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ORIGINAL INVESTIGATION

Role of protein kinase C epsilon (PKCε) in the reduction of ethanol reinforcement due to mGluR5 antagonism in the nucleus accumbens shell

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Abstract
Rationale The type 5 metabotropic glutamate receptor (mGluR5) and the epsilon isoform of protein kinase C (PKCε) regulate ethanol intake, and we have previously demonstrated that mGluR5 receptor antagonism reduces ethanol consumption via a PKCε-dependent mechanism.
Objectives We explored the potential neuroanatomical substrates of regulation of ethanol reinforcement by this mGluR5-PKCε signaling pathway by infusing selective inhibitors of these proteins into the shell or core region of the nucleus accumbens (NAc).
Methods Male Wistar rats were trained to self-administer ethanol intravenously and received intra-NAc infusions of vehicle or the selective mGluR5 antagonist 3-((2-methyl-1,3-thiazol-4-yl)ethynyl)pyridine (MTEP) alone and in combination with a PKCε translocation inhibitor (εV1–2) or a scrambled control peptide (sεV1–2). The effects of intra-NAc MTEP on food-reinforced responding and open-field locomotor activity were also determined.
Results MTEP (1 μg/μl) had no effect on ethanol or food reinforcement or locomotor activity when infused into either region. MTEP (3 μg/μl) reduced ethanol reinforcement when infused into the NAc shell but not the core, and this effect was reversed by εV1–2 (1 μg/μl) but not sεV1–2 (1 μg/μl). In both regions, this concentration of MTEP did not alter food-reinforced responding or locomotor activity, and infusion of εV1–2 alone did not alter ethanol reinforcement. MTEP (10 μg/μl) reduced locomotor activity when infused into the shell; therefore, this concentration was not further tested on responding for ethanol or food.
Conclusions Blockade of mGluR5 receptors in the NAc shell reduces ethanol reinforcement via a PKCε-dependent mechanism.

Keywords Ethanol · Food · Reinforcement · Self-administration · Intravenous · Microinjection · mGluR5 · PKCε · Locomotor activity

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Introduction

There is substantial evidence indicating that glutamatergic neurotransmission is involved in the reinforcing effects of ethanol. Extracellular levels of glutamate are increased in regions of the brain reward circuitry such as the nucleus accumbens (NAc) and amygdala following ethanol exposure or consumption (Dahchour et al. 2000; Kapasova and Szumlinski 2008; Moghaddam and Bolinao 1994; Quertemont et al. 1998; Roberto et al. 2004; Selim and Bradberry 1996; Szumlinski et al. 2007), which likely results from ethanol-induced decreases in glutamate uptake via glutamate transporters (Melendez et al. 2005). In addition, numerous studies have shown that antagonists of various glutamate receptor subtypes reduce ethanol self-administration in rodents, and several pharmacological compounds acting on glutamate receptors or altering glutamate release are either approved for the use in the treatment of alcoholism or are being investigated for such purposes (reviewed in Gass and Olive 2008; Lawrence 2007; Vengeliene et al. 2008).

Receptors for glutamate can be divided into two categories: ionotropic glutamate receptors (iGluRs, which are ligand-gated ion channels that include the *N*-methyl-D-

aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and kainate receptor subtypes), and metabotropic glutamate receptors (mGluRs), which are G-protein coupled receptors classified as mGluR1–8. A great deal of interest in the role of mGluRs in regulating the self-administration of addictive substances was spawned by a pivotal study in which it was demonstrated that mice lacking the mGluR5 receptor gene do not self-administer cocaine and are indifferent to its locomotor stimulant effects (Chiamulera et al. 2001). A host of other studies have subsequently shown that pharmacological antagonism of mGluR5 receptors reduces ethanol self-administration and relapse-like behaviors in a variety of rodent species and strains (Backstrom et al. 2004; Besheer et al. 2008; Cowen et al. 2005, 2007; Hodge et al. 2006; Lominac et al. 2006; McMillen et al. 2005; Schroeder et al. 2005). It has also been demonstrated that mice lacking mGluR5 receptors consume less ethanol than their wild-type counterparts (Bird et al. 2008), and there is preliminary evidence for an association between polymorphisms in the mGluR5 gene and alcohol dependence in humans (Schumann et al. 2008).

