Running title: RasGRF and cocaine

The inhibition of RasGRF2, but not RasGRF1, alters cocaine reward in mice

Rick E. Bernardi[1#], Anastasia Olevska[1#], Ilaria Morella[2,3#], Stefania Fasano[2,3], Eugenio Santos[4], Riccardo Brambilla[2,3], Rainer Spanagel[1]

[1]Institute of Psychopharmacology, Central Institute of Mental Health, Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany, 68159

[2]Neuroscience and Mental Health Research Institute, Cardiff University, Cardiff, UK, CF24 4HQ

[3]Cardiff University, School of Biosciences, Division of Neuroscience, Cardiff, UK, CF24 4HQ

[4]Centro de Investigación del Cáncer-Instituto de Biología Molecular y Celular del Cáncer (CSIC - Universidad de Salamanca) and CIBERONC, Salamanca, Spain, 37007

*These authors contributed equally to this work.

[#]Corresponding author: Rick E. Bernardi
Central Institute of Mental Health
Institute of Psychopharmacology
J5
68159, Mannheim, Germany
Phone: +49 0621/17036266
Fax: +49 0621/17036255
E-mail: rick.bernardi@zi-mannheim.de

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Abstract

Ras/Raf/MEK/ERK(Ras-ERK) signaling has been implicated in the effects of drugs of abuse. Inhibitors of MEK1/2, the kinases upstream of ERK1/2, have been critical in defining the role of the Ras-ERK cascade in drug-dependent alterations in behavioral plasticity, but the Ras family of small GTPases has not been extensively examined in drug-related behaviors. We examined the role of Ras Guanine Nucleotide Releasing Factor 1(RasGRF1) and 2(RasGRF2), upstream regulators of the Ras-ERK signaling cascade, on cocaine self-administration(SA) in male mice. We first established a role for Ras-ERK signaling in cocaine SA, demonstrating that pERK1/2 is upregulated following SA in C57Bl/6N mice in striatum. We then compared RasGRF1 and RasGRF2 knock-out(KO) mouse lines, demonstrating that cocaine SA in RasGRF2 KO mice was increased relative to wild-type(WT) controls, while RasGRF1 KO and WT mice did not differ. This effect in RasGRF2 mice is likely mediated by the Ras-ERK signaling pathway, as pERK1/2 upregulation following cocaine SA was absent in RasGRF2 KO mice. Interestingly, the lentiviral knockdown of RasGRF2 in the NAc had the opposite effect to that in RasGRF2 KO mice, reducing cocaine SA. We subsequently demonstrated that the MEK inhibitor PD325901 administered peripherally prior to cocaine SA increased cocaine intake, replicating the increase seen in RasGRF2 KO mice, while PD325901 administered into the NAc decreased cocaine intake, similar to the effect seen following lentiviral knockdown of RasGRF2. These data indicate a role for RasGRF2 in cocaine SA in mice that is ERK-dependent, and suggest a differential effect of global versus site-specific RasGRF2 inhibition.

SIGNIFICANCE STATEMENT

Exposure to drugs of abuse activates a variety of intracellular pathways, and following repeated exposure, persistent changes in these pathways contribute to drug dependence. Downstream components of the Ras-ERK signaling cascade are involved in the acute and chronic effects of drugs of abuse, but their upstream mediators have not been extensively characterized. Here we show using a combination of molecular, pharmacological, and lentiviral techniques that the guanine nucleotide exchange factor RasGRF2 mediates cocaine self-administration via an ERK-dependent mechanism, while RasGRF1 has no effect on responding for cocaine. These data indicate dissociative effects of mediators of Ras activity on cocaine reward and expands the understanding of the contribution of Ras-ERK signaling to drug-taking behavior.

Introduction

The extracellular signal-regulated kinases (ERK) cascade (Ras/Raf/MEK/ERK; Ras-ERK) couples activity at cell surface receptors with the activation of transcription factors and subsequent gene expression (Grewal et al., 1999; Mazzucchelli and Brambilla, 2000). Largely known for its regulation of cell proliferation, differentiation and survival (Roberts and Der, 2007; Mandala et al., 2014), the Ras-ERK pathway also plays a critical role in long-term potentiation and memory formation (Brambilla et al., 1997; Jin and Feig, 2010), as well as reinforcement and enduring drug-dependent plasticity (Valjent et al., 2000; Ferguson et al., 2006; Girault et al., 2007). Indeed, this pathway has been implicated in the acute and chronic effects of a variety of drugs of abuse, most notably cocaine and other psychostimulants (Valjent et al., 2000; Lu et al., 2005; Miller and Marshall, 2005).

The majority of studies defining the role of the Ras-ERK pathway in drug-mediated behaviors have been achieved using inhibitors of MEK1/2, the kinases upstream of ERK1/2, while the Ras family of small GTPases has not been extensively examined. Ras family GTPases link extracellular signals to distinct intracellular signaling cascades by switching from an inactive GDP-bound state to an active GTP-bound state, each of which recognizes a distinct set of effector proteins and thereby allows Ras to function as a molecular switch (Takai et al., 2001; Cox and Der, 2010). The activation state of Ras proteins is controlled by two types of regulatory proteins. Guanine nucleotide exchange factors (GEFs) catalyze the release of bound GDP, thus allowing activating GTP to replace it. GTPase activating proteins (GAPs) promote the ability of Ras to hydrolyze GTP to GDP (Boguski and McCormick, 1993; Pamonsinlapatham et al., 2009). Multiple mammalian families of GEFs regulate the Ras activation cycle (Cox and Der, 2010). Particularly important with respect to the Ras-ERK

pathway in neuronal signaling are two members of the Ras guanine nucleotide releasing factor (RasGRF) family of GEFs, RasGRF1 and RasGRF2, that allow certain neurotransmitter receptors to activate Ras in a Ca^{2+}/calmodulin-dependent manner (Farnsworth et al., 1995).

RasGRF1 has been shown to play a role in the neuronal and behavioral responses to both psychostimulants and alcohol (Zhang et al., 2007; Fasano et al., 2009; Parelkar et al., 2009; Ben Hamida et al., 2012). For example, RasGRF1 KO mice show a decrease in the phosphorylation of ERK (pERK) in the ventral striatum in response to cocaine, while RasGRF1 overexpressing mice show an increased pERK in the ventral striatum following cocaine, as well as decreased and increased, respectively, cocaine-mediated locomotor sensitization and conditioned place preference (Fasano et al., 2009). The extent to which RasGRF2 may be involved in drug-mediated behaviors has to date been primarily restricted to alcohol studies. A single-nucleotide polymorphism in the gene encoding RasGRF2 has been associated with alcohol consumption (Schumann et al., 2011) and further linked to alcohol-related reward anticipation (Stacey et al., 2012) and binge drinking in male adolescents (Stacey et al., 2016), and RasGRF2 KO mice demonstrated a loss in alcohol-induced dopamine increase in the nucleus accumbens (NAc) and reduction in alcohol drinking (Stacey et al., 2012). However, it is unclear whether the involvement of RasGRF2 extends to other drugs of abuse.

The purpose here was to better characterize the role of the Ras-ERK pathway by direct comparison of the contribution of RasGRF1 and RasGRF2 to cocaine-mediated behaviors. We first established a role for the Ras-ERK pathway in cocaine SA by performing immunohistochemistry for pERK1/2, as well as the phosphorylation of (Ser10)-acetylated (Lys14) histone H3 (pAcH3), a nuclear ERK substrate that has demonstrated involvement in

drug-induced neuroadaptations in both rodents and humans (Brami-Cherrier et al., 2009; Damez-Werno et al., 2016; Papale et al., 2016). We then performed SA in RasGRF1 and RasGRF2 KO mice and WT littermate controls, followed by further pERK and pAcH3 analysis. We also performed site-specific lentiviral knockdown of RasGRF1 and RasGRF2 in mice in the NAc, as well as in the dorsal striatum (DS). Finally, we examined the effects of both the peripheral and site-specific inhibition of MEK1/2 on cocaine SA. Our experiments indicate a role for RasGRF2, but not RasGRF1, in cocaine SA in mice.

Material and Methods

Animals

C57Bl/6N mice (Charles River, Germany) and male RasGRF1 (Brambilla et al., 1997) and RasGRF2 (Fernandez-Medarde et al., 2002) KO mice and their WT littermate controls were single-housed in a temperature-controlled (21 °C) environment maintained on a 12-hr light-dark cycle (lights on at 6 a.m.). Food and water was available *ad libitum*. All experiments were performed in accordance with EU guidelines on the care and use of laboratory animals and were approved by the local animal care committee. All behavioral testing was conducted during the light phase between 0800 h and 1700 h.

Drugs

Cocaine hydrochloride (Sigma-Aldrich, Germany) was dissolved in physiological saline (0.9% NaCl) for 0.50 mg/kg/14 µl infusion for SA (Bernardi et al., 2017). PD325901 (Sigma-Aldrich, Germany) was dissolved in 100% dimethyl sulfoxide (DMSO) and diluted to a 20%

DMSO solution with sterile 0.9% NaCl for IP injection of 10 mg/kg (10 ml/kg). For intracranial administration, PD325901 (Carbosynth Limited, UK) was dissolved in 100% dimethyl sulfoxide (DMSO) and diluted to a 0.002% DMSO solution with sterile 0.9% NaCl for intracranial injection of 5 ng/0.5 µl/side. This dose was determined using a comparison of its IC50 to that of other MEK inhibitors commonly used for intracranial injection. The vehicle for all PD325901 experiments consisted of an identical % DMSO solution as that used for the drug.

Lentiviral vector production

The LV-RasGRF1-shRNA, LV-RasGRF2-shRNA and LV-scrambled-shRNA were constructed and prepared as previously described (Bido et al., 2015). Briefly, expression plasmids for Ras-GRF1 and Ras-GRF2 RNA interference were obtained from a commercial source (OriGene, Rockville, MD). The following sequences were used as shRNA inserts against RasGRF1 and RasGRF2, respectively: GACGGCCTGGTCAACTTCTCCAAGATGAG and TAATGCAGAAGTACATTCATCTAGTTCAG. The company provided all scrambled control sequences. The shRNA gene specific expression cassettes (U6-shRNA cassette) were cloned into the pCCLsin.PPT.hPGK.eGFP.PRE lentiviral construct. VSV-pseudotyped third-generation lentiviral vectors (LV) were produced as previously described (Indrigo et al., 2010; Papale and Brambilla, 2014). Western blots demonstrating knockdown of both RasGRF1 and RasGRF2 proteins both *in vitro* and *in vivo* in striatal cells have been shown previously (Bido et al., 2015).

Apparatus & Procedures

157 Cocaine SA

158 Cocaine SA was assessed in 12 operant chambers (Med Associates, USA) housed in light-
159 and sound-attenuating cubicles. Each chamber (24.1 x 20.3 x 18.4 cm) is equipped with two
160 levers (left and right), a food dispenser and a drug delivery system connected via infusion
161 pump (PHM-100, Med-Associates, USA) located outside the cubicle. Operant chambers were
162 controlled using Med-PC IV (Med Associates, USA) software. Mice first underwent lever
163 training with 14 mg sweetened food pellets (TestDiet, USA), as previously described
164 (Bernardi and Spanagel, 2013). Following lever training, mice were implanted with an
165 indwelling intravenous catheter (made in-house) into the jugular vein. Catheter patency was
166 maintained with 0.15 ml heparanized saline (100 i.u./ml) containing Baytril (0.7 mg/ml)
167 administered daily throughout the experiment. After 3d recovery, mice underwent daily 1hr
168 cocaine SA for 7 consecutive days. Cocaine (0.50 mg/kg/14 µl infusion) delivery was
169 contingent upon pressing on the active lever under an FR2 schedule of reinforcement (unless
170 otherwise specified) and paired with the 20s presentation of a blinking light stimulus
171 (Conditioned Stimulus, CS), which also served as a timeout period, during which lever
172 presses were not reinforced. For all experiments, presses on the inactive lever were recorded
173 but had no scheduled consequence.

174

175 Immunohistochemistry

176 Immunohistochemistry was performed following the protocol described in Papale et al, 2016.
177 Free-floating sections were rinsed in TBS and then incubated for 15 min in a quenching
178 solution containing 3% H2O2 and 10% methanol. One hour after blocking in 5% normal goat
179 serum and 0.1% Triton X-100, sections were incubated overnight at 4°C with anti-phospho-
180 p44/42 MAP kinase (Thr202/Tyr204) (1:1000, Cell Signaling Technology Cat# 4370L,
181 RRID:AB_231511), anti-phospho (Ser10)-acetylated (Lys14) histone H3 (1:500, Millipore

Cat# 07–081, RRID:AB_310366), or anti-GFP antibody (1:500, Life Technologies Cat# A11122, RRID: AB_221569). The next day, slices were rinsed in TBS and incubated with biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories Cat# BA-1000, RRID:AB_2313606) for 2 hours at room temperature. Detection of the bound antibodies was carried out using a standard peroxidase-based method (ABC-kit, Vectastain, Vector Labs), followed by incubation with DAB and H2O2 solution. Images were acquired from the dorsal and ventral striatum with a bright field microscope (Leica DM2000LED Macro/micro imaging system) at 20X magnification. Neuronal quantification was carried out using ImageJ software. The total number of pERK1/2- and pAcH3-positive cells was counted in the dorsal and ventral striatum in 2 consecutive rostral sections per mouse.

