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Extracellular Matrix Signaling Through β3 Integrin Mediates Cocaine Cue–Induced Transient Synaptic Plasticity and Relapse

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ABSTRACT

BACKGROUND: Cue-induced relapse to drug use is a primary symptom of cocaine addiction. Cue-induced transient excitatory synaptic potentiation (t-SP) induced in the nucleus accumbens mediates cued cocaine seeking in rat models of relapse. Cue-induced t-SP depends on extracellular signaling by matrix metalloproteases (MMPs), but it is unknown how this catalytic activity communicates with nucleus accumbens neurons to induce t-SP and cocaine seeking.

METHODS: Male Sprague Dawley rats (*N* = 125) were trained to self-administer cocaine, after which self-administration was extinguished and then reinstated by cocaine-conditioned cues. We used a morpholino antisense strategy to knock down the β1 or β3 integrin subunits or inhibitors to prevent phosphorylation of the integrin signaling kinases focal adhesion kinase (FAK) or integrin-linked kinase. We quantified protein changes with immunoblotting and t-SP by measuring dendritic spine morphology and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid/*N*-methyl-D-aspartate glutamate currents. Integrin signaling was stimulated by microinjecting an MMP activator or integrin peptide ligand into the accumbens.

RESULTS: Knockdown of β3 integrin or FAK inhibitor, but not β1 integrin or integrin-linked kinase inhibitor, prevented cue-induced cocaine seeking but not sucrose seeking. β3 integrin knockdown prevented t-SP as measured by preventing the cue-induced increases in both alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid/*N*-methyl-D-aspartate glutamate ratio and spine head diameter. Activating MMP gelatinases with tissue plasminogen activator potentiated cue-induced reinstatement, which was prevented by β3 integrin knockdown and FAK inhibition. Stimulating integrin receptors with the RGD ligand liberated by MMP gelatinase activity also potentiated cued cocaine seeking.

CONCLUSIONS: Activation of MMP gelatinase in the extracellular space is necessary for and potentiates cued cocaine seeking. This extracellular catalysis stimulates β3 integrins and activates FAK to induce t-SP and promote cue-induced cocaine seeking.

Keywords: Cocaine, Drug abuse, Focal adhesion kinase, Integrin, Nucleus accumbens, Relapse, Synaptic plasticity

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The negative social and personal consequences of drug addiction have inspired researchers to identify drug-induced pathogenic neuroplasticity in glutamatergic brain synapses, particularly in the nucleus accumbens (1). Human imaging and rodent studies of cue-induced drug seeking reveal enduring adaptations in accumbens glutamatergic synapses (2–4). Rodent experiments that model cue reactivity studies in humans reveal that drug-conditioned cues elicit drug seeking and transient synaptic potentiation (t-SP) across all drug classes tested to date (cocaine, opioids, and nicotine) (4). t-SP is quantified as postsynaptic enlargement in dendritic spine head diameter (d_h) and increases in the ratio of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) to *N*-methyl-D-aspartate (NMDA) (A/N) receptor currents (5). Importantly, t-SP elicited by drug cues is not recapitulated by cue-induced sucrose seeking (4,6), thereby identifying t-SP as a potential pathological addiction process that is not recruited by conditioned cues motivating biological reward seeking.

In addition to adaptations in the canonical pre- and post-synapse, t-SP and drug seeking induced by conditioned cues require transient remodeling of the extracellular matrix (ECM). The ECM is an extracellular signaling domain composed of proteins that are catalytically activated by matrix metalloprotease (MMP) activity (7). Cues associated with cocaine, heroin, or nicotine self-administration transiently increase MMP-9 activity (6), and MMP-9 activation is required for cue-induced induction of t-SP and drug-seeking behaviors (8). However, it is unknown how MMP-9 activity in the ECM signals into nucleus accumbens medium

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spiny neurons (MSNs) to increase A/N ratio and d_h and thereby promote cue-induced cocaine seeking.

MMP-9 is a gelatinase whose activation is necessary for inducing long-term potentiation in the hippocampus (9,10) and for developing fear conditioning and spatial learning (11,12). Cleavage of gelatin sequences by MMP-9 reveals an arginine-glycine-aspartate (RGD) binding motif that signals into neurons by binding to integrins (13). Integrins are heterodimeric (α and β subunits) transmembrane cell adhesion receptors that support the interface between cell membranes and the ECM (14,15). The β1 and β3 integrin subunits are most abundant in the brain, and acute or repeated cocaine administration changes the amount of β1- and β3-containing integrins in the nucleus accumbens (16,17). Many studies have shown that signaling through either subunit contributes to synaptic plasticity (18–20). Specifically, integrin subunits regulate spine morphology and synaptic strength by linking to actin cytoskeleton and regulating glutamate receptor surface expression through focal adhesion (FAK) and integrin-linked kinases (ILKs) (14,15,21,22). Thus, β1 and β3 integrin subunits and associated kinases may constitute the MMP-9 signaling pathway necessary for cue-induced t-SP and reinstatement.

We tested the hypothesis that MMP-9 acts through the integrin signaling cascade in MSNs of the core subcompartment of the nucleus accumbens (NAcore) to initiate cue-induced t-SP and cocaine seeking. We used an integrin subunit knockdown strategy and FAK or ILK inhibitors (FAKi and ILKi, respectively) to disrupt cued cocaine seeking and t-SP. We also activated NAcore MMP-9 with tissue plasminogen activator (tPA) (23,24) and directly modulated integrins with the RGD peptide liberated by catalytic MMP-9 activity (13). From these studies, we conclude that MMP-9 activation by cocaine-associated cues signals through β3 integrin to activate FAK, produce t-SP, and reinstate cocaine seeking.

METHODS AND MATERIALS

Complete information regarding the methods used in this study can be found in the Supplemental Methods.

Animal Housing and Surgery

One hundred twenty-five male Sprague Dawley rats (250 g; Charles River Laboratories, Wilmington, MA) were individually housed using a 12-hour light/dark cycle with ad libitum food and water. All experimentation occurred during the dark cycle. For most experiments, rats were stereotaxically implanted immediately after catheterization with bilateral guide cannulas aimed at the NAcore. All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Assessment and Accreditation of Laboratory Animal Care.

Drugs and Reagents Used

Cocaine hydrochloride was supplied by the National Institute of Drug Abuse. Morpholinos were purchased from Gene Tools, LLC (Summerton Way, Philomath, OR). The antisense sequences that were used were as follows: β3 integrin morpholino (5′-TC TCTGCCTCAGAACTCGCCCCGCT-3′), β1 integrin morpholino (5′-TGCAAATTCATCTTTTCGCAGCGTC-3′), and standard scrambled control (5′-CCTCTTACCTCAgTTACAATTTATA-3′). MMP-9 inhibitor was purchased from EMD Biosciences Inc. (#444278; Ki = 5 nM; San Diego, CA). The FAK inhibitor prevents autophosphorylation at Y397 and was microinjected at 1, 3, or 10 nmol/0.5 μL per side (FAK inhibitor 14, #3414, Ki = 1 μM; Tocris Bioscience, Minneapolis, MN). The ILK inhibitor prevents phosphorylation at Ser473 and was microinjected at 1 or 10 nmol/0.5 μL per side (Cpd 22, #407331, Ki = 0.6 μM; EMD Millipore, Temecula, CA). All reagents were dissolved in sterile saline, except for MMP-9 inhibitor, which was dissolved in 1% dimethylsufloxide.

