTATAGCATA; Reporter: CCGGTGCTTCTGTGTATC. For pdyn^{lox} mice, the following primers were used for PCR screening: Forward Primer: AGAGTACGTGGATTGTCTACAGAGA; Reverse Primer: GGAAAGGTTGAGAGCTGAGTAATCA; Reporter: CTGGGATCGGATCCTC.

2.3. Stereotaxic injection

Mice were anesthetized with isoflurane and mounted on a stereotaxic alignment system (Model 1900 David Kopf Instruments, CA, USA). A sterile ophthalmic ointment (Puralube; KS, USA) was applied to the eyes to prevent drying. A 30 gauge needle Neuros Syringe (Hamilton, NV, USA) was lowered unilaterally for viral injection (0.5 μ L; AAV1-DIO-GCaMP6m; Addgene # 100,838) for fiber photometry in the VTA (A/P = –3.28 mm; M/L

= ±0.5 mm; D/V -4.5 mm) of DAT^{Cre} or DAT^{Cre} floxed KOR (DFK) or bilaterally for inhibitory opsin (AAV5-DIO-SwiChR_{CA}-eYFP or AAV5-DIO-eYFP; University of North Carolina viral vector core) experiments in DAT^{IRESCre} mice. For experiments targeting the dorsal raphe nucleus (DRN; A/P = -4.5 mm; M/L = -1.09 mm; D/V -3.19 mm; angled 20 °), 0.5 μL of virus (AAV5-DIO-ChR2-eYFP, Addgene # 20,298; or AAV5-DIO-eYFP, Addgene # 27,056) was injected towards the midline. For pdyn^{lox} mice, 0.5 μL of AAV5-Cre (Addgene # 105,553) or AAV5-eGFP (Addgene # 105,547) was injected towards the midline. In pdyn^{Cre} mice, 0.5 μL of AAV5-DIO-ChR2-eYFP was injected unilaterally into the DRN for optogenetic stimulation experiments or 0.5 μL of CAV2-DIO-ZsGreen (provided by Dr. Larry Zweifel; University of Washington) was injected unilaterally into the VTA for retrograde tracing. A chronically implantable fiberoptic cannula (photometry: 400/430 core, 0.57 NA; optogenetic stimulation: 200/240 core, 0.22 NA; Doric Lenses, Quebec, CA) was placed 0.2 mm dorsal to the viral injection site, then C&B metabond (Parkell Inc., NY, USA) and dental cement (Stoelting, IL, USA) was used to secure the cannula to the skull. After injection, the needle was kept at the injection site for 5 additional minutes before removal. Mice without cannula were sutured with 5-0 polypropylene sutures (SharpPoint, PA, USA). Viral infusions occurred at least 4–6 weeks before behavioral experiments, optical stimulation, or fiber photometry.

2.4. Drugs

Cocaine hydrochloride (15 mg/kg) was provided by the National Institute of Drug Abuse Drug Supply Program (Bethesda, MD) and dissolved in saline to be administered intraperitoneally (IP) in a volume of 10 mL/kg.

2.5. Intracardiac perfusions and antigen retrieval

As described in Abraham et al. [41], mice were anesthetized with pentobarbital (Beuthanasia-D) and intracardially perfused with room temperature phosphate-buffered saline (PBS) and chilled 10% formalin. Thereafter, brains were stored overnight in 10% formalin. For antigen retrieval in KORp-IR experiments, brains were sectioned into 5 mm width sections and placed in a small basket in PBS (85–90° C for three min). The brains were agitated every thirty seconds. Immediately after 3-min incubation, the brains were removed from the warmed PBS and immersed in room temperature PBS. Brains were then put in 20% sucrose at 4° C for storage until sectioning.

2.6. Immunohistochemistry

Fixed sections of the midbrain containing the VTA were sliced at 40 μm, then washed in PBS before being placed in blocking solution (PBS containing 5% normal goat serum and 0.3% Triton X-100). VTA slices were incubated in an rabbit anti-KT2 (KOR tail) antibody (1:50 dilution; generated as previously described [42,43]) and chicken anti-tyrosine hydroxylase (TH; 1:1000 dilution; AB9702; MilliporeSigma, Burlington, MA, USA), rabbit anti-KORp antibody (1:25 dilution), mouse anti-Cre (1:500; MAB3120; MilliporeSigma, Burlington, MA, USA), or chicken anti-GFP (1:3000; ab13970; Abcam, Cambridge, UK) solution diluted in blocking buffer (Detailed protocol in Lemos et al. [44]) for 24 h (KT2, GFP, Cre) or 72 h (KORp) on a shaker in a cold room (° C). KORp peptide used for rabbit immunization was generated as described in [41] by Biomatik (Wilmington, DE,

USA). Slices were then washed in PBS and incubated with an AlexaFluor 488 or 555 goat anti-rabbit (A11008; A32732), 488 goat anti-chicken (A11039), or 488 goat anti-mouse (A28175) secondary antibody (1:500 dilution; ThermoFisher Scientific, Waltham, MA, USA) for 2-h covered on the shaker. After two h, the tissue sections were washed in PBS and mounted on Superfrost Plus slides with Vectashield hardset mounting media and imaged at a Leica SP8X Confocal Microscope. To quantify the total number of KT2 and TH positive cells and changes in KORp-IR (previously described in [41]), we used ImageJ. For KT2 experiments, an investigator blinded to treatment conditions counted all immunoreactive cells in each section in a single plane across the rostrocaudal axis of the lateral and medial ventral tegmental area. For the KORp experiments, an investigator blinded to the treatment conditions randomly sampled 30 cells within the VTA in each slide at a single plane. Cells were circled to generate regions of interest (ROIs) and average fluorescence intensity (pixel intensity) within each ROI was recorded using ImageJ. KORp was stained with AlexaFluor 488 and incoming fluorescent fibers and other fluorescent artifacts were excluded from analyses to focus on changes in cell body fluorescence. The average background fluorescence of a sample was recorded by circling an area with no visible cells and recording pixel intensity. The background fluorescence was subtracted from the averaged cell fluorescence within each slide to account for differences in background across animals.