mGluR5 receptors activate the $G\alpha_q$ class of G-proteins, which stimulate one of several phospholipases to hydrolyze phosphoinositol (PI), resulting in the formation of lipid signaling intermediates such as inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG, in turn, can activate various intracellular messengers including protein kinase C (PKC), which consists of a family of at least 11 distinct serine/threonine kinases classified as conventional (α, βI, βII, and γ), novel (δ, ε, η, θ, and μ), or atypical (ζ and ι/λ; Olive and Messing 2004). Upon activation, PKC isoforms bind to an intracellular binding protein specific for each isozyme (termed receptor for activated C-kinase, or RACK), which transports the enzyme to a specific subcellular location in order to exhibit its kinase activity (Mochly-Rosen and Gordon 1998). We previously demonstrated that mGluR5 receptors in the dorsal striatum are specifically coupled to the epsilon isoform of PKC (PKCε) and that systemically administered mGluR5 antagonists reduce ethanol consumption in wild-type mice but not in mice carrying a targeted deletion of the gene-encoding PKCε (Olive et al. 2005). Given these findings, the purpose of the present study was to determine a potential neuroanatomical locus of these observations by examining the effects of site-specific microinjections of an mGluR5 antagonist alone and in combination with a peptide translocation inhibitor of PKCε (εV1–2), which prevents the binding of PKCε to its corresponding RACK, on ethanol self-administration. The NAc core and shell were chosen as target regions due to their well-established involvement in ethanol reinforcement (Gonzales et al. 2004). We also sought to determine if any observed reductions in ethanol self-administration by local mGluR5 antagonism in the NAc could be attributable to locomotor impairments, and whether any such effects were selective for ethanol by assessing effects of intra-NAc infusion of MTEP on food-reinforced responding.

Materials and methods

Subjects

Principles of animals care (http://www.nap.edu/readingroom/books/labrats/) were followed and conformed to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003), and with the approval of an Institutional Animal Care and Use Committee. Male Wistar rats (250–275 g upon arrival, Harlan, Indianapolis, IN, USA) were housed in an animal colony room with temperature and humidity within NIH guidelines and were allowed to recover from transport to the facility for at least 3 days prior to initiation of experiments. Animals were maintained on a reversed 12 h light–dark cycle (lights off at 0700 hours) with ad libitum access to food unless otherwise stated. All experimentation was conducted during the dark phase of the light–dark cycle, with the exception of overnight food training sessions which commenced at approximately 1600 hours and terminated at 0800 hours the following morning.

Self-administration apparatus

Ethanol self-administration was conducted in operant self-administration chambers (ENV-008, Med Associates, St. Albans, VT, USA; Gass and Olive 2007). Chambers were housed in a melamine sound-attenuating cubicle equipped with a house light and exhaust fan designed to mask external noise and odors, and were interfaced to a personal computer (PC). Each chamber was equipped with two stainless steel response levers located on one wall that flanked a 4.2×5-cm food pellet receptacle. Each response lever was located approximately 7 cm above a stainless steel rod floor, and positioned above each lever was a 2.5-cm diameter white stimulus light. Located near the top of each self-administration chamber was a Sonalert speaker that provided an auditory stimulus during reinforcer delivery. A food pellet dispenser and a syringe pump that were interfaced to the computer were located outside each chamber.

Self-administration procedures

Prior to self-administration training, rats that were to be trained to respond for i.v. ethanol were prepared with intravenous catheters into the jugular vein (Gass and Olive

2007). Briefly, rats were anesthetized with 2% isoflurane vaporized in medical grade breathing air at a flow rate of 0.4 L/min. The right jugular vein was isolated and a sterile silastic catheter filled with 100 U/ml heparin was inserted 2.5 cm into the vein. The catheter was secured to the surrounding tissue with sutures, and the opposite end of the catheter was tunneled subcutaneously to the dorsum where it exited the skin between the scapulae. This end of the catheter was secured to the surrounding tissue via sutures and a mesh collar attached to a threaded vascular access port (Plastics One, Roanoke, VA, USA). The access port was sealed with a piece of Tygon tubing closed at one end and a protective cap. During the same surgical procedure, rats also underwent implantation of bilateral stainless steel guide cannulae (26-ga outer diameter, Plastics One) aimed at the NAc core (AP+1.8, ML±1.5, DV −6.0 mm from bregma and skull surface) or shell (AP+1.8, ML±0.75, DV −6.4) according to the atlas of Paxinos and Watson (2005). Guide cannulae were fitted with obturators and a protective cap to prevent contamination and obstruction, and secured to the skull with stainless steel screws and dental cement. The wound was then treated with a topical antibiotic ointment and 2% *w*/*v* xylocaine (Henry Schein Veterinary Supply, Melville, NY, USA). Following surgical procedures, rats were allowed at least 5 days of recovery and received daily intravenous infusions of 70 U/ml heparin (0.2 ml volume) to maintain catheter patency and 100 mg/ml cefazolin (0.1 ml volume) to protect against infection. Catheter patency was tested periodically throughout the experiment by infusion of 10 mg/ml sodium methohexital (0.2 ml volume) and observation of brief loss of postural muscle tone. Animals that were to be tested for effects of intra-accumbal infusions of MTEP on food-reinforced responding or locomotor activity received implantations of microinjection guide cannulae as described above, but did not undergo catheter implantation.