Drug Self-administration and Reinstatement

Seven days after surgery, 125 rats began daily 2-hour self-administration sessions for cocaine, and 11 rats began daily 2-hour self-administration sessions for heroin. During self-administration, each drug was delivered using a fixed-ratio 1 schedule of reinforcement with a 20-second time out after each infusion. Active lever presses resulted in cocaine infusions (0.25 mg/infusion) or heroin infusions (100 μg/infusion for days 1 and 2, 50 μg/infusion for days 3 and 4, and 25 μg/infusion for days 5–12) (25) and simultaneous presentation of a compound light (above the active lever) and tone (2900 Hz) conditioning stimulus. An inactive lever was also provided to control for nonspecific responding. After at least 10 self-administration sessions at ≥10 infusions per day, rats began extinction training, during which programmed consequences were removed from lever pressing. Extinction training lasted at least 10 days, or until 2 consecutive days revealed ≤25 active lever presses.

Five days after the last morpholino microinjection, the light/tone-conditioned cue was restored to active lever pressing, and reinstated lever pressing was quantified. For integrin knockdown experiments, rats were reinstated once and the session lasted 15 minutes, after which tissue was obtained for spine analysis or electrophysiology; alternatively, the session lasted for 120 minutes if full behavioral analysis was to be performed. A total of 125 rats began cocaine self-administration, 11 rats were heroin trained, 18 rats were yoked-saline control (SAL; rats paired with cocaine-trained rats where lever pressing was without consequence and saline was infused when the paired rats self-administered a cocaine infusion), and 16 were eliminated from the study because of catheter failure or microinjection cannula placements outside the NAcore.

Sucrose Self-administration

Sixteen rats were trained in daily 2-hour sessions on a fixed-ratio 1 schedule of reinforcement during which active lever presses resulted in delivery of a sucrose pellet (45 mg; Bio-Serv, Flemington, NJ) paired with light/tone cues. After a minimum of 10 days of 10 pellets or more, rats entered extinction training for 11 to 14 days, followed by a cue-induced reinstatement test. Morpholino microinjections were made as described above.

Microinjection Procedures and Histology

Rats were stereotaxically implanted immediately after catheterization with bilateral guide cannulas aimed above the NAcore (anterioposterior +1.5, mediolateral ±1.7, and dorsoventral −5.5) (26). Bilateral microinjections of 0.5 μL were made into the NAcore over 2 minutes of β3 integrin subunit, β1

integrin subunit, or scrambled control (25 pmol × 5 days); small molecule inhibitors for MMP-9 (0.1 nmol), FAK inhibitor (1.0, 3.0, or 10.0 nmol), ILK inhibitor (1.0, 3.0, or 10.0 nmol), or peptides tPA (1, 10, or 100 ng); and RAD and RGD (1 nmol). At the end of experimentation, rats were perfused, and coronal slices (100-μm thick) of NAcore were mounted and stained with cresyl violet to verify guide cannula placement.

Membrane Fractionation and Western Blotting

Rats were rapidly decapitated and the NAcore was dissected and homogenized. Protein (15 μg) was added to each lane of 10% Bis-Tris gels (Bio-Rad Laboratories, Hercules, CA) and transferred to polyvinylidene difluoride membranes via the Invitrogen iBlot transfer system (Carlsbad, CA). Primary antibodies used were for the β1 integrin subunit (1:500, ab1952; Abcam, Cambridge, United Kingdom), the β3 integrin subunit (1:250, Millipore 05769), ILK (1:1000, ab76468; Abcam), Calnexin (Chemicon International, Temecula, CA), and glyceraldehyde 3-phosphate dehydrogenase (5174; Cell Signaling, Danvers, MA).

Immunohistochemistry on Dendrites and Dendritic Spine Analysis

NAcore tissue slices and DiI-labeling dendrite spine morphology was performed as described previously (27). Confocal Z-series data sets of spine morphology were acquired using a Leica SP5 laser-scanning confocal microscope (Wetzlar, Germany) and images were acquired at 63× oil (1024 × 256 frame; 0.21 μm/Z step). Following deconvolution, individual dendrites were isolated and all image acquisition, cropping, and isolating dendrites was performed by an investigator unaware of the treatment groups (Supplemental Figure S1).

Slice Preparation and Whole Cell Recordings

Rats were anesthetized with ketamine, and 250-μm-thick coronal slices were collected in oxygenated artificial cerebrospinal fluid. Inhibitory synaptic transmission was blocked with picrotoxin (50 μM), and whole-cell patch clamp recordings in voltage clamp mode were performed in the NAcore using glass microelectrodes (1–2 MΩ) filled with a cesium-based internal solution. Postsynaptic currents were evoked at 0.05 Hz with a bipolar stimulating electrode placed ∼300 μm dorsomedial of the recorded cell. The stimulation intensity chosen evoked a ∼50% of maximal AMPA current. Recordings began more than 10 minutes after the cell membrane was ruptured to allow for diffusion of the internal solution into the cell. AMPA currents were first measured at −80 mV, and then the membrane potential was gradually increased to +40 mV. Recordings were resumed 5 minutes later. D(−)-2-Amino-5-phosphonopentanoic acid (D-AP5) (50 μM) was bath-applied to block NMDA currents, and AMPA currents were recorded at +40 mV. NMDA currents were obtained by subtracting the AMPA currents from the total current. All recordings were made by an individual who was blinded to the treatment groups.

In Vivo Zymography

MMP activity was measured using an in vivo zymography assay as described previously (8). Briefly, rats were microinjected with intramolecularly dye-quenched fluorescein conjugated gelatin (Life Technologies, Carlsbad, CA) in the NAcore (reconstituted in phosphate-buffered saline at 1 mg/mL, 1.5 μL/hemisphere at a rate of 0.5 μL/min) with a 15-minute incubation time. Proteolytic cleavage by the gelatinases (MMP-2,9) results in an activity-dependent increase in green fluorescence. Coronal brain sections (100 μm) were imaged using a Leica SP5 laser-scanning confocal microscope using a 10× objective. ImageJ software (National Institute of Mental Health, Bethesda, MD) was used to quantify images.

Statistics

All statistical analyses were conducted using GraphPad software (version 6; Prism, San Diego, CA) or a two-level nested analysis of variance (ANOVA) in Excel (Microsoft, Redmond, WA). Behavioral and A/N ratio data were analyzed by two-way ANOVA followed by Bonferroni post hoc tests for multiple comparisons. Protein was compared using the paired Student *t* test. All spine density, d_h, and immunohistochemistry data were statistically analyzed with a nested ANOVA, and post hoc comparisons were conducted using a Bonferroni post hoc test. All data except behavior were obtained and quantified by individuals unaware of the treatment groups. Complete statistical information can be found in Supplemental Table S1.

