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Protein kinase C ϵ activity regulates mGluR5 surface expression in the rat nucleus accumbens

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Abstract

Type 5 metabotropic glutamate receptors (mGluR5) activate protein kinase C (PKC) via coupling to G $\alpha_q/11$ protein signaling. We have previously demonstrated that the epsilon isoform of PKC (PKC ϵ) is a critical downstream target of mGluR5 in regulating behavioral and biochemical responses to alcohol. Recent evidence suggests that PKC-mediated phosphorylation of mGluR5 can lead to receptor desensitization and internalization. We therefore sought to examine the specific involvement of PKC ϵ in the regulation of mGluR5 surface expression in the nucleus accumbens (NAc), a key regulator of alcohol-associated behaviors. Coronal brain sections from male Wistar rats analyzed for either co-localization of mGluR5 and PKC ϵ via immunohistochemistry, or changes in mGluR5 surface expression and PKC ϵ phosphorylation following local application of PKC ϵ translocation activator or inhibitor peptides and/or an orthosteric mGluR5 agonist. We observed co-localization of mGluR5 and PKC ϵ in the NAc. We also showed that intra-NAc infusion of PKC ϵ translocation inhibitor ϵ V1-2 increased mGluR5 surface expression under baseline conditions. Stimulation of mGluR5 with an orthosteric agonist DHPG, dose-dependently increased ERK1/2 and PKC ϵ phosphorylation as well as mGluR5 internalization in acute NAc slices. Finally, we observed that activation of PKC ϵ translocation with Tat- ψ eRACK peptide mediates agonist-independent mGluR5 internalization, while PKC ϵ translocation inhibitor ϵ V1-2 prevents agonist-dependent internalization of mGluR5 in NAc slice preparations. These findings suggest that the subcellular localization of mGluR5 in the NAc is regulated by PKC ϵ under basal and stimulation conditions, which may influence the role of mGluR5-PKC ϵ signaling in alcohol-related behaviors.

GRAPHICAL ABSTRACT

The current study suggests a novel functional interaction between mGluR5 and PKC ϵ in the rat nucleus accumbens as evidenced by (1) co-localization of mGluR5 and PKC ϵ , and (2) the

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

ROLE OF AUTHORS

Both authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, and critical revision of the article: MS and MFO.

heart failure (Ferreira et al. 2011), progression of various types of tumors (Jain and Basu 2014), as well as in behavioral response to alcohol (Hodge et al. 1999; Migues et al. 2010; Shirai et al. 2008; Zeidman et al. 2002), and more recently, cocaine (Miller et al. 2016).

With specific regards to alcohol, it has been shown that brief exposure of PKC ϵ -expressing cell-lines to alcohol causes enzyme translocation from perinuclear regions to the cytoplasm (Gordon et al. 1997). In animals, binge alcohol drinking elevates phospho-Ser729-PKC ϵ levels in both the NAc and the central nucleus of the amygdala in mice (Cozzoli et al. 2015). This latter study further demonstrated that inhibition of PKC ϵ translocation within both brain regions reduced binge alcohol consumption in a manner dependent on intact function of mGluR1/5. Likewise, a previous study by our group demonstrated that PKC ϵ is a downstream signaling target of mGluR5 that mediates the ability of mGluR5 antagonists to reduce voluntary alcohol intake when administered systemically (Olive et al. 2005) or into the NAc (Gass and Olive 2009). These findings suggest that ventral striatal mGluR5 receptors are coupled to PKC ϵ , and that in turn activity of PKC ϵ is critical for mGluR5 regulation of alcohol-related behaviors. However, the nature and the cellular mechanisms of PKC ϵ -dependent regulation of mGluR5 remain unclear.

In general, activation of PKC results in phosphorylation a number of cellular substrates, including mGluR1/5 (Mao et al. 2008). PKC-mediated phosphorylation of mGluR1/5 can then lead to receptor desensitization and internalization (Gereau and Heinemann 1998; Ko et al. 2012; Lee et al. 2008). Interestingly, decreased surface expression of mGluR5 in the striatum following a prolonged withdrawal from cocaine self-administration (Knackstedt et al. 2014) coincides with increased phosphorylation of PKC ϵ in the same tissue (Schwendt and Olive 2012). In order to investigate the relationship between PKC ϵ activation and mGluR5 surface expression, the present study sought to determine whether: 1) mGluR5 and PKC ϵ co-localize in the rat NAc, 2) whether stimulation of mGluR5 in vivo increases PKC ϵ activation as measured by phosphorylation of PKC ϵ Ser729 in this region, and 3) whether activation or inhibition of PKC ϵ translocation alters mGluR5 surface expression in the NAc. As abnormal function of mGluR5 has been implicated in the pathophysiology of many neurological and psychiatric disorders, including anxiety, schizophrenia, fragile X mental retardation syndrome and drug addiction, understanding and manipulating mGluR5-PKC ϵ functional interactions may be utilized in the development of novel neurobiologically-based therapies (Olive 2009).

