Archival Report

Biological Psychiatry: GOS

ERK2 Signaling in the Nucleus Accumbens Facilitates Stress Susceptibility and Cocaine Reinstatement

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ABSTRACT

BACKGROUND: Second-messenger signaling within the mesolimbic reward circuit plays a key role in the negative effects of stress and the underlying mechanisms that promote drug abuse. Because the nucleus accumbens (NAc) integrates reward valence, we investigated how ERK2 (extracellular signal-regulated protein kinase-2) signaling affects the development of stress-related comorbidities, including negative affect and drug sensitivity.

METHODS: We assessed how chronic unpredictable stress influenced the phosphorylation of ERK2-signaling proteins within the NAc of male Sprague Dawley rats. Using a herpes simplex virus, we either upregulated or downregulated NAc ERK2 activation and evaluated behavioral responses to stress-eliciting stimuli (elevated plus maze, open field, forced swim test) and cocaine-seeking behavior (conditioned place preference, self-administration). We also examined ERK2-mediated modifications in spine morphology of medium spiny neurons within the NAc.

RESULTS: Chronic unpredictable stress increased the phosphorylation of ERK2-signaling proteins within the NAc. Viral-mediated activation of NAc ERK2 enhanced susceptibility to both depression- and anxiety-related stimuli and increased cocaine-seeking behavior (conditioned place preference and reinstatement). These behavioral changes were associated with an increase in stubby and mushroom spines of NAc medium spiny neurons. Conversely, downregulation of ERK2 activation attenuated affect-related behavioral responses in the forced swim test and blunted cocaine's rewarding effects without influencing NAc spine morphology.

CONCLUSIONS: NAc ERK2 contributes to stress-induced behavioral deficits, including anxiety- and depression-like phenotypes, while promoting cocaine-seeking behavior. These findings suggest that ERK2 signaling in the NAc plays a role in the comorbidity of these related syndromes.

https://doi.org/10.1016/j.bpsgos.2024.100416

The mesocorticolimbic dopamine system, which includes the ventral tegmental area (VTA), nucleus accumbens (NAc), and prefrontal cortex, primarily mediates the rewarding and reinforcing effects of drugs of abuse (1–3). However, when dysregulated by stress, this system also contributes to behavioral maladaptation (4). Clinical evidence shows that perturbations in NAc function are common in individuals chronically affected by affective disorders (5) and substance use disorders (6,7). Interestingly, dopamine release from the VTA to the NAc occurs in response to both rewarding and aversive stimuli (8,9), making this circuit complex and capable of mediating maladaptive behaviors. Major depressive disorder and drug use frequently co-occur in a bidirectional manner: drug abuse increases the risk of developing mood disorders, and individuals with major depressive disorder are at higher risk of developing drug-seeking behaviors (10,11). This suggests that stress-induced mechanisms may overlap, promoting alterations in both mood and drug reward (i.e., cross-sensitization). This perspective is supported by rodent models of stress-induced mood disorders and drug-taking behavior (9,12–15). For example, a priming injection of cocaine or exposure to an acute stressor can reinstate drug-seeking behavior in rodents that had previously extinguished such behaviors (16,17). Furthermore, chronic stress induces persistent molecular and cellular changes that resemble those observed after prolonged exposure to various drugs of abuse (18–21).

While the precise mechanisms that underlie these similarities remain elusive, second-messenger signaling pathways, including ERK (extracellular signal-regulated protein kinase), have been consistently implicated in these processes (22–24). ERK signaling in the VTA and other brain regions is well-understood (23,25,26), with studies revealing its pivotal role in cocaine-induced neuroplasticity and behavioral adaptations (27). In particular, ERK activation in the striatum is essential for cocaine-induced locomotor sensitization (28–30). Because NAc function is critical for encoding drug valence and driving drug-seeking behaviors (31), assessing ERK modulation in this area is key to understanding affect-related behaviors and drug sensitivity, particularly in cocaine addiction.

ISSN: 2667-1743

One of the key neuronal populations within the NAc are medium spiny neurons (MSNs), which are highly responsive to both stress and drug exposure. MSNs play a crucial role in mesocorticolimbic communication, and their morphology can be altered by external stimuli including chronic stress and drug use (32). These alterations can affect the overall function of the NAc, influencing behaviors associated with mood and addiction. Therefore, investigating how ERK signaling impacts MSNs is critical to understanding how molecular and structural changes in the NAc mediate these behaviors.

ERK has 2 main isoforms, ERK1 and ERK2, which are thought to have distinct functions in the brain, although their specific roles remain incompletely characterized. This lack of differentiation is largely due to the significant genetic similarity between the isoforms and the current limitations of pharmacological tools to distinguish between them (33,34). Nevertheless, several studies suggest that ERK2 is more directly involved in drug-related mechanisms than ERK1 (23,25,35,36). Additionally, ERK2 has been implicated in stress adaptation and is considered an integral mediator of effective antidepressant treatment, which further supports its role in modulating affective-related behaviors (25,26,37,38).

The goal of the current study was to investigate how direct manipulation of ERK2 activity within the NAc influences affect-related behavioral responses and sensitivity to drugs of abuse, namely cocaine. We also examined how ERK2 modulation impacts the morphology of MSNs in the NAc, given their central role in mesolimbic communication and behavior.

METHODS AND MATERIALS

Animals

Male Sprague Dawley rats weighing 275 to 300 g upon arrival (Charles River Laboratories) were used in this study. The rats were housed in clear polypropylene cages with wood shavings in an animal colony maintained at 23 °C to 25 °C under a 12-hour light/dark cycle (lights on from 7:00 AM–7:00 PM). Food and water were provided ad libitum. All procedures strictly followed the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (39) and were approved by the Institutional Animal Care and Use Committee. For further details on experimental group assignments, please refer to Table 1.