Lentivirus microinjections

Mice were anesthetized by isoflurane (4% for induction, 1% for maintenance), secured in a stereotaxic frame (David Kopf Instruments, Tujunga, USA), and the lentiviral vectors (LV-RasGRF1-shRNA, LV-RasGRF2-shRNA and LV-scrambled-shRNA) were bilaterally injected into the NAc (AP +1.20; ML +/-0.90; DV -4.75, relative to Bregma) or DS (AP +0.00; ML +/-2.20; DV -3.30, relative to Bregma) in a volume of 0.5 µl/hemisphere over 2 min (at a rate of 0.125 µl/min). After the infusion, needles were left in place for additional 2 min to allow for diffusion. Mice were then sutured and allowed to recover from surgical procedures. After the completion of behavioral experiments, mice were anesthesized with isoflurane and perfused with 5 ml ice-cold phosphate-buffered saline (PBS) and 5 ml 4% paraformaldehyde (PFA) in PBS; their brains were removed and kept in 4% PFA in PBS over night for post-fixation, and then transferred into 30% sucrose in PBS solution for at least 24 hours. Coronal sections (30 µm) were subsequently taken at the level of the NAc or DS with a cryostat (Leica Microsystems, Wetzlar, Germany), mounted onto polarized glass slides and

eGFP expression was imaged using fluorescent microscopy. The extension of virus spread was assessed manually based on the mouse brain atlas (Paxinos and Franklin, 2004). Animals with placements outside of the NAc or DS, with unilateral expression or extensive mechanical damage were excluded. It must be noted here that no distinction was made between core and shell or dorsolateral and dorsomedial striatum in terms of virus injections, due to the difficulty in targeting one region specifically in mice.

Cannula implantation and microinjections

Following food training, mice were anesthetized by isoflurane (4% for induction, 1% for maintenance), secured in a stereotaxic frame (David Kopf Instruments, USA), and cannula (Plastics One, USA) were mounted above the NAc (C235GS, double cannula, AP +1.20; ML +/-0.75; DV -2.75, relative to Bregma) or DS (C315GS, bilateral single cannula, AP +0.00; ML +/-2.20; DV -1.80, relative to Bregma) secured using screws and dental cement and dummy cannulae (C235DCS and C315DCS for NAc and DS, respectively, Plastics One, USA) were inserted to protect the cannulae. Mice were then allowed to recover from surgical procedures for 7 days. Dummy cannulae were removed prior to and replaced following daily SA sessions to habituate the animals to the handling procedure. Microinjections of PD325901 were conducted using internal cannula (C235IS and C315IS for NAc and DS, respectively, Plastics One, USA) that extended beyond the cannula guide (2 mm for NAc and 1.5 mm for DS) in a volume of 0.5 µl/hemisphere over 2 min (at a rate of 0.125 µl/min) under isoflurane anesthesia. After the infusion, needles were left in place for additional 2 min to allow for diffusion. After the completion of behavioral experiments, all mice were anesthesized with isoflurane, cannulae were injected with coomassie blue dye, and brains were removed and flash-frozen in isopentane for placement verification. Coronal sections (30 µm) were assessed as to cannula placement using the mouse brain atlas (Paxinos and Franklin, 2004). Animals

with placements outside of the NAc or DS were excluded. It must be noted here that no distinction was made between core and shell or dorsolateral and dorsomedial striatum in terms of intracranial injections, due to the difficulty in targeting one region specifically in mice.

Experimental design and statistical analysis

Statistical analyses were conducted using SPSS software (StatSoft, USA). All SA data was performed using three-way [number of presses: genotype/treatment (between subjects) x lever (within subjects) x day (within subjects)] or two-way ANOVAs [number of reinforcers: genotype/treatment (between subjects) x day (within subjects)], followed by Bonferroni-corrected independent samples t-tests, where indicated. Cocaine intake data following intracranial PD325901 microinjection was compared using independent samples t-tests, where indicated. Immunohistochemical data in RasGRF2 KO mice and controls were conducted using a two-way ANOVA [genotype (between subjects) x treatment (between subjects)], followed by independent samples t-tests, where indicated. Immunohistochemical data in all other experiments were compared using independent samples t-tests, except where data was non-normal, in which a Mann-Whitney U-Test was used (where indicated). Significance was set at $p < .05$.

Results

pERK1/2 and pAcH3 are increased in C57Bl/6N mice following cocaine SA

We previously showed that acute cocaine resulted in an increase in the number of pERK1/2- and pAcH3-positive cells in the ventral and dorsal striatum (Papale et al, 2016). Here we sought to determine the role of the Ras-ERK pathway in cocaine SA using these indicators.

We first performed immunochemistry for pERK1/2- and pAcH3-positive cells in the ventral and dorsal striatum on the 7th day of SA. C57Bl/6N mice underwent cocaine (n = 5) or saline (n = 6) SA (as described above), with animals sacrificed 30 min following the first infusion achieved on day 7. Following testing, mice were anesthesized with isoflurane and transcardially perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA) in PBS. Brains were removed and placed in 4% PFA in PBS for 24h for post-fixation, and then transferred into 20% sucrose in PBS for at least 24h and finally into 30% sucrose in PBS until processing.

pERK1/2 analysis

Following cocaine SA, C57Bl/6N mice demonstrated an increase in the number of pERK1/2-positive cells in response to cocaine on day 7 relative to saline controls in both the ventral and dorsal striatum. Figure 1A shows the mean number of pERK1/2-positive cells (± SEM) in the ventral striatum following cocaine or saline SA in C57Bl/6N mice. A Mann-Whitney U-Test revealed that distributions of the number of pERK1/2-positive cells in the ventral striatum in animals that underwent cocaine and saline SA differed significantly [$U < .0005$, $Z = -2.7$, $p = .004$]. Figure 1B shows the mean number of pERK1/2-positive cells (± SEM) in the dorsal striatum following cocaine or saline SA in C57Bl/6N mice. An independent samples t-test revealed that animals that underwent cocaine SA demonstrated a significant increase in the number of pERK1/2-positive cells in the dorsal striatum relative to saline controls [$t(4.6) = 4.8$, $p = .006$]. Figure 1C shows representative slices of pERK1/2-positive cells from the ventral and dorsal striatum of C57Bl6N mice that underwent cocaine or saline SA.

pAcH3 analysis

Following cocaine SA, C57Bl/6N mice demonstrated an increase in the number of pAcH3-positive cells in response to cocaine on day 7 relative to saline controls in both the ventral and dorsal striatum. Figure 1D shows the mean number of pAcH3-positive cells (± SEM) in the ventral striatum following cocaine or saline SA in C57Bl/6N mice. An independent samples t-test revealed that animals that underwent cocaine SA demonstrated a significant increase in the number of pAcH3-positive cells in the ventral striatum relative to saline controls [$t(5.0) = 5.3$, $p = .003$]. Figure 1E shows the mean number of pAcH3-positive cells (± SEM) in the dorsal striatum following cocaine or saline SA in C57Bl/6N mice. An independent samples t-test revealed that animals that underwent cocaine SA demonstrated a significant increase in the number of pAcH3-positive cells in the dorsal striatum relative to saline controls [$t(9) = 5.0$, $p = .001$]. Figure 1F shows representative slices of pAcH3-positive cells from the ventral and dorsal striatum of C57Bl6N mice that underwent cocaine or saline SA.

These data suggest that pERK1/2 and pAcH3 are increased as a result of cocaine SA mice, suggesting that these downstream components of the Ras-ERK pathway may be critical for cocaine reward.

Cocaine SA was increased in RasGRF2 KO mice, but unaffected in RasGRF1 mice

To determine the contribution of RasGRF1 and RasGRF2 to cocaine SA, RasGRF1 and RasGRF2 KO lines were used. These lines have been previously demonstrated to show impaired cocaine-related behaviors and alcohol intake, respectively (Fasano et al., 2009; Stacey et al., 2012). RasGRF1 KO mice (n = 11) and WT littermate controls (n = 10), and RasGRF2 KO mice (n = 19) and WT littermate controls (n = 14), underwent SA for 7d as described above.

RasGRF1 KO mice and WT controls did not differ in cocaine SA. Figure 2A shows the mean (± SEM) responding on the active and inactive levers during 7 daily 1hr sessions of cocaine SA in RasGRF1 KO mice and WT controls. A three-way ANOVA (lever x day x genotype) revealed significant main effects of lever [$F(1,19) = 51.8$, $p < .0005$], indicating a distinction between the active and inactive levers, and day [$F(2.1,39.1) = 4.8$, $p = .024$], but no other significant effects [$Fs < 1$], indicating no difference in lever responding between KO and WT mice. Figure 2B shows the mean (± SEM) number of cocaine infusions received during 7d of cocaine SA in RasGRF1 KO mice and WT controls. A two-way ANOVA (day x genotype) revealed no significant effects of genotype [$Fs < 1$], indicating no difference in cocaine intake in RasGRF1 KO mice relative to WT controls.

RasGRF2 KO mice and WT controls differed in cocaine SA, with RasGRF2 KO mice demonstrating an increase in responding on the cocaine-associated lever and subsequent cocaine intake. Figure 2C shows the mean (± SEM) responding on the active and inactive levers during 7 daily 1hr sessions of cocaine SA in RasGRF2 KO mice and WT controls. A three-way ANOVA (lever x day x genotype) revealed significant main effects of lever [$F(1,31) = 201.5$, $p < .0005$], indicating a distinction between the active and inactive levers, and a significant main effect of genotype [$F(1,31) = 12.8$, $p = .001$], and importantly, a lever x genotype interaction [$F(1,31) = 6.3$, $p = .017$], indicating a difference between KO and WT controls over 7d of cocaine SA. No other effects reached significance [$Fs < 1$, except lever x day: $F(2.4,74.4) = 2.4$, $p = .088$]. Independent samples t-tests confirmed that RasGRF2 KO mice responded more on the active lever than control mice [$t(31) = 3.6$, $p = .001$; Bonferroni-corrected $\alpha = .025$] across days, but the two groups did not differ on inactive lever pressing [$t(31) = 1.6$, $p = .119$; Bonferroni-corrected $\alpha = .025$], indicating a selective increase in responding on the cocaine-associated lever by RasGRF2 KO mice relative to littermate

controls. Figure 2D shows the mean (± SEM) number of cocaine infusions received during 7d of cocaine SA in RasGRF2 KO mice and WT controls. A two-way ANOVA (day x genotype) revealed a significant main effect of genotype [$F(1,31) = 9.0$, $p = .005$], but no other significant effects [day: $F(2.9,91.0) = 1.1$, $p = .363$; day x genotype: $F(2.9,91.0) = 1.9$, $p = .142$], indicating an increase in cocaine intake in RasGRF2 KO mice relative WT controls.