MATERIALS AND METHODS

Animals

Adult male Wistar rats (Charles River Laboratories, Raleigh, NC, USA) weighing 275–300 g were single-housed in a temperature and humidity controlled vivarium on a reversed 12-hour light/dark cycle with food and water available *ad libitum*. All animal procedures in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by an Institutional Animal Care and Use Committee.

Drugs and Tat peptides

(RS)-3,5-dihydroxyphenylglycine (DHPG) was purchased from Abcam Biochemicals (Cambridge, MA, USA) and dissolved in artificial cerebrospinal fluid (ACSF; in mM: 125 NaCl, 1 CaCl₂, 2.5 KCl, 4 MgCl₂, 25 NaHCO₃, 1.25 NaH₂PO₄, 0.4 ascorbic acid and 10 D-glucose). Peptides were custom synthesized by AnaSpec (Fremont, CA, USA) and dissolved in ACSF at concentrations were based on results of previously published studies (Gass and Olive 2009; Liron et al. 2007). To facilitate cell permeability, peptides were modified at their N-terminus by attaching either myristoyl acid (CH₃(CH₂)₁₂COOH) or Tat peptide sequence YGRKKRRQRRR (Tat₄₅₋₅₇). Peptides utilized here were: the PKCε translocation activator HDAPIGYD (Tat-ψeRACK), the PKCε translocation inhibitor EAVSLKPTT (Tat-εV1-2), or scrambled control peptides (Tat-sc). Peptide inhibiting PKCε translocation was also used in its myristoylated form (Myr-εV1-2). Concentrations of DHPG and PKCε peptides used in this study were based on previously published information (Bajo et al. 2008; Mao et al. 2005; Tronson et al. 2010; Wang et al. 2013).

Immunohistochemistry (Experiment 1)

Rats were anesthetized with sodium pentobarbital (100 mg/kg) and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% w/v paraformaldehyde (pH=7.4) in PBS. Brains were then removed, post-fixed overnight at 4°C and cryoprotected in a 30% w/v sucrose/PBS solution for at least 48 hrs at 4°C. Next, 40 μm thick sections were cut on a cryostat, and free-floating sections were then pre-blocked with PBS containing 0.1% Tween 20 (PBST) and 5% v/v normal donkey serum for 2 hr. Sections were then incubated with rabbit anti-mGluR5 (Neuromics, Edina, MN, USA) and mouse anti-PKCε (BD Transduction Laboratories, San Jose, CA, USA) primary antisera overnight at 4°C under gentle agitation. On the following day, sections were washed in PBST and incubated with AlexaFluor488-conjugated donkey anti-mouse IgG (1:500) and AlexaFluor568-conjugated donkey anti-rabbit IgG (1:500) secondary antisera (Jackson ImmunoResearch, West Grove, PA, USA) for 4 hr at room temperature. Finally, sections were washed, mounted onto microscope slides with mounting media containing an anti-fade reagent, coverslipped, sealed, and stored in darkness. Immunoreactivity in the NAc visualized on a Leica SP5 confocal laser scanning microscope equipped with a krypton/argon laser at 10–40× magnification. Images were obtained at a 400 Hz scan speed at an 8-bit pixel resolution of 1024 × 1024. AlexaFluor488 was excited at 488 nm with an emission filter range of 495–520 nm, and AlexaFluor568 was excited at 568 nm with an emission filter range of 575–620 nm. Pixels from AlexaFluor488 wavelengths were assigned a green color, pixels from AlexaFluor594 wavelengths were assigned a red color, and overlapping pixels were assigned a yellow color. For assessment of the degree of co-localization of mGluR5 and PKCε, a total of n=6 images each of the NAc core and shell subregions were obtained, and the number of co-localized cells were divided by the total number of mGluR5-positive cells to yield a percent co-localization value. All primary antibodies used in this study are described in detail in Table 1.

Stereotaxic surgery and intracranial peptide administration (Experiment 2)

Rats were anesthetized with ketamine HCl (87.5 mg/kg, IM) and xylazine (5 mg/kg, IM). Ketorolac (3 mg/kg, IP) was administered pre-operatively to provide analgesia. Animals were then placed into a small animal stereotaxic instrument (Stoelting Instruments, Wood Dale, IL, USA) and guide cannulas were implanted and secured with dental cement. Cannulas (20 gauge; Plastics One, Roanoke, VA, USA) were implanted 2 mm above the infusion target according to following coordinates: NAc (+1.8 mm, AP; ± 2.5 mm, ML 60 angle; -7.0 mm, DV) relative to bregma according to (Paxinos and Watson 2007). Animals were allowed to recover for 5 days prior to the intracranial infusions of myristoylated peptides. For the infusion, two 33-gauge microinjectors that extended 2 mm beyond the tip of the guide cannula were bilaterally inserted into the NAc. Scrambled myristoylated control peptide) or Myr-eV1-2 peptides (5 pmol) were infused at a rate of 0.5 μ l/minute in a total volume of 0.5 μ l/side (NAc) using an infusion pump (Harvard Apparatus, Holliston, MA, USA). After the infusion was completed, the microinjectors were left in place for an additional 1 minute to allow for diffusion of peptides. Animal were sacrificed 20 min following the infusion and acute NAc slices were prepared as described below.