Chronic Unpredictable Stress

Previous studies have shown that chronic unpredictable stress (CUS) increases activation of the ERK2-signaling cascade in the VTA of adult male rats (25). To assess whether 4 weeks of CUS would similarly alter the phosphorylation of ERK1/2 and their downstream substrates, MSK (mitogen- and stress-activated kinase-1) and p90-RSK (90 kDa ribosomal S6-kinase), we employed a comparable approach in the NAc. Specifically, rats were exposed to 1 stressor per day in a randomized manner for 4 weeks, ensuring that there was no acclimation to the stress schedule as previously described (25,40) (Figure 1). This detail is crucial because the controllability of stressors has been shown to significantly influence the deleterious effects of stress (41) (Figure 1). The stressors included alternating periods of food or water deprivation (overnight), continuous cage shaking (1 hour on an automatic shaker), social isolation (24 hours), forced swim stress (FST) (15 minutes in 18 °C water; limited to 3 exposures), continuous overnight illumination (12 hours), overnight cage flooding (12 hours), exposure to cold temperature (1 hour at 4 °C), and acute restraint stress (1 hour) using plastic restraint bags (DecapiCones) as previously described (25,40).

Table 1. Summary of Experimental Groups

Experimental Group	Intervention	*n* per Group	Experimental Groups
Stress Exposure	CUS	9–10	CUS and control
Viral Manipulations: Behavioral Testing Beginning 3 Days Postinfusion			
Cohort 1A	EPM, OFT, FST days 1 and 2[a]	11–12	HSV-GFP, HSV-wtERK2, HSV-dnERK2
Cohort 1B	Locomotion[a]	4–5	HSV-GFP, HSV-wtERK2, HSV-dnERK2
Cohort 3	CPP	6–8	HSV-GFP, HSV-wtERK2, HSV-dnERK2
Cohort 4	Self-administration	8	HSV-GFP, HSV-wtERK2, HSV-dnERK2
Cohort 5	Spine analysis	5–6	HSV-GFP, HSV-wtERK2, HSV-dnERK2

CPP, conditioned place preference; CUS, chronic unpredictable stress; EPM, elevated plus maze; FST, forced swim test; GFP, green fluorescent protein; HSV, herpes simplex virus; OFT, open field test.

[a]Performed on day 4 postviral infusion.

Western Immunoblots

Protein from NAc tissue punches was isolated using an Illustra TriplePrep kit (GE Healthcare) according to the manufacturer's instructions and stored at −80 °C until use. See Supplemental Methods and Materials and Table S1 for more details regarding the antibodies used.

Viral Transfection Surgery, Histology, and Transgene Detection

Viral transfection was performed via bilateral microinjections (1.0 μL per side over 10 minutes) of a herpes simplex virus (HSV) vector encoding GFP (green fluorescent protein), an overexpression vector (HSV-wtERK2, hereafter referred to as wtERK2), or a dominant negative HSV-dnERK2 (hereafter referred to as dnERK2) into the NAc at coordinates (anteroposterior: +1.7, lateral: ±2.5, dorsoventral: −6.7 mm from bregma), angled at 10° from the midline. To confirm viral infusion into the NAc, we verified HSV expression within the targeted region (Figure 2A). Behavioral testing began 3 days after surgery, during the time of maximal transgene expression (42) (Table 1). Representative images of targeting and infusion can be seen in Figure 2A. Please see Supplemental Methods and Materials for full details.

Elevated Plus Maze

The elevated plus maze (EPM) was used to assess anxiety-like behavior (43). Briefly, at the start of the test (day 3 postviral infusion), rats were positioned in the central area facing one of the open arms and were allowed to explore freely for 5

Chronic Unpredictable Stress (CUS): Sample Schedule		
Day	Stressor	Duration
1	Forced swimming	15 min
2	Wet bedding	Overnight
3	Overcrowding	1 hour
4	Cold box	1 hour
5	Isolation	Overnight
6	Wet bedding	Overnight
7	Lights on	Overnight
8	White noise	Overnight
9	Food deprivation	Overnight
10	Restraint	1 hour

