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Pain relief produces negative reinforcement through activation of mesolimbic reward–valuation circuitry

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Relief of pain is rewarding. Using a model of experimental postsurgical pain we show that blockade of afferent input from the injury with local anesthetic elicits conditioned place preference, activates ventral tegmental dopaminergic cells, and increases dopamine release in the nucleus accumbens. Importantly, place preference is associated with increased activity in midbrain dopaminergic neurons and blocked by dopamine antagonists injected into the nucleus accumbens. The data directly support the hypothesis that relief of pain produces negative reinforcement through activation of the mesolimbic reward–valuation circuitry.

motivated behavior | incision | in vivo microdialysis | immunohistochemistry | ventral tegmental area

Reinforcement of behaviors that maximize benefit (positive reinforcement) and reduce loss or injury (negative reinforcement) is crucial for survival. Whereas positive reinforcement can be produced by activation of mesolimbic dopaminergic pathways, the neural circuits that underlie negative reinforcement are not well understood. Ongoing pain can be "unmasked" in animals using conditioned place preference (CPP). Thus, in the presence of ongoing pain, pairing manipulations that are not rewarding in the absence of pain, such as peripheral nerve block (PNB) or intrathecal administration of ω-conotoxin or clonidine, with a previously neutral context elicits CPP (1–3). CPP resulting from pain relief is a measure of negative reinforcement.

Human functional imaging studies have shown that offset of an acute noxious stimulus (4, 5) or placebo analgesia (6) activates brain regions that overlap extensively with those implicated in appetitive rewards, in particular the ventral tegmental area (VTA), and its dopaminergic projections to the nucleus accumbens (NAc) (5, 6). Manipulations that disrupt mesolimbic dopamine transmission attenuate food or drug reward-induced CPP (7, 8). Electrophysiological recordings from dopaminergic neurons in the VTA demonstrate phasic neuronal activation by primary food or liquid rewards, by rewarding drugs, and reward-predicting cues (9). Similarly, immunohistochemical studies show increased expression of the immediate early gene cFOS in the VTA in response to rewarding drugs, providing further support for an enhanced neuronal activity (10–13). The NAc can be anatomically and functionally divided into core and shell regions that respectively receive projections from the lateral and medial VTA (14). In vivo microdialysis measurements or fast-scan voltammetry demonstrate that appetitive rewards promote an efflux of dopamine in the NAc (15, 16). It has been suggested that NAc neurons signal reward value and participate in behavioral decision making (17–21).

We hypothesized that relief of ongoing pain would activate the mesolimbic dopamine pathway and that such activation is necessary for negative reinforcement. We tested this hypothesis directly in rats with incisional injury-induced pain (22) subsequently relieved by peripheral nerve block.

Results

Incision of the skin and underlying hind-paw tissue induced time-dependent, observable pain behaviors, including guarding (avoidance of touching the floor with the injured area) and thermal hypersensitivity (decreased response latencies to a noxious thermal stimulus). As demonstrated previously (22), evoked pain hypersensitivity was prominent at 24 h and still present, although diminished, at 96 h postincision. Peripheral nerve block (PNB) with popliteal fossa (PF) lidocaine injection given 24 h postincision resulted in strong preference for the chamber paired with PNB, demonstrating negative reinforcement. In contrast, in sham-operated animals or at 96 h postincision, when evoked hypersensitivity is still present, pairing PNB with the context did not produce CPP (Fig. 1 *A* and *B*). PNB at the site contralateral to the injured hind paw did not result in CPP (Fig. S1), confirming that lidocaine at the dose used for PF injection does not produce systemic effects on pain relief, as demonstrated previously (23). These data suggest that relief of postsurgical, ongoing (i.e., spontaneous) pain is rewarding.