Slice preparation and treatment (Experiment 3 and 4)

After rapid decapitation, rat brains were removed and briefly chilled in ice-cold ACSF oxygenated with 95% O₂/5% CO₂. Next, 2 mm-thick coronal slices containing the NAc were prepared using the rat brain matrix (ASI instruments, Warren, MI, USA). The NAc tissue was then bilaterally dissected using a 2-mm micropunch (Harris-Unicore, Ted Pella, Redding, CA, USA) as illustrated on Figure 2A and cut into 250- μ m slices using a McIlwain Tissue Chopper (Stoelting, Wood Dale, IL, USA). Acute NAc slices were equilibrated for 1 hour at 22°C in oxygenated ACSF. After equilibration, slices were incubated with Tat peptides (5 μ M) for 45 min at 37°C in oxygenated ACSF (see: Ster et al. 2009, for more details). Alternatively, after 15 min incubation with Tat peptides, 100 μ M DHPG was added to subsets of slices for an additional 30 mins. Immediately after incubation with peptides or DHPG, slices were washed with ice-cold ACSF and subjected to surface biotinylation as described below. Incubation times and conditions were based on the previously published literature (Choe and Wang 2001; Lee et al. 2008; Jin et al. 2013). In addition to slice preparation as described above, in Experiment 3 the presence of peptide infusion site in the dissected NAc tissue was verified as follows: first, 2mm coronal slice had to contain the signs of microinjection point of entry into the brain, and second, the presence of cannulea tracks in NAc slices was visually confirmed.

Surface biotinylation (Experiments 2–4)

Surface biotinylation was conducted as described in our previous studies (Knackstedt and Schwendt 2016; Knackstedt et al. 2014) with a few modifications. Briefly, NAc slices were biotinylated with 1 mg/ml EZ link NHS-SS-Biotin/ACSF (Thermo Fisher Scientific, Rockford, IL, USA) for 1 hr at 4°C. Next, biotinylation reaction was quenched with a 100 mM glycine/ACSF buffer. Biotinylated slices were homogenized in a lysis buffer (25 mM HEPES, 150 mM NaCl, 1% Triton X-100, 0.1% SDS) supplemented with protease and phosphatase inhibitors. Following incubation for 1 hr at 4°C, insoluble debris was removed

by centrifugation. An equal aliquot of pre-cleared solubilized total protein fraction (T) was saved and frozen at -80°C . Biotinylated proteins were captured by incubating with Neutravidin agarose beads (Thermo Fisher Scientific) overnight at 4°C . After non-biotinylated, intracellular (I) proteins were removed, bound biotinylated surface (S) proteins were recovered from the beads with elution buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, and 50 mM dithiothreitol, pH 6.8) and incubation at 95°C for 10 min. Protein concentration in total, intracellular and surface fractions was determined using BCA assay (Thermo Fisher Scientific) and proteins of interest were analyzed by immunoblotting as described below.

Immunoblotting (Experiments 2–4)

Equal aliquots from each fraction separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked for 1 hr in 5% milk/Tris-buffered saline and probed overnight at 4°C with the following primary antibodies diluted in 5% milk/Tris-buffered saline containing 0.1% Tween-20: calnexin (Enzo Life Sciences, Farmingdale, NY, USA), syntaxin1a (Abcam, Cambridge, MA, USA), mGluR5, PKC ϵ , phospho-(Ser729)-PKC ϵ (all from Millipore, Billerica, MA, USA) and ERK1/2 and phospho-ERK1/2 (both from Cell Signaling Technology, Danvers, MA, USA). After incubation with an appropriate horseradish peroxidase-conjugated secondary antiserum (Jackson ImmunoResearch, West Grove, PA, USA), immunoreactive bands on the membranes were detected by ECL+ chemiluminescence reagents on an X-ray film (GE Healthcare, Piscataway, NJ, USA). Subsequently, blots were stripped and re-probed with calnexin and syntaxin-1a antibodies to monitor biotinylation of intracellular proteins and to normalize for unequal loading and/or transfer of proteins. The integrated band density of each protein sample was measured using NIH Image J software version 1.32j (RRID: SCR_003070). All primary antibodies used in this study are described in detail in Table 1.

Statistical analysis

Immunoblotting data, represented by integrated density of individual protein bands, were normalized to the density of calnexin or syntaxin-1a immunoreactivity within the same sample. Differences between treatment groups were analyzed by Student's t-test or by one-way ANOVA followed by multiple comparisons vs. control group (Bonferroni test). For immunohistochemical co-localization data, a single value for each NAc subregion in each tissue section was obtained and averaged across all sections, and data were analyzed using a Student's t-test. For all statistical analyses used, the alpha level was set at 0.05. Sigma Plot software version 10 (RRID:SCR_003210, Systat Software, San Jose, CA, USA) was used to analyze all data.

RESULTS

Experiment 1: Co-localization of mGluR5 and PKC ϵ in the NAc

Immunoreactivity for both mGluR5 and PKC ϵ were highly cytoplasmic within cell bodies in the NAc, with occasional immunoreactivity in singular processes extended from the cell soma (Fig. 1). PKC ϵ immunoreactivity was found to be co-localized with that of mGluR5 in approximately one-third of mGluR5-positive cells (NAc core $32.06 \pm 3.91\%$, NAc shell

$32.24 \pm 1.37\%$), and there were no significant differences in the percentage of cells demonstrating co-localization of these two antigens ($t(6) = -0.04$, $p > 0.05$).