RESULTS

Knockdown of β3, but Not β1, Integrin Prevented Cued Cocaine Seeking

We used a morpholino knockdown strategy to determine if integrins containing β3 or β1 subunits were mediating cue-induced t-SP and cocaine seeking. Morpholinos have been used to modulate drug seeking (28,29) and are synthetic antisense oligonucleotides with a backbone consisting of a morpholine ring and phosphorodiamidate group that increases in vivo stability and cell penetration (30). Five daily microinjections of morpholino antisense or control scrambled-sequence morpholino (Scr) were made into the NAcore in opposite hemispheres of drug-naïve rats to validate the efficacy and specificity of the morpholino sequences at reducing β3 or β1 integrin subunit expression. Five days after the last microinjection, β3 or β1 was reduced compared with Scr by ∼70% or ∼60%, respectively (Figure 1B, C and Supplemental Figure S2). The β3 morpholino selectively reduced the β3 subunit without altering the β1 subunit or ILK. In contrast, the morpholino targeting the β1 subunit caused a compensatory elevation in β3 integrin and ILK.

To determine if knockdown of either β subunit altered cue-induced cocaine seeking and t-SP, rats were trained to self-administer cocaine, and lever pressing was extinguished (Figure 1D). A light/tone compound stimulus was paired with cocaine infusions during self-administration, and lever pressing during extinction training yielded neither cocaine nor light/tone stimulus. Rats received five daily bilateral infusions of β1 integrin, β3 integrin, or Scr morpholino into the NAcore during extinction training. Five days after the last morpholino microinjection, the light/tone-conditioned cue was restored to active

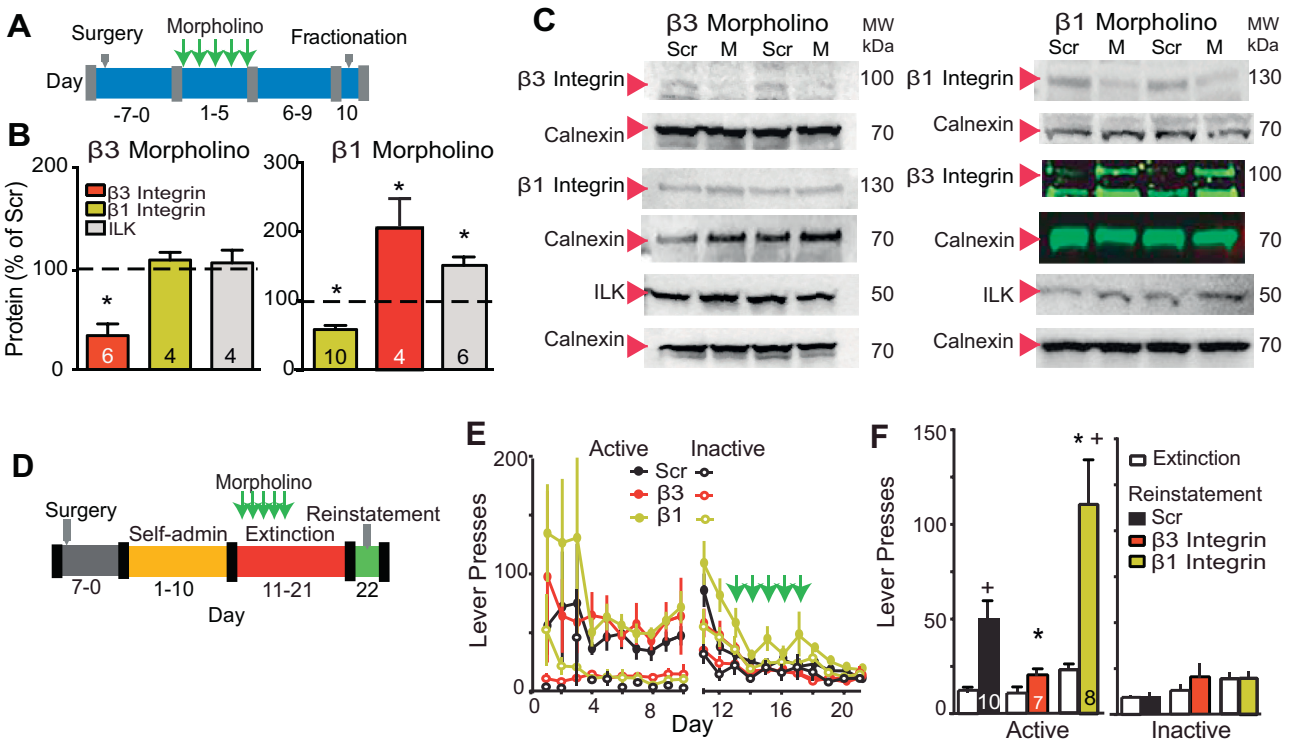

Figure 1. Knockdown of β3 integrin, but not β1 integrin, blocked cue-induced cocaine seeking. **(A)** Treatment protocol for morpholino experiments to assess the efficacy of morpholino-knockdown of β3 or β1 integrin. **(B)** Scrambled sequence (Scr) or β integrin morpholino were microinjected into opposite hemispheres, allowing within-animal comparison. After β3 morpholino treatment, β3 subunit expression was reduced (paired Student *t* test, $t_5 = 8.91$, $p < .001$) without changing the β1 subunit or integrin-linked kinase (ILK). After β1 morpholino treatment, the β1 subunit expression was reduced (*t* test, $t_5 = 5.75$, $p < .01$), but β3 subunit ($t_5 = 3.98$, $p = .050$) and ILK ($t_5 = 4.24$, $p = .008$) expression were elevated. **(C)** Representative Western blots of each protein and the respective calnexin or glyceraldehyde 3-phosphate dehydrogenase loading control are shown below each bar. Note that because of the relatively weak β3 integrin antibody, a fluorescent secondary was used to augment detection after β1 morpholino treatment. $^{*}p < .05$. **(D)** A diagram of the experimental timeline for drug self-administration. **(E)** Time course of active and inactive lever pressing for self-administration and extinction in cocaine-treated rats used in Figure 1 was equivalent between treatment groups. **(F)** Cue-induced reinstatement of cocaine seeking was reduced after knockdown of β3 subunit compared to Scr morpholino and potentiated after β1 integrin knockdown (two-way repeated measures analysis of variance, extinction/reinstatement $F_{1,22} = 20.05$, $p < .001$; Scr/β3/β1 $F_{2,22} = 8.63$, $p = .002$; interaction $F_{2,22} = 4.78$, $p = .019$). All data are shown as mean ± SEM, and *n* is shown within the bars. $^{*}p < .05$, compared with Scr or Scr/reinstatement, using a Bonferroni post hoc test. $^{+}p < .05$, compared to extinction responding within each treatment group. M, morpholino; MW, molecular weight.

lever pressing, and reinstated lever pressing was quantified over 120 minutes (Figure 1E). Cocaine infusions and active and inactive lever pressing during self-administration and extinction training were statistically equivalent between animals assigned to the Scr, β3 integrin, or β1 integrin morpholino groups (Figure 1E and Supplemental Figure S3A), and all rats had bilateral microinjection cannula tips in the NAcore (Supplemental Figure S3B).

Exposing extinguished (EXT) rats to cocaine-associated cues reinstated active lever pressing in the Scr-treated animals compared with active lever pressing averaged over the last 2 days of extinction training (Figure 1F). Morpholino knockdown of β3 integrin abolished reinstated lever pressing, while β1 integrin knockdown potentiated pressing (Figure 1F). These data support a necessary role by β3-containing integrins in signaling cue-induced cocaine seeking. The reduction in cocaine seeking after β3 integrin knockdown was without effect on inactive lever pressing (Figure 1F) or open field motor activity (Supplemental Figure S3C). The paradoxical increase in reinstated seeking after β1 knockdown may arise from the compensatory increase in β3 integrin and ILK (Figure 1B).