These findings suggest that RasGRF2, but not RasGRF1, is important in mediating cocaine reward in mice during SA. Because previous studies have demonstrated a *decrease* in alcohol reward in RasGRF2 KO mice (Stacey et al., 2012), these data also suggest that RasGRF2 KO results in a decrease in putative cocaine reward that in terms of IV SA is compensated for by an increase in intake.

pERK1/2 and pAcH3 are inhibited in RasGRF2 KO mice following cocaine SA

As RasGRF2 KO in mice altered cocaine SA, immunohistochemical analyses were performed to determine whether RasGRF2 KO affected pERK1/2- and pAcH3 activation during cocaine SA, which we showed above likely mediates cocaine reward. The numbers of pERK1/2- and pAcH3-positive cells were measured in the ventral and dorsal striatum of RasGRF2 KO mice and WT littermate controls on the 7th day of SA. RasGRF2 KO (cocaine, n = 4; saline, n = 5) and WT controls (cocaine, n = 4; saline, n = 5) underwent cocaine or saline SA as described above, with animals sacrificed 30 min following the first reinforcer achieved on day 7. Following testing, mice were anesthesized with isoflurane and transcardially perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA) in PBS. Brains were removed and placed in 4% PFA in PBS for 24h for post-fixation, and then transferred into 20% sucrose in PBS for at least 24h and finally into 30% sucrose in PBS until processing.

pERK1/2 analysis

Following cocaine SA, WT mice of the RasGRF2 line demonstrated an increase in the number of pERK1/2-positive cells in response to cocaine on day 7 relative to saline controls in both the ventral and dorsal striatum, consistent with the data from C57Bl/6N mice shown in Figure 1. The increase in the number of pERK1/2-positive cells on day 7 was not present in either the ventral or dorsal striatum of RasGRF2 KO mice. Figure 3A shows the mean number of pERK1/2-positive cells (± SEM) in the ventral striatum in RasGRF2 KO mice and WT controls that were sacrificed on day 7 after cocaine or saline SA. A two-way ANOVA (genotype x treatment) of the number of pERK1/2-positive cells revealed significant main effects of genotype [$F(1,14) = 8.2$, $p = .012$] and treatment [$F(1,14) = 6.6$, $p < .022$], and a significant genotype x treatment interaction [$F(1,14) = 7.0$, $p = .019$]. An independent samples t-test revealed an attenuation of the number of pERK1/2-positive cells in RasGRF2 KO mice that underwent cocaine SA, relative to WT controls [$t(6) = 2.9$, $p = .027$]. Figure 3B shows the mean number of pERK1/2-positive cells (± SEM) in the dorsal striatum in RasGRF2 KO mice and WT controls that were sacrificed on day 7 after cocaine or saline SA. A two-way ANOVA (genotype x treatment) of the number of pERK1/2-positive cells revealed significant main effects of genotype [$F(1,14) = 18.8$, $p = .001$] and treatment [$F(1,14) = 34.0$, $p < .0005$], and a significant genotype x treatment interaction [$F(1,14) = 13.3$, $p = .003$]. An independent samples t-test revealed an attenuation of the number of pERK1/2-positive cells in RasGRF2 KO mice that underwent cocaine SA, relative to WT controls [$t(6) = 3.6$, $p = .011$]. Figure 3C shows representative slices of pERK-positive cells from the ventral and dorsal striatum of RasGRF2 KO mice and WT controls that underwent cocaine or saline SA.

pAcH3 analysis

Following cocaine SA, WT mice of the RasGRF2 line demonstrated an increase in the number of pAcH3-positive cells in response to cocaine on day 7 relative to saline controls in both the ventral and dorsal striatum, consistent with the data from C57Bl/6N mice shown in Figure 1. The increase in the number of pAcH3-positive cells on day 7 was not present in either the ventral or dorsal striatum of RasGRF2 KO mice. Figure 3D shows the mean number of pAcH3-positive cells (± SEM) in the ventral striatum in RasGRF2 KO mice and WT controls that were sacrificed on day 7 after cocaine or saline SA. A two-way ANOVA (genotype x treatment) of the number of pAcH3-positive cells revealed significant main effects of genotype [$F(1,14) = 7.9$, $p = .014$] and treatment [$F(1,14) = 22.3$, $p < .0005$], and a significant genotype x treatment interaction [$F(1,14) = 14.8$, $p = .002$]. An independent samples t-test revealed an attenuation of the number of pAcH3-positive cells in RasGRF2 KO mice that underwent cocaine SA, relative to littermate controls [$t(6) = 3.3$, $p = .017$]. Figure 3E shows the mean number of pAcH3-positive cells (± SEM) in the dorsal striatum in RasGRF2 KO mice and littermate controls that were sacrificed on day 7 after cocaine or saline SA. A two-way ANOVA (genotype x treatment) of the number of pAcH3-positive cells revealed significant main effects of genotype [$F(1,14) = 7.4$, $p = .017$] and treatment [$F(1,14) = 6.0$, $p = .028$], and a significant genotype x treatment interaction [$F(1,14) = 11.8$, $p = .004$]. An independent samples t-test revealed an attenuation of the number of pAcH3-positive cells in RasGRF2 KO mice that underwent cocaine SA, relative to littermate controls [$t(6) = 3.2$, $p = .019$]. Figure 3F shows representative slices of pAcH3-positive cells from the ventral and dorsal striatum of RasGRF2 KO mice and WT controls that underwent cocaine or saline SA.

These data suggest that RasGRF2 mediates cocaine reward through an ERK-dependent pathway. Furthermore, these data are supportive of the hypothesis that the increase in SA

404 demonstrated in RasGRF2 KO mice results from an inhibition of pERK-mediated reward and
405 a consequent compensatory increase in cocaine intake.
406
407 **Lentiviral-mediated knockdown of RasGRF2 into the NAc and DS decreased cocaine**
408 **intake**
409 Here we sought to determine the contribution of RasGRF2 specifically in the NAc on cocaine
410 SA in mice using lentiviral-mediated knockdown of RasGRF2. Because RasGRF1 KO mice
411 showed no difference in cocaine SA as compared to littermate wild-type controls, we used
412 lentiviral-mediated knockdown of RasGRF1 in the NAc as a control. Four weeks following
413 microinjections of LV-RasGRF2-shRNA (n = 9) or LV-scrambled-shRNA (n = 11), or LV-
414 RasGRF1-shRNA (n = 12) or LV-scrambled-shRNA (n = 13), into the NAc, mice underwent
415 7d of cocaine SA as described above. In a separate experiment, we further determined
416 whether microinjections of LV-RasGRF2-shRNA into the DS affected cocaine SA in mice.
417 Four weeks following microinjections of LV-RasGRF2-shRNA (n = 9) or LV-scrambled-
418 shRNA (n = 6) into the DS, C57Bl/6N mice underwent 7d of cocaine SA as described above.
419 Mice from this DS group were sacrificed immediately after the final SA session on day 7 so
420 that in addition to virus and placement verification, alterations in pERK activity during
421 cocaine SA using immunohistochemistry could also be assessed.
422
423 Microinjections of LV-RasGRF2-shRNA into the NAc resulted in a decrease in cocaine
424 intake during SA, relative to LV-scrambled-shRNA controls. Figures 4A and 4B show
425 representative viral eGFP expression images using fluorescent microscopy and DAB staining,
426 respectively, of the LV-RasGRF2-shRNA and LV-scrambled-shRNA constructs for the NAc.
427 Figure 4C shows the mean (± SEM) responding on the active and inactive levers during 7
428 daily 1hr days of cocaine SA following NAc microinjection of LV-RasGRF2-shRNA and

LV-scrambled-shRNA constructs. A three-way ANOVA (lever x day x treatment) revealed a significant main effect of lever [$F(1,18) = 61.4$, $p < .0005$], indicating a distinction between the active and inactive levers, but no other significant effects [$Fs < 1$, except lever x treatment: $F(1,18) = 1.5$, $p = .238$; lever x day: $F(6,108) = 2.1$, $p = .063$; lever x day x treatment: $F(6,108) = 1.3$, $p = .267$; treatment: $F(1,18) = 2.5$, $p = .132$], indicating no significant difference in active lever-specific responding as a function of treatment. Figure 4D shows the mean (± SEM) number of cocaine infusions received during 7d of cocaine SA. A two-way ANOVA (day x treatment) indicated a main effect of treatment [$F(1,18) = 4.8$, $p = .043$], but no other significant effects [$Fs < 1$], indicating a decrease in cocaine intake resulting from the LV-RasGRF2-shRNA microinjection.

Microinjections of LV-RasGRF1-shRNA into the NAc did not affect cocaine SA, relative to LV-scrambled-shRNA controls. Figures 5A and 5B show representative viral eGFP expression images using fluorescent microscopy and DAB staining, respectively, of the LV-RasGRF1-shRNA and LV-scrambled-shRNA constructs for the NAc. Figure 5C shows the mean (± SEM) responding on the active and inactive levers during 7 daily 1hr days of cocaine SA following NAc microinjection of LV-RasGRF1-shRNA and LV-scrambled-shRNA constructs. A three-way ANOVA (lever x day x treatment) revealed significant main effects of lever [$F(1,23) = 86.8$, $p < .0005$], indicating a distinction between the active and inactive levers, and a lever x day interaction [$F(3.8,87.2) = 3.0$, $p = .032$], but no other significant effects [$Fs < 1$], indicating no difference in lever responding as a function of treatment. Figure 5D shows the mean (± SEM) number of cocaine infusions received during 7d of cocaine SA following NAc microinjection of LV-RasGRF1-shRNA and LV-scrambled-shRNA constructs. A two-way ANOVA (day x treatment) revealed no significant effects [Fs

453 < 1], indicating no difference in cocaine intake resulting from LV-RasGRF1-shRNA
454 treatment relative to the scrambled control.
455
456 Microinjections of LV-RasGRF2-shRNA into the DS resulted in a decrease in cocaine intake
457 during SA, relative to LV-scrambled-shRNA controls. Figures 6A and 6B show
458 representative viral eGFP expression images using fluorescent microscopy and DAB staining,
459 respectively, of the LV-RasGRF2-shRNA and LV-scrambled-shRNA constructs for the DS.
460 Figure 6C shows the mean (± SEM) responding on the active and inactive levers during 7
461 daily 1hr days of cocaine SA following DS microinjection of LV-RasGRF2-shRNA and LV-
462 scrambled-shRNA constructs. A three-way ANOVA (lever x day x treatment) revealed
463 significant main effects of lever [$F(1,13) = 104.0$, $p < .0005$], indicating a distinction between
464 the active and inactive levers, a main effect of treatment [$F(1,13) = 6.6$, $p = .023$], a lever x
465 day interaction [$F(1.9,24.6) = 7.0$, $p = .004$], and only a trend toward a lever x treatment
466 interaction [$F(1,13) = 4.5$, $p = .054$], but no other significant effects [day: $F(2.6,33.9) = 2.2$, p
467 $= .110$; day x treatment: $F(2.6,33.9) = 1.5$, $p = .233$; lever x day x treatment: $F(1.9,24.6) = 2.3$,
468 $p = .128$], indicating no significant difference in active lever-specific responding as a function
469 of treatment. Figure 6D shows the mean (± SEM) number of cocaine infusions received
470 during 7d of cocaine SA following DS microinjection of LV-RasGRF2-shRNA and LV-
471 scrambled-shRNA constructs. A two-way ANOVA (day x treatment) revealed a main effect
472 of treatment [$F(1,13) = 10.7$, $p = .006$], but no other significant effects [$Fs < 1$, except day:
473 $F(2.4,31.0) = 1.4$, $p = .258$], indicating a decrease in cocaine intake resulting from the LV-
474 RasGRF2-shRNA microinjection.
475
476 Immediately following the final session of cocaine SA, LV-RasGRF2-shRNA mice
477 demonstrated a decrease in the number of pERK1/2-positive cells in response relative to LV-

scrambled-shRNA controls in the dorsal striatum. Figure 7 shows the mean number of pERK1/2-positive cells (± SEM) in the dorsal striatum in LV-RasGRF2-shRNA and LV-scrambled-shRNA mice that were sacrificed on day 7 after cocaine SA, as well as representative slices of pERK-positive cells from the dorsal striatum of these groups An independent samples t-test revealed a decrease in the number of pERK1/2-positive cells in LV-RasGRF2-shRNA mice that underwent cocaine SA, relative to LV-scrambled-shRNA controls [$t(13) = 6.3$, $p < .0005$]. These data are representative of the ability of LV-RasGRF2-shRNA to inhibit Ras-ERK signaling in striatal tissue as measured by pERK activation.

These data confirm a role for RasGRF2 in the NAc, as well as in the DS, in cocaine SA in mice. Because LV-RasGRF2-shRNA reduced cocaine intake, in contrast to the increase in intake demonstrated in RasGRF2 KO mice, further examination is required.

Peripheral PD325901 increased, while intra-NAc PD325901 decreased, cocaine SA in mice.

As our results above show, there were opposing results on cocaine intake in RasGRF2 KO mice (increased) and animals administered LV-RasGRF2-shRNA (decreased). We sought to clarify this inconsistency using the selective MEK inhibitor PD325901 administered both peripherally and site-specifically during cocaine SA. We previously showed that PD325901 crosses the blood brain barrier and inhibits the increased pERK1/2 and pAcH3 associated with acute cocaine (Papale et al, 2016). For peripheral PD325901 administration, C57Bl/6N mice underwent 7d of cocaine SA under an FR1 schedule of reinforcement following pretreatment with vehicle (n = 9) or PD325901 (10 mg/kg; n = 11) 30 min prior to the start of each daily session. For immunohistochemical confirmation of the effect of PD325901 on pERK1/2

signaling, a separate group of C57Bl/6N mice underwent 7d of cocaine SA under an FR2 schedule of reinforcement following pretreatment with vehicle (n = 5) or PD325901 (10 mg/kg; n = 5) 30 min prior to the start of each daily session, with animals sacrificed 30 min following the first infusion achieved on day 7. For intracranial injection, mice implanted with cannulae aimed at either the NAc (vehicle, n = 10; PD325901, n = 9) or DS (vehicle, n = 6; PD325901, n = 7) underwent 7d of cocaine SA under an FR2 schedule of reinforcement. PD325901 was injected 30 min prior to the cocaine SA session on day 7, and mice were given additional SA sessions on days 8 and 9.