To determine whether the mesolimbic reward circuit is necessary for PNB-induced CPP, we investigated whether CPP would be prevented by inactivation of dopaminergic neurons in the VTA. Microinjection of lidocaine (4%, wt/vol; 0.5 μL per side) to block neuronal activity, including inhibition of fibers of passage, in the VTA 10 min before PNB prevented PNB-induced CPP (Fig. 1*C*). Moreover, VTA baclofen, a $GABA_B$ receptor agonist (25 ng/0.2 μL per side), known to inhibit firing of dopaminergic neurons and reduce NAc dopamine release (24–26), also abolished PNB-induced CPP. In the absence of PNB, pairing VTA baclofen treatment with a chamber had no effect on place preference (Fig. S1). In contrast, although endogenous opioids in the VTA underlie the positive reinforcing effects of addictive drugs (27), pretreatment of the VTA with a nonselective opioid receptor antagonist, naloxone (3 μg/0.5 μL per side), did not prevent PNB-induced CPP (Fig. 1*C*).

Activation of VTA dopaminergic neurons in injured rats following PNB was investigated using immunohistochemistry and expression of c-FOS, a marker of neuronal activity (28). In coronal brain sections the number of FOS-positive cells was counted along the anteroposterial axes of the VTA identified by staining of catecholaminergic (dopaminergic) neurons with tyrosine hydroxylase (TH) (Fig. 2 and Fig. S2). PNB in injured rats increased the number of FOS-expressing cells in posterior regions of the VTA at −5.8 to −6.3 mm from bregma. Incision injury itself, or PNB in sham rats, did not change FOS expression (sham/saline, 37 ± 8; sham/lidocaine, 41 ± 10; incision/saline, 39 ± 6; incision/lidocaine, 62 ± 8; n = 4–5 rats). Confocal images acquired within

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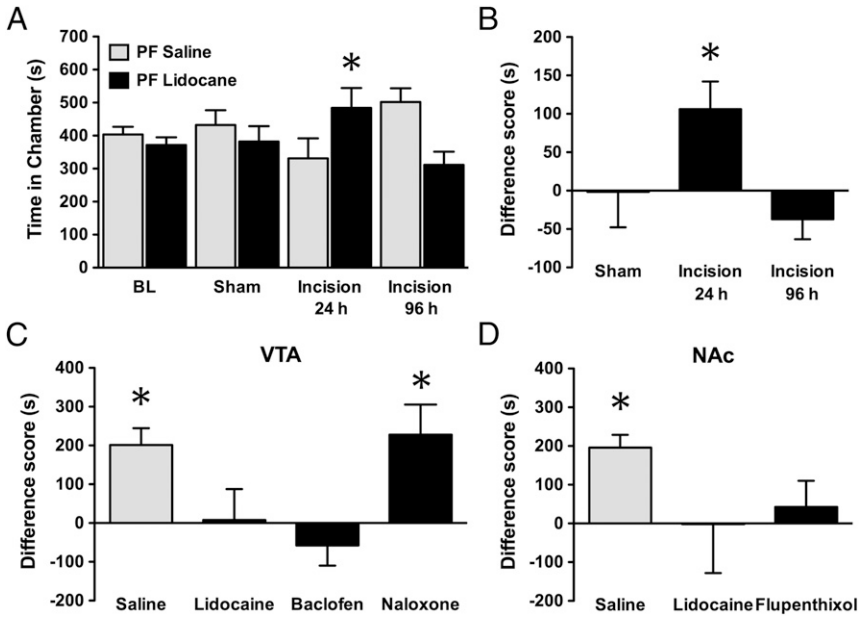

Fig. 1. PNB in rats with incisional pain produced VTA- and NAc-dependent CPP. PNB in injured rats 24 h, but not 96 h, following incision of the hind paw induced CPP as demonstrated by (*A*) increased time spent in chambers paired with popliteal fossa (PF) lidocaine and (*B*) difference scores. (*C*) Pretreatment with lidocaine or baclofen, but not naloxone, in the VTA 10 min before PNB abolished CPP. (*D*) Pretreatment with NAc lidocaine or flupenthixol blocked CPP. Mean ± SEM, $n = 9$–13, $^*P < 0.05$, Student's paired t test.