β3 Integrin Knockdown Did Not Alter Cued Sucrose Seeking

We next determined if β3 integrin signaling in the NAcore was selective for regulating drug seeking or was also necessary for cued reinstatement of seeking a biological reward. Rats were trained to self-administer sucrose pellets, and active lever pressing was extinguished in combination with β3 or Scr morpholino microinjections (Figure 1D). The β3 and Scr groups demonstrated equivalent amounts of active lever pressing during self-administration and extinction training and consumed an equal number of pellets over the training period (Figure 2A). All microinjection cannulas were bilaterally targeted into the NAcore (Figure 2B). Sucrose-trained rats showed robust cue-induced reinstatement of active lever pressing, and there was no

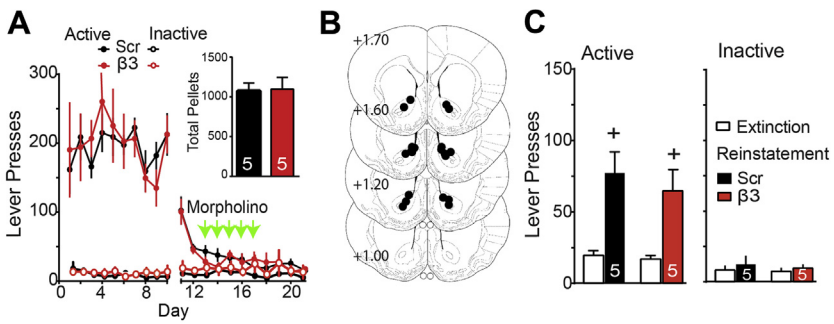

Figure 2. Morpholino knockdown of the β3 integrin subunit did not alter cued sucrose seeking. **(A)** Time course of active lever pressing for self-administration and extinction in sucrose-trained rats was equivalent between treatment groups. (*Inset*) Equivalent pellets taken by each treatment group. **(B)** Location of microinjection cannula tips in the core subcompartment of the nucleus accumbens (NAcore) corresponding to the data shown in panels **(A)** and **(C)**. **(C)** Sucrose-trained rats show cue-induced reinstatement and no difference between treatment groups (extinguished/reinstatement $F_{1,8}$ = 22.95, $p < .001$; scrambled sequence [Scr]/β3 $F_{1,8}$ = 0.51, $p = .494$; interaction $F_{1,8} = 0.20$, $p = .667$). Data shown as mean ± SEM, and *n* is shown within the bars. $^{+}p < .05$ compared with extinguished lever presses using a Bonferroni post hoc test.

difference between rats that were pretreated with β3 or Scr morpholino (Figure 2C). These data reveal that in the NAcore, β3 integrin subunits are recruited to mediate cue-induced cocaine but not sucrose seeking.

β3 Integrin Knockdown Blocked Cue-Induced t-SP: A/N Ratio

Fifteen minutes after initiating cue-induced drug seeking for a variety of addictive drugs, the A/N ratio in NAcore MSNs is elevated, and it returns to baseline levels within 120 minutes (4). The increase in A/N ratio is positively correlated with the number of cue-induced active lever presses and depends on the activation of MMP-9 (5,8). To determine if cued increases in A/N ratio result from stimulating β3 integrin, rats were trained to self-administer cocaine, the behavior was extinguished, and morpholino injections were made into the NAcore during extinction training (Figure 1D). Rats assigned to the Scr or β3 integrin groups showed equal active lever pressing and drug infusions during cocaine self-administration, SAL, and extinction training (Figure 3A–C). NAcore slices were obtained 24 hours after the last extinction session for SAL and cocaine extinction groups, or 15 minutes after beginning a cued reinstatement trial. Cue-induced active lever pressing over the 15 minutes before making tissue slices was reduced in the β3 morpholino compared with the Scr treatment group without altering inactive lever presses (Figure 3D). Figure 3A–D shows behavior for rats that were then randomly assigned to quantify A/N ratio (Figure 3) or spine morphology (Figure 4). The reinstated lever pressing in Figure 3D was measured for 15 minutes compared with Figure 1F, where behavior was recorded for 120 minutes. Whole-cell patch clamp recordings were made from MSNs and revealed increased A/N ratio during 15 minutes of cue-induced reinstatement in Scr-treated rats compared with SAL control rats. The cued increase in A/N ratio was abolished in reinstated rats that were pretreated with β3

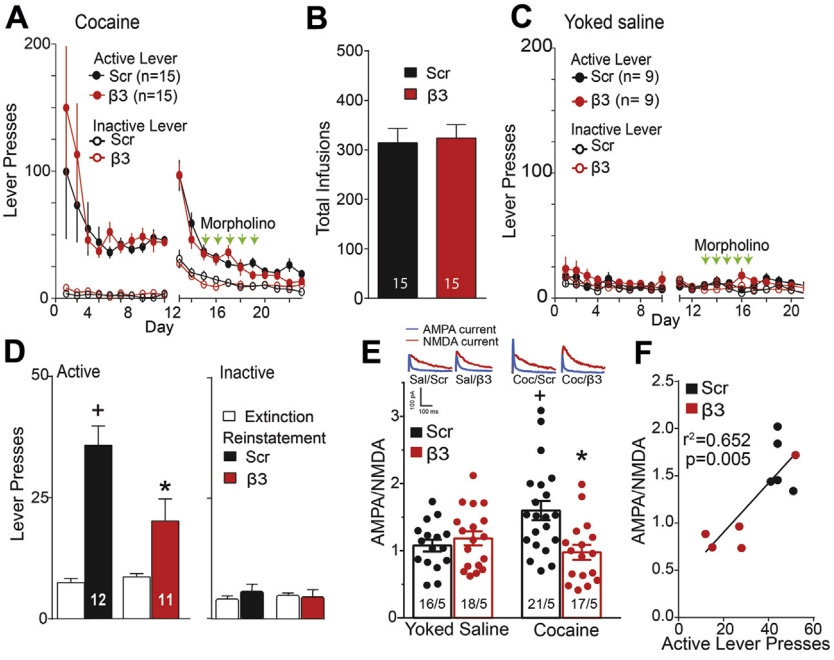

Figure 3. β3 integrin knockdown prevented synaptic potentiation initiated by cued cocaine seeking. **(A)** Time course of active and inactive lever pressing for self-administration and extinction in cocaine-treated rats (Coc) used for amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) to *N*-methyl-D-aspartate (NMDA) (A/N) ratio (Figure 3) and spine morphology measurement (Figure 4) was equivalent between treatment groups. **(B)** An equivalent number of infusions taken by each treatment group. **(C)** Time course of active and inactive lever pressing for yoked-saline (Sal) and extinction in rats used for A/N and spine morphology measurement. **(D)** Cue-induced reinstatement of cocaine seeking increased lever presses over 15 minutes before rats were euthanized for morphological measurements and A/N ratio measurements, and this was reduced by β3 integrin morpholino (extinguished/reinstatement $F_{1,21}$ = 48.81, $p < .001$; scrambled sequence [Scr]/β3 $F_{1,21}$ = 4.78, $p = .040$; interaction $F_{1,21}$ = 8.66, $p = .008$). **(E)** Two-way analysis of variance revealed elevated A/N ratio during 15-minute cue-induced reinstatement in Scr morpholino treated rats compared with β3 morpholino and Sal animals (Scr/β3 $F_{1,68}$ = 4.647, $p < .05$; Sal/Coc $F_{1,68}$ = 1.743, $p = .191$; interaction $F_{1,68}$ = 9.297, $p = .010$). *n* in bars represents the number of cells recorded over number of animals in each condition. Representative traces are shown for each treatment. Data are shown as mean ± SEM. **(F)** The A/N ratio measured after 15 minutes of cued reinstatement was correlated with the number of active lever presses. $^{*}p < .05$ compared with Scr/reinstatement using a Bonferroni post hoc test. $^{+}p < .05$ compared with extinguished or Scr/Sal.

