PKMζ in the nucleus accumbens acts to dampen cocaine seeking.

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Abstract

The constitutively active, atypical protein kinase C, protein kinase M-ζ (PKMζ), is exclusively expressed in the brain and its expression increases following exposure to drugs of abuse. However, the limitations of currently available tools have made it difficult to examine the role of PKMζ in cocaine addiction. The current study demonstrates that constitutive deletion of PKMζ potentiates cue-induced reinstatement of cocaine seeking and increases both food and cocaine taking, without affecting cue-driven food seeking in both male and female mice. Conditional deletion of PKMζ within the nucleus accumbens recapitulated the increase in cocaine taking and seeking seen in the constitutive knockout mice, but only in male animals. Site-specific knockdown of PKMζ in the nucleus accumbens had no effect on cocaine or natural reward behaviors in female mice. Western blot analysis of nucleus accumbens tissue revealed an increase in GluA1 AMPA receptor subunit expression in male mice, but not females, following conditional deletion of PKMζ. Taken together these results indicate that PKMζ may act to dampen addictive phenotypes by controlling the AMPAR subunit composition. This is the first study demonstrate a compensatory role for PKMζ in addiction-like behavior. Furthermore, these results indicate that PKMζ is playing divergent roles in reward seeking in males and females.

Keywords: cocaine, PKMζ, self-administration, AMPA, nucleus accumbens, sex differences

Introduction

PKMζ, a constitutively active form of protein kinase C, has been extensively studied for its supposed role in memory maintenance. Early work in this area demonstrated that application of PKMζ is sufficient to induce long-term potentiation (LTP) in vitro (Ling *et al*, 2002). Stimulation protocols to induce LTP also increase PKMζ (Hrabetova and Sacktor, 1996; Sacktor *et al*, 1993). Furthermore, PKMζ has been shown to be involved in memory processes in vivo. PKMζ is upregulated following learning and memory tasks (Hsieh *et al*, 2017; Li *et al*, 2011). Inhibition of PKMζ has been shown to disrupt memory maintenance. Administration of a PKMζ inhibitor, Zeta Inhibitory Peptide (ZIP), into the hippocampus blocked the ability of rats to recall a previously learned fear response 24hrs later (Pastalkova *et al*, 2006). Similar, long-lasting disruption of memories has been seen with spatial memory following ZIP infusion into the hippocampus (Pastalkova *et al*, 2006; Serrano *et al*, 2008), taste memory following ZIP infusion in the insular cortex (Shema *et al*, 2007), and fear memory following ZIP infusions in the amygdala (Migues *et al*, 2010; Serrano *et al*, 2008).

More recent data has shed doubt on the necessity of PKMζ for learning and memory. No LTP or memory deficits were found in two independently generated lines of PKMζ knockout mice (Lee *et al*, 2013a; Volk *et al*, 2013). Furthermore, when given ZIP, PKMζ knockout mice show memory impairments (Lee *et al*, 2013a). These findings indicate that the memory dampening effects of ZIP are not due to its actions on PKMζ. However, the ability of experience to upregulate PKMζ expression suggests it may still be playing a role in behavioral and synaptic plasticity. Since many past experiments utilized the non-specific inhibitor, ZIP, previous studies examining the role of PKMζ are difficult to interpret.

Despite the mixed evidence for the importance of PKMζ in memory maintenance, there does appear to be a role for PKMζ in motivation and reward processing. PKMζ KO mice consume more alcohol than their wildtype controls in a drinking in the dark paradigm (Lee *et al*, 2014). Very few studies have examined reward-

related behaviors in PKMζ KO mice. Therefore, the aim of these studies was to determine if the ability of PKMζ to dampen reward extends beyond the published effects on alcohol. To achieve this we utilized constitutive PKMζ knockout mice to examine the role of PKMζ in cocaine self-administration, extinction and reinstatement of cocaine seeking. Additional studies examined how conditional knockdown of PKMζ within the nucleus accumbens affected cocaine taking and seeking. Our results suggest that PKMζ reduces cocaine taking and seeking and the mechanism of action may be sex-specific.