the posteromedial VTA at bregma −5.8 mm (rectangle in Fig. 2*A*) demonstrate that increased FOS immunoreactivity occurred preferentially in TH-positive (dopaminergic) neurons (sham/saline, 20 ± 5; sham/lidocaine, 17 ± 7; incision/saline, 18 ± 2; incision/lidocaine, 32 ± 8; $n = 4$–5 rats).

In vivo microdialysis in awake and freely moving rats was performed to investigate potential efflux of dopamine in the NAc shell of incised rats following PNB. Basal NAc dopamine levels did not differ between incised and sham-treated animals [4.68 ± 0.45 pg/30 μL ($n = 15$) and 4.71 ± 0.45 pg/30 μL ($n = 13$), respectively]. An increase from baseline was detected in injured rats during the 120-min time period following PNB, with peak levels in the 30- to 60-min fraction elevated by 63 ± 24% (Fig. 3*A*). The area under the curve of percent change from baseline was significantly higher only in incised rats given PNB 24 h, but not 96 h, postincision (Fig. 3*B*). Administration of cocaine (20 mg/kg, i.p.) after the testing induced a robust increase (about 500%) in extracellular dopamine in both incised and sham animals and served as a positive control.

In line with dopamine efflux in the NAc following pain relief, we further investigated whether inhibiting the dopaminergic activity of the NAc would prevent PNB-induced CPP (Fig. 1*D*). Microinjection of lidocaine (4%, wt/vol; 0.5 μL per side) into the NAc shell 10 min before PNB in injured rats abolished CPP. More specifically, blockade of dopamine signaling by a nonselective dopamine receptor antagonist, flupenthixol (3 μg/0.5 μL per side) (29), also prevented CPP. This directly demonstrates that dopaminergic transmission in the nucleus accumbens is required for the negative reinforcement (manifested as CPP) that results from pain relief. In the absence of PNB, pairing NAc flupenthixol treatment with a chamber had no effect on place preference (Fig. S1). These results implicate mesolimbic dopaminergic signaling in pain-relief–induced CPP.

Discussion

Pain is a subjective and multidimensional experience with sensory, affective, and cognitive components. The subjective unpleasantness of pain is essential to the human experience but difficult to evaluate in laboratory animals. In agreement with our previous findings in other models of experimental pain (1, 3, 30, 31), our current behavioral data demonstrate that the removal of the aversive state resulting from ongoing postsurgical nociceptive input by PNB elicits CPP. Importantly, no CPP was observed following PNB in sham-treated rats or 96 h after the incision surgery, when injury-induced ongoing pain was likely to be greatly diminished. Thus, as we have previously shown, ongoing (i.e., spontaneous) pain can be unmasked by demonstrating that its removal is rewarding using CPP (1, 3, 30, 31).

Findings that relief of ongoing incisional, neuropathic, osteoarthritic, and inflammatory pain (1, 3, 30) produces CPP confirms that pain relief elicits reward. However, direct neurochemical evidence for the activation of the brain reward circuitry during pain relief has not previously been established directly. Here, we show that (*i*) peripheral nerve block at 24 h, but not 96 h, produces CPP in animals with hind-paw incision consistent with ongoing surgery-induced pain; (*ii*) activation of dopaminergic projections from the VTA to NAc is necessary for PNB-induced CPP; (*iii*) dopaminergic transmission in the NAc is required for PNB-induced CPP; and (*iv*) in contrast, PNB-induced CPP does not require endogenous opioids in the VTA. These findings are consistent with functional magnetic resonance imaging studies in humans showing increased activation of the NAc at the offset of an acute noxious stimulus (pain relief) (5).