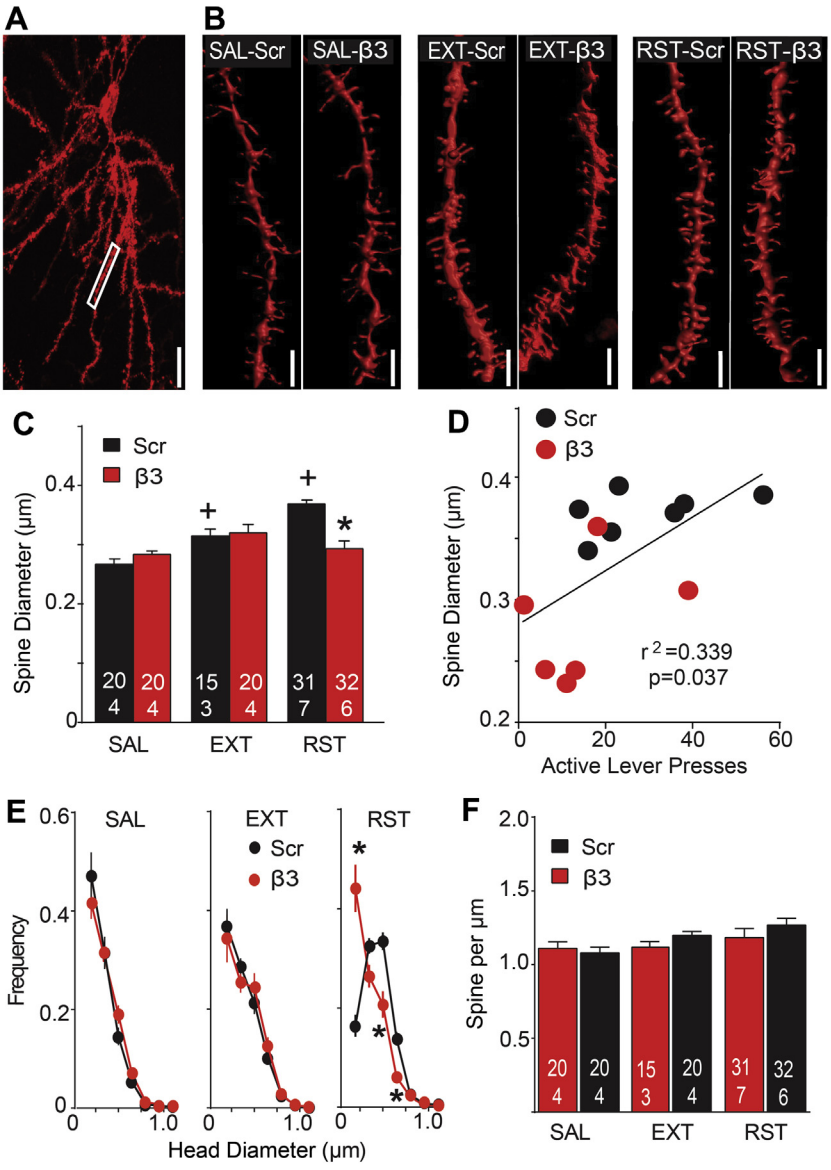

Figure 4. β3 integrin knockdown blocked increases in dendritic spine head diameter (d_h) during cue-induced cocaine seeking. **(A)** Representative DiI-filled medium spiny neuron. Box shows area sampled from this neuron for spine analysis. Scale bar = 20 μm. **(B)** Representative dendritic segments from each treatment group. Scale bar = 2 μm. **(C)** d_h was increased during 15-minute reinstatement and β3 morpholino treatment inhibited the increase in d_h. Nested analysis of variance revealed an overall interaction between groups for d_h ($F_{5,22} = 6.68$, $p < .001$). **(D)** Correlation between d_h and active lever presses during 15-minute cue-induced reinstatement. **(E)** The decreased d_h after β3 integrin treatment was associated with a shift in the frequency distribution compared with scrambled sequence (Scr) treatment only in reinstated (RST) animals (2-way analysis of variance with repeated measures over d_h [Scr/β3 $F_{1,61} = 0.26$, $p = .612$; d_h $F_{6,366} = 95.58$, $p < .001$; interaction $F_{6,366} = 20.61$, $p < .001$]). *n* in bars represents the number of neurons quantified over number of animals in each condition. Three to seven dendrite segments from separate neurons were analyzed from each animal. **(F)** No change was measured in spine density between any of the treatment groups. Data are shown as mean ± SEM. $^{*}p < .05$ compared with Scr/RST using Bonferroni post hoc. $^{+}p < .05$ compared with Scr/yoked-saline (SAL) in each morpholino group. EXT, extinguished.

morpholino (Figure 3E). Supporting a relationship between β3 integrin knockdown and A/N ratio in regulating cued reinstatement, increased A/N ratio was correlated with the number of reinstated lever presses (Figure 3F).

β3 Integrin Knockdown Blocked Cue-Induced t-SP: Spine Morphology

Self-administration, extinction, and cued reinstatement data are shown in Figure 3 together with rats used for measuring labeled neurons A/N ratio. To quantify spine density and d_h, Z-series confocal images were made of diolistically labeled neurons in the NAcore (Figure 4A and Supplemental Figure S1). Cued cocaine seeking increased d_h in the reinstated compared with the EXT or SAL rats that were pretreated with Scr morpholino, and the increase in d_h during reinstatement was abolished in rats that were pretreated with β3 morpholino (Figure 4B, C). Supporting a relationship between β3 integrin knockdown and d_h in regulating cued reinstatement, reinstated lever pressing was positively correlated with d_h (Figure 4D). Analysis of d_h frequency distribution revealed no effect of β3 morpholino in SAL or EXT rats. However, the decrease in thin spines ($d_h < 0.20$ μm) and increase in thick spines ($0.35 > d_h < 0.65$ μm) elicited during cued reinstatement in rats pretreated with Scr control was eliminated after β3 morpholino, resulting in a d_h frequency distribution equivalent to SAL and EXT rats (Figure 4E). No difference in spine density was found between any of the treatment groups (Figure 4F).