Following recovery from surgical procedures, all animals were limited to access to 20 g of food per day for the remainder of the experiments to facilitate operant self-administration performance. In our experience, this mild food restriction results in body weights equivalent to 90% of those under free-feeding conditions while still allowing weight gain, and results in more robust patterns of ethanol and food self-administration than those under free-feeding conditions (see also Oei and Singer 1979). To initiate operant responding, rats were placed in the self-administration chambers for a single 16-h overnight training session whereby each press on the designated active lever delivered a 45-mg food pellet (Bio-Serv, Frenchtown, NJ, USA) into the food receptacle on a fixed-ratio 1 (FR1) schedule of reinforcement. Each food pellet delivery was accompanied by illumination of the stimulus light for 1 s and was followed by a 4-s timeout period, during which additional active lever presses were recorded but produced no programmed consequences. Presses on the designated inactive lever were recorded but produced no consequences at any time during the experiment. Approximately 24 h following the initial overnight training session, rats were then placed into 60 min self-administration sessions conducted 5 days/week (i.e., Mondays through Fridays). For the ethanol self-administration group, each press on the active lever resulted in delivery of ethanol (1% *v*/*v*, delivered in a volume of 0.03 ml over a 1-s period, equivalent to ~1 mg/kg/infusion) on a FR1 schedule of reinforcement. For the food self-administration group, each press on the active lever resulted in delivery of a 45-mg food pellet on a FR1 schedule of reinforcement. Each ethanol infusion or food pellet delivery was followed by a 4-s timeout period, during which additional active lever presses were recorded but produced no programmed consequences. Ethanol was delivered to the vascular access port by polyethylene tubing housed in a stainless steel spring tether that was attached to a single channel liquid swivel mounted on top of the self-administration chamber. Each ethanol infusion or food pellet delivery was accompanied by concurrent illumination of the stimulus light and presentation of an auditory stimulus (~65 dB, 2,900 Hz) for 1 s. For the ethanol self-administration group, each session was preceded by intravenous infusion of 0.1 ml of 70-U/ml heparin and was followed by infusion of 0.1 ml of 100 mg/ml cefazolin.

Microinjection procedures

During the maintenance phase of ethanol or food self-administration, animals were handled prior to each session and had their obturators removed and immediately replaced to habituate them to microinjection needle insertion procedures. Following stabilization of responding for ethanol or food reinforcement (defined as less than 15% variance in the number of active lever presses across two consecutive days), microinjection procedures were commenced. For each microinjection, animals then had their obturators removed, and clean microinjection needles (33-ga outer diameter) were inserted to extend 1 mm beyond the ventral tip of the guide cannulae. Test substances were then infused at a rate of 0.5 μl/min over a period of 1 min using a syringe pump (Harvard Apparatus, Holliston, MA, USA) while the animal was placed unrestrained in a small plastic tub. Following infusion, microinjection needles remained in place for an additional 1 min to allow for diffusion of the substances into the surrounding tissue, and then the microinjection needle was removed and replaced with the obturator. Animals were then immediately placed into the self-administration apparatus and allowed to self-administer ethanol or food for 60 min. Microinjections were performed only on Tuesdays through Fridays, since it was

found that ethanol- and food-reinforced responding was more variable on Mondays. If a significant treatment effect was observed during the session following the microinjection, at least one treatment-free self-administration session was allowed to ascertain that ethanol- or food-reinforced responding had returned to baseline levels.

Monitoring of locomotor activity

To exclude the possibility that any observed reductions in ethanol or food self-administration following infusion of MTEP into the NAc were a result of impairment of motor activity, a separate group of animals underwent implantation of guide cannulae aimed at the NAc core and shell (as described above) and were examined for open-field locomotor activity. Following habituation to microinjection procedures as described above, animals were then placed into locomotor activity monitoring chambers (20.3×15.9×21.3 cm, $L \times W \times H$, Med Associates) equipped with eight sets of infrared photobeams interfaced to a PC. For the first two days, animals were habituated to the testing apparatus in 60 min sessions. Next, animals received bilateral infusions of artificial cerebrospinal fluid vehicle (aCSF, see below) at a flow rate of 0.5 μl/min for 1 min and were then placed into the testing apparatus for 60 min for monitoring of horizontal locomotor activity. On the next day, animals received bilateral infusions of MTEP (1, 3 or 10 μg/μl) and were then placed into the testing apparatus for 60 min for monitoring of locomotor activity. During each test session, locomotor activity (beam breaks) was assessed in 10-min intervals.

Histology verification of microinjection cannulae placement

Following experimental procedures, animals were anesthetized with isoflurane and euthanized by decapitation. Brains were then removed, immersed in 10% *v*/*v* formalin for at least 1 week at 4°C, and then immersed in a 30% (*w*/*v*) sucrose solution for at least 48 h at 4°C. Brains were then cut into 40-μm coronal sections on a cryostat (Leica CM1900, Leica Microsystems, Bannockburn, IL), mounted onto microscope slides, and stained with cresyl violet for histological verification of cannulae placement under light microscopy.