Behavioral, electrophysiological (9), and in vivo microdialysis (15, 16) studies have consistently demonstrated activation of mesolimbic dopaminergic neurons by natural rewards, rewarding drugs, and reward-predicting cues (11, 32, 33). Distinct patterns of neuronal activity are consistent with the role of the VTA in positive reinforcement and learned appetitive behavior (34). Our data showing activation of VTA dopaminergic neurons and dopamine efflux in the NAc indicate that a population of midbrain dopamine neurons is activated following termination of an aversive state and may underlie negative reinforcement. Importantly, because PNB-induced CPP is prevented by blockade of mesolimbic dopamine neurotransmission, activation of VTA neurons appears to be necessary for reinforced learning.

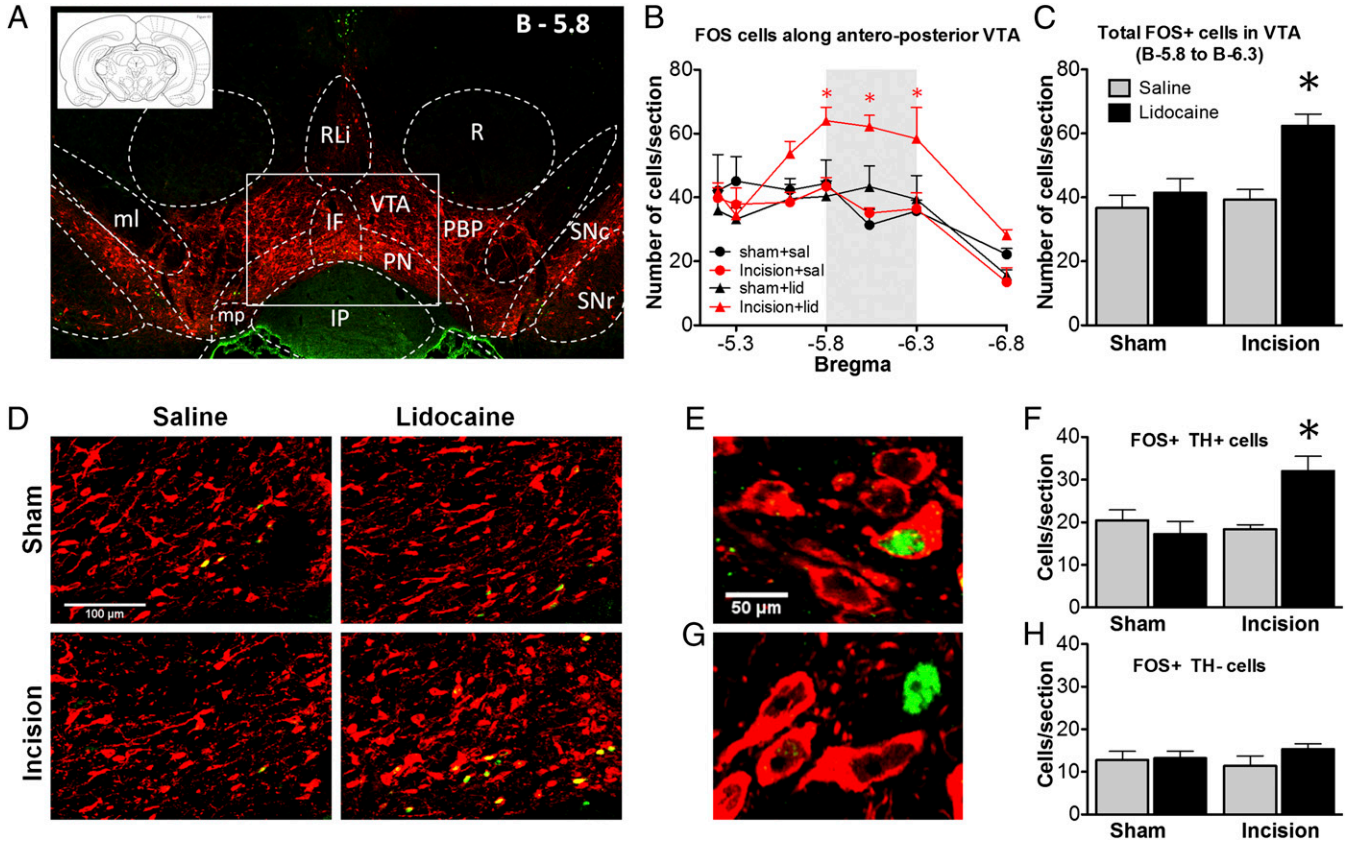

Fig. 2. PNB increased the number of FOS-positive dopaminergic neurons in the VTA. (*A*) A coronal brain section at bregma −5.8 mm outlining the VTA identified by tyrosine hydroxylase labeling (red). (*B*) The average number of FOS+ cells (green) per section was calculated between bregma −5.2 and −6.8 mm. (*C*) The number of FOS+ cells increased significantly at bregma −5.8 to −6.3 mm only in incised rats following PNB. (*D*) Confocal images within the medial VTA (rectangle in Fig. 2*A*). The number of dopaminergic FOS+/TH+ cells (*E*) increased in incised rats after PNB (*F*); the number of nondopaminergic FOS+/TH- cells (*G*) did not change (*H*). Mean ± SEM, n = 4–5 rats/group, $^*P < 0.05$; two-way ANOVA with Bonferroni multiple comparisons test (*B*), one-way ANOVA with Tukey's multiple comparisons test (*C* and *F*).