Inhibiting FAK Phosphorylation Prevented Cue-Induced Drug Seeking

Two kinases mediating β3 integrin signaling are FAK and ILK. The phosphorylation of FAK on Tyr397 (31) or ILK at

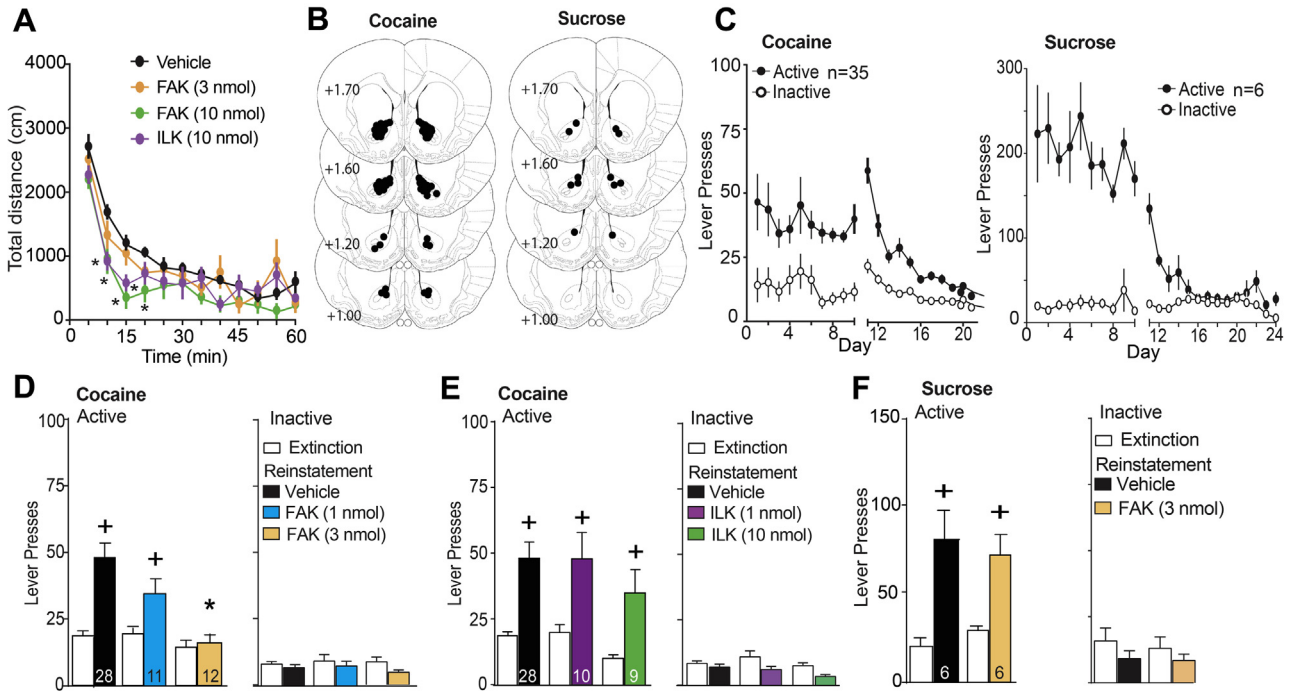

Figure 5. Inhibiting focal adhesion kinase (FAK), but not integrin-linked kinase (ILK), prevented cue-induced cocaine and heroin seeking but not sucrose seeking. **(A)** Novel open field locomotor activity revealed decreased activity after the highest doses of FAK inhibitor (FAKi) or ILK inhibitor (ILKi) (10 nmol) microinjected 10 minutes before the test compared with vehicle (two-way analysis of variance repeated measures over time, inhibitor $F_{3,21} = 4.27$, $p = .017$; time $F_{11,231} = 44.43$, $p < .001$; interaction $F_{33,231} = 4.61$, $p = .017$). Intra–core subcompartment of the nucleus accumbens (NAcore) FAKi (3 nmol) microinjection did not alter locomotor activity. **(B)** Histological verification of NAcore guide cannula for cocaine and sucrose-seeking experiments. Circles drawn on the modified stereotaxic atlas indicate the location of the tip of the microinjector (2 mm below the end of the guide cannula). Numbers refer to the millimeters from the bregma. **(C)** Time course of active and inactive lever pressing for self-administration and extinction in cocaine- and sucrose-treated rats. **(D)** FAKi bilateral microinjection into the NAcore prevented cue-induced cocaine seeking (2-way analysis of variance, repeated measures over extinction/reinstatement, dose $F_{2,48} = 8.60$, $p < .001$; extinguished/reinstated $F_{1,48} = 16.57$, $p < .001$; interaction $F_{2,48} = 5.54$, $p = .007$). No statistical difference was observed in inactive lever presses during cued-induced cocaine-seeking after vehicle or FAKi microinjections in the NAcore (left panel). **(E)** ILKi microinjection into the NAcore did not prevent cocaine seeking (dose $F_{2,44} = 1.93$, $p = .16$; extinguished/reinstated $F_{1,44} = 30.50$, $p < .001$; interaction $F_{2,44} = 0.08$, $p = .92$). No statistical difference was observed in inactive lever presses during cue-induced cocaine-seeking after vehicle or ILKi microinjections in the NAcore (left panel). **(F)** FAKi (3 nmol) microinjection in the NAcore did not prevent the cue-induced reinstatement in sucrose-trained rats (vehicle/FAK $F_{1,10} = 0.01$, $p = .99$; extinguished/reinstated $F_{1,10} = 34.96$, $p < .001$; interaction $F_{1,10} = 1.049$, $p = .33$). No statistical difference was observed in inactive lever presses during cued-induced cocaine-seeking after vehicle or FAKi microinjections in the NAcore (left panel). Rats were reinstated two or three times each in random order with vehicle and one or two doses of inhibitor. *n* in bars represents the number of animals in each condition. Data are shown as mean ± SEM. $^{+}p < .05$ compared with extinguished using a Bonferroni post hoc test. $^{*}p < .05$ compared with vehicle using a Bonferroni post hoc test.

Ser473 (32) activates the kinases, and enzyme selective small molecule inhibitors of phosphorylation were used to determine which kinase mediated the capacity of β3 integrin to initiate t-SP and reinstate cocaine seeking. An initial study conducted in drug-naïve rats determined that bilateral microinjection of an FAK inhibitor or ILK inhibitor into the NAcore reduced locomotor activity at the highest dose tested (10 nmol/side) (Figure 5A). Accordingly, lower doses were evaluated for effects on cued drug seeking to avoid motor depression. Rats were implanted with microinjection cannulas over the NAcore (Figure 5B and Supplemental Figure S4B for heroin) and trained to self-administer cocaine, sucrose, or heroin, and the behavior was then extinguished (Figure 5C and Supplemental Figure 4A). Using a randomized crossover design between vehicle and FAKi treatment, NAcore microinjection of FAKi (1 or 3 nmol/side) 10 minutes before initiating cued reinstatement dose-dependently inhibited active lever pressing in cocaine-trained rats (Figure 5D), without altering inactive lever responding (Figure 5D) or locomotor activity in an open field (Figure 5A). The specificity of FAK involvement in cued drug seeking was further demonstrated by showing that FAKi (3 nmol/side) microinjection also reduced cued heroin seeking but not cue-induced reinstatement of sucrose seeking (Figure 5G and Supplemental Figure S2C).