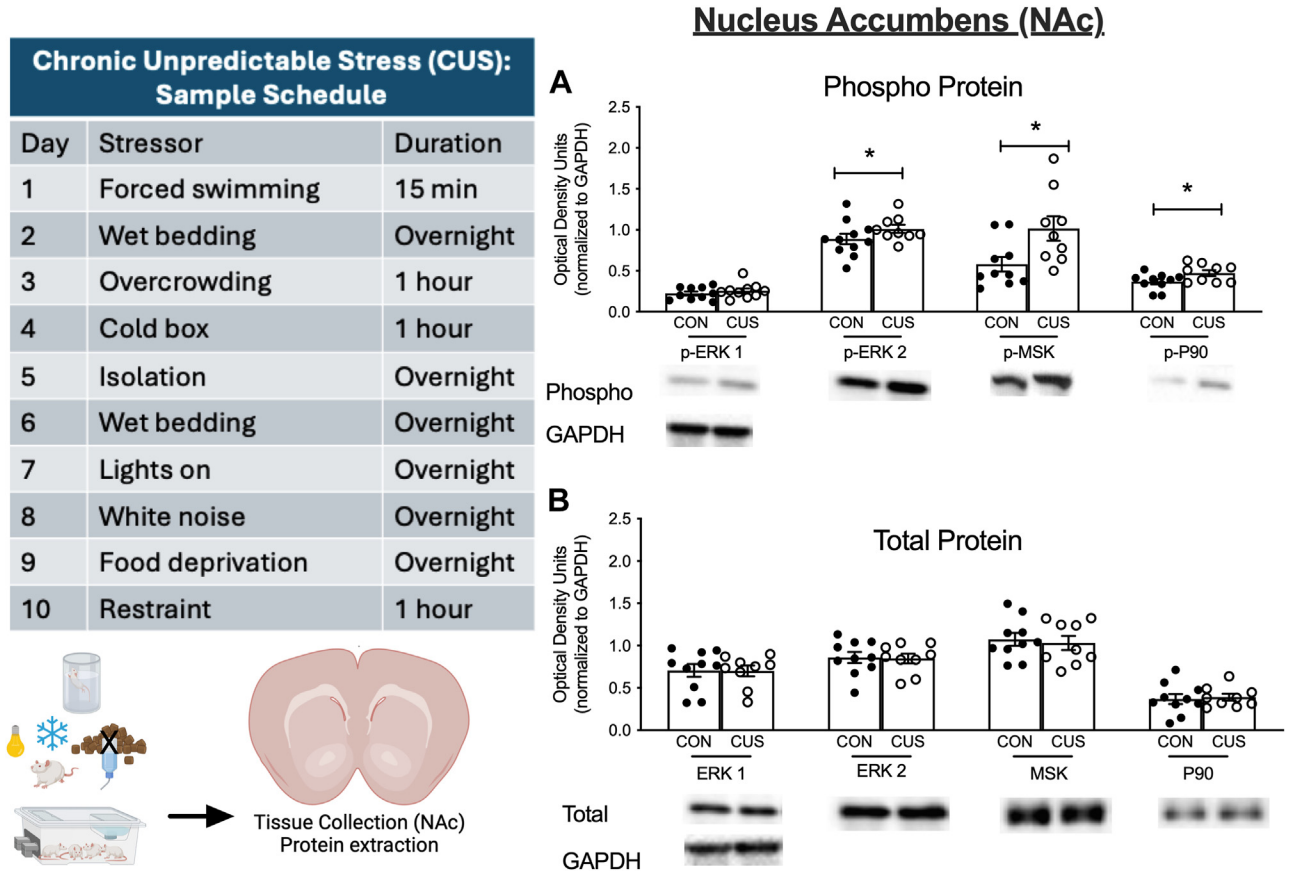

Figure 1. CUS (n = 9–10/group) increases phosphorylation of ERK2-related proteins in the NAc of adult male Sprague Dawley rats. Rats were exposed to 4 weeks of randomized stressors, including overcrowding, sleep disruption (light cycle changes, cage tilting), cold exposure, and water or food deprivation. **(A)** NAc tissue punches were collected 24 hours after the final behavioral test (forced swim test) to assess levels of phosphorylated ERK1/2, MSK, p90-RSK, and GAPDH. CUS significantly increased phosphorylated ERK2, MSK, and p90-RSK levels compared with nonstressed controls, while total protein levels remained unchanged **(B)**. Protein levels were normalized to their respective GAPDH values. *Significantly different from CON, $p < .05$. CUS, chronic unpredictable stress; CON, nonstressed control; ERK, extracellular signal-regulated kinase; MSK, mitogen and stress activated kinase-1; P90, 90 kDa ribosomal S6-kinase.

minutes. Time spent in the open arms and total distance traveled were measured using Noldus Ethovision XT. Full details are provided in Supplemental Methods and Materials.

Open Field Test

As a second measure of anxiety-like behavior, a separate group of rats was placed in an open field box (63 × 63 cm; Tru Scan; Coulbourn Instruments) 3 days after viral infusion. The rats were left in the open field test (OFT) for 10 minutes, during which the time spent in the center of the apparatus (31 × 31 cm) and total distance traveled were recorded. Anxiety-like behavior was determined by comparing the time spent exploring the periphery of the arena to the time spent in the center.

Forced Swim Test

The FST is a 2-day procedure designed to assess depression-like behavior (44). On day 1, rats were placed in plastic cylinders (75 × 30 cm) filled with 25 °C water and forced to swim for 15 minutes (3 days postviral infusion). Initially, they engaged in escape-like behaviors but eventually adopted a posture of immobility, making only the movements necessary to keep their head above water. After the swim, rats were removed from the water, dried, and placed in a warm enclosure for 30 minutes. All cylinders were emptied and rinsed between trials. On day 2 (24 hours later), rats were retested for 5 minutes under identical conditions (4 days postviral infusion). The latency to become immobile and total immobility were measured (45). Latency to immobility was defined as the time at which the rat first adopted a stationary posture and stopped attempting to escape. To qualify as immobility, the rat's posture had to be clearly visible and sustained for ≥2.0 seconds (46).

Basal Locomotor Activity After Day 1 of Forced Swimming

To control for potential confounding effects on FST performance, spontaneous locomotor activity was measured 24 hours after the first FST exposure (day 4 postviral infusion). Rats were allowed to explore an automated open field box (as described above) for 60 minutes, and the total distance traveled was recorded (47,48).