The midbrain VTA is an anatomically and functionally heterogeneous region composed of several nuclei including the parabrachial pigmented area (PBP), paranigral nucleus (PN), rostral linear nucleus raphe (RLi), interfascicular nucleus (IF), and caudal linear nucleus raphe (CLi) (Fig. 2*A*). The activation of posteromedial VTA dopaminergic neurons, which primarily target the medial accumbens shell, appears to promote positive affective states (14). Accordingly, the present study found enhanced cFOS expression in the posteromedial division of the VTA. In agreement with cellular targets of these neurons, dopamine levels were increased in the medial shell of the NAc. Thus, pain relief reward appears to share many similarities with appetitive rewards. However, as indicated by our findings with VTA naloxone, activation of a reward circuit by pain relief does not appear to involve an endogenous opioid system in the VTA, suggesting that there may be important differences as well (27, 35, 36).

Acute noxious stimuli have been reported to excite some and inhibit other midbrain dopamine neurons (37), suggesting multiple populations of neurons support different aspects of aversive

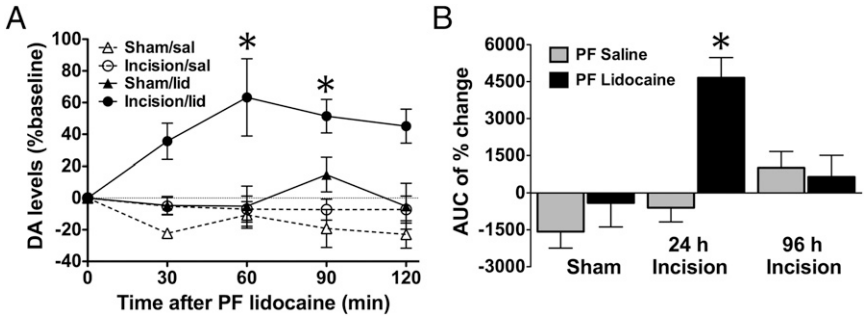

Fig. 3. PNB increased the extracellular dopamine (DA) levels in the NAc shell of rats with incisional pain. (*A*) Extracellular dopamine levels increased above basal levels following PNB only in incised animals. (*B*) Area under the curve (AUC) of percent change from baseline demonstrated significant dopamine release within 120 min after PNB given 24 h, but not 96 h, following incision. Mean ± SEM, n = 3–8 rats/group, $^*P < 0.05$; one-way ANOVA with Tukey's multiple comparisons test.