Vehicle- and PD325901-administered mice differed in cocaine intake following peripheral administration. Figure 8A shows the mean (± SEM) responding on the active and inactive levers during 7 daily 1hr sessions of cocaine SA following IP administration of PD325901 or vehicle. A three-way ANOVA (lever x day x treatment) revealed a significant main effect of lever [$F(1,18) = 62.4$, $p < .0005$], indicating a distinction between the active and inactive levers, a significant lever x day interaction [$F(3.3,59.1) = 4.0$, $p = .01$], and a main effect of treatment [$F(1,18) = 4.9$, $p = .04$], but no other significant effects [$Fs < 1$, except lever x treatment: $F(1,18) = 3.0$, $p = .10$; day x treatment: $F(3.0,54.7) = 1.4$, $p = .249$]. Figure 8B shows the mean (± SEM) number of cocaine infusions received during 7d of cocaine SA following IP administration of PD325901 or vehicle. A two-way ANOVA (treatment x day) revealed significant main effects of treatment [$F(1,18) = 15.8$, $p = .001$] and day [$F(3.0,54.4) = 3.4$, $p = .024$], and a significant treatment x day interaction [$F(3.0,54.4) = 3.9$, $p = .013$].

PD325901-treated C57Bl/6N mice demonstrated a decrease in the number of pERK1/2-positive cells in response to cocaine on day 7 relative to vehicle-treated mice in both the ventral and dorsal striatum. Figure 8C shows the mean number of pERK1/2-positive cells (±

SEM) in the ventral striatum on day 7 in C57Bl/6N mice administered either vehicle or PD325901 prior to daily SA sessions. An independent samples t-test revealed that animals administered PD325901 demonstrated a significant decrease in the number of pERK1/2-positive cells in the ventral striatum relative to vehicle controls [$t(8) = 3.8$, $p = .005$]. Figure 8D shows the mean number of pERK1/2-positive cells (± SEM) in the dorsal striatum on day 7 in C57Bl/6N mice administered either vehicle or PD325901 prior to daily SA sessions. An independent samples t-test revealed that animals administered PD325901 demonstrated a significant decrease in the number of pERK1/2-positive cells in the dorsal striatum relative to vehicle controls [$t(4.0) = 5.6$, $p = .005$]. Figure 8E shows representative slices of pERK1/2-positive cells from the ventral and dorsal striatum of C57Bl6N mice administered vehicle or PD325901. PD325901 treatment also resulted in an increase in the number of cocaine infusions in these mice (*data not shown*); a two-way ANOVA (treatment x day) revealed a significant main effect of treatment [$F(1,8) = 5.4$, $p = .048$], but no significant effect of day [$F(1,8) = 2.8$, $p = .136$] or treatment x day interaction [$F < 1$] for days 1-6, and no difference on the shortened day 7 [independent samples t-test: $t(8) = 1.9$, $p = .090$].

Vehicle- and PD325901-administered mice differed in cocaine intake following intra-NAc administration. Figure 9A shows the mean (± SEM) number of cocaine infusions received during 9d of cocaine SA, with intra-NAc administration of PD325901 or vehicle conducted 30 min prior to the session on day 7. A two-way ANOVA (treatment x day) of days 1-6 revealed no difference between PD325901 or vehicle groups prior to intra-NAc microinjections [$Fs < 1$ except day: $F(5,85) = 13.2$, $p < .0005$]. Independent samples t-tests of the number of cocaine infusions received on days 7-9 indicate that PD325901-injected mice showed a decrease in cocaine intake relative to vehicle-treated mice on day 8 [$t(17) = 2.8$, $p = .012$], but not day 7 or 9 [day 7: $t(17) = 1.1$, $p = .290$; day 9: $t(17) = 1.3$, $p = .228$]. Figure 9B

shows a representative image from a cannula-mounted mouse showing dye injected into the cannula tract in the NAc.

Vehicle- and PD325901-administered mice did not differ in cocaine intake following intra-DS administration. Figure 9C shows the mean (± SEM) number of cocaine infusions received during 9d of cocaine SA, with intra-DS administration of PD325901 or vehicle conducted 30 min prior to the session on day 7. A two-way ANOVA (treatment x day) of days 1-6 revealed no difference between PD325901 and vehicle groups prior to intra-DS microinjections [Fs < 1]. Independent samples t-tests of the number of cocaine infusions received on days 7-9 indicate that PD325901- and vehicle-treated mice showed no difference in cocaine intake on any of the three days [day 7: $t(11) = 0.1$, $p = .891$; day 8: $t(11) = 1.3$, $p = .237$; day 9: $t(11) = 0.7$, $p = .525$]. Figure 9d shows a representative image from a cannula-mounted mouse showing dye injected into the cannula tract in the DS.

These data confirm somewhat our previous findings in RasGRF2 KO mice and LV-RasGRF2-shRNA mice. Consistent with the RasGRF2 KO line, peripheral PD325901 resulted in an increase in cocaine SA, suggesting that general, or systemic, alterations in Ras-ERK signaling may result in a decrement in cocaine reward that is overcome by increasing cocaine intake. Consistent with our findings with LV-RasGRF2-shRNA in mice, PD325901 administered into the NAcc reduced cocaine intake, albeit temporarily, suggesting that focused Ras-ERK inhibition may decrease cocaine reward.

Discussion

Here we sought to extend the understanding of the role of the Ras-ERK pathway in general, and RasGRF1 and RasGRF2 more specifically, in drug-mediated behaviors using operant responding for cocaine in mice. Cocaine SA resulted in an increase in pERK1/2- and pAcH3-positive cells in both the ventral and dorsal striatum relative to controls. RasGRF2 KO mice demonstrated an increase in cocaine SA, while RasGRF1 KO in mice had no effect, suggesting that RasGRF2 is more relevant to the primary reinforcing properties of cocaine than RasGRF1. Increases in pERK1/2- and pAcH3-positive cells in the ventral and dorsal striatum resulting from cocaine SA in WT mice were absent in RasGRF2 KO mice, suggesting an important role of the Ras-ERK signaling cascade in cocaine reinforcement during cocaine SA. Finally, microinjection of LV-RasGRF2-shRNA, but not LV-RasGRF1-shRNA, into both the NAc and DS reduced cocaine intake during SA relative to LV-scrambled-shRNA controls, although this effect was opposite to that seen in RasGRF2 KO mice. Importantly, daily pre-session peripheral administration of the MEK inhibitor PD325901 replicated both the behavioral and molecular effects demonstrated in RasGRF2 KO mice, increasing cocaine intake and decreasing pERK1/2 expression during SA. In contrast, intra-NAc administration of PD325901 resulted in a similar decrease in cocaine intake to that demonstrated following LV-RasGRF2-shRNA administration, albeit only temporarily, while intra-DS PD325901 had no effect. These data suggest that RasGRF2 plays an important role in cocaine reward in mice that can be differentiated on the global and more focal levels.

Our demonstration of an increase in the number of pERK1/2-positive cells in both the dorsal and ventral striatum in response to cocaine relative to controls is consistent with other studies

demonstrating pERK activation following acute and repeated peripheral injections of cocaine (Valjent et al., 2004; Papale et al., 2016). Furthermore, the increase in the number of pAcH3-positive cells in both the ventral and dorsal striatum is consistent with increases in the expression of pERK1/2. Histone H3 is a nuclear ERK substrate activated by mitogen and stress-activated protein kinase 1 (MSK1) that has been linked to the transcriptional activation of IEGs such as c-*fos* (Thomson et al., 1999; Clayton and Mahadevan, 2003), which have been demonstrated to be critical to the enduring plasticity associated with drugs of abuse (Berke and Hyman, 2000; Chandra and Lobo, 2017). Following activation in the cytoplasm, pERK translocates to the nucleus, where it activates MSK1, which subsequently phosphorylates Histone H3 (Brami-Cherrier et al., 2009). The findings of increased pERK1/2 and pAcH3 are consistent with a role for the Ras-ERK pathway in the striatum in cocaine SA.

Cocaine SA in RasGRF2 KO mice resulted in an increase in SA relative to WT mice. In contrast, cocaine SA was not affected in RasGRF1 KO mice, suggesting dissociation between the actions of RasGRF1 and RasGRF2 on cocaine reward. Previous work has demonstrated both impaired conditioned sensitization and CPP in response to cocaine in RasGRF1 KO mice relative to controls (Fasano et al., 2009). These data suggest that RasGRF2 is more relevant for cocaine reward, while RasGRF1 may mediate conditioned responding following the learning of associations between cocaine and cocaine-associated cues and environments.

The increase in cocaine SA in RasGRF2 KO mice likely reflects a decrease in the magnitude of the cocaine reinforcer, requiring an increase in cocaine intake to achieve a similar putative subjective effect, similar to that demonstrated following a reduction in the dose of cocaine (e.g., Thomsen and Caine, 2006). This effect is similar to that seen with peripheral injections of dopamine D1-receptor (D1R) antagonists. The D1R antagonist SCH-23390 has been

627 consistently demonstrated to increase cocaine SA when administered systemically (Haile and
628 Kosten, 2001; Caine et al., 2007) in rodents. Previous research has specifically implicated
629 D1R-mediated signaling (Valjent et al., 2000; Zhang et al., 2004; Bertran-Gonzalez et al.,
630 2008)-- in concert with NMDA receptor activation (Jiao et al., 2007; Ren et al., 2010)-- via
631 subsequent Ras-ERK activation as a critical factor in the regulation of cocaine-dependent
632 synaptic plasticity in the striatum (Girault et al., 2007; Cerovic et al., 2013). In addition to a
633 clear postsynaptic role for Ras-ERK signaling resulting from dopaminergic activity, RasGRF2
634 has also been identified as part of the proteome of the dopamine transporter (Maiya et al.,
635 2007), suggesting a potential presynaptic role for RasGRF2, such as influencing DA release
636 (Bloch-Shilderman et al., 2001).
637
638 Our pERK1/2 data in the RasGRF2 line confirm the likelihood that RasGRF2 mediates its
639 effects via the Ras-ERK pathway. RasGRF2 KO mice did not show the increase in pERK1/2-
640 positive cells in the ventral and dorsal striatum demonstrated in WT littermates and in our first
641 experiment, a likely mechanistic explanation for the increase in SA demonstrated in RasGRF2
642 KO mice. The increased pAcH3 outlined in the first experiment was also replicated in
643 RasGRF2 WT controls, but absent in RasGRF2 KO mice. These findings further implicate
644 this ERK substrate in cocaine SA in mice. Previous studies have demonstrated that an acute
645 injection of cocaine resulted in an increase in pAcH3 (Brami-Cherrier et al., 2005; Bertran-
646 Gonzalez et al., 2008), an effect not present in MSK1 KO mice (Brami-Cherrier et al., 2005)--
647 which also lacked a c-Fos response-- and impaired when preceded by the MEK inhibitor
648 PD325901 (Papale et al., 2016). Our findings suggest that the inhibition of a cocaine-induced
649 increase in pAcH3 is at least in part mediated through the Ras-ERK pathway via RasGRF2.
650

In contrast to our findings in RasGRF2 KO mice, cocaine intake decreased following microinjection of LV-RasGRF2-shRNA into the NAc relative to LV-scrambled-shRNA. It is not entirely clear why the response to cocaine differed in these animals, but may result from a focal knockdown of RasGRF2, in contrast to the global disruption of RasGRF2 in KO animals. In fact, it has been previously hypothesized that the knockout of RasGRF2 in mice resulted in a reduced excitability of VTA DA neurons and subsequent generalized disruption of dopaminergic signaling, which caused a reduction in alcohol consumption in RasGRF2 KO mice relative to controls (Stacey et al., 2012). This hypothesis is consistent with the alteration in basal extracellular DA levels demonstrated in RasGRF2 KO mice (Stacey et al., 2012). In terms of the studies reported here, a disruption in dopaminergic signaling may have resulted in a minor decrease in cocaine reward that was overcome by increased cocaine intake. In contrast, knockdown of RasGRF2 in the NAc likely resulted in a focused, disruptive effect in a brain region that specifically mediates putative cocaine reward (Wise and Bozarth, 1985; Koob and Volkow, 2010). For example, previous studies have demonstrated that minor decreases in cocaine reward result in compensatory increases in cocaine intake, while further decreases result in a reduction of intake (De Wit and Wise, 1977; Ettenberg et al., 1982; Caine and Koob, 1994). These divergent findings may implicate brain areas outside of the striatum in the effects of the RasGRF2 KO on cocaine reward. As already mentioned, RasGRF2 may play an important role on DAT-containing neurons (Bloch-Shilderman et al., 2001). Nonetheless, our data confirm that RasGRF2 is involved in cocaine reward during SA. In contrast, LV-RasGRF1-shRNA administration had no effect on cocaine SA relative to LV-scrambled-shRNA controls, consistent with our findings in RasGRF1 KO mice.