Drugs

Artificial cerebrospinal fluid (aCSF, consisting of (in mM) Na 150, K 3.0, Ca 1.4, Mg 0.8, P 1.0, Cl 155, pH=7.4, Harvard Apparatus) was used as the vehicle for all test substances administered. MTEP hydrochloride was obtained from Alexis Biochemicals (San Diego, CA, USA) and was dissolved by sonication in a vehicle consisting of 20% *w*/*v* 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, St. Louis, MO, USA), followed by dilution to 1, 3 or 10 μg/μl in aCSF. Concentrations of MTEP are expressed as the weight of the salt. The PKCε peptide translocation inhibitor εV1–2 (EAVSLKPT) and its scrambled control sεV1–2 (LSETKPAV) (Gray et al. 1997; Johnson et al. 1996) were custom synthesized by Sigma–Genosys (St. Louis, MO, USA) and *N*-myristoylated to facilitate cell penetrance (see Eichholtz et al. 1993; O'Brian et al. 1991; Ward and O'Brian 1993). These peptides were dissolved in aCSF at a concentration of 1 μg/μl and co-administered with MTEP in the same solution. Ethanol (95% *v*/*v*) was obtained from the MUSC Pharmacy Distribution Services and diluted to 1% *v*/*v* in physiological saline for intravenous infusion. The concentrations and combinations of test substances were administered in a repeated measures counterbalanced design to control for possible order effects.

Statistical analysis

Data were analyzed using SigmaStat version 3.5 (Systat Inc., San Jose, CA, USA). Effects of infusions of test substances on ethanol- or food-reinforced responding were analyzed by a separate one-way repeated measures analysis of variance (ANOVA) for each brain region and for each measure (i.e., number of active or inactive lever presses and the number of ethanol infusions or food pellets delivered per session). Effects of infusions of test substances on open-field locomotor activity were analyzed by a two-way repeated measures ANOVA for each brain region, with treatment (i.e., concentration of MTEP infused) and time (in 10-min bins) as factors. Each ANOVA was followed by post hoc Holm-Sidak pairwise multiple comparisons of effects of test substance infusion against aCSF vehicle treatment. All data are stated or depicted as mean ± SEM. *P* values less than 0.05 were considered to be significant for all tests.

Results

Effects of intra-accumbens infusion of MTEP on locomotor activity

To control for the possibility that any observed effects of MTEP infusion into the NAc on ethanol or food self-administration might be attributable to alterations in motor activity, separate groups of animals (n=5–6 per group) received infusions of aCSF vehicle or MTEP (1, 3, or 10 μg/μl) into the NAc shell or core prior to assessment of locomotor activity in 10-min time bins. These data are

presented in Fig. 1. A significant effect of time was observed in all animals (NAc shell, $F_{5,40}$=23.93, p<0.05; NAc core, $F_{5,52}$=13.82, p<0.05), with higher locomotor activity observed in the first 10-min time bin followed by gradual decrease during the remaining time bins. A significant interaction between treatment and time bin was observed for animals receiving MTEP into the NAc shell ($F_{15,40}$=2.49, p<0.05), and post hoc analyses revealed that the 10 μg/μl dose suppressed locomotor activity during time bins 4 and 5 (Fig. 1a). There was no significant effect of treatment or a treatment × time bin interaction produced by MTEP infusion into the core (p>0.05), although there appeared to be a trend towards suppression of locomotor activity during time bin 4 (see Fig. 1b). In light of the locomotor suppressant effects of the 10 μg/μl MTEP, this dose was not further tested on ethanol- or food-reinforced responding.

Blockade of mGluR5 receptors in the NAc shell, but not the core, attenuates intravenous ethanol reinforcement via a PKCε-dependent mechanism

The results of experiments examining the effects of local infusion of MTEP and/or the PKCε translocation inhibitor εV1–2 into the NAc shell (n=8) or core (n=10) on intravenous ethanol reinforcement are presented in Fig. 2. A significant effect of treatment on active (i.e., ethanol-reinforced) lever presses ($F_{5,34}$=4.39, p<0.005) and the number of ethanol infusions delivered ($F_{5,34}$=3.589, p< 0.05) was observed in the NAc shell group (Fig. 2a), and post hoc analysis revealed that treatment with the 3 μg/μl concentration of MTEP (MTEP3) significantly reduced the number of active lever presses and ethanol infusions delivered as compared with vehicle-treated animals. When co-infused with the PKCε translocation inhibitor εV1–2 into the NAc shell, MTEP (MTEP3+εV1–2) failed to reduce the number of active lever presses or ethanol infusions delivered. However, when co-infused with the scrambled control version of εV1–2 (MTEP3+sεV1–2) into this region, the ability of MTEP to reduce the number of active lever presses and ethanol infusions was again observed. Infusion of εV1–2 (1 μg/μl) alone had no effect on the number of active lever presses or ethanol infusions delivered when infused into the NAc shell, and none of the treatments altered the number of inactive (i.e., non-reinforced) lever presses (p>0.05). When test substances were infused into the NAc core (Fig. 2b), none of the treatments had any effect on the number of active or inactive lever presses or the number of ethanol infusions delivered (all p>0.05).