processing (38). These diverse patterns of neuronal activity are likely reflected in the observed complexity of dopaminergic signaling in experimental models of tonic pain (39, 40). In the present study, we found no change in the number of FOS-positive neurons in incised versus sham rats. Further, the basal dopamine levels in the NAc of incised and sham rats did not differ. Thus, 24 h following incisional surgery, before PNB-induced pain relief, we saw no net change in mesolimbic dopamine transmission. Numerous factors may account for this observation, including (*i*) the sensitivity of our analytical technique, (*ii*) the nonchronic nature of the injury, or (*iii*) involvement of VTA neurons that project to other sites beyond the NAc.

How afferent nociceptive pathway(s) modulate the reward circuit is not known. In the rat, major ascending nociceptive pathways from lamina I of the lumbar spinal cord terminate in the lateral parabrachial nucleus (PBN) or in the thalamic nuclei (41, 42). Midbrain dopaminergic neurons receive direct nociceptive inputs from projections in the PBN (43). The anterior cingulate cortex (ACC) receives prominent inputs from nociceptive neurons in the thalamus, and activation of the ACC through the spinothalamic pathway contributes to the aversiveness of nociceptive stimuli. Accordingly, functional imaging studies have implicated the ACC in processing the unpleasant affective aspects of pain in humans (44). The ACC projects directly or via other limbic regions such as the amygdala to the mesolimbic reward system (45, 46). Recent work has identified a circuit that includes the lateral habenula neurons that indirectly inhibit midbrain dopamine neurons (47). Inhibition of this input pathway would disinhibit VTA dopamine neurons.

In conclusion, we demonstrate that relief of ongoing postsurgical pain following PNB produces CPP and activates the mesolimbic dopaminergic circuit implicated in positive reinforcement. PNB elicits efflux of dopamine in the NAc shell only in the setting of injury-induced, ongoing pain. Additionally, inhibition of dopaminergic neurons in the VTA and dopamine signaling in their projection target in the NAc shell prevented PNB-induced CPP, providing direct evidence for a causal relationship between activation of this mesostriatal circuit and the negative reinforcing effect of pain relief. These data indicate that activation of the VTA to NAc dopamine signaling contributes to both positively and negatively reinforced behavior.

Materials and Methods

Animals. Adult, male Sprague-Dawley rats (250–350 g; Harlan) were used. All procedures were performed in accordance with the policies of the National Institutes of Health guidelines for laboratory animals under protocols approved by the University of Arizona Institutional Animal Care and Use Committee. Rats were housed three per cage on a 12-h light–dark cycle with food and water provided for ad libitum consumption.

Intracranial VTA and NAc Cannulation. Stereotaxic surgeries were performed in anesthetized rats (i.p. ketamine/xylazine 80/12 mg/kg; Western Medical Supply/Sigma) according to the brain atlas. Two 26-gauge guide cannulas (Plastics One) were directed toward the following coordinates: VTA [anteroposterior (AP), bregma −5.8 mm; mediolateral (ML), midline ±0.6 mm; dorsoventral (DV), skull −8.0 mm], NAc shell (AP, bregma +1.5 mm; ML, midline ±1.0 mm; DV, skull −6.5 mm). For microdialysis, a single guide cannula (AG-8; EICOM Corp.) was implanted into the left NAc (AP, bregma +1.7 mm; ML, midline −1.0 mm; DV, skull −6.0 mm). Stainless steel dummy cannulas were inserted to keep the guide free of debris. After surgery, rats were housed individually and allowed to recover for 5–7 d.