Interestingly, microinjection of LV-RasGRF2-shRNA into the DS also resulted in a decrease in cocaine intake. Beyond its well-known role in the control of habitual behavior following

extensive drug-taking (Everitt and Robbins, 2005; Pierce and Vanderschuren, 2010), some previous research has also indicated a role for the DS in the acute reinforcing properties of cocaine. For example, the magnitude of the dopaminergic response to self-administered cocaine in drug-naïve rats was shown to be similar in the NAc and DS (D'Souza and Duvauchelle, 2006). In addition, Veeneman et al. (2012) demonstrated that the DA receptor antagonist α-flupenthixol administered into the dorsolateral striatum (DLS) altered cocaine SA in rats even after limited exposure (see also Kantak et al., 2002), and disconnection studies between the NAc shell and DLS with α-flupenthixol demonstrated that these serial connections mediate cocaine reinforcement during early cocaine exposure (Veeneman et al., 2015). A role for the DS in cocaine reinforcement is consistent with our molecular results above, in which cocaine SA increased pERK activation in the DS in addition to the VS, effects impaired in KO mice. Further studies will need to clarify whether this effect of RasGRF2 inhibition is due to a serial connection between the VS and DS or results from another as yet unspecified role of the DS in cocaine SA.

Daily SA sessions preceded by administration of the MEK inhibitor PD325901 increased cocaine intake while simultaneously decreasing pERK1/2 levels in the ventral and dorsal striatum. These findings replicated those seen in RasGRF2 KO mice, suggesting that RasGRF2 exerts its effects via the Ras-ERK signaling cascade and confirming the likelihood that a global inhibition of Ras-ERK signaling may result in a loss in cocaine reward that is overcome by a compensatory increased intake. In terms of cocaine, few, if any, studies have demonstrated alterations in cocaine intake specifically during SA resulting from the inhibition of activity of components of the Ras-ERK signaling pathway, such as impairment of MEK (but see Miszkiel et al., 2014). However, numerous previous studies have demonstrated the involvement of MEK, and the subsequent activity of ERK, in other cocaine-related behaviors,

such as CPP (e.g., Valjent et al., 2000; Miller and Marshall, 2005; Papale et al., 2016), which support an inhibitory role of Ras-ERK signaling blockade on cocaine reward. For example, we previously demonstrated that PD325901 administered prior to a test for cocaine conditioned place preference (CPP) resulted in the long-term inhibition of CPP and a complete attenuation of an acute cocaine-induced increase in pERK1/2 in the ventral striatum (Papale et al., 2016). SL327, like PD325901 one of only a few available blood-brain barrier penetrating MEK inhibitors, was also shown to increase alcohol SA in mice when administered prior to daily sessions (Faccidomo et al., 2009) and was interpreted as a compensatory increase in responding due to a decrease in alcohol reward. In addition, RasGRF2 KO mice have previously been shown to demonstrate a loss in alcohol-induced dopamine increase in the NAc and dorsal striatum (Stacey et al. 2012), suggesting a reduction in alcohol reinforcement and consistent with a modulatory role of the Ras-ERK pathway in drug-mediated behaviors.

The site-specific administration of PD325901 into the NAc, but not DS, resulted in a decrease in cocaine intake. PD administered into the NAc failed to have an immediate effect, but during the next session on day 8 resulted in a temporary decrease in cocaine intake that was no longer significant on day 9. A lack of effect on day 7 is not surprising given that the relatively stressful injection procedures likely masked any potential differences between the groups. The decrease in intake the following day is consistent with the pharmacokinetics of PD325901, which showed 50% inhibition of pERK at approximately 24h in rodent brain at the dose used in the current study (Iverson et al., 2009). Furthermore, this effect is consistent with the decrease in cocaine intake following LV-RasGRF2-shRNA, again suggesting that a NAc-specific inhibition of Ras-ERK signaling resulted in an impairment in cocaine SA. That PD325901 had no effect on cocaine intake may be surprising given that LV-RasGRF2-

shRNA in the DS resulted in a reduction in intake similar to that seen with LV-RasGRF2-shRNA in the NAc. As noted above, several studies have suggested a role for the DS in cocaine reward during early exposure, and RasGRF2 knockdown prior to cocaine SA in mice may have inhibited the acquisition of SA, while MEK inhibition at a single administration after several days of SA failed to affect the maintenance of cocaine SA. However, further disentanglement of the specific role of RasGRF2 in the DS during cocaine SA is required.

In summary, we used a combination of molecular, pharmacological, and lentiviral techniques to demonstrate that RasGRF2, but not RasGRF1, is involved in cocaine reinforcement associated with operant SA. These studies further implicate the role of the Ras-ERK pathway in the effects of drugs of abuse and indicate that RasGRF2 may be a risk factor in the cocaine use that may ultimately lead to dependence in humans.

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963 Figures legends
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965

966 Figure 1. Cocaine SA increased pERK1/2 and pAcH3 in C57Bl6/N mice. Cocaine (n = 5)
967 SA resulted in an increase in the number of pERK1/2-positive cells relative to saline controls
968 (n = 6) in both the (A) ventral and (B) dorsal striatum. Data represent mean number of
969 pERK1/2-positive cells (± SEM) in each condition. (C) Representative slices showing pERK-
970 positive cells from the ventral and dorsal striatum of C57Bl6N mice that underwent cocaine
971 or saline SA. Cocaine SA resulted in an increase in the number of pAcH3-positive cells
972 relative to saline controls in both the (D) ventral and (E) dorsal striatum. (F) Representative
973 slices showing pAcH3-positive cells from the ventral and dorsal striatum of C57Bl6N mice
974 that underwent cocaine or saline SA. Mice were sacrificed for immunohistochemistry on day
975 7 of SA, 30 min following the 1st injection of cocaine. *$p < .05$; **$p < .005$
976

977 Figure 2. Cocaine SA in RasGRF1 and RasGRF2 KO mice and WT controls. (A) RasGRF1
978 KO mice (n = 11) did not differ in lever responding relative to WT controls (n = 10). Data
979 represent mean number of presses (± SEM) on the active and inactive levers during 7 daily 1
980 hr sessions of cocaine SA (0.50 mg/kg/14 µl infusion). (B) RasGRF1 KO mice did not differ
981 in the number of cocaine reinforcers achieved relative to WT controls. Data represent mean
982 number of cocaine reinforcers (± SEM) achieved during 7 daily 1 hr sessions of cocaine SA
983 (0.50 mg/kg/14 µl infusion). (C) RasGRF2 KO mice (n = 19) demonstrated an increase in
984 responding on the cocaine-associated lever relative to WT controls (n = 14). Data represent
985 mean number of presses (± SEM) on the active and inactive levers during 7 daily 1 hr sessions
986 of cocaine SA (0.50 mg/kg/14 µl infusion). (D) RasGRF2 KO mice demonstrated an increase
987 in the number of cocaine reinforcers achieved relative to WT controls. Data represent mean

number of cocaine reinforcers (± SEM) achieved during 7 daily 1 hr sessions of cocaine SA (0.50 mg/kg/14 µl infusion). #p < .05, main effect of genotype; **p < .005

Figure 3. Cocaine SA resulted in an increase in pERK1/2 and pAcH3 in WT, but not KO, mice of the RasGRF2 line. Cocaine SA resulted in an increase in the number of pERK1/2-positive cells relative to saline controls in both the (A) ventral and (B) dorsal striatum in WT mice (cocaine, n = 5; saline, n = 5), an effect not present in RasGRF2 KO mice (cocaine, n = 5; saline, n = 5). Data represent mean number of pERK1/2-positive cells (± SEM) in each condition. (C) Representative slices showing pERK-positive cells from the ventral and dorsal striatum of RasGRF2 KO mice and WT controls that underwent cocaine or saline SA. Cocaine SA resulted in an increase in the number of pAcH3-positive cells relative to saline controls in both the (D) ventral and (E) dorsal striatum in WT mice, an effect not present in RasGRF2 KO mice. (F) Representative slices showing pAcH3-positive cells from the ventral and dorsal striatum of RasGRF2 KO mice and WT controls that underwent cocaine or saline SA. Mice were sacrificed for immunohistochemistry on day 7 of SA, 30 min following the 1st injection of cocaine. *p < .05

Figure 4. LV-RasGRF2-shRNA microinjected into the NAc decreased cocaine intake. (A) Representative images of viral eGFP expression in the NAc following microinjections of RasGRF2-shRNA (left) or LV-scrambled-shRNA (right) using fluorescent microscopy. (B) Representative images of viral eGFP expression in the NAc following microinjections of RasGRF1-shRNA (left) or LV-scrambled-shRNA (right) using DAB staining. (C) Mice microinjected with LV-RasGRF2-shRNA (n = 9) and LV-scrambled-shRNA (n = 11) did not differ significantly in lever responding. Data represent mean number of presses (± SEM) on the active and inactive levers during 7 daily 1 hr sessions of cocaine SA (0.50 mg/kg/14 µl

infusion). (D) The number of cocaine reinforcers achieved differed significantly between the groups across daily sessions, with the LV-RasGRF2-shRNA group earning less reinforcers than the LV-scrambled-shRNA group. Data represent mean number of reinforcers (± SEM) achieved during 7 daily 1 hr cocaine SA sessions (0.50 mg/kg/14 µl infusion). *$p < .05$, main effect of treatment

Figure 5. LV-RasGRF1-shRNA microinjected into the NAc had no effect on cocaine SA. (A) Representative images of viral eGFP expression in the NAc following microinjections of RasGRF1-shRNA (left) or LV-scrambled-shRNA (right) using fluorescent microscopy. (B) Representative images of viral eGFP expression in the NAc following microinjections of RasGRF1-shRNA (left) or LV-scrambled-shRNA (right) using DAB staining. (C) Mice microinjected with LV-RasGRF1-shRNA (n = 12) and LV-scrambled-shRNA (n = 13) did not differ significantly in lever responding. Data represent mean number of presses (± SEM) on the active and inactive levers during 7 daily 1 hr sessions of cocaine SA (0.50 mg/kg/14 µl infusion). (D) The number of cocaine reinforcers achieved did not differ significantly between the groups across daily sessions. Data represent mean number of reinforcers (± SEM) achieved during 7 daily 1 hr cocaine SA sessions (0.50 mg/kg/14 µl infusion).

Figure 6. LV-RasGRF2-shRNA microinjected into the DS decreased cocaine intake. (A) Representative images of viral eGFP expression in the DS following microinjections of RasGRF2-shRNA (left) or LV-scrambled-shRNA (right) using fluorescent microscopy. (B) Representative images of viral eGFP expression in the DS following microinjections of RasGRF2-shRNA (left) or LV-scrambled-shRNA (right) using DAB staining. (C) Mice microinjected with LV-RasGRF2-shRNA (n = 9) and LV-scrambled-shRNA (n = 6) did not differ significantly in lever responding. Data represent mean number of presses (± SEM) on

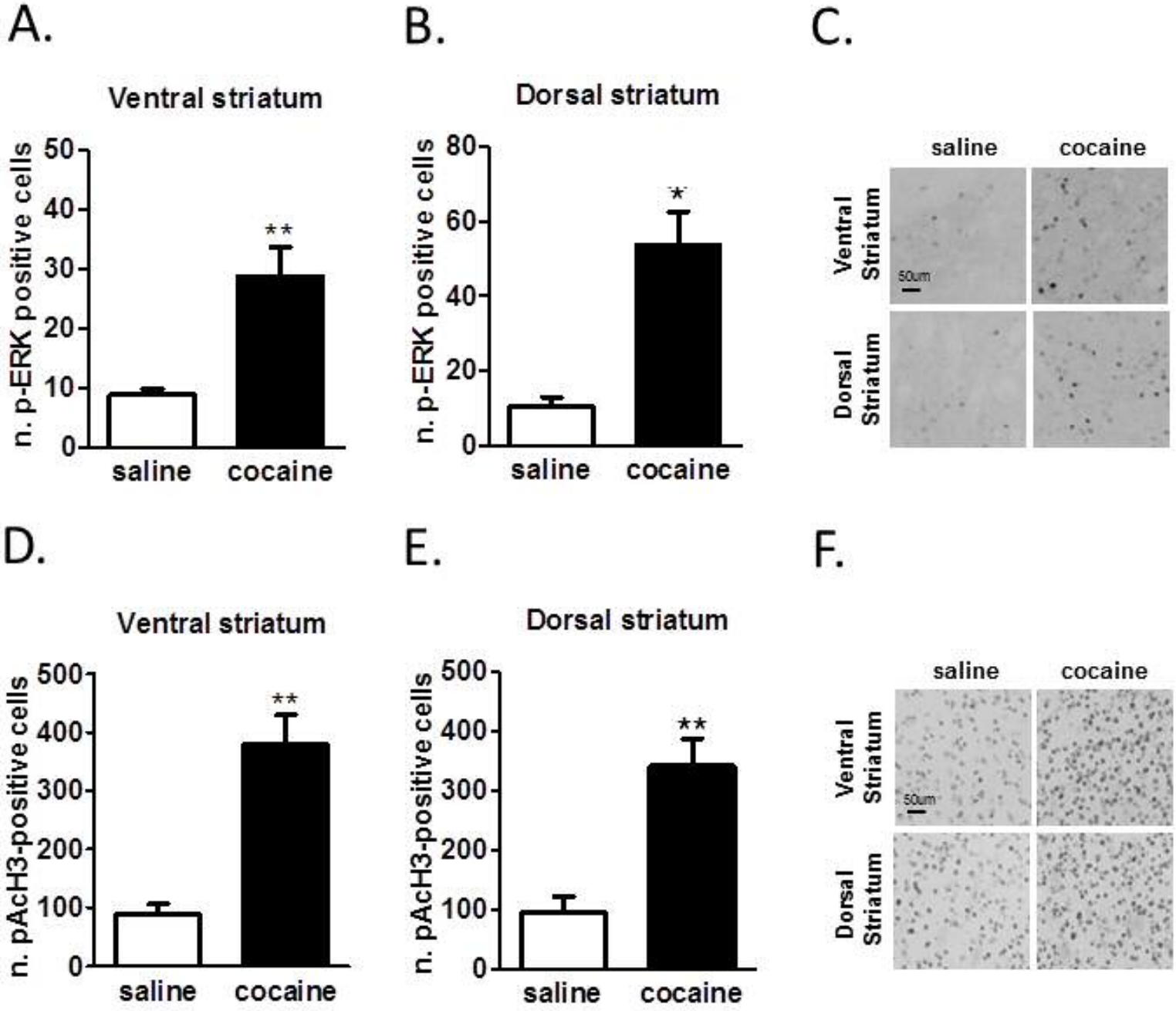
the active and inactive levers during 7 daily 1 hr sessions of cocaine SA (0.50 mg/kg/14 µl infusion). (D) The number of cocaine reinforcers achieved differed significantly between the groups across daily sessions, with the LV-RasGRF2-shRNA group earning less reinforcers than the LV-scrambled-shRNA group. Data represent mean number of reinforcers (± SEM) achieved during 7 daily 1 hr cocaine SA sessions (0.50 mg/kg/14 µl infusion). *p < .05, main effect of treatment