Experiment 2: Inhibition of PKC ϵ translocation in vivo increases surface expression of mGluR5 in the NAc

mGluR5 protein was detected in both surface and intracellular fractions obtained by surface biotinylation of acute NAc slices (Fig. 2A). Under the mild reducing conditions used in this study, the dimer was the predominant form of mGluR5 detected. As the mGluR5 dimer is thought to represent mature, functional form of mGluR5 (Brock et al. 2007), in the remainder of this study (Experiments 2–4), only the dimeric form of mGluR5 was analyzed. The presence of the membrane protein syntaxin-1a in the surface fraction and limited or no presence of known intracellular proteins (PKC ϵ and calnexin) in the same fraction demonstrated that biotinylation selectively labelled cell surface proteins (Fig. 2A). The presence of syntaxin-1a in both surface membrane and intracellular fractions corresponds with its known role in presynaptic exo- and endocytosis (e.g. Wu et al. 1999; Yu et al. 2006). Microinfusion of the myristoylated PKC ϵ translocation inhibitor peptide Myr- ϵ V1-2 (5 pmol/ μ l) into the NAc resulted in increased surface expression of mGluR5 in NAc slices, as compared to local application of a scrambled control Myr peptide (Fig. 2B; $t(6) = 2.67$, $p < 0.05$). This increase in mGluR5 surface expression was accompanied by reduced levels of intracellular mGluR5 (Fig. 2C, $t(6) = 2.79$, $p < 0.05$) without a change in total receptor levels (data not shown), suggesting that inhibition of PKC ϵ translocation promotes insertion of intracellular mGluR5 receptors into the membrane under basal (unstimulated) conditions.

Experiment 3: Activation of mGluR5 dose-dependently increases mGluR5 internalization as well as ERK1/2 and PKC ϵ phosphorylation in acute NAc slices

Incubation of NAc slices with the Group I mGluR agonist DHPG dose-dependently altered the surface expression of mGluR5, as well as the phosphorylation state of two kinases, ERK1/2 and PKC ϵ , known to be phosphorylated in response to mGluR5 activation (Choe and Wang 2001; Olive et al. 2005). With regards to mGluR5 surface expression, one-way ANOVA analysis revealed a main effect of DHPG treatment ($F(2,10) = 4.67$, $p < 0.05$). Post-hoc analysis revealed that mGluR5 surface expression was reduced in NAc slices treated with 100 μ M DHPG when compared to vehicle-treated slices (Fig. 3A; $p < 0.05$). There was also a main effect of DHPG treatment on phosphorylation of ERK1/2 in the NAc ($F(2,10) = 17.96$, $p < 0.01$). Treatment with 100 μ M (but not 10 μ M) DHPG increased phosphorylation of ERK1/2 in the NAc slices when compared to vehicle-treated slices (Fig. 3B; $p < 0.01$). One-way ANOVA analysis revealed a main effect of DHPG treatment on phosphorylation of PKC ϵ at Ser729 ($F(2,10) = 7.22$, $p < 0.05$). Again, increased phosphorylation was observed only in the NAc slices treated with the highest (100 μ M) concentration of DHPG (Fig. 3C; $p < 0.05$).

Experiment 4a: PKC ϵ activation mediates agonist-independent and agonist-dependent internalization of mGluR5 in acute NAc slices

In this experiment, the effects of the PKC ϵ translocation inhibitor (Tat- ϵ V1-2) or activator (Tat- ψ ϵ RACK) on surface trafficking of mGluR5, were studied in NAc slices under baseline conditions and following DHPG treatment. Under baseline conditions (no mGluR5 agonist

present), one-way ANOVA revealed a main effect of Tat peptides on surface ($F(3,18) = 6.89$, $p < 0.01$) and intracellular ($F(3,18) = 6.83$, $p < 0.01$) expression of mGluR5 receptors (Fig. 4A). Post hoc analysis showed that there was a significant decrease in mGluR5 surface expression in NAc slices treated with Tat- ψ eRACK, when compared to slices treated with non-functional scrambled Tat peptide (Tat-sc; $p < 0.05$). Further, there was a significant increase in intracellular mGluR5 levels ($p < 0.01$ vs. Tat-sc), indicating internalization of mGluR5 in response to Tat- ψ eRACK. There was no difference in mGluR5 surface or intracellular levels between ACSF and Tat-sc treatments. Next, the effects of Tat peptides on mGluR5 surface trafficking in the presence of an agonist (100 μ M DHPG) were evaluated. One-way ANOVA revealed the significant effect of DHPG/Tat peptide treatment on surface ($F(3,18) = 4.93$, $p < 0.05$) and intracellular ($F(3,18) = 8.39$, $p < 0.01$) mGluR5 levels (Fig. 4B). Post-hoc analysis showed that DHPG alone increased internalization of mGluR5; demonstrated by the decrease in surface mGluR5 levels (Tat-sc-DHPG vs. Tat-sc; $p < 0.05$) and simultaneous increase in intracellular mGluR5 levels (Tat-sc-DHPG vs. Tat-sc; $p < 0.01$). The effect of DHPG was prevented by pre-treatment with Tat-eV1-2 peptide (Tat-sc + DHPG vs. Tat-eV1-2 + DHPG; n.s.). Interestingly, combined treatment with DHPG and Tat- ψ eRACK also increased internalization of mGluR5 (decreased surface levels; $p < 0.05$) and (increased intracellular levels; $p < 0.01$), but the effect was not different from the effects of DHPG treatment alone (Tat- ψ eRACK + DHPG vs. Tat-sc + DHPG; n.s.).