Incisional Injury Pain Model. Incision injury of the skin plus deep tissue, including fascia and underlying muscle, was done as described by Brennan et al. (22). Rats were anesthetized with 2% (vol/vol) isoflurane, a 1-cm longitudinal incision was made through the skin of the left hind paw, and the plantaris muscle was elevated and incised longitudinally. The cut skin was stitched with two 5–0 nylon sutures and the wound site treated with neomycin. Sham animals were anesthetized and the left hind paw was cleaned, but no incision was made. Peripheral nerve block was achieved by injecting 200 μL of saline or lidocaine (4%, wt/vol; Qualitest Pharmaceuticals) into PF under light isofluorane anesthesia (30).

Brain Microinjection. Bilateral intracranial microinjections of 4% (wt/vol) lidocaine hydrochloride (0.5 μL per side), baclofen (25 ng/0.2 μL per side; Sigma), flupenthixol (3 μg/0.5 μL per side; Sigma), or naloxone hydrochloride (3 μg/0.5 μL per side; Tocris) in the VTA or NAc were done using injectors extended 1 mm beyond the guide cannula. All animals were euthanized via CO_2 overdose at the end of the experiments and the placement of the guide cannulas/injection sites was confirmed with histology methods. Data from animals with misplaced cannulas were removed from the analyses.

Guarding Behavior. Assessment of guarding behavior was done as previously described (30). Rats were observed for 10 s each at 1-min intervals and scored 0–2 (0, injured hind-paw area was touching the mesh and the area was blanched or distorted by the mesh; 1, the injured hind paw touched the mesh without blanching or distortion; 2, the injured hind paw was completely off the mesh). For each rat a cumulative score was obtained by adding 30 scores during the 30-min testing period. All testing was done by an experimenter blinded to the treatment conditions.

Thermal Hypersensitivity. Nociceptive withdrawal thresholds to noxious radiant heat were determined using the Hargreaves test. Rats were allowed to acclimate within a Plexiglas enclosure on a clear glass plate for 30 min. A radiant heat source was directed onto the plantar surface of the left hind paw. A motion detector halted both heat lamp and timer when the paw was withdrawn. Baseline latencies were established at 20 s. A maximal cutoff of 30 s was used to prevent tissue damage.

Conditioned Place Preference Procedures. A single trial conditioning protocol was used for CPP as previously described (2, 30). All rats underwent handling by the experimenter before the preconditioning phase. On preconditioning day, rats were placed into the CPP boxes with access to all chambers; time spent in each chamber was determined by an automated process and analyzed across 15 min to verify no preconditioning chamber preference. Following preconditioning, rats received incision or sham surgeries and were placed back into their home cages overnight. On conditioning day (24 h postincision), rats received a saline (200 μL) injection into the PF and were immediately (within 2 min) placed into the appropriate pairing chamber. Four hours later, rats received a lidocaine injection (4%, wt/vol; 200 μL) into the PF and were placed into the opposite chamber. Chamber pairings were counterbalanced. To determine the role of the VTA or NAc in PNB-induced CPP, rats with VTA or NAc cannulas received saline injection into the VTA or NAc followed 10 min later by PF saline injection and immediate placement into the appropriate pairing chambers. Four hours later, rats received treatment drug injection into the VTA or NAc followed in 10 min by PF lidocaine injection and placement into the opposite chambers. The conditioning time was 30 min in each chamber. On test day, 20 h following the afternoon pairing, rats were placed in the CPP box with access to all chambers and behavior was recorded for 15 min for analysis for chamber preference. Difference scores were calculated as test time minus preconditioning time spent in the PF–lidocaine paired chamber.