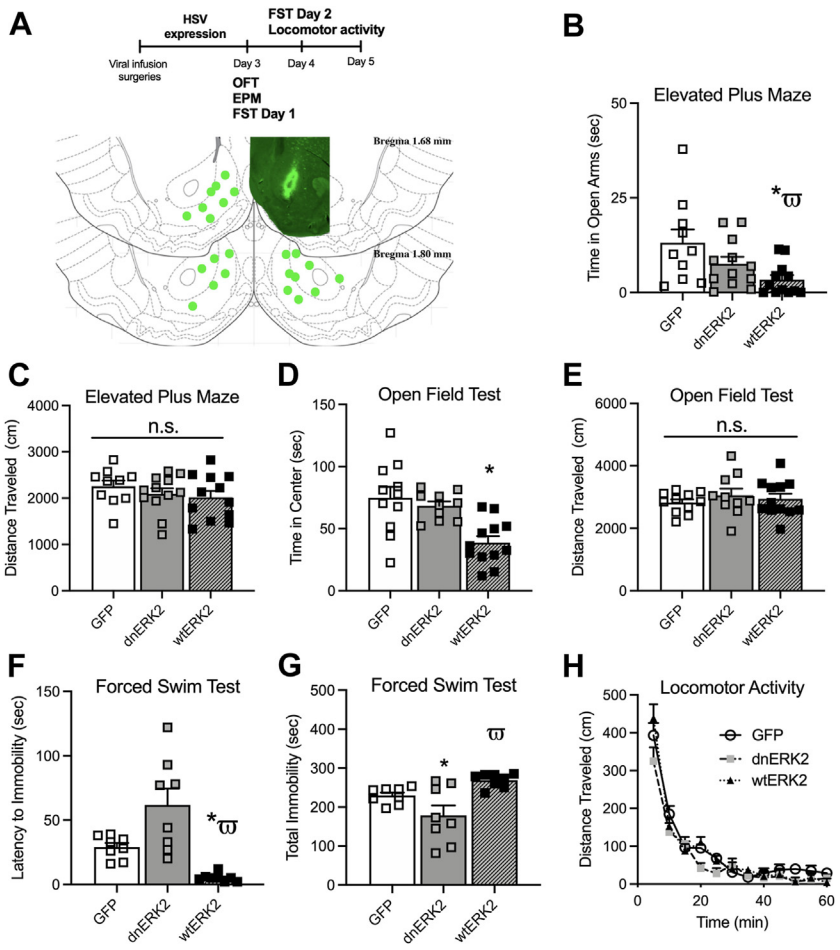

Figure 2. Virally mediated activation of ERK2 in the nucleus accumbens regulates anxiety- and depression-like behavior. Testing timeline and schematic of surgical placements within the nucleus accumbens **(A)**. In the elevated plus maze, wtERK2 significantly decreased amount of time spent in the open arms **(B)** without influencing total distance traveled **(C)**. In the open field test, wtERK2 significantly decreased amount of time spent in the center **(D)** without influencing total distance traveled **(E)**. Interestingly, on day 2 of the FST, rats infused with wtERK2 showed a significantly shorter latency to immobility compared with both GFP- or dnERK2-infused rats **(F)**, with wtERK2 infusion promoting a corresponding increase in total immobility. **(G)** No differences were found in total locomotion in rats infused with either dnERK2 or wtERK2 when tested in an open chamber locomotion test **(H)**. n = 8–12/group; $^{*}p < .05$ compared with GFP-infused rats, $^{\omega}p < .05$ compared with dnERK2-infused rats; error bars indicate + SEM. EPM, elevated plus maze; ERK, extracellular signal-regulated kinase; FST, forced swim test; GFP, green fluorescent protein; HSV, herpes simplex virus; n.s., not significant; OFT, open field test.

Conditioned Place Preference

Conditioned place preference (CPP) for cocaine was conducted in a 3-chamber apparatus (24). On the preconditioning day (day 0), rats were allowed to explore the entire apparatus for 30 minutes to establish baseline preferences for the compartments (side compartments: 35 × 27 × 25 cm; middle compartment: 10 × 27 × 25 cm). Rats that exhibited no spontaneous preference (unbiased procedure) were used, which accounted for over 90% of the animals. Bilateral HSV microinjections were administered into the NAc, and the rats recovered for 2 days before conditioning trials (days 3 and 4). During the conditioning trials, rats received an intraperitoneal saline injection (1 mL/kg) and were confined to one of the compartments of the apparatus for 30 minutes. After 3 hours, rats received cocaine (0.0, 5.0, or 10.0 mg/kg, intraperitoneally; Sigma-Aldrich) and were confined to the opposite-side compartment for 1 hour. Conditioning trials were counterbalanced such that half of the rats received the drug in one compartment, and the other half received the drug in the opposite compartment. On the final test day (day 5), rats were allowed to explore the apparatus freely for 30 minutes.

Intravenous Catheterization and Intracranial Cannulation

Jugular catheterization and intracranial cannulation were performed in a single procedure under halothane anesthesia (1%–2.5%). Silastic catheters pretreated with tridodecylmethyl ammonium chloride heparin were secured in the jugular vein using Mersilene surgical mesh. Immediately following the catheter implantation, each rat was then placed into a stereotaxic instrument, and bilateral guide cannula were inserted into the NAc (anteroposterior: +1.7, mediolateral: ±1.5, dorsoventral: −5.7; from bregma). Once inserted, the guide cannula was fixed in place with dental cement, and dummy stylets were placed into the guide cannula. Catheters were flushed daily with 0.1 mL heparinized saline, and rats recovered for 4 to 7 days before experiments began.

Cocaine Self-Administration, Extinction, and Reinstatement Procedures

Self-administration procedures were carried out in operant conditioning chambers (Med-Associates) equipped with 2 levers and an infusion pump. Rats were initially trained to lever press for sucrose pellets on a FR1 (fixed ratio 1) schedule to

facilitate acquisition of cocaine self-administration. Following surgery recovery, rats self-administered intravenous cocaine (0.5 mg/kg/100 μL injection) on a FR1 reinforcement schedule for 2 hours daily for at least 3 days. This was followed by a FR5 schedule for 5 additional days. Cocaine injections were delivered over 5 seconds concurrent with the illumination of a cue light above the active lever and was followed by a 15-second time out during which the house light remained off, and responding produced no consequence. Inactive lever responses produced no consequence throughout testing. After cocaine self-administration, rats underwent daily 6-hour extinction sessions. During extinction, responses on the lever previously paired with cocaine (drug-paired lever) and on the inactive lever were recorded but had no programmed drug or cue delivery. We tested the ability of wtERK2, dnERK2, or GFP (control) vectors to influence reinstatement of cocaine seeking using a repeated testing design. The day prior to reinstatement testing (day 15), rats received bilateral (2.0 μL/side) micro infusions of the vectors into the NAc over 10 minutes through a 33-gauge injection cannulae extending 1 mm beyond the guide cannula. The injectors were left in place for an additional 2 minutes to allow diffusion into the tissue. Reinstatement testing began 24 hours after the infusion and continued for 4 days during maximal expression. Each reinstatement session was initiated with 2 hours of extinction conditions followed by a 2-hour reinstatement test period. Cue-induced reinstatement was measured in the first reinstatement test by the noncontingent (priming) presentation of cocaine-associated cues delivered every 2 minutes for the first 10 minutes. Responding to the drug-paired lever resulted in response-contingent cue delivery throughout the 2-hour test. Cocaine-primed reinstatement was tested on the next 3 days by experimenter-administered vehicle or cocaine (5 and 15 mg/kg, intraperitoneally) immediately prior to the 2-hour reinstatement test in a counterbalanced order. Responses at both the drug-paired and inactive levers were recorded but produced no cue or drug delivery during testing.