Blockade of mGluR5 receptors in the NAc shell or core has no effect on food-reinforced responding

In order to determine if the observed effects of infusion of MTEP into the NAc shell were specific for ethanol-reinforced responding, we conducted a separate set of experiments to determine the effects of intra-NAc infusion of MTEP on food-reinforced responding. The results of these experiments are shown in Fig. 3. When infused into the NAc shell or core (n=4–5 per group), MTEP (1 or 3 μg/μl, abbreviated as MTEP1 and MTEP3, respectively), had no effect on the number of active (i.e., food-reinforced) lever presses, number of food pellets delivered, or the number of inactive (i.e., non-reinforced) lever presses (all p>0.05).

Figure 4a shows diagrams of coronal sections of the rat brain with the location of tips of microinjection guide cannulae in all experimental animals as determined from post-mortem histological analysis. One rat was excluded from analysis due to placement of the guide cannulae outside the intended target region. Figure 4b shows photo-micrographs of stained brain sections with tracts left by microinjection needles.

Discussion

In the present study, we demonstrated that local infusion of the selective mGluR5 receptor antagonist MTEP into the NAc shell reduces the reinforcing effects of ethanol, providing a

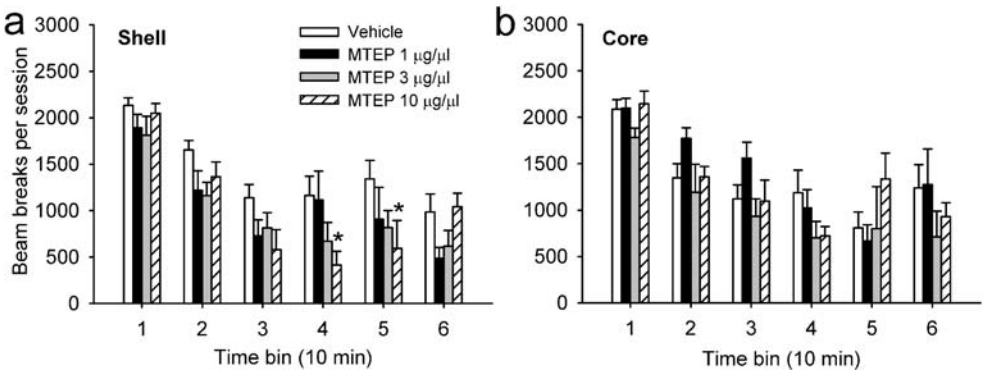

Fig. 1 Effects of local infusion of aCSF vehicle or MTEP (1, 3 or 10 μg/μl) into the NAc shell (**a**) and core (**b**) on open-field locomotor activity in 10-min time bins. Data are presented as mean ± SEM; n=5–6 per group. *p<0.05 vs. vehicle-treated animals within the same time bin

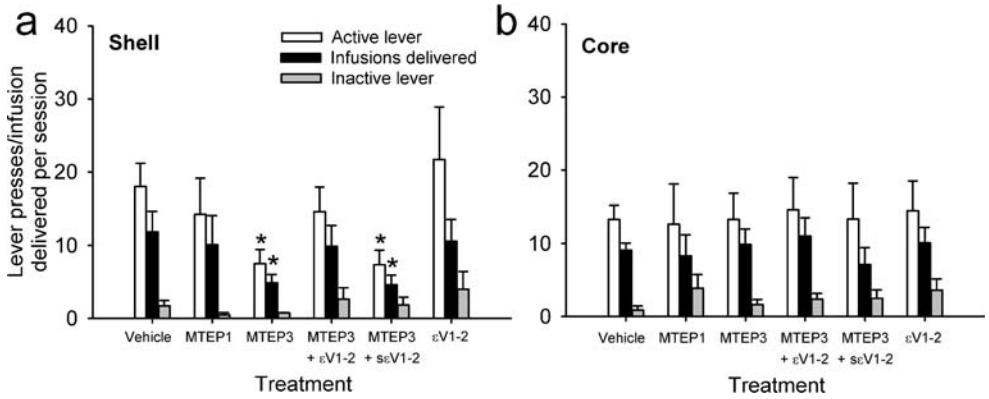

Fig. 2 Effects of local infusion of MTEP and/or the PKCε peptide translocation inhibitor εV1–2 into the NAc shell (**a**) and core (**b**) on the number of active (ethanol reinforced) lever presses, ethanol infusions delivered, and inactive (non-reinforced) lever presses during 60-min ethanol self-administration sessions. Concentrations of MTEP infused were 1 μg/μl (MTEP1) or 3 μg/μl (MTEP3). Concentrations of εV1–2 or a scrambled control peptide (sεV1–2) were 1 μg/μl each; n=8–10 per group. Data are presented as mean ± SEM. *p<0.05 vs. the number of active lever presses or ethanol infusions delivered following infusion of vehicle