2.7. Stress potentiation of conditioned place preference

To test cocaine CPP, a balanced three-chamber apparatus was used [21]. Mice were given a pretest on day 1 and then cocaine (15 mg/kg) was paired with the less preferred side during conditioning on days 2 and 3. Saline was paired with the alternative chamber side four hours after the cocaine conditioning session. To induce stress, mice were exposed to a modified forced-swim test as previously described [21,22,36,41]. Briefly, the modified-Porsolt forced-swim paradigm used a 2-day procedure in which mice swam in 30 °C water in a 5 L opaque beaker for 15 min the first day within ten minutes following the pre-conditioning preference test, and four 6-min swims (separated by 6 min each) 10 min before the first cocaine conditioning session on Day 2. During day 4 (posttest), mice were allowed to freely explore the apparatus. Preference score was determined by subtracting time in the drug-paired compartment during posttest from time in the drug-paired compartment during pretest (post-pre).

2.8. Optogenetic stimulation

A small nestlet was placed under the head of the mouse prior to connecting the incoming fiberoptic patchcord to the indwelling fiberoptic cannula. Mice were placed into a novel cage with fresh bedding and allowed to freely explore with patchcord attached. On laser exposure days, mice received 473 nm light source (10 mW incoming laser power; OEM Laser, Midvale, UT) controlled through a waveform generator (Grass Instruments). For optical inhibition, laser was on for a thirty-minute session where mice received a 100 ms pulse of laser light every 3 s. For optical stimulation experiments, mice received 5 s of 20 Hz (10 ms pulse) laser on and 5 s laser off cycled over a 30 min session, based on Al-Hasani et al. (2015) [45] showing optically elicited dynorphin release with 20 Hz stimulation. Mice were perfused within ten minutes of the termination of optical stimulation in the DRN.

2.9. Fiber photometry

We used a real-time signal processor (RZ5P; Tucker-Davis Technologies) connected to Synapse Software (Fiber Photometry) to set frequency of light stimulation and record input from photodetectors. The RZ5P was connected to a light emitting diode (LED) driver (Doric Lenses) that controlled the power of a 465 nm and 560 nm Doric LED. The LED was attached with a low autofluorescence patchcord (400/430) to a Fluorescent MiniCube (Doric Lenses) with dichroic mirrors. Optical patchcords connected the MiniCube with a pigtailed rotary joint (FRJ; Doric Lenses) that allowed free animal movement and Newport (Irvine, CA) visible 2151 Femtowatt Photodetectors connected to the RZ5P for data collection. Prior to photometry sessions, patchcords were bleached with light for at least 4 h to minimize autofluorescence. Power of the LED at the fiber tip was set to 30 μW and was tested prior to the start of each session. Signals were collected at a sampling frequency of 1017 Hz. Each of the sessions were downsampled by a factor of 100 and normalized to a five-minute baseline period in the beginning of the recording. The sessions were then smoothed using a moving average filter (100 s window) to remove high frequency noise and detrended to remove linear drift. The control channel (560 nm) was fitted to the signal (465 nm) channel using a least-squares method and subtracted to remove motion artifacts. Each recording session started with a 5 min baseline recording period prior to behavioral manipulations to calculate fluorescent change from baseline ($\Delta F/F$; change in fluorescence/baseline fluorescence) and each trial period was set to start at zero. Calcium events were defined as having a peak width greater than 1 s and peak height greater than 2.9x standard deviation from the mean, based on Calipari et al. [46].

2.10. Statistical analysis

All data are presented as mean \pm s.e.m. Individual data points are shown when possible. Mice were removed from analyses if no viral expression was found postmortem. We used two-tailed t-tests, one-way and two-way ANOVA (incorporating repeated measures where appropriate), and performed post-hoc tests as specified in text. Photometry data were analyzed through custom-built MATLAB software (MathWorks Inc.; Natick, Massachusetts, USA). Behavioral data were analyzed using Ethovision XT 11.5 (Noldus; VA, USA) and statistical analyses were performed with Prism 9.0 (GraphPad Software; CA, USA).

3. Results

We first assessed the distribution of KORs in the VTA using antibodies targeting either tyrosine hydroxylase (TH), an enzyme involved in the synthesis of dopamine, or an epitope within the C-terminal tail of the KOR “KT2” [42,43]. We determined the number of dopamine neurons (TH⁺) that expressed KOR (Fig. 1A,B), as well as the number of neurons showing only KOR or TH⁺ immunoreactivity. In a total of n = 6764 cells surveyed in n = 3 C57B6/J male mice, we found that a majority (6101) showed immunoreactivity for both KOR and TH. This confirmed previous observations suggesting that KOR is expressed in a majority of VTA dopamine neurons [27,34,47].

Our prior studies [21,25,36] have demonstrated that repeated forced swim stress prior to drug exposure enhances the expression of conditioned drug preference. In addition to

serotonergic contributions to this effect [23,25,48] and based on VTA KOR-dependent aversion reported in Ehrlich et al. [16], we hypothesized that dopaminergic signaling was also likely necessary for potentiation of cocaine reward. We tested the necessity of KOR activation in dopamine neurons for stress potentiation of cocaine reward using a conditioned place preference assay and compared control (DAT^{Cre}) to mice with a conditional deletion of KORs in dopamine neurons (Fig. 1C, D). There was a significant interaction between repeated forced swim stress (rFSS) exposure and genotype ($F(1,41) = 4.104, p = 0.0493$). In control (DAT^{Cre}) mice, there was a significant increase in preference score in mice receiving swim stress ($n = 13; p = 0.008$) compared to those that did not receive swim stress ($n = 12$). However, when KORs were deleted from dopamine neurons (by crossing DAT^{Cre} with floxed KOR mice; DFK), rFSS ($n = 11$) did not significantly increase expression of cocaine preference compared to no rFSS mice ($n = 9$). This demonstrated that KOR activation in dopamine neurons is required for stress potentiation of reward. We were then interested in characterizing the calcium dynamics of dopamine neurons during and after stress that may contribute to these changes in behavior.