Spine Density Analysis

For the spine analysis, rats were perfused with 4% paraformaldehyde 3 days after viral microinjections, and brain sections (100 μm) were prepared using a vibratome (Leica VT 1000s). Sections were incubated with an anti-GFP antibody (Millipore), mounted, coded (to blind the rater to experimental conditions), and imaged on a confocal microscope (Zeiss LSM 710). Secondary and tertiary dendrites of NAc MSNs were imaged under a 100× oil-immersion objective at a resolution of 0.027 × 0.027 × 0.3 μm. Seven to 10 neurons were imaged per animal (average total dendritic length = 400 um per animal, n = 5–6). Dendritic length was measured using ImageJ (National Institutes of Health), and spine numbers and types were analyzed using NeuronStudio (Mount Sinai School of Medicine).

Statistical Analysis

Data analysis was performed using a mixed-design analysis of variance (between- and within-group variables) followed by Fisher's post hoc tests. Student's t tests were used for planned comparisons. Data are expressed as mean + SEM. Reinstatement data were analyzed using a mixed-design 2-factor analysis of variance with repeated measures on extinction baseline versus cue presentation (cue reinstatement) or cocaine dose (cocaine reinstatement). Interaction effects were followed by simple main effects analyses and Bonferroni post hoc tests. Statistical significance was set at $p < .05$. Total animal numbers used in each experiment are summarized in Table 1.

RESULTS

CUS Increases ERK2 Signaling Within the NAc

Twenty-four hours after the final behavioral assessment (FST), protein was extracted from NAc tissue punches to assess CUS-induced changes in ERK2 and its related signaling molecules (Figure 1). A Student's t test revealed significant differences in phosphoprotein expression between CUS-exposed rats and their nonstressed control counterparts (Figure 1A). Specifically, increased phosphorylated levels of ERK2 ($t_{17} = 2.246$, $p = .0382$), MSK ($t_{17} = 2.557$ $p = .0204$), and p90-RSK ($t_{17} = 2.193$, $p = .0425$) were observed in the NAc as a function of CUS exposure. Post hoc comparisons using Fisher's least significant difference (LSD) test following significant analysis of variance results confirmed pairwise differences between groups. No significant differences were observed in phospho-ERK1 levels ($t_{17} = 0.2804$, $p = .7826$). Total protein levels for ERK1, ERK2, MSK, and p90-RSK remained unchanged between stressed and nonstressed rats (n = 9–10/group) (Figure 1B) (ERK1 [$t_{17} = 0.06039$, $p = .9526$]; ERK2 [$t_{17} = 0.1422$, $p = .8886$]; MSK [$t_{17} = 0.3765$ $p = .7112$]; p90-RSK [$t_{17} = 0.3004$, $p = .7675$]).

Viral Overactivation of NAc ERK2 Induces Anxiogenic-Like Behavior in the EPM

To assess the role of ERK2 signaling in mood-related behaviors, rats were microinjected with GFP, dnERK2, or wtERK2 in the NAc and tested in the EPM 3 days later. Time spent in the open arms ($F_{2,31} = 4.5$, $p < .05$) varied as a function of viral treatment. A Fisher's LSD test revealed that rats infused with wtERK2 spent significantly less time spent in the open arms than GFP controls ($p < .05$) (Figure 2B). Total distance traveled in the EPM was not significantly affected by wtERK2 or dnERK2 compared with GFP controls (Figure 2C).

Viral Overactivation of NAc ERK2 Induces Anxiogenic-Like Behavior in the OFT

To further assess anxiety-like behavior, a separate group of rats was tested in the OFT after receiving microinjections of GFP, dnERK2, or wtERK2 into the NAc. Time spent in the center of the arena varied significantly by virus treatment ($F_{2,30} = 9.9$, $p < .01$) (Figure 2D). Fisher's LSD test revealed that wtERK2 infusions significantly decreased time spent in the center compared with GFP controls ($p < .05$), while dnERK2 had no significant effect. Total distance traveled was not significantly different between groups, indicating that viral manipulations did not affect overall locomotion ($p > .05$) (Figure 2E).

NAc ERK2 Modulates Despair-Related Behavior in the FST

To evaluate stress sensitivity, behavioral responses in the FST were analyzed 3 days after viral infusions. Latency to immobility varied significantly as a function of viral treatment ($F_{2,21}$ = 13.8, $p < .01$) (Figure 2F). Fisher's LSD test revealed that dnERK2 increased latency to immobility compared with GFP controls, while wtERK2 decreased this latency ($p < .05$). Total immobility also varied by virus type ($F_{2,21}$ = 8.2, $p < .01$) (Figure 2G), with Fisher's LSD test revealing that wtERK2 significantly increased total immobility, while dnERK2 decreased total immobility ($p < .05$).

Basal Locomotor Activity After Day 1 of FST

To ensure that differences in FST performance were not influenced by changes in locomotor activity, a separate group of rats was tested for spontaneous locomotion 24 hours after day 1 of the FST. No significant differences in total distance traveled were found among the GFP, dnERK2, or wtERK2 groups ($p > .05$) (Figure 2H).