Immunohistochemistry. Two hours after treatment rats were anesthetized by i.p. ketamine/xylazine and transcardially perfused with 4% (wt/vol) paraformaldehyde. Coronal brain sections (30 μm thick) were cut in a Microm HM 525 cryostat and mounted on Superfrost Plus microscope slides. Brain tissue was permeabilized with 0.2% Triton X-100, blocked with 5% (wt/vol) normal goat serum and incubated overnight with the mixture of primary antibodies of rabbit polyclonal anti-cFOS (sc-52, 1:25,000; Santa Cruz) and mouse monoclonal anti-tyrosine hydroxylase (MAB55280, 1:3,000; Millipore). The sections were incubated with biotinylated anti-rabbit antibody followed by the ABC complex (Vectastain Elite ABC kit; Vector Laboratories, Inc.) and tyramide signal amplification detection (TSA Plus Fluorescein Kit; Perkin-Elmer). TH was visualized with anti-mouse Alexa Fluor 555 (1:1,000; Molecular Probes, Invitrogen). Slides were mounted in Vectashield mounting medium (Vector Laboratories, Inc.) and examined under an Olympus BX51 microscope equipped with a Hamamatsu C8484 digital camera. Confocal images were obtained with a Zeiss LSM520 laser scanning confocal microscope using the 488- and 543-nm excitation wavelengths. Micrographs of 10–15 sections per rat 150 μm apart within the bregma −5.20 to −6.80 mm were analyzed for FOS expression. In the ImageJ software the VTA area including the PBP, PN, RLi, IF, and CLi was outlined according to the TH staining. FOS-positive nuclear puncta within the outlined area were counted manually by an observer blinded to the treatment conditions. Coexpression

of FOS and TH was evaluated within the medial VTA at bregma −5.80 mm (rectangle in Fig. 2*A*) in two to four sections per each rat using the Zeiss LSM520 confocal microscope images. Cells with nuclear FOS staining and a visible cytoplasmic TH staining (Fig. 2*E*) were counted as dopaminergic; FOS-positive cells with no clear cytoplasmic TH staining (Fig. 2*G*) were counted as nondopaminergic.

In Vivo Microdialysis and HPLC Quantification of Dopamine. Microdialysis was done in awake, freely moving animals. The microdialysis probe (A-I-8-02; Eicom) was inserted into the NAc with 2 mm of semipermeable membrane projecting beyond the guide cannula (Fig. S3) and perfused at 1.25 μL/min with artificial cerebrospinal fluid (aCSF: 147.0 mM NaCl, 2.8 mM KCl, 1.2 mM $MgCl_2$, and 1.2 mM $CaCl_2$). After a 90-min washout period, two baseline and four treatment fractions (30 min/fraction) were collected into prechilled (4 °C) Eppendorf tubes containing 1.0 μL of 40× antioxidant solution [6.0 mM L-cysteine, 2.0 mM oxalic acid, and 1.3% (vol/vol) glacial acetic acid] (48). All rats were then injected with cocaine (20 mg/kg, i.p.) and dialysates were collected for additional 90 min. Fractions were analyzed using an Agilent 1100 HPLC system with a 5020 guard cell, MD-150 column and Coulochem III 5014B electrochemical detector (ESA). The guard cell was set at 350 mV, electrode 1 at −150 mV, and electrode 2 at 250 mV. Standard curve was obtained from seven serial dilutions of dopamine (2.5–160 pg in 20 μL aCSF plus antioxidant mixture). The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the formulas LOD = 3.3 (SDr/S) and LOQ = 10 (SDr/S), where the SD of the response SDr (SD of *y* intercepts of regression lines) and the slope of the standard curve S was determined from the measurements of 10 independent standard curves (Fig. S3). The data from rats that failed to generate dopamine efflux following cocaine treatment were excluded. Dopamine concentrations were expressed as percent of the corresponding baseline level.

Statistical Analysis. Statistical analyses were calculated using GraphPad Prism 5 software. Results were expressed as mean ± SEM. Two-way ANOVA with Bonferroni multiple comparisons test or one-way ANOVA with Tukey's multiple comparison post hoc tests was used for between-groups comparison. Student's paired *t* test was used to analyze the difference scores for the CPP data. Significance was set at $P < 0.05$.

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