We measured the effect of rFSS and cocaine CPP on calcium activity in dopamine neurons in mice with KOR conditionally deleted from dopamine neurons (DAT^{Cre} floxed KOR; DFK $n = 5$) and intact control mice (DAT^{Cre} $n = 5$ no rFSS, 6 with rFSS; Fig. 2A). AAV1-DIO-GCaMP6m was injected into the VTA of all mice and a fiber was implanted above the injection site to record bulk calcium activity in DAT^{Cre} neurons (Fig. 2B; **Supplement 1A**). There were no significant differences in the magnitude of calcium transients during pre-test (Fig. 2C) or in F/F during the first 15-min swim stress exposure between groups (Fig. 2D). However, KOR-mediated differences in neuronal calcium activity were observed during Day 2 of rFSS exposure (Fig. 2E). During Day 2 swim stress periods, mice with intact KOR showed significant differences in calcium activity during swim stress periods 1,3, and 4 compared to DFK mice (Genotype x Time interaction; Day 2 Swim 1: $F(359,3231) = 1.30, p = 0.0002$; Swim 3: $F(359,3231) = 1.79, p < 0.0001$; Swim 4: $F(359,3231) = 1.96, p < 0.0001$; **Supplement 1B**). There was no significant difference in immobile time during the session between groups (**Supplement 1C**). When mice were removed from the swim stress chamber (post-swim 1–4), there was a significant difference in calcium activity in control (DAT) mice compared to DFK mice during post-swim periods 2, 3, and 4 (Post 2: $F(359,3231) = 1.45, p < 0.0001$; Post 3: $F(359,3231) = 1.66, p < 0.0001$; Post 4: $F(359,3231) = 2.17, p < 0.0001$). We quantified the overall change in fluorescence from the first minute of the session to the last minute of each test session (Fig. 2F) and found that during the fourth swim and post-swim period, there was a significant difference between activity during stress compared to activity in the post-swim period (Genotype x Time interaction: $F(7,63) = 3.19, p = 0.006$; Sidak's post hoc: $p = 0.0036$). This was also reflected by the total number of calcium events (Fig. 2G) that were also significantly increased ($F(7,63) = 2.88, p = 0.011$) compared to the first swim session during relief periods 2 (Sidak's post hoc: $p = 0.047$), 3 ($p < 0.0001$), and 4 ($p = 0.004$) in control mice. We confirmed that there was a significant main effect of conditioning (Fig. 2H) on the number of calcium events in the drug-paired chamber ($F(1,13) = 11.1, p = 0.006$; Sidak's post hoc: DAT rFSS $p = 0.029$). Our calcium measurements suggested that rFSS-induced KOR activation in dopamine neurons produced a period of inhibition followed by increased calcium activity in dopamine neurons. This

post-inhibitory rebound in dopamine neurons may enhance associative learning and promote reward seeking behaviors.

We hypothesized that inhibition of dopamine neurons would be sufficient to mimic the effects of stress on cocaine reward preference (Fig. 3A). We tested this using a modified blue- and red-light responsive chloride channel opsin (Step-waveform inhibitory ChannelRhodopsin2; SwiChR [49]) to directly inhibit dopamine neurons (Fig. 3B). DAT^{Cre} Mice were injected with AAV5-DIO-eYFP or AAV5-DIO-SwiChR in the VTA (Fig. 3C). We first confirmed that SwiChR inhibition of dopamine neurons could produce aversion, as observed with other inhibitory opsins [7,50,51]. Mice (n = 4 eYFP; n = 4 SwiChR) received dopamine neuron inhibition (100 ms pulse every 3 s) when crossing into their preferred chamber, and inhibition was terminated with a 100 ms pulse of red light when mice crossed over to the other chamber side (Fig. 3D; **Supplement 1D**). SwiChR inhibition produced significant real-time aversion to the light-paired chamber compared to control mice injected with AAV5-DIO-eYFP (Group X Time interaction: $F(2,12) = 4.07$, $p = 0.045$; Sidak's post hoc: Laser Day 1 $p = 0.039$, Laser Day 2 $p = 0.005$). We then tested how dopamine neuron inhibition would alter cocaine CPP. Mice (n = 6 eYFP; n = 6 SwiChR) received laser stimulation (100 ms pulse every 3 s) during cocaine conditioning sessions (Fig. 3E). Mice that were treated with eYFP and cocaine showed a significantly higher preference than mice treated with SwiChR and cocaine concurrently ($t_{10} = 2.684$, $p = 0.023$). Together with our real time preference data, this demonstrated that SwiChR inhibition of dopamine neurons is aversive and blocks cocaine CPP.

The temporal relationship between aversive and rewarding events is critical for stress potentiation of reward [18,20,21,22]. We tested whether pre-treatment with SwiChR inhibition could recapitulate the effects of stress on cocaine conditioned place preference potentiation (Fig. 3F). We found that mice (n = 7) that received a 30-min session of SwiChR inhibition 30-min prior to cocaine conditioning showed a significant increase in cocaine CPP compared to eYFP treated mice (n = 8; $t_{13} = 2.25$, $p = 0.042$). This demonstrated that prior inhibition of dopamine neurons was sufficient to enhance cocaine reward learning and suggests that the inhibitory actions of KOR activation could initiate signaling mechanisms that result in stress priming of dopamine neuron activity.

To determine the neural circuits involved in controlling stress-mediated enhancements in drug reward, we identified sources of dynorphin into the VTA. Prodynorphin-Cre (Pdyn^{Cre}) mice (n = 3) were injected in the VTA with a retrograde canine adenovirus (CAV2) which produced Cre-dependent expression of a green fluorescent protein (CAV2-DIO-ZsGreen) [39] to label dynorphin inputs to the VTA (Fig. 4A). Following six weeks of viral expression, we observed significant labeling of prodynorphin^{Cre} expressing neurons largely localized in the medial DRN (Fig. 4B) that project into the VTA, as previously reported in Fellinger et al. [39]. We then tested the contribution of dynorphin release from this neuron population to stress-mediated alterations in reward behaviors.