Materials and Methods

Subjects. Wildtype studies: Male and female c57/BL6 mice, bred in house, were utilized for the PKMζ expression studies. Constitutive deletion: The current study utilized PKMζ knock-out mice as described previously (Volk *et al*, 2013). Heterozygous PKMζ KO mice on a C57bl/6 background were mated resulting in mutant and wildtype littermates. Conditional deletion: Mice homozygous for the Cre/lox-conditional allele of PKMζ (flox/flox) were bred on a C57bl/6 background as described previously (Volk et al., 2013). Mice (2-6 months old, 20-40g; age matched across group) were group housed until the start of the behavioral experiments at which point they were individually housed. All animals were housed in a temperature and humidity controlled animal care facility with a 12 hr light/dark cycle (lights on a 7:00 A.M.). All procedures were approved by the Temple University Animal Care and Use Committee.

Adeno-associated virus constructs. The adeno-associated virus (AAV) expressing Cre recombinase (AAV2/9.CMV.PI.CRE, titer $2.84*10^{13}$ gc/µl) and the AAV expressing green fluorescent protein (eGFP) (AAV2/9.CMV.eGFP, titer $3.74*10^{13}$ gc/µl) were generated by the Univ. of Pennsylvania Vector Core. AAVs were diluted in sterile phosphate-buffered saline (PBS) for microinjections.

Intraaccumbal microinjections. $PKM\zeta_{flox/flox}$ mice (6-8 weeks) are anesthetized with isoflurane and AAV (0.5µL, $1*10^{9}$ GC/µl) is injected into the accumbens through a

30 gauge needle at a rate of 0.1 μl/min. Stereotaxic coordinates for the nucleus accumbens are (from Bregma) anterior-posterior 1.5, lateral +/- 1.0, dorso-ventral -5.0. Following recovery, mice remain in the home cage for 6 weeks prior to behavioral testing.

Drugs.

Cocaine was obtained from the National Institutes of Drug Abuse Drug Supply Program (Bethesda, MD) and dissolved in sterile 0.9% saline.

Operant Food Training. Prior to catheterization, mice were trained to perform an operant response for sucrose pellets. The mice were placed in operant chambers (Med-Associates) where they learned to spin a wheel manipulandum to receive the sucrose pellet. When the pellet was delivered, a cue light above the active wheel was illuminated, a 2900 Hz tone played, and the house light turned off. This was followed by an 8s time-out where the house light remained off and spinning the wheel had no programed consequences. Mice were able to self-administer up to 50 pellets per 60 min operant session. The mice were food restricted to approximately 90% of their free feeding weight throughout the course of the operant training. They were returned to ad libitum feeding three days into the cocaine self-administration sessions.

Jugular Catheterization Surgery. Mice were anesthetized with 80 mg/kg ketamine and 12 mg/kg xylazine. An indwelling silastic catheter was placed into the right jugular vein and sutured in place. Then the catheter was threaded subcutaneously over the shoulder blade and was routed to a mesh back mount platform (Strategic Applications, Inc.) that secured the placement. The catheters were flushed daily with 0.1 ml of antibiotic (Timentin, 0.93 mg/ml) dissolved in heparinized saline. The catheters were sealed with plastic obturators when not in use.

Cocaine Self-administration. Mice were given 3-4 days to recover from surgery before beginning behavioral testing. The cocaine self-administration testing was

measured over 2 hr sessions (6 days per week) in the same chamber used for the operant food training. During testing, responding on the active wheel delivered an intravenous cocaine injection (0.6 mg/kg/infusion,) paired with the same cues as the food training. After 10 day of cocaine self-administration, cocaine-seeking was extinguished by replacing the cocaine with 0.9% saline. During extinction, the light and tone cues were not present. Daily 2 hr extinction continued until the mice met the extinction criterion of <25% of their responding during the self-administration (average of the last three days). 24 h after meeting the extinction criterion animals underwent a cue-induced reinstatement session. The light and tone were presented non-contingently for 20 s every 2 min during the first 10 min of the session. For the remaining 110 min, the cues were presented following responses on the active wheel, just as was done during the cocaine self-administration sessions. During reinstatement, the mice received saline infusions following active responses. Saline-yoked mice were placed in identical operant chambers however their responding had no programmed consequences. Instead, they received saline infusions along with cue presentations when their partner mouse received cocaine.