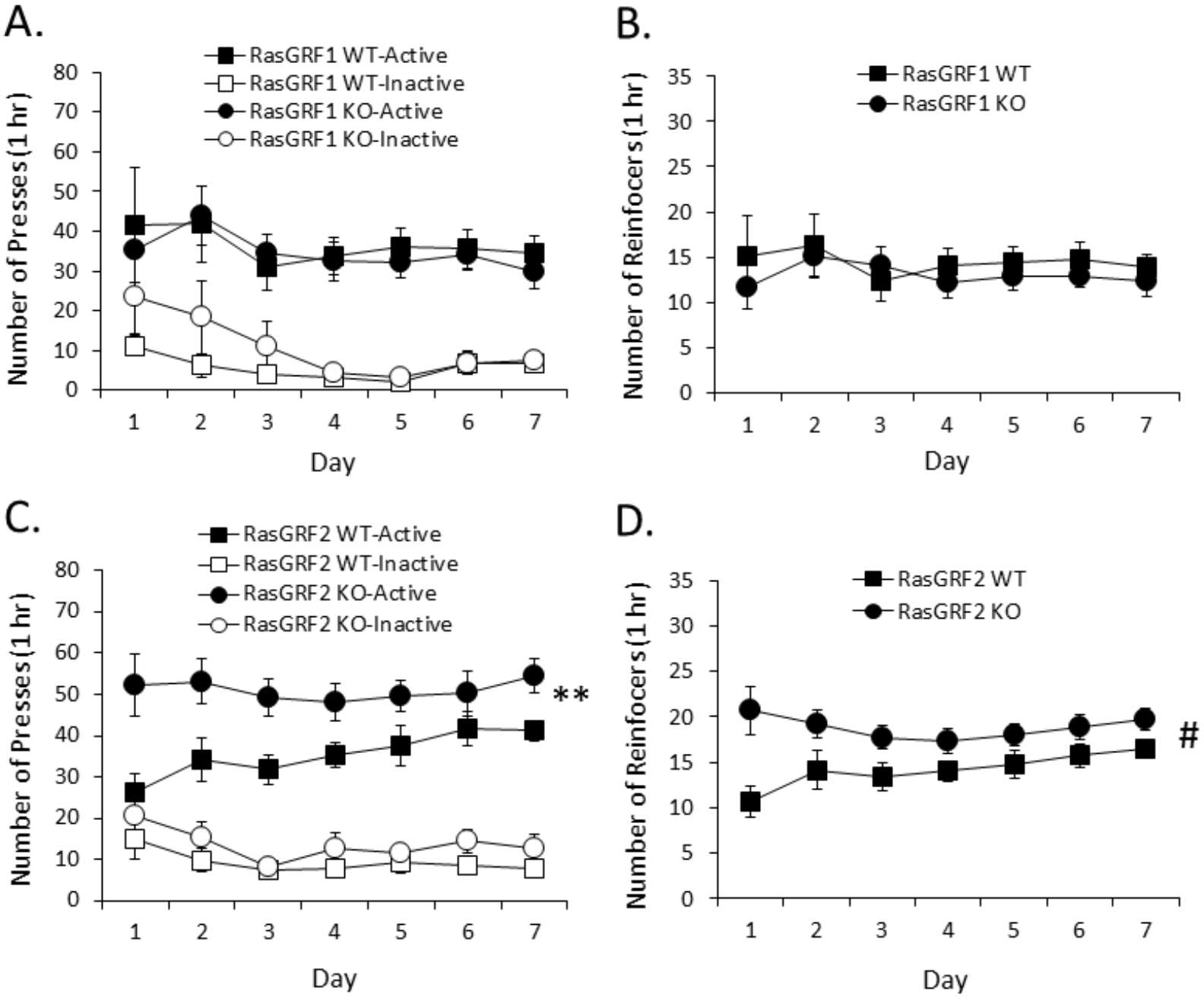
Figure 7. LV-RasGRF2-shRNA decreased pERK. LV-RasGRF2-shRNA (n = 9) resulted in a decrease in the number of pERK-positive cells as compared to LV-scrambled-shRNA (n = 6) in the dorsal striatum in mice sacrificed immediately following the final cocaine SA session on day 7. Data represent mean number of pERK1/2-positive cells (± SEM) in each condition, with representative slices showing pERK-positive cells from the dorsal striatum of LV-RasGRF2-shRNA and LV-scrambled-shRNA below. ***p < .0005

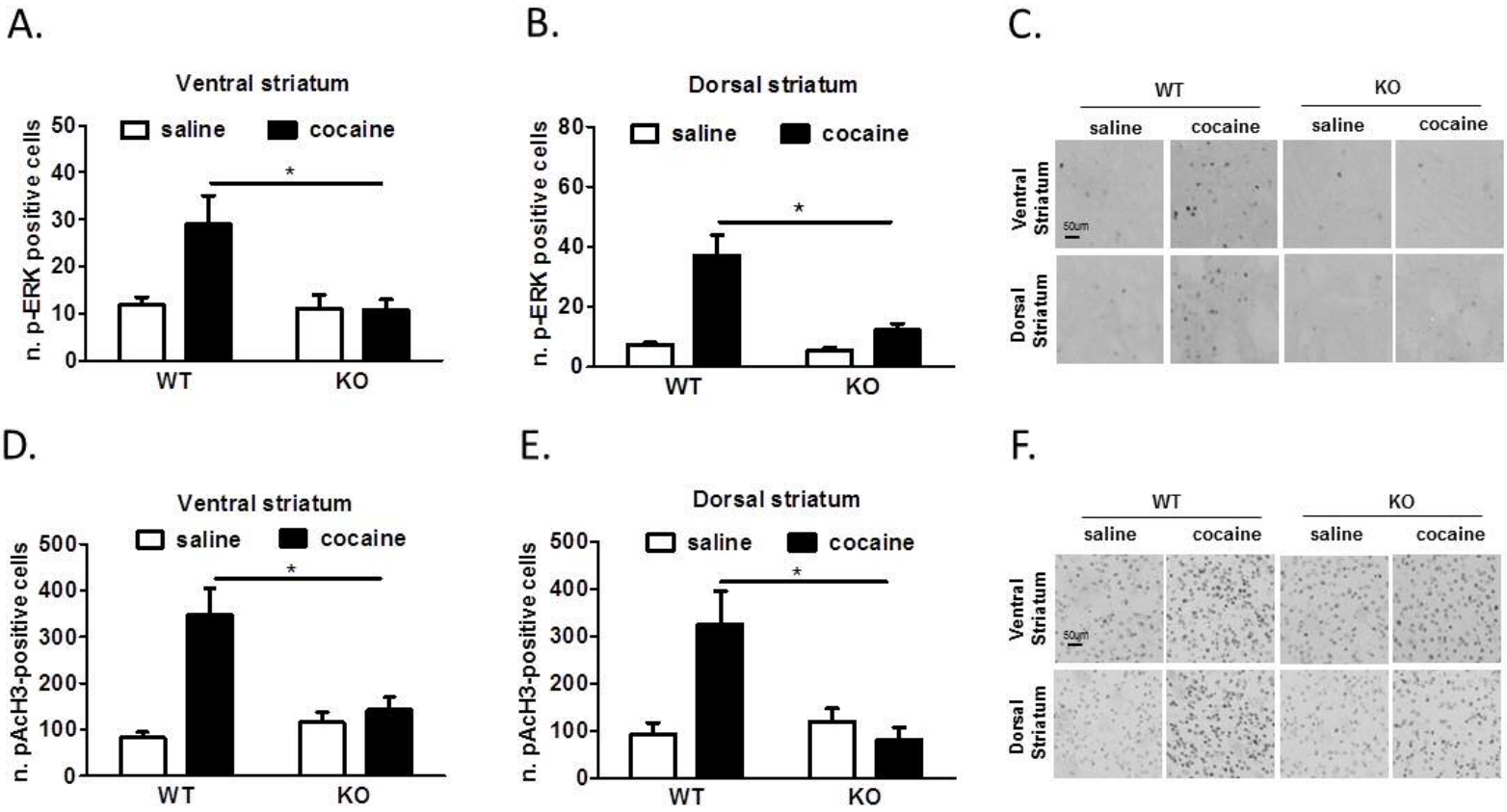
Figure 8. PD325901 administered IP increased cocaine intake and decreased pERK. (A) Mice treated with IP vehicle (VEH; n = 9) or PD325901 (PD, 10 mg/kg; n = 11) prior to daily cocaine SA sessions did not differ significantly in lever responding. Data represent mean number of presses (± SEM) on the active and inactive levers during 7 daily 1 hr sessions of cocaine SA (0.50 mg/kg/14 µl infusion). (B) The number of cocaine reinforcers achieved differed significantly between the groups across daily sessions, with the PD group earning more reinforcers than the VEH group. Data represent mean number of reinforcers (± SEM) achieved during 7 daily 1 hr cocaine SA sessions (0.50 mg/kg/14 µl infusion). PD325901 (n = 5) decreased the number of pERK1/2-positive cells relative to vehicle (n = 5) in both the (C) ventral and (D) dorsal striatum following pretreatment prior to 7 daily cocaine SA sessions. Data represent mean number of pERK1/2-positive cells (± SEM) in each condition. (E)