We first confirmed that DRN neurons release dynorphin into the VTA area by optogenetically stimulating DRN dynorphin neurons and measuring KOR activation in the VTA (Fig. 4C) with a phospho-selective antibody (KORp; Fig. 4D). We also measured

the effect of endogenous dynorphin release evoked by rFSS on KORp immunoreactivity in the VTA. There was a significant effect of treatment ($F(3,15) = 25.8, p < 0.0001$). Phosphorylation of KOR in the VTA was significantly increased (Fig. 4E) after DRN optogenetic stimulation (473 nm; 10 mW; 20 Hz; 5 s on/5 s off) with Channelrhodopsin2 (ChR2; $n = 4; p = 0.0001$) compared to an eYFP control group ($n = 4$). Swim stress-elicited dynorphin release increased KOR phosphorylation ($n = 6$ rFSS; $n = 5$ no rFSS) in the VTA compared to a no rFSS group (Sidak's post hoc $p < 0.0001$). Together, these experiments demonstrate that dynorphin-containing neurons in the DRN are functionally connected to VTA neurons to activate KORs.

We then tested the effect of virally-mediated deletion of prodynorphin from the DRN on stress potentiation of cocaine reward. Floxed prodynorphin mice ($pdyn^{lox/lox}$) were injected with AAV5-Cre into the DRN ($DRN^{pdyn^{-/-}}$) and given >4 weeks for viral expression to proceed (Fig. 4F). Control mice received an injection of AAV5-eGFP into the DRN. Mice then underwent cocaine CPP training with rFSS ($n = 10$ control, $n = 8$ $DRN^{pdyn^{-/-}}$) or without rFSS ($n = 11$ control, $n = 8$ $DRN^{pdyn^{-/-}}$) prior to the first cocaine conditioning session (Fig. 4G). There was a significant interaction ($F(1, 33) = 4.63, p = 0.039$) between treatment and genotype (Fig. 4H). Control mice showed a significant (Sidak's post hoc: $p = 0.0379$) stress-mediated increase in cocaine CPP with rFSS compared to no rFSS. However, deletion of dynorphin from the DRN blocked stress-induced increases in cocaine reward.

4. Discussion

Our results show that dynorphin neurons in the dorsal raphe neuron project into the ventral tegmental area and activate KORs in the VTA, which are required for stress-mediated enhancements in cocaine reward learning. Further, we observed that inhibition of dopamine neurons by stress produces post-inhibitory rebound periods which likely promotes associative learning. These effects contribute to motivating behavior to escape stressful situations and to seek rewarding stimuli to mitigate the aversive effects of stress.

It has been previously demonstrated that stress can alter functioning of both serotonergic [25] and dopaminergic neurons [16] through KOR activation, and our data indicate that dynorphin neurons in the DRN may be a critical node for coordinating the effect of stress between serotonergic and dopaminergic neurons. Dynorphin released from neurons in the DRN is likely to have effects on local KORs within the DRN that have been characterized on SERT neurons [52] and our data extend this observation by showing that DRN dynorphin neurons can also project to the VTA and are functionally connected to the VTA as observed with KORp measurements. This projection is also known to produce effects on fear generalization [39] indicating that there may be aversive and reward learning elements that are altered by activity in this system. The contribution of other dynorphin inputs to the VTA to stress-induced changes in learning is not well characterized. Although striatal dynorphin neurons have some projections to the VTA, stimulation of these neurons did not produce KOR mediated changes in the VTA [53]. Similarly, although dynorphin neurons in the bed nucleus stria terminalis project to the VTA, they did not affect discrimination learning [39]. Lateral hypothalamic sources of dynorphin input to the VTA has been shown to alter cocaine reward, and the co-release of orexin and dynorphin from these neurons

likely contributes to these effects [54–56]. Interactions between the DRN and VTA have been characterized for glutamatergic and serotonergic populations [57,58], but our data suggest a subset of these populations that may be distinct from non-dynorphinergic DRN inputs to the VTA. Single cell transcriptomic data from the DRN indicate that dynorphin is primarily expressed in serotonergic neurons [59,60], but the interactions between serotonin and dynorphin release in the VTA remains unknown. Glutamatergic neurons (VGLUT3) also project to the VTA from the DRN [57], but SwiChR inhibition of these neurons blunted cocaine conditioned place preference [25], suggesting that transient inhibition of populations involved in positive valence is not sufficient to generate potentiation like effects. Instead, our data suggest multiple points of integration between DRN and VTA that are reflected by changes in downstream structures, including the striatum [16,25] and cortex [33,41]. Understanding the interactions between serotonergic and dopaminergic systems during stress and following stressful periods may help to better delineate the actions of KOR on reward learning behaviors.

We aimed to measure the KOR-mediated effects of stress on dopamine neuron activity using bulk calcium recordings. We observed both decreases and increases in calcium activity following repeated stress that were dependent on intact KOR in dopamine neurons. These slow changes in activity may reflect altered tonic activity, although we were not able to directly test this hypothesis. Dopamine neuron activity can increase in response to aversive events [4,6] and after the removal of an aversive stimulus [61,62]. Inhibition of dopamine neurons can lead to rebounding actions in a subset of dopamine neurons [63]. This pattern of responding may be important for shaping behaviors during and after stress, such as enabling both pauses in behavior during stress and motivating escape or avoidance behaviors after the termination of a stress. Circuit mechanisms of this type may also be important for encoding the emotional context in which learning occurs and enable better learning of cues that predict stress. Repeated stress may lead to a decreased ability to recover from inhibition and eventually lead to anhedonialike adaptations observed in dopamine neurons following KOR activation [64]. It is unknown whether a system that is not as tonically active as dopamine neurons would be expected to show similar effects following KOR activation, but our initial evidence in glutamatergic and serotonergic neurons [25] suggests that the effect is likely specific to a few systems. We have previously reported that optical inhibition of serotonin neurons with SwiChR can potentiate cocaine reward. It is currently unknown whether SwiChR or other inhibitory chloride channel opsins potentiate activity of dopamine or serotonin neurons following inhibition. SwiChR inhibition has not been reported to produce rebounding activity after termination of light delivery in the cortex [65] although this effect may depend on the particular population measured. Direct effects of SwiChR inhibition on behavior also decay within minutes of light delivery [66]. Although we did not directly measure electrophysiological changes thirty minutes after inhibition, prior research suggests that dopamine neurons could enter a primed state after inhibition or stress [67]. Our results suggest important considerations for the time course of inhibitory opsin activity when measuring behaviors.