Viral Overactivation of NAc ERK2 Enhances Cocaine CPP

The effects of virally mediated overactivation of NAc ERK2 cocaine CPP are shown in Figure 3. Three days after the GFP,

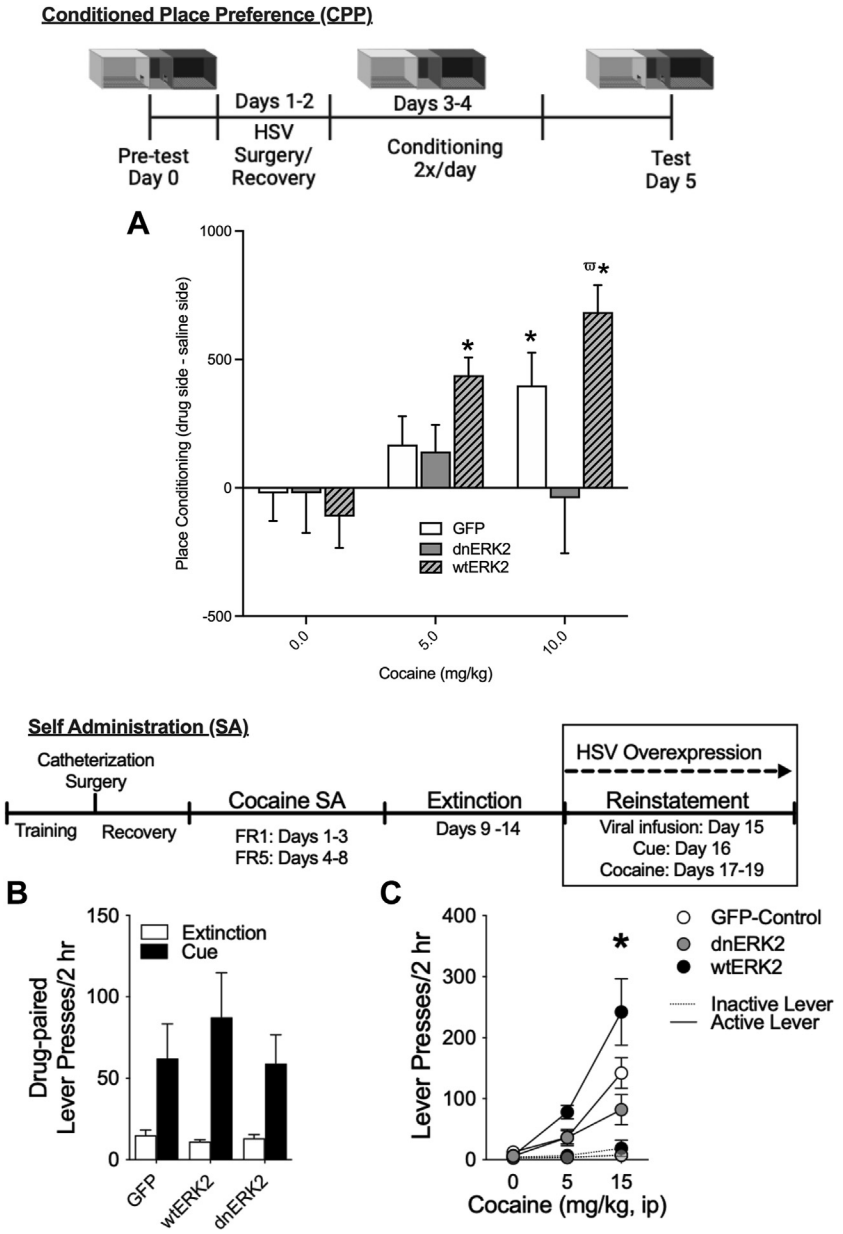

Figure 3. ERK2 modulation in the nucleus accumbens regulates cocaine-seeking behavior. Cocaine-mediated place conditioning protocol. Control GFP rats showed reliable CPP to 10.0 mg/kg of cocaine but not 5.0 mg/kg. Interestingly, wtERK2 rats showed robust place preference at both 5.0 and 10.0 mg/kg of cocaine, whereas dnERK2 blunted CPP acquisition to both cocaine doses **(A)**. Self-administration was used to assess cocaine motivation via acquisition, extinction training, and subsequent reinstatement testing during viral overexpression. Acquisition and extinction data are available in Figure S1. While there was no effect of ERK2 on cue-induced reinstatement **(B)**, wtERK2 significantly enhanced cocaine-primed reinstatement **(C)** n = 6–8/group; $^{*}p < .05$ compared with vehicle-treated rats within the same viral group, $^{\varpi}p < .05$ compared with dnERK2-infused within the same cocaine dose; error bars indicate + SEM. ERK, extracellular signal-regulated kinase; FR, fixed ratio; GFP, green fluorescent protein; HSV, herpes simplex virus; ip, intraperitoneal.

dnERK2, or wtERK2 viral infusion, rats were tested on cocaine (0.0, 5.0, 10.0 mg/kg) CPP (Figure 3A). Time spent in the cocaine-paired compartment varied significantly based on viral treatment ($F_{2,57} = 4.2$, $p < .05$), drug treatment ($F_{2,57} = 7.3$, $p < .01$), and the interaction between these factors (virus × drug interaction: $F_{6,85} = 2.7$, $p < .05$). Fisher's LSD post hoc tests revealed that wtERK2-treated rats spent significantly more time in the cocaine-paired compartment at both 5.0 and 10.0 mg/kg doses compared with GFP controls ($p < .05$). Rats conditioned to saline, regardless of virus type (GFP, dnERK2, wtERK2), did not show a preference for either compartment. As expected, GFP-infused rats spent significantly more time in the cocaine-paired environment at the 10 mg/kg dose ($p < .05$), but they did not show a preference at the 5 mg/kg (subthreshold) dose. However, Fisher's LSD post hoc tests revealed that rats that received wtERK2 microinjections into the NAc spent significantly more time in environments paired with both moderate doses of cocaine (5.0 and 10.0 mg/kg; $p < .05$). Conversely, rats that received dnERK2 infusions did not consistently approach the cocaine-paired compartments ($p > .05$), indicating that dnERK2 attenuated the rewarding effects of cocaine.