Experiment 4b: The effects of PKC ϵ activation on agonist-dependent/-independent phosphorylation of ERK1/2 and PKC ϵ in acute NAc slices

Phosphorylation of ERK1/2 and PKC ϵ was analyzed in total (T) NAc lysates obtained in Experiment 4a. Under baseline conditions (e.g., no mGluR5 agonist present), one-way ANOVA revealed a main effect of Tat peptides on phosphorylation of ERK1/2 ($F(3,18) = 9.97$, $p < 0.01$; Fig 5A). Post hoc analysis showed that there was a significant increase in phospho-ERK1/2 levels in NAc slices treated with Tat- ψ eRACK, when compared to slices treated with non-functional scrambled Tat peptide (Tat-sc; $p < 0.01$). There was no difference in phospho-ERK1/2 levels between ACSF- and Tat-sc treated groups. Phosphorylation of PKC ϵ was not altered in any of the treatment groups ($F(3,18) = 0.74$, n.s.; Fig 5A). Next, the effects of Tat peptides on ERK1/2 and PKC ϵ phosphorylation were evaluated in the presence of an mGluR5 agonist (100 μ M DHPG). One-way ANOVA revealed the significant effect of DHPG/Tat peptide treatment on phosphorylation of both ERK1/2 ($F(3,18) = 11.69$, $p < 0.01$) and PKC ϵ ($F(3,18) = 6.49$, $p < 0.01$, Fig 5B). Post-hoc analysis showed increased ERK1/2 phosphorylation in all samples treated with DHPG (regardless of Tat-peptide treatment; Tat-sc + DHPG $p < 0.01$; Tat-eV1-2 + DHPG $p < 0.05$; $p < 0.05$; Tat- ψ eRACK + DHPG $p < 0.01$; all vs. Tat-sc). Further, post hoc analysis of phospho-PKC ϵ levels revealed increased phosphorylation of this kinase in samples treated with DHPG alone (Tat-sc-DHPG vs. Tat-sc; $p < 0.05$) or with combination DHPG and Tat- ψ eRACK peptide (Tat- ψ eRACK + DHPG vs. Tat-sc; $p < 0.01$). Interestingly, the effect of DHPG was prevented by pre-treatment with Tat-eV1-2 peptide (Tat-sc + DHPG vs. Tat-eV1-2 + DHPG; n.s.).

DISCUSSION

The present study demonstrates anatomical and functional interactions between mGluR5 and PKC ϵ in the NAc. First, we show that both of these proteins co-localize in the same cell populations in this region, with no differences in the relative amount of co-localization between NAC subregions (shell or core). While previous studies have shown that mGluR5 and PKC ϵ are both highly expressed in the NAc (Romano et al. 1995; Saito et al. 1993; Shigemoto et al. 1993), to our knowledge this is the first demonstration that these proteins are co-expressed by the same cell types (Figure 1). This finding was not surprising, since PKC signaling is a known downstream target of mGluR5 via coupling to G_{q/11} proteins (Niswender and Conn 2010). We found that PKC ϵ was expressed in approximately one-third of mGluR5-positive cells, suggesting that surface expression of mGluR5 in other cells of the NAc is potentially regulated by PKC ϵ -independent mechanisms. Previous studies utilizing neuronal tracing and in situ hybridization or immunohistochemical approaches have revealed that mGluR5 receptors are expressed in approximately 80% of striatal projection neurons (Lu et al., 2009), yet a significant percentage (65%) of cholinergic interneurons of the striatum also express mGluR5 (Tallaksen-Greene et al., 1998). Future studies are needed in order to determine the neurochemical phenotype and projection targets of NAc neurons that co-express mGluR5 and PKC ϵ .

Previous evidence suggest that constitutive PKC ϵ activity can be detected in rodent brain tissue (Bajo et al. 2008; Bazzi and Nelsestuen 1988; Cozzoli et al. 2015; Olive et al. 2005), and that PKC-dependent phosphorylation of mGluR5 receptors is associated with their internalization (Lee et al. 2008). Therefore we examined whether inhibition of PKC ϵ translocation, which is critical for its activation, increases surface expression of mGluR5 in the NAc (Experiment 2). We found that microinfusion of the PKC ϵ translocation inhibitory peptide (Myr- ϵ V1-2) resulted in increased levels of surface mGluR5, concurrent with decreased intracellular levels of this protein. These results suggest that under basal (non-stimulated) conditions, PKC ϵ plays a role in maintaining an intracellular pool of mGluR5. Similar observations have been reported for regulation of dopamine, serotonin, and amino acid receptors and transporters by PKC and its respective RACK (Barnes 2000; Lee et al. 2004; Nissen-Meyer and Chaudhry 2013; Schmitt and Reith 2010; Steiner et al. 2008). Further studies are needed to determine if other PKC isozymes in the NAc also regulate the subcellular localization of mGluR5 receptors.