Interactions between KOR and dopamine neuron activity is complex due to the distribution of KOR in the VTA and inputs and outputs of the VTA. We observed extensive colocalization of KOR with tyrosine hydroxylase, however different approaches suggest

less widespread distribution [68] or different patterns of KOR expression [69,70]. Although the detected KOR presence in dopamine neurons may be lower than observed with immunohistochemistry because of assay differences, we find that KOR activation within the VTA has a potent influence on dopamine neuron activity and behavior. In addition to dopamine neurons, KOR can modulate the activity of GABAergic neurons [71] and glutamatergic inputs to the VTA [72]. Dynorphin released in the VTA could have extensive effects on the function of these independent populations and may also produce self-regulatory or autocrine effects on dynorphin release. For example, KOR and dynorphin expression may overlap within the DRN to regulate dynorphin neuron projection activity to the VTA. Dynorphin release within the striatum also regulates serotonergic [23] and dopaminergic terminals [16] and distinct cellular mechanisms are likely involved in potentiating or suppressing release of neurotransmitters at these sites after KOR activation. This suggests a complex system of dynorphin/KOR interactions acting to alter dopamine and serotonin signaling throughout the brain that could contribute to altering reward behaviors.

Our findings suggest that KOR antagonists could promote stress resilience by decreasing the dynamic range of dopamine neuron activity during stress. KOR activation in both serotonergic and dopaminergic systems is known to contribute to aversion and stress-mediated changes in cocaine reward, suggesting that dynorphin neurons in the DRN that project to the VTA may be a critical population that coordinates activity between these systems during stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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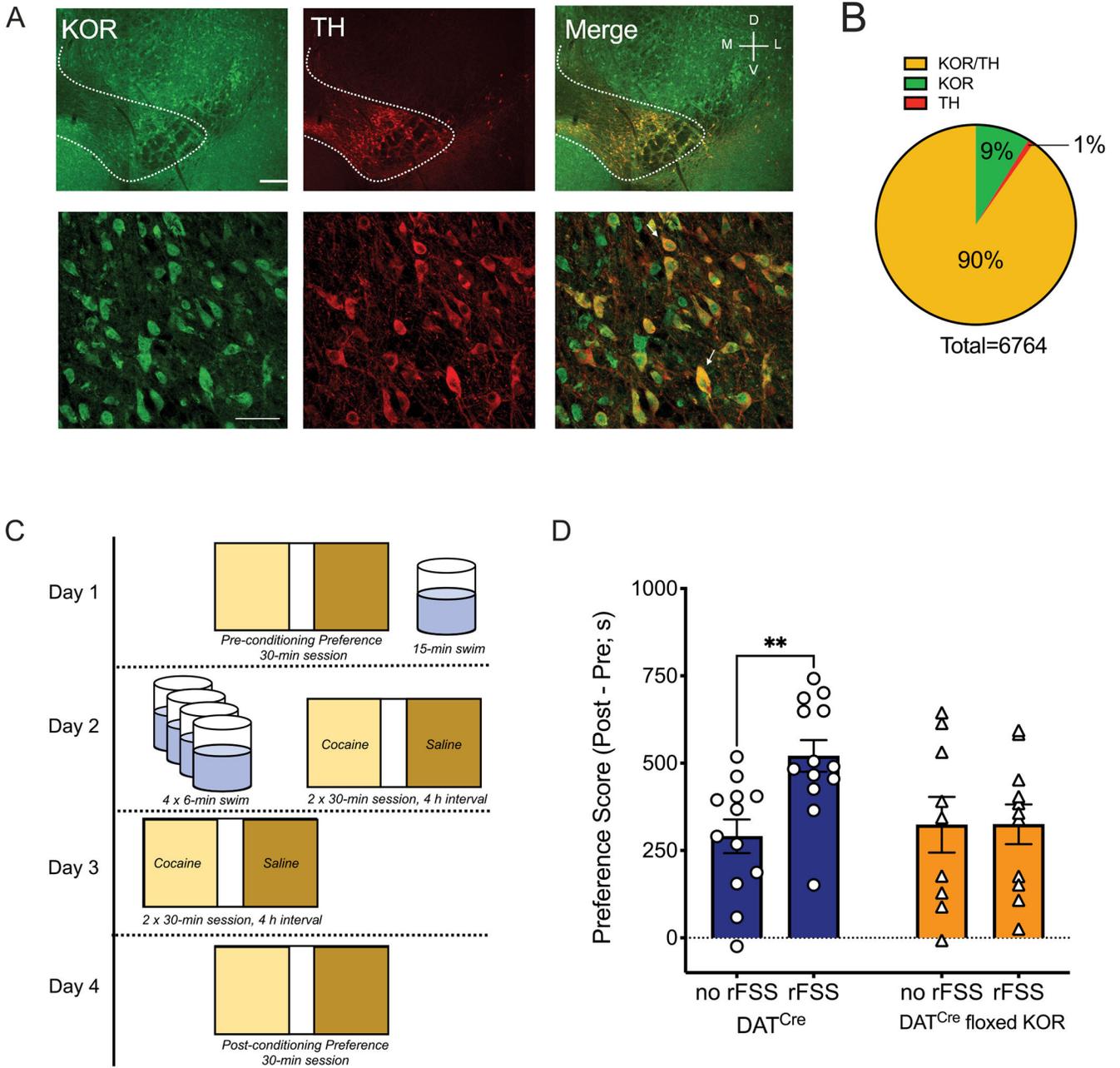