potential neuroanatomical locus where mGluR5 receptors regulate ethanol self-administration. These effects appeared to be specific to the shell subregion of the NAc, since no effects of MTEP were observed when infused into the NAc core. In addition, these effects appeared to be selective for ethanol and not due to impairments in motor activity, as similar doses of MTEP infused into the NAc had no effect on food reinforcement or open-field locomotor activity. However, a high (10 μg/μl) concentration of MTEP reduced locomotor activity when infused into the NAc shell, which precluded testing the effects of this dose on ethanol- or food-reinforced responding. We also demonstrated that the ability of intra-NAc MTEP to attenuate ethanol self-administration is dependent on the activity of PKCε, as the inhibitory effects of MTEP on ethanol-reinforced responding were reversed by co-infusion of the PKCε peptide translocation inhibitor εV1–2. These latter observations are consistent with our previous findings that systemic administration of mGluR5 antagonists reduces ethanol consumption in wild-type but not PKCε-deficient mice (Olive et al. 2005). Given that mGluR5 receptors are known to be coupled to PKC activity (Conn and Pin 1997; Hermans and Challiss 2001), these data collectively suggest that PKCε is a critical downstream signaling target of mGluR5 receptors that regulates the ability of mGluR5 antagonists to attenuate ethanol reinforcement.

Given that our laboratory and other investigators have shown that both pharmacological antagonism of mGluR5 receptors (Backstrom et al. 2004; Besheer et al. 2008; Cowen et al. 2005, 2007; Hodge et al. 2006; Lominac et al. 2006; McMillen et al. 2005; Schroeder et al. 2005) and genetic deletion of PKCε (Choi et al. 2002; Hodge et al. 1999; Olive et al. 2000) reduce ethanol self-administration in rodents, it might be expected that inhibition of the function of both mGluR5 receptors and PKCε within the NAc might produce additive or synergistic effects in reducing ethanol consumption. However, such effects were not observed in our study, as we showed that co-infusion of εV1–2 into the NAc actually reversed the ability of locally applied MTEP to reduce ethanol consumption. The reasons for this phenomenon are currently unclear but may be due to a regulatory role of PKCε in governing the activity of mGluR5 receptors. For example, it has been shown that PKC phosphorylates the mGluR5 receptor, which mediates the rapid desensitization

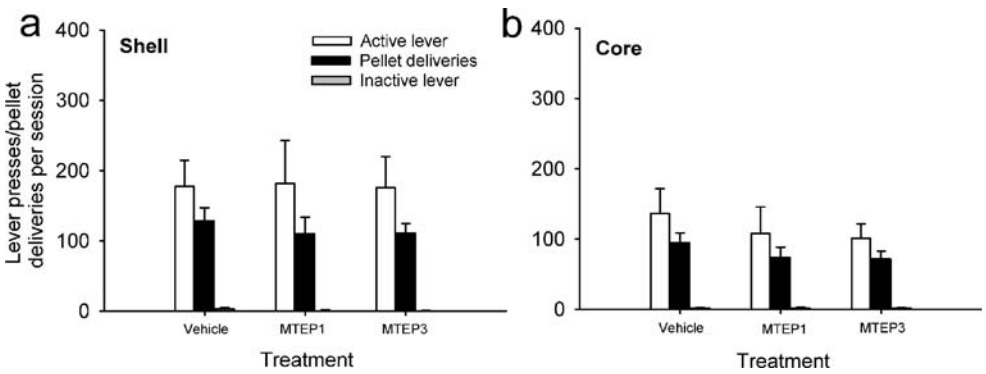

Fig. 3 Effects of local infusion of MTEP into the NAc shell (**a**) and core (**b**) on the number of active (food reinforced) lever presses, food pellets delivered, and inactive (non-reinforced) lever presses during 60-min food self-administration sessions. Concentrations of MTEP infused were 1 μg/μl (MTEP1) or 3 μg/μl (MTEP3); n=4–5 per group. Data are presented as mean ± SEM