The bilateral microinjection of ILKi into the NAcore 10 minutes before cued-induced cocaine reinstatement did not alter reinstated cocaine seeking compared with vehicle injection (Figure 5E), even at a dose (10 nmol) that decreased locomotor activity (Figure 5A). Inactive lever pressing was not altered by any dose of ILKi (Figure 5E). Taken together, these data are consistent with FAK signaling, but not ILK signaling, being downstream from MMP-9 and β3 integrin in mediating cue-induced reinstatement of cocaine and heroin, but not of sucrose seeking.

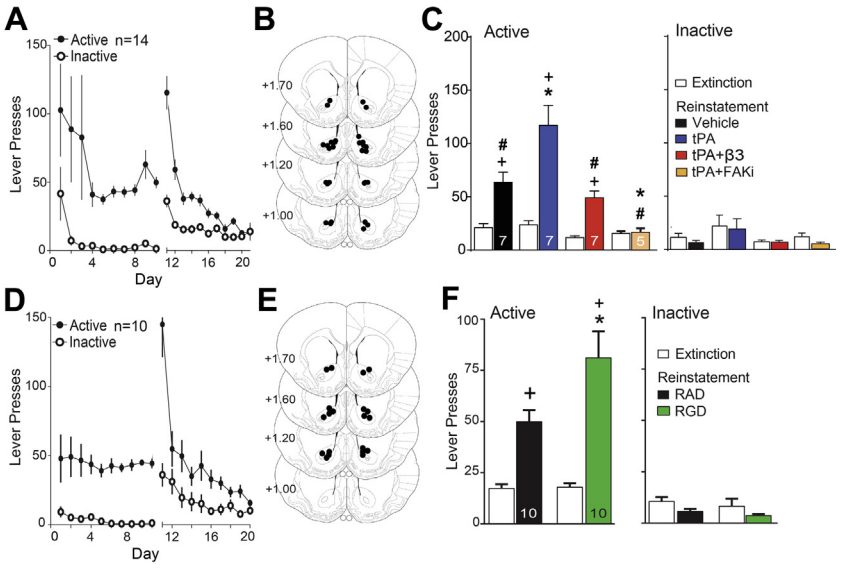

Figure 6. Stimulating matrix metalloproteinases with tissue plasminogen activator (tPA) or integrins with RGD peptide potentiated cue-induced cocaine seeking. **(A)** Time course of active and inactive lever pressing for self-administration and extinction in cocaine-treated rats for tPA experiment. **(B)** Location of microinjection cannula tips in the core subcompartment of the nucleus accumbens (NAcore) for all rats used in Figure 6C. Numbers refer to millimeters to the bregma. **(C)** tPA bilateral microinjection into the NAcore potentiates cue-induced cocaine seeking (two-way analysis of variance, repeated measures over extinction/reinstatement, vehicle/tPA/β3/focal adhesion kinase inhibitor [FAKi] $F_{3,22} = 10.05$, $p < .001$; extinguished/reinstated $F_{1,22} = 40.66$, $p < .001$; interaction $F_{3,22} = 6.99$, $p = .002$) and β3 integrin or FAKi microinjections in the NAcore prevented tPA-induced potentiation in cued-cocaine seeking. No statistical difference was observed in inactive lever presses during cued-induced cocaine-seeking after vehicle, tPA, β3 integrin, or FAKi microinjections in the NAcore. **(D)** Time course of active and inactive lever pressing for self-administration and extinction in cocaine-treated rats for RAD/RGD experiment. **(E)** Location of microinjection cannula tips in the NAcore for rats used in Figure 6F. **(F)** RGD (1 nmol) microinjection in the NAcore potentiates cue-induced reinstatement in cocaine-trained rats (RAD/RGD $F_{1,18} = 4.52$, $p = .047$; extinguished/reinstated $F_{1,18} = 47.31$, $p < .001$; interaction $F_{1,18} = 4.81$, $p = .042$). No statistical difference was observed in inactive lever presses during cue-induced cocaine-seeking after RAD or RGD microinjections in the NAcore. $^{+}p < .05$ compared with extinguished using a Bonferroni post hoc test. $^{*}p < .05$ compared with vehicle or RAD using a Bonferroni post hoc test. $^{\#}p < .05$ compared to tPA treatment in **(C)** using a Bonferroni post hoc test.

MMP Activity Potentiated Cue-Induced Reinstatement by Signaling Through β3 Integrin

Drug cues increase MMP-9 activity in the NAcore, and MMP-9 inhibition prevents both t-SP and cued reinstatement (8). MMP-9 is a gelatinase that catalytically liberates RGD peptide sequences from brain ECM proteins, and RGD is a high-affinity integrin binding motif (13). To further link MMP-9 activity with β3 integrin signaling, we used tPA to activate MMP-9 (23,24). Gelatinase (MMP-2,9) activity was quantified using in vivo zymography after fluorescein isothiocyanate–quenched gelatin

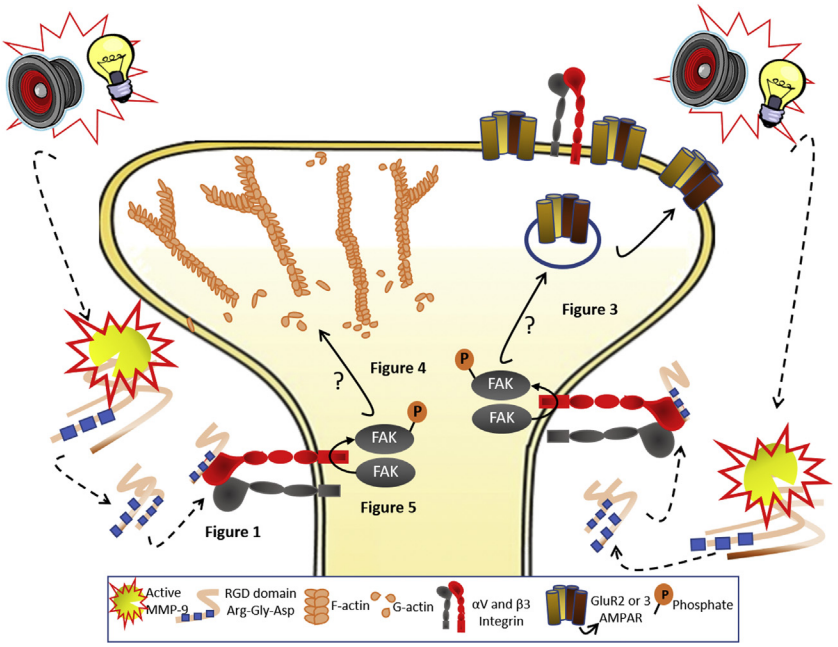

Figure 7. Schematic illustration of the matrix metalloproteinase-9 (MMP-9) to β3 integrin to focal adhesion kinase (FAK) signaling pathway in the core subcompartment of the nucleus accumbens that is proposed to mediate increases in transient excitatory synaptic potentiation and that is necessary for cue-induced cocaine seeking. Drug-paired cues (tone and light) increase MMP-9 activity through the spillover of synaptic glutamate and increased nitric oxide production [not shown, see (4,50)]. MMP-9 catalytically exposes RGD binding domains during 15 minutes of cued cocaine seeking. RGD binds to and stimulates the β3 integrin subunit to phosphorylate FAK, which initiates spine head expansion and increases in amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) to *N*-methyl-D-aspartate ratio. How FAK signals to transient excitatory synaptic potentiation was not studied (indicated by the question marks), but FAK promotes LIM kinase phosphorylation (51) to inactive cofilin via Ser3 phosphorylation and thereby promote elongation of F-actin to increase d_h. In addition, β3 integrin–dependent increases in AMPA currents may arise from stabilizing GluA2 subunit–containing AMPA receptors (AMPARs) through a protein complex between β3 and GluA2 (19). P, phosphorylation.