Fig. 1.
A. Representative images for KOR and tyrosine hydroxylase (TH) immunoreactive neuron overlap in the VTA of C57BL6/J male mice. Dashed line indicates VTA. Upper left panel shows image of the VTA with immunoreactivity for KOR in green (scale bar: 250 μ m). Lower left panel shows image of region with KOR+ neurons (scale bar: 100 μ m). Upper and lower center panels show images of TH immunoreactivity. Upper and lower right panels show the overlap between TH and KOR staining (merge). White arrows in lower right panel indicate overlapping TH and KOR staining. **B.** Total percentage of neurons with overlapping expression of KOR and TH, KOR alone, or TH alone is shown. **C.** Schematic

for stress potentiation of cocaine CPP. On Day 1, control (DAT^{Cre}) mice or conditional knockout mice (DAT^{Cre} crossed with floxed KOR mice; DFK) received a 30-min test for chamber preference (Pre-conditioning Preference). Within ten minutes of the end of the initial preference test, mice received a 15-min forced swim stress exposure (Day 1 of rFSS) or brief handling (no rFSS). The following day, mice received four six-min swim stress exposures with a six min interval between each stress period (Day 2 rFSS). Within ten min following the last stress exposure, mice underwent a 30-min cocaine conditioning session. Four h later, mice received a 30-min saline conditioning session in the alternative chamber. On Day 3, mice received 30-min cocaine and saline conditioning sessions separated by 4 h. On Day 4, mice were again tested for chamber preference. **D.** Preference score is presented as the average of the time in the cocaine-paired chamber prior to conditioning subtracted from time in the cocaine-paired chamber following conditioning. Stress potentiates cocaine CPP in control mice, but not in mice with genetic ablation of KORs from DAT^{Cre} neurons. Error bars indicate S.E.M. ** $p < 0.01$ (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

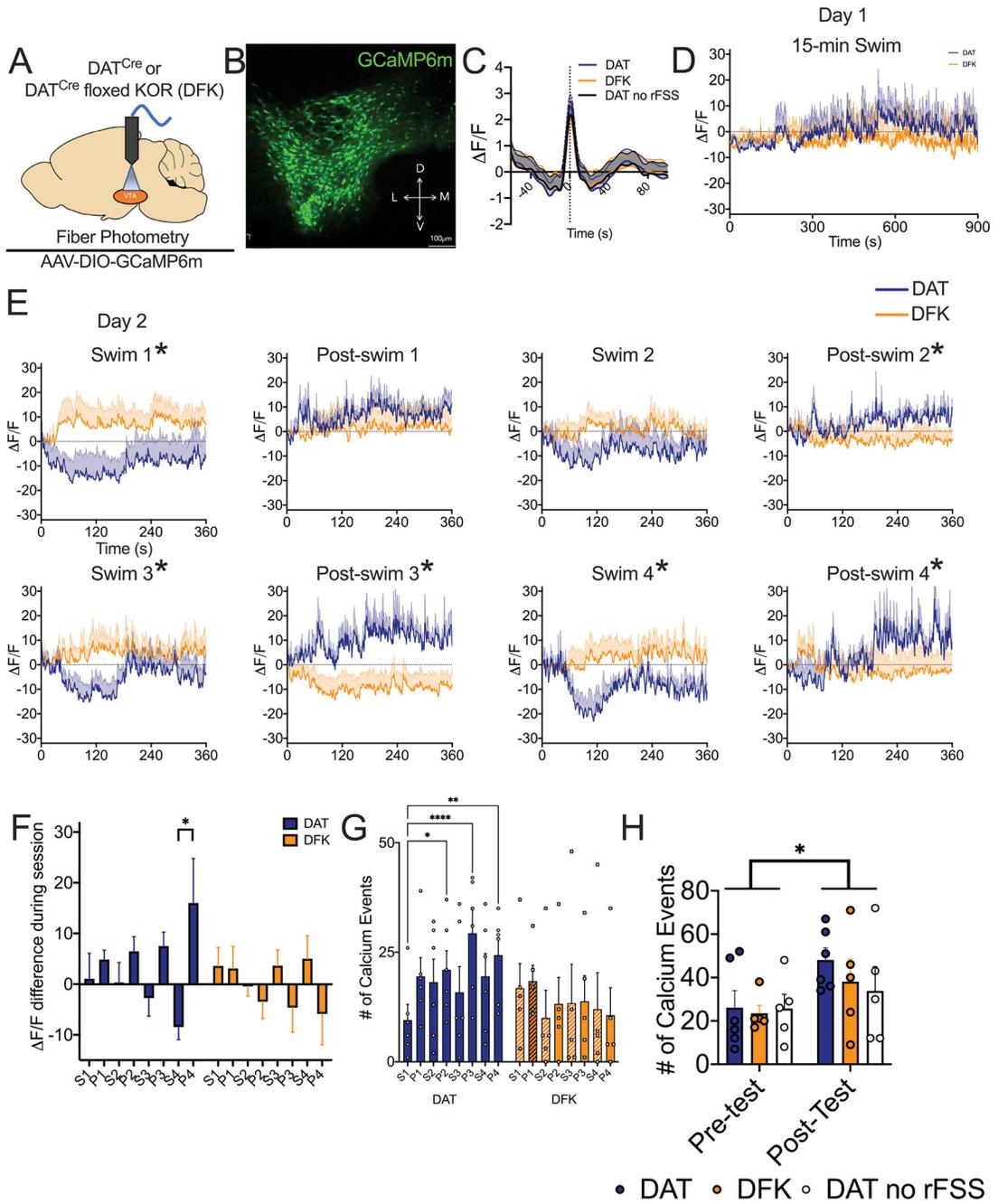


Fig. 2.
A. Schematic for experimental protocol. Mice with intact KOR (DAT; DAT KOR^{+/+}) or with KOR genetically ablated from dopamine neurons (DFK; DAT KOR^{-/-}) were injected in the VTA with AAV1-DIO-GCaMP6m. **B.** Expression of GCaMP6m in VTA is shown. **C.** Average of calcium transients ($\Delta F/F$) during pre-conditioning preference test is shown, demonstrating that there was no significant baseline difference in the shape or magnitude of calcium transients between groups. **D.** There was no significant difference between DAT or DFK mice during the 15-min swim period on the first day of conditioning. **E.** Panel E shows