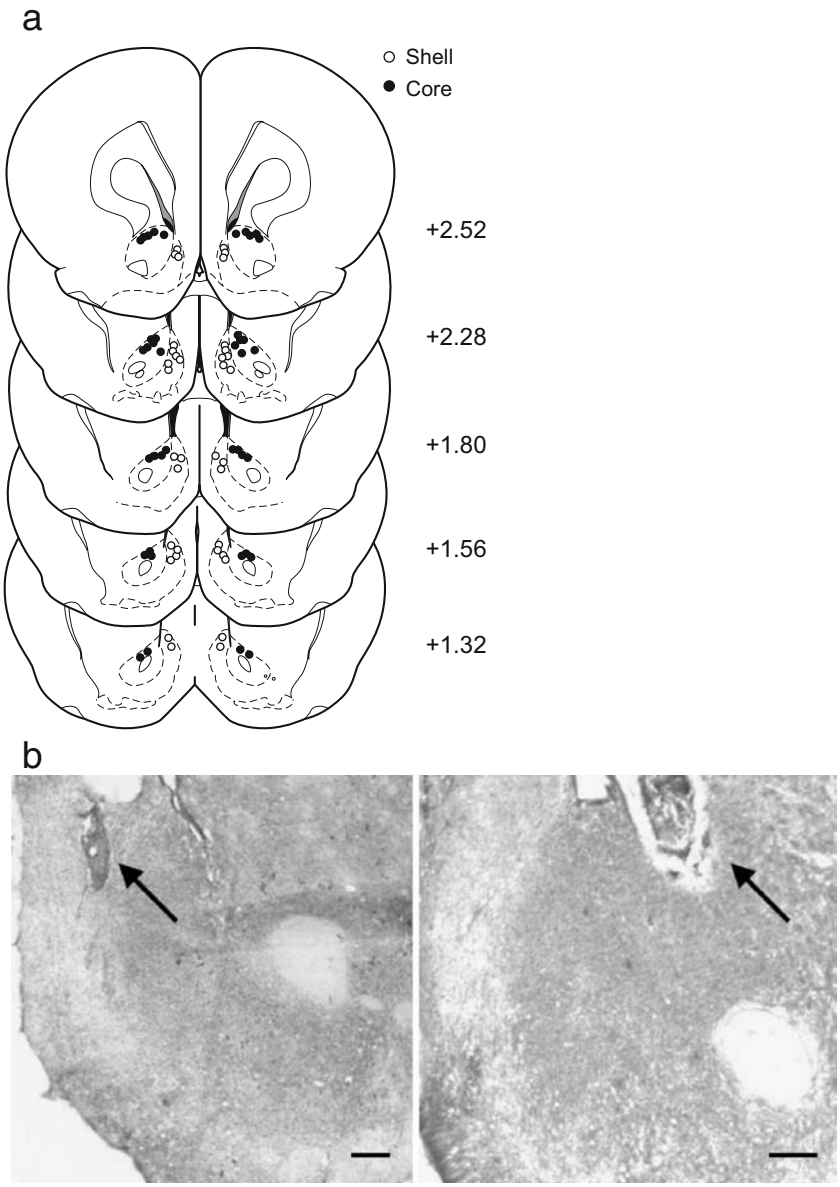

Fig. 4 **a** Coronal diagrams of the rat brain showing the location of the tips of microinjection guide cannulae in the NAc shell (*open circles*) and core (*filled circles*). Cannulae placement was determined from postmortem histological analysis of cresyl violet stained tissue sections. Numbers along side each coronal diagram represent the distance (in millimeters) of the section from bregma. Diagrams taken from the atlas of Paxinos and Watson (2005). **b** Photomicrographs of microinjection guide cannulae tips (*arrows*) in histological sections from the NAc shell (*left*) and core (*right*). *Scale bar*=250 μm

of mGluR5 function in response to pharmacological stimuli (Alagarsamy et al. 1999, 2002; Gereau and Heinemann 1998; Mao et al. 2008). In addition, it has recently been demonstrated that PKC competes with calmodulin for phosphorylation and regulation of the surface expression of mGluR5 receptors (Lee et al. 2008). It is therefore possible that PKCε may phosphorylate and thereby regulate the desensitization and surface expression of mGluR5. Thus, in the presence of the PKCε translocation inhibitor εV1–2, mGluR5 function and/or cell surface expression may be altered so that the receptor becomes unresponsive to pharmacological antagonism. Investigations into this possibility are currently ongoing in our laboratory.

In addition, since it has been previously demonstrated that mice lacking PKCε exhibit reduced ethanol consumption as compared to wild-type littermates (Choi et al. 2002; Hodge et al. 1999; Olive et al. 2000), one might anticipate that local infusion of εV1–2 alone into a brain region known to be involved in ethanol reinforcement might reduce ethanol consumption. However, in the present study, we observed no effect of administration of εV1–2 alone into either the NAc core or shell. When infused into the shell, two of the animals actually exhibited increases in ethanol-reinforced responding, but the remainder of the animals' behavior remained unchanged (data not shown) and the number of ethanol infusion delivered was unaltered (Fig. 2a). Thus, the ability of εV1–2 to reverse the inhibitory effects of intra-NAc MTEP on ethanol reinforcement is not due to εV1–2-induced generalized increases in active lever pressing. These data suggest that PKCε activity

alone in the NAc does not regulate the reinforcing effects of ethanol and that reduced ethanol consumption exhibited by PKCε-deficient mice is likely a result of the absence of PKCε activity in regions of the brain other than the NAc, such as the amygdala (Lesscher et al. 2009).