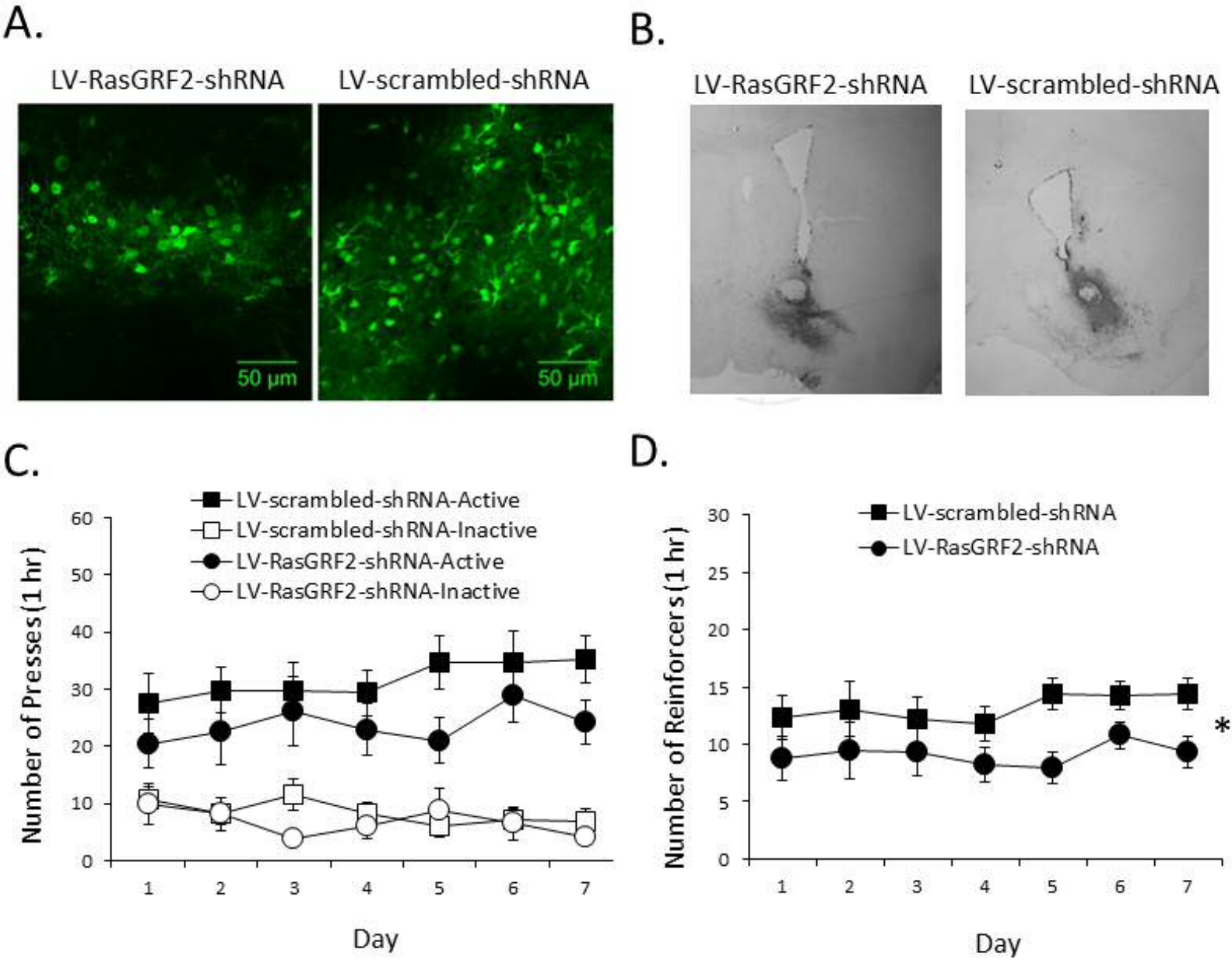
Representative slices showing pERK-positive cells from the ventral and dorsal striatum of C57Bl6N mice that received vehicle or PD325901. Mice were sacrificed for immunohistochemistry on day 7 of SA, 30 min following the 1st injection of cocaine. **p < .005, main effect of treatment; ##p = .005

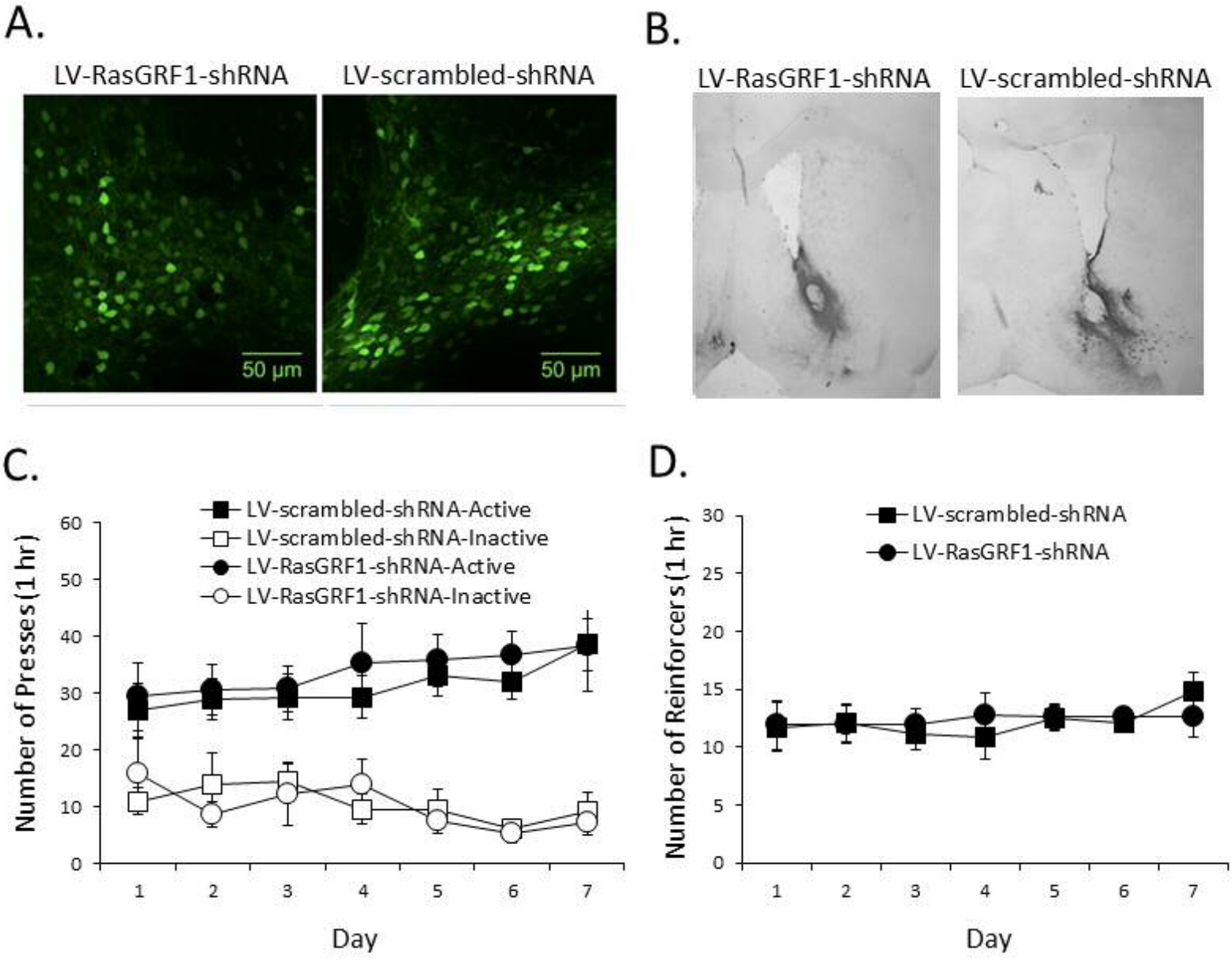
Figure 9. Intra-NAc, but not intra-DS, PD325901 decreased cocaine intake. (A) The number of cocaine reinforcers achieved differed between groups administered vehicle (VEH; n = 10) or PD325901 (PD; n = 9) into the NAc only on day 8, with the PD325901 group earning less reinforcers than the vehicle group. Data represent mean number of reinforcers (± SEM) achieved during 9 daily 1 hr cocaine SA sessions (0.50 mg/kg/14 µl infusion). PD325901 (5 ng/0.5 µl /side) or vehicle was administered into the NAc 30 minutes prior to the day 7 SA session; inset shows a representative image from a cannula-mounted mouse showing dye injected into the cannula tract in the NAc (black arrows denote location of catheter tip). (B) The number of cocaine reinforcers achieved differed between groups administered vehicle (VEH; n = 6) or PD325901 (PD; n = 7) into the DS only on day 8, with the PD325901 group earning less reinforcers than the vehicle group. Data represent mean number of reinforcers (± SEM) achieved during 9 daily 1 hr cocaine SA sessions (0.50 mg/kg/14 µl infusion). PD325901 (5 ng/0.5 µl /side) or vehicle was administered into the DS 30 minutes prior to the day 7 SA session; inset shows a representative image from a cannula-mounted mouse showing dye injected into the cannula tract in the DS (black arrows denote location of catheter tip). *p < .05

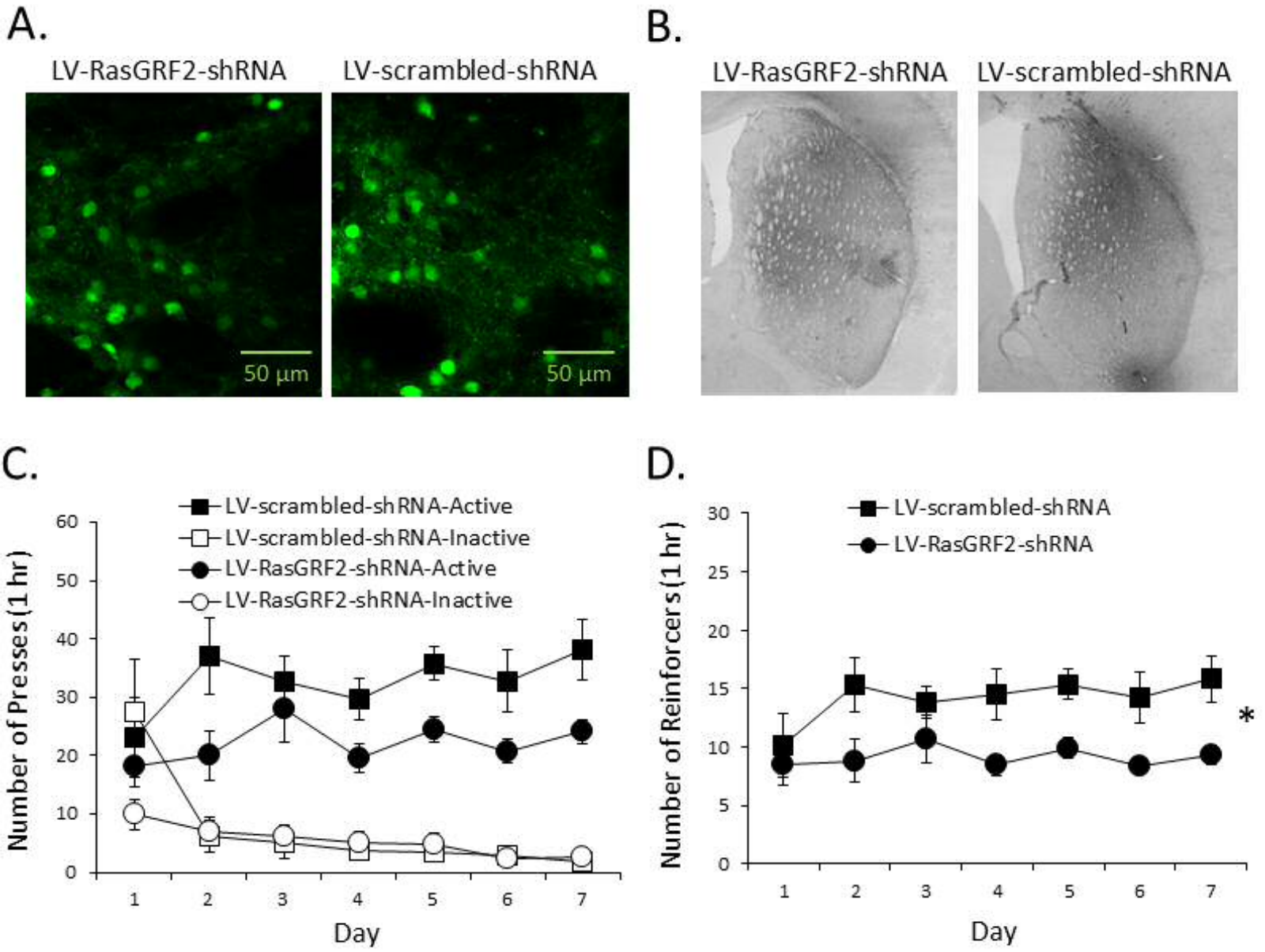

A.
Ventral striatum
n. p-ERK positive cells
50
40
30
20
10
0
**
saline
cocaine
B.
Dorsal striatum
n. p-ERK positive cells
80
60
40
20
0
*
saline
cocaine
C.
saline
cocaine
Ventral Striatum
Dorsal Striatum
50um
D.
Ventral striatum
n. pAcH3-positive cells
500
400
300
200
100
0
**
saline
cocaine
E.
Dorsal striatum
n. pAcH3-positive cells
500
400
300
200
100
0
**
saline
cocaine
F.
saline
cocaine
Ventral Striatum
Dorsal Striatum
50um