calcium activity on Day 2 during each 6-min swim period and in the 6 min period following removal from the swim chamber. Swims and post-swim periods are shown sequentially starting at the upper left through upper right, then lower left through lower right. There were significant differences in $\Delta F/F$ between DAT and DFK mice during Swims 1, 3, and 4, and Post-swim periods 2, 3, and 4. **F.** Change in fluorescence during each period is shown, based on the difference between the first and last minute of each swim period. Swim is shown as S1–4 and post-swim is P1–4. There was a significant difference between Swim 4 and Post-swim 4. **G.** The number of calcium transients during each swim and post-swim period is shown (S1–4, P1–4 as above). There was a significant increase in the number of calcium transients on post-swim 2, 3, and 4 compared to the first swim in the DAT group, but not in the DFK group. **H.** Transient number in drug-paired context during pre-conditioning and post-conditioning test is shown. Conditioning produced an overall significant increase in transient number in both DAT and DFK groups. Error bars or dashed lines indicate S.E.M. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

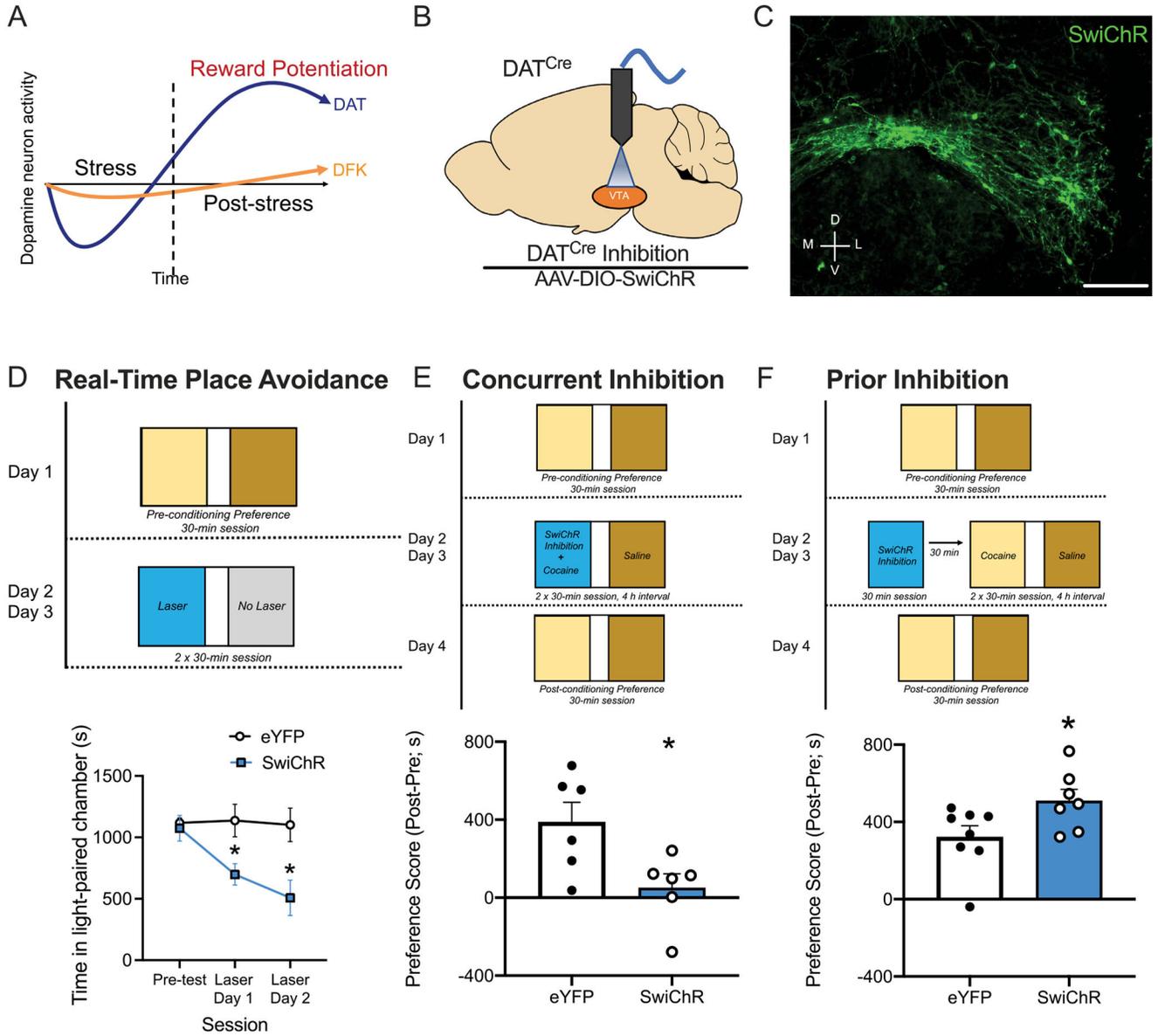


Fig. 3.