In addition to regulation of mGluR5 trafficking by constitutive PKC ϵ activity, we also sought to determine if pharmacological activation of mGluR5 receptors results in activation of PKC ϵ and other known intracellular signaling targets of this receptor (Experiment 3). Indeed, we observed that mGluR1/5 agonist DHPG dose-dependently increased levels of phosphorylated PKC ϵ at the Ser729 residue, as well as phosphorylated forms of ERK1/2. These findings are consistent with those of prior studies that have established phospho-Ser729 PKC ϵ as marker of activation of this PKC isozyme (Cenni et al. 2002; Parekh et al. 1999), and ERK1/2 as a signaling target of mGluR5 (Mao et al. 2005; Thandi et al. 2002). These findings are also consistent with our previous observations in the dorsal striatum where pharmacological activation of mGluR5 with the orthosteric agonist CHPG also increases phosphorylation of PKC ϵ at Ser729 as well as other activation sites such as

Thr566, but not other PKC isoforms (Olive et al. 2005). In general, phosphorylation of PKC ϵ at Ser729 site has been associated with changes in intracellular location of PKC ϵ and priming of the kinase for activation. Some studies suggest that phospho-Ser729 PKC ϵ is translocated from cytosol to the plasma membrane (e.g. Schonhoff et al. 2009), a process guided by RACK proteins (Fenton et al. 2009). Therefore, phosphorylation and translocation of PKC ϵ to the membrane may bring activated PKC ϵ to the proximity of mGluR5 receptors where it can alter receptor trafficking via direct phosphorylation of this receptor (see Ko et al. 2012).

Since mGluR5 surface expression may depend on both constitutive and inducible activity of PKC ϵ , we next investigated PKC ϵ -dependent regulation of mGluR5 subcellular localization under both baseline and pharmacologically-induced activation conditions (Experiment 4a). We observed that local application of the PKC ϵ translocation activator ψ eRACK promoted internalization of mGluR5 in acute NAc slices under non-stimulated conditions, as evidenced by increased intracellular protein and decreased surface levels, but had no effect on DHPG-stimulated receptor internalization. However, pretreatment with the PKC ϵ translocation inhibitor Tat-eV1-2 prevented DHPG-dependent mGluR5 internalization. Thus, PKC ϵ appears to regulate the subcellular localization of mGluR5 under both non-stimulated and stimulated conditions, consistent with prior reports using isozyme non-selective PKC activators and inhibitors (Gereau and Heinemann 1998; Ko et al. 2012; Lee et al. 2008). When comparing the effects peptide PKC ϵ inhibitors on mGluR5 surface expression under non-stimulated conditions, we noted a discrepancy between the *in vivo* effects of Myr-eV1-2 (Fig. 2) and *ex vivo* effects of Tat-eV1-2 (Fig. 4). Specifically, while Myr-eV1-2 significantly increased mGluR5 surface levels; Tat-eV1-2 had a small but statistically non-significant increase. The observed efficacy of Myr-eV1-2 *in vivo* is in agreement with our previous study that has shown mGluR5-dependent effects of Myr-eV1-2 on alcohol reinforcement after its intra-NAc infusion (Gass and Olive 2009). Other studies have shown that administration of myristoylated inhibitory peptides can block the activity of various kinases, including protein kinase C isoforms (Eichholtz et al. 1993; Miguez et al. 2010; Zeidman et al. 2002) and also alter receptor trafficking (Miguez et al. 2010). However, peptide myristoylation also promotes membrane association which may limit peptide diffusion various subcellular compartments (Nelson et al. 2007). Therefore, in more recent studies, Tat-conjugation is often preferred, as it offers increased cell penetrance and distribution relative to myristoylation (Reissmann 2014). Therefore, the unexpected efficacy of myristoylated PKC ϵ pseudosubstrate peptides (vs. Tat-conjugated peptides) on mGluR5 trafficking is not likely due to higher cellular penetration of Myr-eV1-2. We speculate that other factors specific to *in vivo* conditions, such as higher rates of internalization, or higher baseline activity of PKC ϵ are contributing to this phenomenon. Future studies are therefore needed to directly compare the effects of myristoylation vs. Tat-conjugation on mGluR trafficking *in vivo* and *ex vivo*.