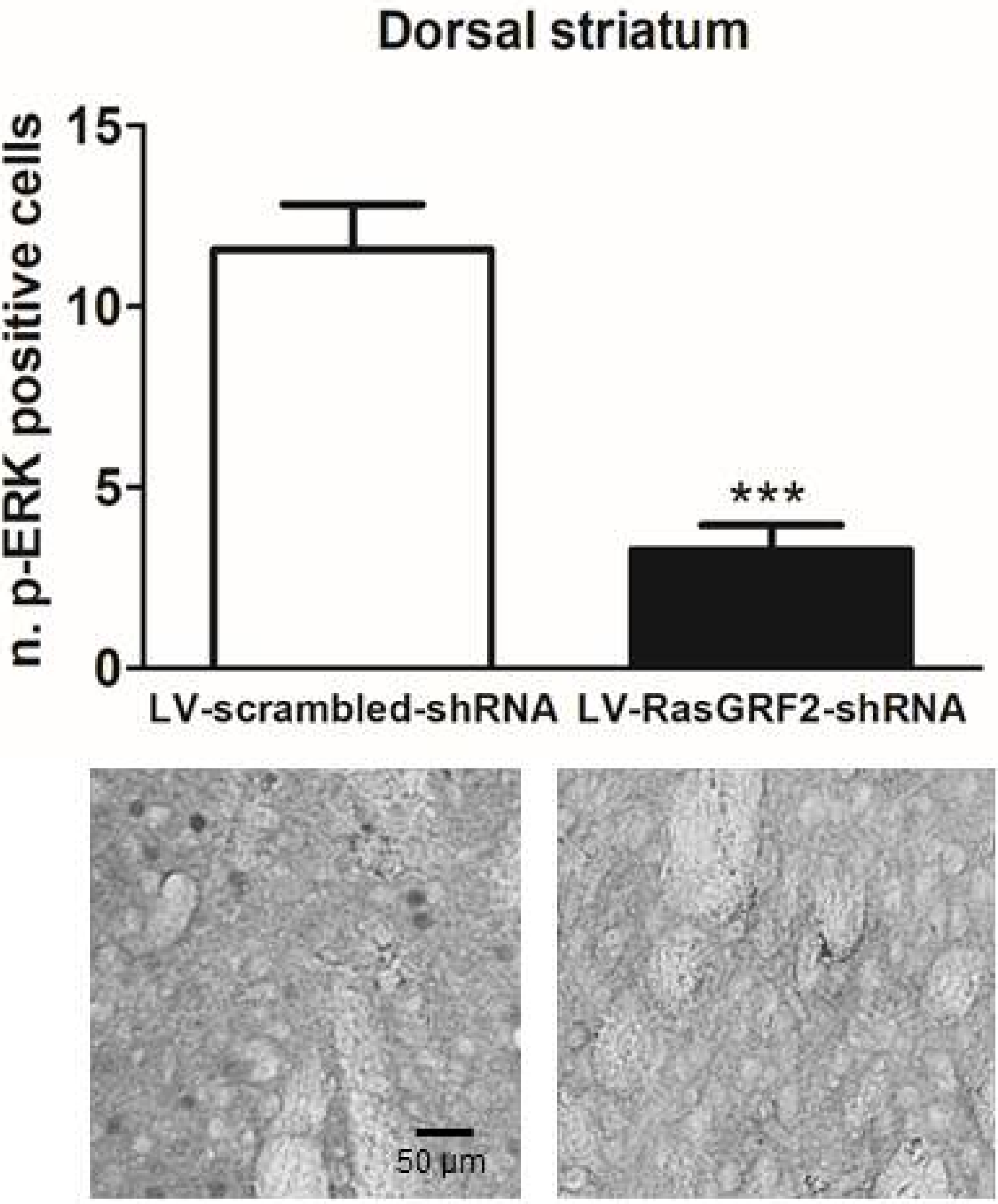

A.
RasGRF1 WT-Active
RasGRF1 WT-Inactive
RasGRF1 KO-Active
RasGRF1 KO-Inactive
Number of Presses (1 hr)
Day
B.
RasGRF1 WT
RasGRF1 KO
Number of Reinfocers (1 hr)
Day
C.
RasGRF2 WT-Active
RasGRF2 WT-Inactive
RasGRF2 KO-Active
RasGRF2 KO-Inactive
**
Number of Presses (1 hr)
Day
D.
RasGRF2 WT
RasGRF2 KO
#
Number of Reinfocers (1 hr)
Day

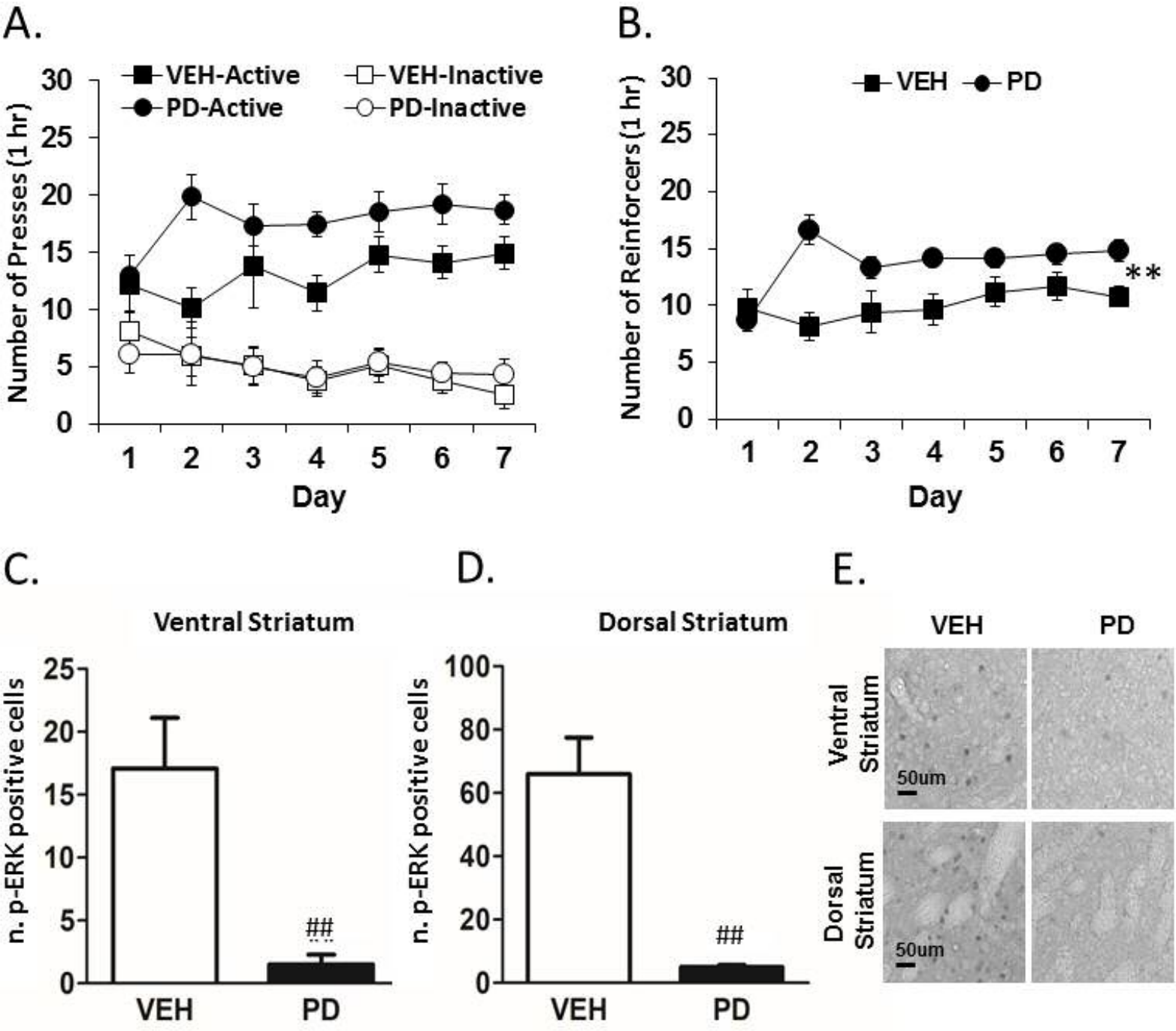

A.
Ventral striatum
n. p-ERK positive cells
saline
cocaine
*
WT
KO
B.
Dorsal striatum
n. p-ERK positive cells
saline
cocaine
*
WT
KO
C.
WT
KO
saline
cocaine
saline
cocaine
Ventral Striatum
Dorsal Striatum
50um
D.
Ventral striatum
n. pAcH3-positive cells
saline
cocaine
*
WT
KO
E.
Dorsal striatum
n. pAcH3-positive cells
saline
cocaine
*
WT
KO
F.
WT
KO
saline
cocaine
saline
cocaine
Ventral Striatum
Dorsal Striatum
50um


A.
LV-RasGRF2-shRNA
LV-scrambled-shRNA
50 µm
50 µm
B.
LV-RasGRF2-shRNA
LV-scrambled-shRNA
C.
LV-scrambled-shRNA-Active
LV-scrambled-shRNA-Inactive
LV-RasGRF2-shRNA-Active
LV-RasGRF2-shRNA-Inactive
Number of Presses (1 hr)
Day
D.
LV-scrambled-shRNA
LV-RasGRF2-shRNA
Number of Reinforcers (1 hr)
*
Day

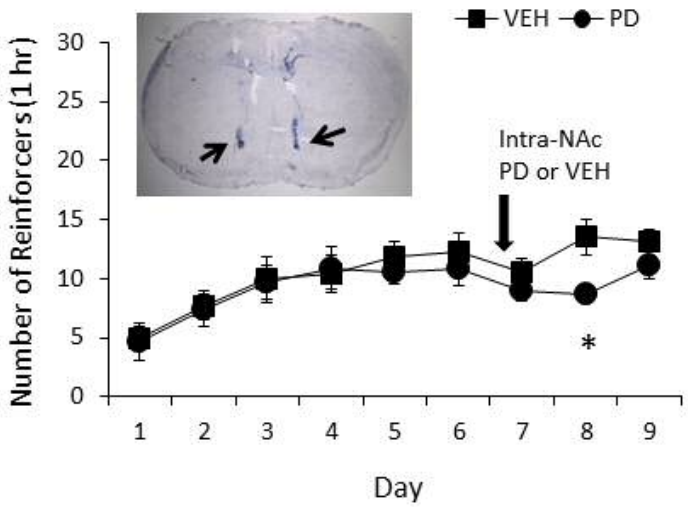

A.
LV-RasGRF1-shRNA
LV-scrambled-shRNA
50 µm
50 µm
B.
LV-RasGRF1-shRNA
LV-scrambled-shRNA
C.
LV-scrambled-shRNA-Active
LV-scrambled-shRNA-Inactive
LV-RasGRF1-shRNA-Active
LV-RasGRF1-shRNA-Inactive
Number of Presses (1 hr)
Day
D.
LV-scrambled-shRNA
LV-RasGRF1-shRNA
Number of Reinforcers (1 hr)
Day


A.
LV-RasGRF2-shRNA
LV-scrambled-shRNA
50 µm
50 µm
B.
LV-RasGRF2-shRNA
LV-scrambled-shRNA
C.
LV-scrambled-shRNA-Active
LV-scrambled-shRNA-Inactive
LV-RasGRF2-shRNA-Active
LV-RasGRF2-shRNA-Inactive
Number of Presses (1 hr)
Day
D.
LV-scrambled-shRNA
LV-RasGRF2-shRNA
Number of Reinforcers (1 hr)
Day
*

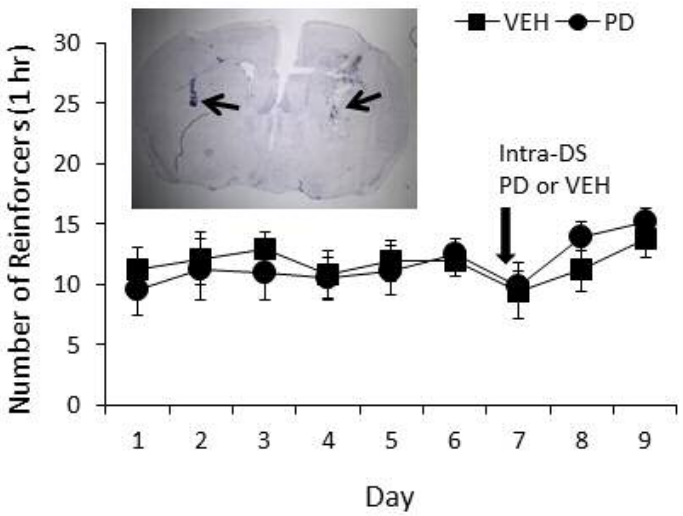

Dorsal striatum
n. p-ERK positive cells
15
10
5
0
***
LV-scrambled-shRNA
LV-RasGRF2-shRNA
50 µm


A.
VEH-Active
VEH-Inactive
PD-Active
PD-Inactive
Number of Presses (1 hr)
Day
B.
VEH
PD
Number of Reinforcers (1 hr)
**
Day
C.
Ventral Striatum
n. p-ERK positive cells
##
VEH
PD
D.
Dorsal Striatum
n. p-ERK positive cells
##
VEH
PD
E.
VEH
PD
Ventral Striatum
50um
Dorsal Striatum
50um

A.


VEH
PD
Number of Reinforcers (1 hr)
30
25
20
15
10
5
0
Intra-NAc
PD or VEH
*
1
2
3
4
5
6
7
8
9
Day

B.


VEH
PD
Number of Reinforcers (1 hr)
30
25
20
15
10
5
0
Intra-DS
PD or VEH
1
2
3
4
5
6
7
8
9
Day