A. A conceptual model for stress potentiation of cocaine reward is shown. We observed that stress inhibited dopamine neurons, but this inhibition did not persist. When stress was removed, dopamine neuron calcium activity rebounded above baseline. This effect was not present in mice with KOR conditionally deleted from dopamine neurons (DFK). When drug reward was administered during the period after stress, mice developed a greater preference for the drug-paired chamber. **B.** Schematic for SwiChR inhibition experiments is shown. Mice received a bilateral cannula targeted towards the VTA after injection with AAV-DIO-SwiChR. **C.** Expression of SwiChR in DAT neurons is shown in green. Scale bar shows 100 μm. **D.** Real Time Place Avoidance. Schematic for experiment is shown in upper panel. Mice that received SwiChR inhibition significantly decreased time in light paired chamber (during Laser 1 and Laser 2 sessions) compared to mice injected with eYFP. **E.** Concurrent

inhibition. Upper panel shows schematic. Mice received cocaine conditioning paired with SwiChR inhibition. Lower panel shows that SwiChR inhibition of dopamine neurons during conditioning blunted cocaine CPP. **F. Prior Inhibition.** Upper panel shows schematic. Mice receiving SwiChR inhibition for 30 min, 30 min prior to cocaine conditioning showed a significantly higher preference for the cocaine-paired floor. Error bars indicate S.E.M. $*p < 0.05$ (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

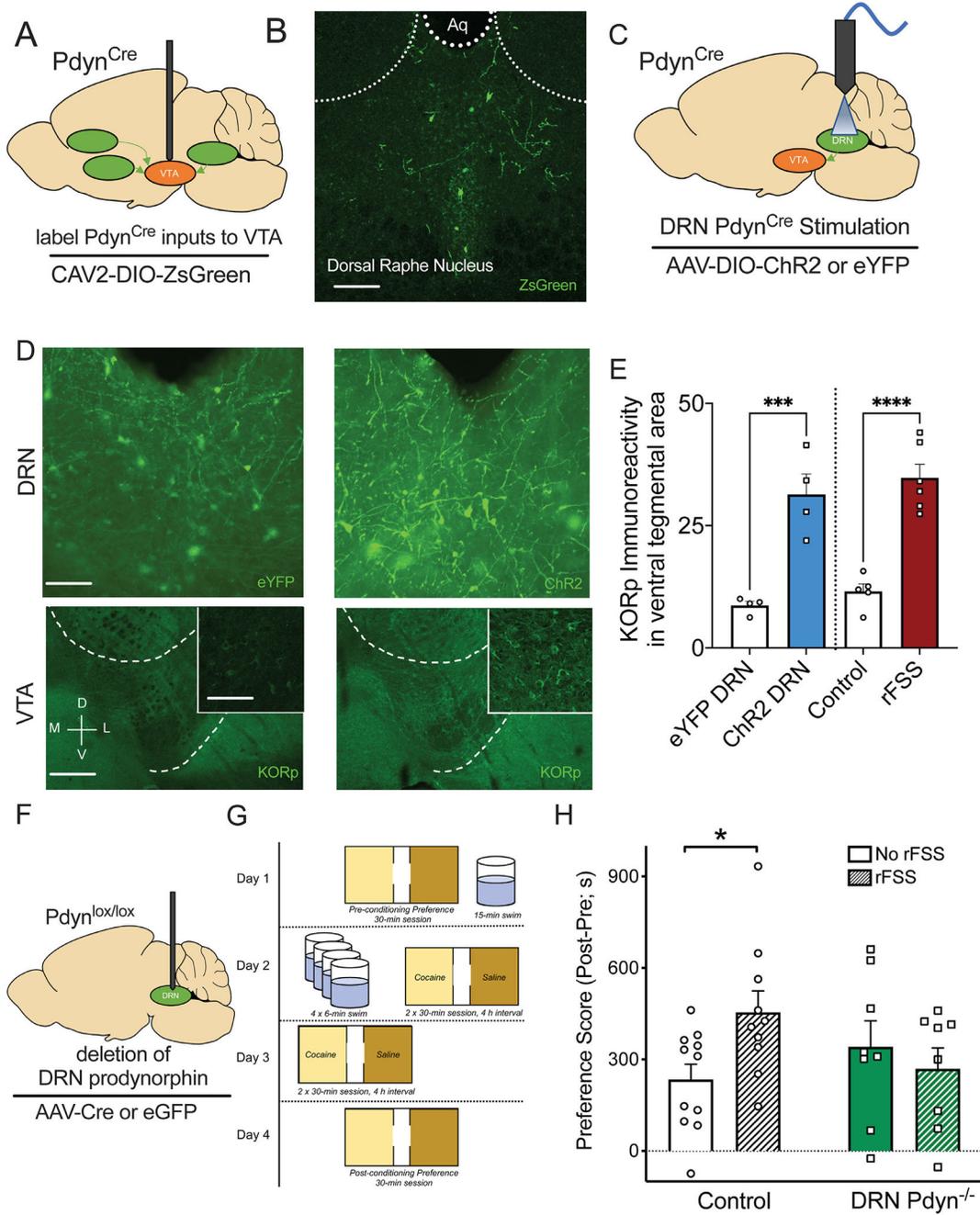


Fig. 4. **A.** Schematic for tracing dynorphin inputs to the VTA is shown. **B.** Representative image of ZsGreen-expressing pdyn^{Cre} DRN neurons. Aqueduct is labeled as Aq, dashed lines indicate DRN. 100 μ m scale bar is shown. **C.** Schematic for optogenetic stimulation of pdyn^{Cre} neurons in the DRN. **D.** Representative images showing dynorphin neurons in the DRN (top row) labeled with eYFP or ChR2 (150 μ m scale bar), and phosphorylated KOR immunofluorescence in the ventral tegmental area (bottom row; dashed lines show VTA) 150 μ m scale bar, 20 μ m scale bar on inset. **E.** Immunohistochemistry

showed that phosphorylation of KOR in the VTA was significantly increased after DRN optogenetic stimulation with ChR2 compared to an eYFP control group. **F.** Schematic for dynorphin^{lox/lox} injection. **G.** Schematic for CPP potentiation procedure. **H.** There was a significant increase in preference for cocaine in control mice, but deletion of prodynorphin from the DRN blocked stress potentiation of cocaine CPP. Error bars indicate S.E.M. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).