Viral Overactivation of NAc ERK2 Facilitates Cocaine Reinstatement

Self-administration and extinction responses were similar across treatment groups before viral infusion (Figure S1). Viral manipulation had no direct effect on cue-induced reinstatement of cocaine seeking (group × cue: $F_{2,52} = 0.5836$, $p > 1.0$) (Figure 3B.). However, wtERK2 markedly enhanced, while dnERK2 attenuated, cocaine-primed reinstatement (group × cocaine dose: $F_{4,52} = 3.72$, $p < .01$) (Figure 3C). Bonferroni's post hoc test revealed that wtERK2 significantly increased cocaine-seeking behavior at both the 5 mg/kg and 15 mg/kg doses ($p < .05$), whereas dnERK2 significantly reduced cocaine-induced reinstatement compared with GFP controls ($p < .05$).

Viral Overactivation of NAc ERK2 Increases Stubby and Mushroom Spine Density

To assess synaptic plasticity, we measured spine density within the NAc after viral manipulations (Figure 4). Spines were categorized based on their distinct phenotypes as mushroom, stubby, or thin. Viral treatment significantly affected both mushroom ($F_{2,16} = 3.93$, $p < .05$) (Figure 4A) and stubby ($F_{2,16} = 3.6$, $p < .05$) (Figure 4B) spine densities but not thin spines ($F_{2,16} = 0.2663$, $p > .05$) (Figure 4C). Post hoc analysis using Fisher's LSD test confirmed that wtERK2-infused rats exhibited significantly higher densities of mushroom and stubby spines than GFP-infused rats ($p < .05$).

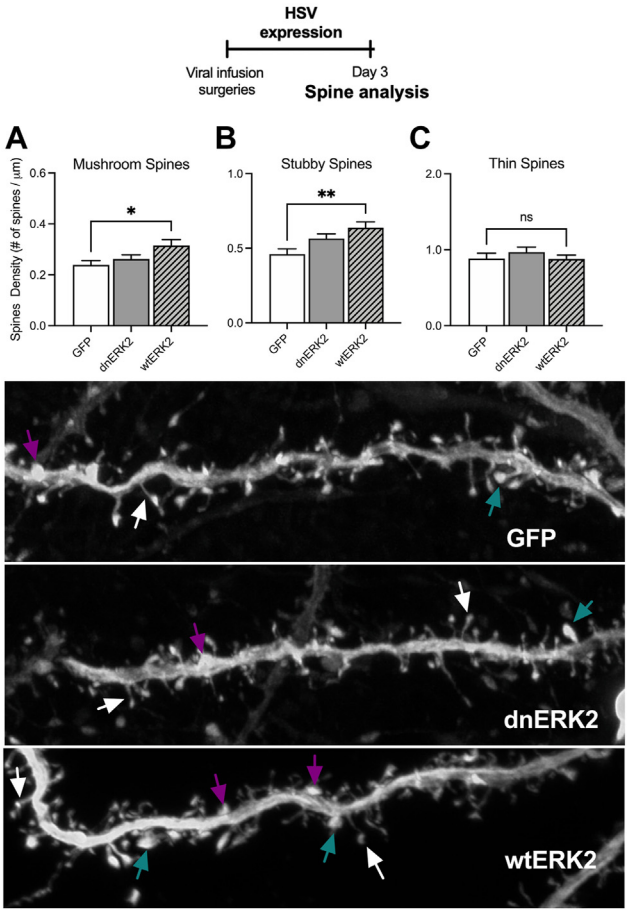

Figure 4. ERK2 activity in the nucleus accumbens regulates synaptic plasticity. wtERK2 significantly increases mushroom **(A)** and stubby **(B)** spine density without influencing thin spines **(C)**. n = 5–6/group. *Significantly different from GFP, $^*p < .05$, $^{**}p < .01$; error bars indicate + SEM. Teal arrows: mushroom spines, purple arrows: stubby spines, white arrows: thin spines. ERK2, extracellular signal-regulated kinase 2; GFP, green fluorescent protein; HSV, herpes simplex virus; ns, not significant.

DISCUSSION

The current study was designed to investigate the role of NAc ERK2 signaling in the development of stress-induced comorbid depression- and drug-related behaviors. We assessed whether virally mediated up- or downregulation of ERK2 activity within the NAc could influence behavioral assessments of mood or drug reward. Our findings demonstrate that ERK2 activation in the NAc influences both anxiety- and depression-like behaviors, as well as sensitivity to cocaine's reinforcing properties.

Specifically, we found that adult rats exposed to 4 weeks of CUS exhibited an increase in phosphorylated ERK2 together with corresponding increases in phosphorylation of its downstream signaling molecules MSK and p90-RSK. Importantly, total ERK2 protein levels remained unchanged, indicating that the effects of CUS were driven by ERK2 activity (signaling) rather than by changes in ERK2 expression. This distinction is crucial because it suggests that stress facilitates behavioral changes through the activation of ERK2-dependent pathways not by increasing the overall amount of ERK2 protein. Using a viral approach to mimic stress-induced ERK2-MSK-RSK phosphorylation (25), we observed that overactivation of ERK2 in the NAc decreased latency to immobility in the FST and reduced overall effort in the task, which is indicative of a despair-related phenotype. Additionally, ERK2 overactivation led to an anxiogenic-like response, with less time spent in the open arms of the EPM and in the center of the OFT. Together,

these behavioral responses suggest increased susceptibility to stress as a direct result of elevated ERK2 signaling.

Conversely, downregulating ERK2 activity in the NAc increased latency to immobility and reduced total immobility in the FST, reflecting an antidepressant-like effect (behavioral results are summarized in Figure S2). This highlights a bidirectional role for ERK2 in both despair- and antidepressant-like behaviors, consistent with findings in other reward-related brain regions like the VTA (25). It is important to note that ERK2 signaling, rather than expression, plays this modulatory role because the viral manipulations specifically influenced ERK2 phosphorylation. Interestingly, downregulation of ERK2 activity did affect behavior in the EPM or OFT, which may not be contradictory because depression- and anxiety-like responses do not always align following stress exposure (49). Furthermore, antidepressant drugs can reduce total immobility in the FST while having varying effects on anxiety-like behavior in other tests (48,50). Given the short-term nature of our testing, it is unsurprising that we did not observe anxiolytic effects following dnERK2 infusions.