In addition to surface trafficking (Experiment 4a), phosphorylation of ERK1/2 and PKC ϵ were also analyzed in total NAc lysates (Experiment 4b). With regards to ERK1/2, immunoblot analysis revealed both mGluR5 agonist-independent and agonist-dependent cellular mechanisms that contribute to ERK1/2 phosphorylation in the NAc. Increases in phospho-ERK1/2 levels were observed both after administration of the PKC ϵ translocation

activator ψ εRACK (no agonist present, Figure 5a), as well as in all samples treated with DHPG (Figure 5b). While the ability of DHPG to induce ERK1/2 phosphorylation is in agreement with our other experiments (Experiment 3, Figure 3b) and with previously published studies (e.g. Mao et al. 2005), a direct link between PKCε activity/translocation and ERK1/2 phosphorylation in the brain has not been previously demonstrated. In one study, upregulation of phospho-ERK1/2 was observed in neonatal myocytes overexpressing PKCε (Heidkamp et al. 2011). Interestingly, DHPG-induced ERK1/2 phosphorylation was not altered by pretreatment with the PKCε translocation inhibitor εV1-2. This is not surprising, as activation of several different signaling pathways downstream from mGluR5 can lead to phosphorylation of ERK1/2 after DHPG treatment (e.g., CaMKII, Choe and Wang, 2001). In contrast, inhibition of PKCε translocation partially reversed DHPG effects on PKCε phosphorylation, suggesting that translocation is an important for full activation and phosphorylation of PKCε. It should be noted, however, that downstream signaling of another group I mGluR (mGluR1) can also be regulated by PKCε, at least in tumor tissue (Marin et al. 2006). In the NAc, mGluR1 is expressed at much lower levels than mGluR5 (Kerner et al. 1997; Tallaksen-Greene et al. 1998; Testa et al. 1994), therefore mGluR1 was not investigated in the current study.

In summary, our findings indicate that mGluR5 and PKCε are co-localized in subpopulations of cells in both the NAc core and shell, and that the subcellular localization of mGluR5 in the NAc is regulated by PKCε under basal and stimulated conditions. It is important for future studies to explore potential interactions between mGluR5 and PKCε in other addiction-related brain regions such as the amygdala or prefrontal cortex, where it has been demonstrated that either of these proteins regulate drug-seeking and related behaviors (Cozzoli et al. 2016; Lesscher et al. 2009; Miller et al. 2016). Given the known role of mGluR5 signaling in the NAc in alcohol- and other drug-related behaviors such as drug intake and relapse, these findings pose important questions for potential targeting of mGluR5-PKCε signaling in the treatment of substance use disorders.

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SIGNIFICANCE STATEMENT

This study shows that the epsilon isoform of protein kinase C (PKC ϵ), an enzyme that regulates numerous alcohol sensitivity and intake, regulates the surface expression of the type 5 metabotropic glutamate receptor (mGluR5) under baseline and activated conditions. This was observed in the nucleus accumbens, a key brain region involved in reward and motivation. These findings establish bidirectional activity between mGluR5 and PKC ϵ , which may lead to new insights on how these signaling pathways regulate alcohol-related behaviors as well as other aspects of substance abuse and addiction.

Fig. 1. Co-localization of mGluR5 and PKC ϵ immunoreactivity in the NAc
 (A) Diagram of coronal rat brain section (adapted from (Paxinos and Watson 2007)) with highlighted area within the NAc used for immunohistochemical analysis. (B–E) PKC ϵ (green) and mGluR5 (red) immunoreactivity at 10 $\times$ (B, C) and 40 $\times$ magnification (D, E). (F) Co-localization (yellow) of immunoreactivity primarily within the cell soma. Scale bar = 100 μ m in B, C and = 20 μ m in D–F. NAc – nucleus accumbens, ac – anterior commissure.

Fig. 2. Intra-NAc infusion of the PKCε translocation inhibitor Myr-εV1-2 increases mGluR5 surface expression under baseline (non-stimulated) conditions

(A) Rat brain atlas coordinates (adapted from Paxinos and Watson 2007) depicting the site of intra-NAc infusions in relation to the site of tissue dissection used for slice preparation. Representative immunoblots show the distribution of mGluR5 and other proteins total (T), surface (S) and intracellular (I) fraction as detected by surface biotinylation assay in acute NAc slices. The presence of the membrane protein syntaxin-1a in the surface fraction and limited or no presence of known intracellular proteins (PKCε and calnexin) in the same fraction demonstrates that biotin selectively labelled surface proteins. (B) Intra-NAc application of the PKCε translocation inhibitor Myr-εV1-2 (5 pmol/μl) increased surface mGluR5 levels left panel, and decreased intracellular mGluR5 levels (right panel) in acute NAc slices. No changes in total mGluR5 levels were detected. Representative immunoblots (top inset) show the distribution of mGluR5 in total (T), surface (S) and intracellular (I) fraction as detected by surface biotinylation assay in acute NAc slices. $n = 4$ per group,

* $p < 0.05$ vs. scrambled control peptide. NAc – nucleus accumbens, stx1a – syntaxin-1a, (d) – mGluR5 dimer, (m) – mGluR5 monomer.