microinjection into the NAcore in drug-naïve animals. tPA produced dose-dependent increases in fluorescence that were abolished by intra-NAcore pretreatment with an MMP-9 inhibitor (Supplemental Figure S5). Rats were trained to self-administer cocaine, and the behavior was then extinguished (Figure 6A). The lowest dose of tPA (1 ng) that increased gelatinase activity was microinjected bilaterally into the NAcore (Figure 6B). tPA microinjection potentiated cue-induced cocaine seeking compared with vehicle, and the potentiated response was inhibited when rats were pretreated with β3 integrin morpholino or FAKi (Figure 6C). The partial inhibition by β3 morpholino may arise from tPA's producing effects in addition to activating the MMP9-β3-FAK signaling cascade (23,24) or because morpholino treatment did not abolish protein expression (Figure 1B, C). There was no effect of tPA, β3 morpholino, or FAKi on inactive lever responding (Figure 6C).

We also modulated β3 integrin directly using the RGD peptide integrin ligand that is liberated by MMP-2,9-mediate catalysis and that produces long-term potentiation—like changes in excitatory postsynaptic currents and spine morphology (33–36). After cocaine self-administration and extinction (Figure 6D), rats were randomly assigned to a crossover design for cue-induced reinstatement testing. Animals were microinjected 10 minutes before the reinstatement session with either RAD peptide control or RGD into the NAcore (Figure 6E). RGD (1 nmol) potentiated cue-induced cocaine seeking compared with RAD microinjections without altering inactive lever responding (Figure 6F). This dose of RGD does not affect locomotor activity (17).

DISCUSSION

The capacity of cues associated with addictive drug use to induce drug seeking contributes to relapse vulnerability (1). Cue-induced drug seeking in animal models of relapse causes t-SP in NAcore MSNs that is correlated with the intensity of behavioral responding for the cue (5,8) and is produced only by addictive drug–associated cues, not sucrose cues (4). These discoveries point to the importance of understanding how signaling in the NAcore initiated by cues is translated into t-SP. Activation of the extracellular enzyme MMP-9 is initiated by synaptic glutamate spillover during cued cocaine seeking and is necessary for t-SP (8). We show that MMP-9 signaling through β3 integrin to FAK is required for t-SP and cue-induced cocaine and heroin seeking but not for sucrose seeking. The proposed signaling cascade between MMP-9 activation by drug cues and t-SP is shown in Figure 7.

β1 and β3 Integrins in Synaptic Plasticity and Addiction

Heterodimeric integrins are mediators of MMP-catalyzed signaling in the ECM, and integrin binding induces both morphological and electrophysiological plasticity at excitatory synapses (37). The two primary β integrin subunits in brain, β1 and β3 integrin, determine intracellular signaling pathways, while α subunits determine extracellular binding partners. The β3 subunit exclusively pairs with the αV subunit, while the β1 subunit pairs promiscuously with different α subunits, including αV (38). Activated MMP-9 catalytically creates RGD-containing peptide ligands that are selective for integrins containing the αV subunit (39). Many studies have shown that β1 integrin stimulation regulates spine and dendrite morphology (20,40) and NMDA or AMPA plasticity (37,41–43). Moreover, MMP-9 activity can signal through β1 integrin to modulate spine morphology—although the primary effect is a decrease in d_h and spine density (44), not the increase observed during cue-induced t-SP. β1 integrins have been linked to the effects of addictive drugs in three preclinical studies. β1 integrin is necessary for the enduring increases in accumbens spine density produced by chronic noncontingent methamphetamine administration (45), knockdown of β1 integrin signaling augments cocaine-induced locomotion (40), and withdrawal from chronic noncontingent cocaine is associated with elevated β1 integrin in the nucleus accumbens (16). Given this literature, we were surprised that knocking down β3 integrin, not β1 integrin, prevented cue-induced reinstatement of cocaine seeking and the associated t-SP in NAcore MSNs. In fact, consistent with augmenting cocaine locomotion (40), knockdown of β1 integrin in the NAcore potentiated cued reinstatement, possibly resulting from compensatory upregulation of the β3 subunit and ILK.

Finding obligatory involvement of β3 integrin in cue-induced t-SP and cocaine seeking is consistent with studies showing that, like β1 integrins, β3 integrins modulate some forms of synaptic plasticity. For example, modulating β3 integrin levels affects spine maturation and homeostatic synaptic scaling, as well as GluA2 AMPA receptor expression in the hippocampus (19,34,46). The importance of β3 integrins over β1 integrins in cue-induced t-SP could reflect that the literature is largely derived from experimentation in the hippocampus, and perhaps β3 integrins are more preferentially involved in NAcore synaptic plasticity. Given the relative abundance of β1 over β3 protein in the NAcore (16), this seems unlikely. An intriguing possibility is that β1 integrins signal the induction of more enduring forms of synaptic plasticity, while β3 integrins are more involved in transient forms of plasticity, such as the cue-induced t-SP mediating cocaine seeking (37,46). For example, rats trained to self-administer cocaine show transient fluctuations in β3 integrin, not β1 integrin, during reinstated cocaine seeking (17). In addition, β3 integrin is involved in regulating homeostatic synaptic scaling, while β1 integrin is more frequently associated with long-term potentiation (46). Finally, β1 integrins are involved in enduring hippocampal plasticity and memory consolidation, while β3 integrins regulate transient acute stress responses (37,47).

β3 Signaling Through FAK, but Not ILK, in t-SP and Reinstated Drug Seeking

β3 integrin signaling through either ILK or FAK can link integrin signaling to the actin cytoskeleton and modulate synaptic plasticity (21,32). In contrast to the selective role for FAK in β3 integrin signaling cocaine reinstatement, cocaine behavioral sensitization studies implicate ILK in both augmented locomotion and increases in NAcore spine density after withdrawal from daily noncontingent injections of cocaine (48,49). The differences in kinase involvement may arise from using distinct models of addiction (sensitization vs. cued drug seeking). This is consistent with the hypothesis above that the β3-FAK

cascade signals transient plasticity, such as t-SP induced by drug cues, while the β1-ILK cascade signals enduring plasticity, such as the increased spine density produced by daily cocaine injections. This can also account for the lack of effect by β3 integrin knockdown on spine morphology or A/N ratio in SAL rats, because the knockdown is long lasting over the course of days, rather than being the effect of cue on behavior, which typically endures for 60 minutes.

Conclusions

We found that t-SP and cue-induced cocaine seeking requires MMP-9 signaling into MSNs via the β3-FAK signaling cascade. Drug-associated cues can promote relapse to drug use in human addiction. Identifying β3 integrin to FAK signaling as a necessary component of the cascade that mediates cue-induced cocaine and heroin, but not sucrose seeking, provides new potential pharmacological targets for treating addiction.

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CG-K and PWK designed the research, CG-K, DN, A-CB, SS, VCC, and CM performed the research, CG-K and PWK analyzed the data, and CG-K and PWK wrote the article.

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