Both chronic and acute exposure to cocaine have been shown to alter neurotrophin signaling in the NAc, thereby triggering downstream effectors, including ERK2 (23,51). We expanded on this by showing that increasing ERK2 activity not only promotes stress susceptibility but also lowers the threshold for cocaine reward (25,26,52). Conversely, reducing ERK2 signaling diminished depression-like behavior and blunted sensitivity to cocaine reward (25,52). To further explore ERK2's role in drug behavior, rats were trained to self-administer cocaine, underwent extinction training, and were tested for reinstatement following viral transfection. The reinstatement of cocaine-seeking behavior was strongest in wtERK2-infused rats, which is consistent with previous studies that have shown that stress exposure can influence both the acquisition and extinction of drug-associated behaviors. Specifically, both acute and chronic stress can reestablish cocaine or methamphetamine seeking in rats, even after an extended period of extinction (53,54).

Stress experienced at different life stages can also impact the acquisition of cocaine self-administration and delay extinction (55,56). The temporal limitations of the HSV constructs used in this study make it challenging to investigate ERK2's role in long-term processes such as acquisition and extinction. Future studies utilizing longer-lasting viral approaches, such as AAVs (adeno-associated viruses), will be critical to understanding the long-term role of ERK2 signaling in drug-related behaviors. However, our findings support the notion that ERK2 signaling contributes to these processes. For example, activation of a subpopulation of heteromeric D1- and D2-expressing MSNs in the NAc has been shown to attenuate ERK2 signaling and disrupt cocaine CPP by influencing both acquisition and extinction of cocaine seeking (57).

Cocaine and stress also affect the distribution complexity of dendritic spines within the NAc (58–61). Given ERK2's established role in memory and synaptic plasticity (62–64), we sought to determine whether manipulating ERK2 activity directly alters dendritic spine density, which could mediate the observed behaviors. We found that only wtERK2 overexpression significantly increased dendritic spine density compared with GFP controls. Specifically, there was an enrichment of more mature spine types—stubby and mushroom spines—compared with thin spines.

Notably, increasing ERK2 activity impaired tasks that rely on learning and memory, such as CPP, reinstatement of drug seeking, and, to a lesser extent, performance in the FST (65–67). The absence of cue-induced reinstatement effects suggests that ERK2 activity does not affect the learning and memory of cocaine-associated cues, but rather influences the adaptive response to cocaine reexposure. Increased ERK2 activity in the NAc may enhance cocaine's rewarding properties upon reexposure, which provides insights into ERK2's role in the habitual aspects of drug addiction and mood disorders. Individual differences in ERK2 signaling could predispose individuals to drug- or stress-triggered environments by priming reward-related learning in the NAc.

Despite these findings, this experimental approach has some limitations. While HSV-mediated modulation provides an effective way to investigate ERK2 signaling, it may not fully capture the broader biological consequences of chronic stress exposure and related synaptic changes. Additionally, the viral vectors used do not differentiate between cytoplasmic or nuclear ERK regulation, so it remains unclear whether ERK2's phosphorylation occurs within the cytoplasm or nucleus. Given ERK2's role in long-term potentiation and synaptic plasticity, it is plausible that nuclear ERK2 elevations promote protein synthesis and facilitate spine maturation as observed in our study. The temporal restriction of HSV expression also limits the behavioral testing window in this design. Moreover, the exclusion of female rats in the current investigation represents a significant limitation. Depression is more prevalent in women, and sex differences in stress responses and drug effects are well documented (68,69). Future studies should include both sexes to provide a more comprehensive understanding of these mechanisms.

Conclusions

Our findings demonstrate a critical role for ERK2 signaling within the NAc in mediating both mood-related and cocaine-seeking behaviors. Viral upregulation of ERK2 activity in the NAc led to increased depression- and anxiety-like behaviors, heightened sensitivity to cocaine relapse, and increased dendritic spine density. Importantly, these effects were driven by ERK2 activity, as evidenced by the phosphorylation of ERK2 and its downstream targets, rather than by changes in ERK2 expression. These data suggest that ERK2 signaling within the NAc facilitates vulnerability to both stress-related stimuli and drugs of abuse, highlighting an overlapping mechanism that contributes to comorbid affective and substance use disorders. This underscores the need for a deeper understanding of second-messenger signaling pathways, such as ERK2, in the NAc, which plays a central role in integrating mood regulation and drug reward. Further exploration of these signaling mechanisms will provide valuable insights into how stress and drug exposure converge to disrupt normal neural function and how interventions that target these pathways may offer therapeutic potential for treating psychiatric comorbidities.

ACKNOWLEDGMENTS AND DISCLOSURES

This research was supported by funding from the National Institute on Drug Abuse (Grant No. R01DA046794 [to CAB-G]), the Brain & Behavior Research Foundation (Grant Nos. 30609 [to EMP] and 31194 [to LFP]), and the Leon Levy Foundation (New York Academy of Medicine) (to LFP).

LFP and SDI conceived the experimental design. LFP, SDI, BLW, and EMP performed the viral infusion surgeries and subsequent behavioral testing. LFP and SDI performed the CPP tests, and RKB and DMD conducted the self-administration tasks. EJN and CAB-G provided resources and helped with data analysis and interpretation. LFP, EMP, and SDI wrote the final manuscript.

Data are available upon reasonable request to the corresponding author.

A previous version of this article was published as a preprint on bioRxiv: https://www.biorxiv.org/content/10.1101/2023.10.03.560689v1.

The authors report no biomedical financial interests or potential conflicts of interest.

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Received Mar 28, 2024; revised Oct 9, 2024; accepted Oct 19, 2024.

Supplementary material cited in this article is available online at https://doi.org/10.1016/j.bpsgos.2024.100416.

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