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Fig. 3. The mGluR5 agonist DHPG dose-dependently increased ERK1/2 and PKCε phosphorylation as well as mGluR5 internalization in acute NAc slices
(Top panels) Representative immunoblot images of mGluR5, phospho-(Thr202/Tyr204)-ERK1/2 and phospho-(Ser729)-PKCε signal as detected in total lysate (T) or in surface (S) fraction prepared from acute NAc slices treated with DHPG (10, 100 μM) or vehicle. *(Lower panels)* Quantitative analyses of surface mGluR5 (A), phospho-ERK1/2 (B) and phospho-PKCε (C) levels as detected in acute NAc slices treated with vehicle or DHPG, showed a dose-dependent reductions in surface mGluR5 and increased phosphorylation of ERK1/2 and PKCε. n = 3–4 per group, *p<0.05, **p<0.01 vs Veh. (d) – mGluR5 dimer, pERK1/2 – phospho-ERK1/2

Fig. 4. PKC ϵ activation mediates agonist-independent and agonist-dependent internalization of mGluR5 in acute NAc slices
(Top panels) Representative immunoblot images of mGluR5 signal as detected in total lysate (T), surface (S) and intracellular (I) fractions prepared from acute NAc slices treated with: 5 μ M Tat-sc, Tat- ϵ V1-2 and Tat- $\psi\epsilon$ RACK in combination with ACSF vehicle (**A**) or 100 μ M DHPG (**B**). *(Center and lower panels)* Quantitative analyses of surface and intracellular mGluR5 levels normalized to total mGluR5 levels in acute NAc revealed that: PKC ϵ translocation activator $\psi\epsilon$ RACK promotes internalization of mGluR5 in acute NAc slices (**A**), while pretreatment with the PKC ϵ translocation inhibitor Tat- ϵ V1-2 prevented DHPG-dependent mGluR5 internalization (**B**). $n = 5-6$ per group, * $p < 0.05$, ** $p < 0.01$ vs Tat-sc. (d) – mGluR5 dimer.

Fig. 5. PKC ϵ activation and agonist-dependent/-independent phosphorylation of ERK1/2 and PKC ϵ in acute NAc slices

(*Top panels*) Representative immunoblot images of phospho-(Thr202/Tyr204)-ERK1/2 and phospho-(Ser729)-PKC ϵ signal as detected in total lysate (T) prepared from acute NAc slices treated with: 5 μ M Tat-sc, Tat-eV1-2 and Tat- ψ ϵ RACK in combination with ACSF vehicle (**A**) or 100 μ M DHPG (**B**). (*Center and lower panels*) Quantitative analyses of phospho-ERK1/2 revealed that treatment of acute NAc slices with Tat- ψ ϵ RACK (**A**) or DHPG (**B**) increased phosphorylation of ERK1/2. Agonist-dependent ERK1/2 phosphorylation was not altered by Tat-eV1-2 or Tat- ψ ϵ RACK. DHPG also induced PKC ϵ phosphorylation that was prevented by Tat-eV1-2 pretreatment (**B**). $n = 5-6$ per group, * $p < 0.05$, ** $p < 0.01$ vs Tat-sc. (d) – mGluR5 dimer. pERK1/2 – phospho-ERK1/2

TABLE 1

Primary antibodies used in this study.

Antigen	Description of immunogen	Source, host species, catalog No., lot No., RRID	Antibody dilution
mGluR5 IB	KLH-conjugated synthetic peptide corresponding to the cytoplasmic domain of mouse mGluR5	EMD Millipore, rabbit polyclonal, ab5675, LV1738270, AB_2295173	1:7,500
mGluR5 IHC	Synthetic peptide corresponding to 13 amino acid C-terminal sequence of rat mGluR5 (LIIRDYQTSSSSL)	Neuromics, rabbit polyclonal, RA13104, 1000041, AB_2572407	1:200
pERK1/2 IB	Synthetic phosphopeptide corresponding to residues surrounding Thr202/Tyr204 of human p44 MAP kinase	Cell Signaling Technology, rabbit polyclonal, 9101L, 27, AB_331647	1:2,500
ERK1/2 IB	Synthetic peptide corresponding to a sequence in the C-terminus of rat p44 MAP kinase	Cell Signaling Technology, rabbit polyclonal, 9102L, 19, AB_823494	1:5,000
pPKC ϵ IB	Synthetic phosphopeptide corresponding to residues surrounding Ser729 (CKGF[pS]YFGEDL) of human PKC ϵ	EMD Millipore, rabbit polyclonal, 06-821, 28-041, AB_310257	1:10,000
PKC ϵ IB	KLH-conjugated synthetic peptide corresponding to the C-terminal variable (V5) region, amino acids 726–737 (KGFSYFGEDLMP) of mouse PKC ϵ	EMD Millipore, rabbit polyclonal, 06-991, JBC1395563, AB_310328	1:5,000 (immunoblotting);
PKC ϵ IHC	Synthetic peptide corresponding to amino acids 1–175 of human PKC ϵ	BD Transduction Laboratories, mouse monoclonal, 610085, 11, AB_397492	1:500
Calnexin IB	Synthetic peptide corresponding to the sequence near the C-terminus of dog calnexin	Enzo Life Sciences, rabbit polyclonal, ADI-SPA-860, 11021101, AB_10616095	1:25,000
Syntaxin 1a IB	Synthetic peptide conjugated to KLH derived from within residues 1 – 100 of Rat Syntaxin.	Abcam, rabbit polyclonal, ab41453, 730259, AB_956343	1:25,000

IB – immunoblotting, IHC - immunohistochemistry