Both mGluR5 and PKCε are highly expressed in the NAc (Romano et al. 1995; Saito et al. 1993; Shigemoto et al. 1993; Testa et al. 1994; Young 1988). Although the relative abundance of these proteins in the core and shell of the NAc are the same, as is the localization of mGluR5 to postsynaptic dendrites and spines across these two compartments (Mitrano and Smith 2007), our findings that mGluR5 antagonism in the NAc shell, but not the core, reduces ethanol self-administration suggests a degree of functional heterogeneity of these receptors within this region with regard to ethanol reinforcement. The NAc receives dense glutamatergic innervation from numerous brain regions including the medial prefrontal cortex, amygdala, and hippocampus (Heimer et al. 1997). In addition, the NAc receives substantial dopaminergic input from ventral tegmental area (VTA) of the midbrain, and activation of this pathway is thought to underlie the reinforcing properties of ethanol (Gonzales et al. 2004; Pierce and Kumaresan 2006). Accumbal dopamine release and associated plasticity is regulated by local glutamatergic transmission (Imperato et al. 1990), including mGluR5 receptor activity (Homayoun et al. 2004; Lominac et al. 2006; Schotanus and Chergui 2008). Since ethanol produces a more robust increase in extracellular dopamine in the shell region as compared with the core (Howard et al. 2008), the ability of mGluR5 antagonism in the NAc shell to preferentially regulate ethanol reinforcement may be due to changes in glutamatergic modulation of ethanol-induced dopamine release in NAc shell. While systemic administration of mGluR5 antagonists (Lominac et al. 2006) and genetic deletion of PKCε (Olive et al. 2000) both abolish ethanol-stimulated mesolimbic dopamine release, additional microdialysis studies are needed to determine if these effects can be mimicked by local inhibition of mGluR5 and/or PKCε in the NAc.

Another possible mechanism by which mGluR5 receptors in the NAc shell preferentially regulate ethanol reinforcement is by modulating the flow of efferent information from this region to its target structures. mGluR5 receptors have been found to be primarily expressed by accumbal neurons that project to the ventral pallidum (VP) and, to a lesser degree, the VTA (Lu et al. 1999). The ventral striatopallidal pathway has been extensively characterized as a limbic–motor interface that subserves the execution of motivated behaviors (Groenewegen et al. 1996; Kalivas and Nakamura 1999; Koob 1999; Mogenson et al. 1983). Differences in the projection topography of the ventral striatopallidal pathway have been documented, with the NAc shell projecting primarily to the ventromedial part of the VP, while the NAc core projects mainly to the dorsolateral part of the VP (Chang and Kitai 1985; Groenewegen and Russchen 1984). Further studies are needed to determine if the different anatomical efferent connections of the NAc core versus shell contribute to the observed differences in local blockade of mGluR5 receptors on ethanol reinforcement.

It should be noted that in the current study, we employed an intravenous method of ethanol reinforcement as opposed to the standard oral self-administration paradigm. Intravenous ethanol self-administration, which has been used by our laboratory as well as numerous other investigators (Gass and Olive 2007; Grupp 1981; Hyytia et al. 1996; Kuzmin et al. 1999; Lyness and Smith 1992; Oei and Singer 1979; Sinden and Le Magnen 1982; Takayama and Uyeno 1985), offers the advantage of providing a more direct measurement of the reinforcing effects of ethanol (since ethanol is infused directly into the bloodstream) without the potential confounds of taste or caloric factors associated with oral self-administration. However, the amount ethanol self-administered intravenously by rats is much lower (approximately 0.01 g/kg/session) than that administered orally by outbred rat strains (approximately 0.3–0.6 g/kg/session), which is likely attributable to the differences in rates of ethanol absorption into the brain, rates of elimination, and rates of acetaldehyde formation. Nonetheless, it is clear from the present study that low-dose intravenous ethanol functions as a reinforcer, and it has recently been reported that similar low doses of intravenous ethanol serve as a reinforcer in the runway paradigm (Steffensen et al. 2009). In this study, low-dose intravenous ethanol increased the firing rate of GABAergic neurons in the ventral midbrain, although the authors did not identify whether these neurons were local tonic inhibitory GABAergic interneurons or GABAergic projection neurons. Clearly, whether these low doses of ethanol interact with the known molecular targets of ethanol (i.e., GABA-A and *N*-methyl-D-aspartate glutamate receptors) or other as yet unidentified substrates of ethanol reinforcement are unknown and warrant further investigation. Given that other investigators have shown that mGluR5 antagonism in the NAc reduces binge-like ethanol intake in mice (Cozzoli et al. 2007), these data collectively suggest that the NAc is a neuroanatomical site where mGluR5 receptors regulate both intravenous and oral ethanol self-administration.

Finally, it could be argued that the reductions in intravenous ethanol self-administration produced by mGluR5 blockade in the NAc shell actually reflect an increase in the reinforcing effects of ethanol, thereby necessitating less ethanol to be self-administered in order to achieve the same pharmacological effects. This is a possibility that needs to be clarified by other measures of reinforcing efficacy of ethanol,

such as the progressive ratio paradigm. However, given that systemic administration of mGluR5 antagonists reduce the reinforcing efficacy of ethanol in the progressive ratio paradigm (Besheer et al. 2008), we hypothesize that intra-NAc blockade of mGluR5 receptors produces a reduction in the reinforcing effects of ethanol.

In summary, we have demonstrated that mGluR5 receptors in the NAc shell mediate the reinforcing effects of ethanol and that this phenomenon is mediated by the activity of PKCε. Future studies are needed to determine precisely how PKCε governs the ability of mGluR5 antagonists to reduce ethanol consumption (such as by regulation of receptor desensitization or cell surface expression). In addition, assessment of the role of other regions of the brain where ethanol reinforcement is regulated by signaling between mGluR5 and PKCε is also warranted.

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