Western Blot. Whole-cell tissue from naïve viral-injected animals was processed for Western blot as described previously (Briand *et al*, 2014). For all samples, protein concentration was quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein (30 µg) were loaded and separated in 10% Tris-Glycine gels (Invitrogen) and transferred to nitrocellulose membranes using the i-Blot dry transfer system (Invitrogen). Membranes were blocked with Li-Cor blocking buffer. Membranes were incubated for 48 hours at 4 °C with selective antibodies to: PKMζ (1:500 Millipore Sigma SAB4502380), GluA1 (1:1000, Millipore Sigma MAB2263), GluA2 (1:1000, Millipore Sigma 07-598) and GAPDH (1:2000, Cell Signaling). Membranes were then incubated with fluorescent secondary antibodies (1:5000, IR-dye 680 or IR-dye 800), before being imaged on an Odyssey fluorescent scanner (Li-cor Biosciences). To ensure equal loading, GAPDH expression was used as a loading control. Separate blots were used for each protein of interest.

RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction. Mice were killed by cervical dislocation directly from their home cages 14 days following cocaine self-administration or yoked saline experience for the evaluation of early gene expression changes. Brains were rapidly removed, whole nucleus accumbens and hippocampi hand-dissected, and frozen in liquid nitrogen. RNA was extracted from hippocampal tissue using TRIzol/chloroform (Invitrogen) and the RNeasy Mini kit (Quiagen). cDNA was synthesized from RNA using an oligo(dT) primer (Operon) and Superscript II reverse transcriptase (Invitrogen). Quantitative real-time polymerase chain reaction (QPCR) was carried out using the SYBR-green master mix (Applied Biosystems) and 300 nM primers (final concentration) on the Stratagene MX3000 using MXPro QPCR software. Cycling parameters were 95°C for 10 min followed by 40 cycles of 95°C (30 sec) and 60°C (1 min), ending with a melting curve analysis to assess the amplification of a single amplicon. All reactions were performed in triplicate, with the median cycle time used for analysis. TATA-box binding protein (TBP) was used as a housekeeping gene against whose levels all experimental genes were normalized. Primer sequences are as follows:

Gene	Forward Primer	Reverse Primer
PKMz	CCTTCTATTAGATGCCTGCTCTCC	TGAAGGAAGGTCTACACCATCGTTC
TBP	CAGCAATCAACATCTCAGCAA	GGGGTCATAGGAGTCATTGGT

Data Analysis.

All analyses were performed using Graphpad Prism 7.0 software (Graphpad Software). Behavioral data were analyzed using two-tailed Student's *t*-test, or two-way ANOVA with *Sidak*'s post hoc as appropriate. Statistical significance for all tests was set at $\alpha = 0.05$.

Results

Cocaine Self-Administration and Abstinence Leads to an Increase in PKMζ mRNA and protein expression within the Nucleus Accumbens.

Wildtype mice underwent ten days of training to acquire operant responses for food followed by 10 days of cocaine self-administration. Saline yoked mice were placed in identical operant chambers however their actions had no programmed consequences. They received saline infusions along with cue presentations when their partner received cocaine. Both cocaine-experienced and saline-yoked mice were then placed in their home cages for 14 days prior to brain dissection. Cocaine-self administration experience led to an increase in PKMζ mRNA and protein expression within the nucleus accumbens (mRNA: $t(8)=5.21$, $p=.0008$, n=5-6/group; protein: $t(22)=2.089$, $p=.048$, n=12; Figure 1a,b). No differences were seen in PKMζ mRNA or protein expression in the hippocampus (mRNA: $t(12)=1.029$, $p=.324$, n=5-9/group; protein: $t(22)=1.50$, $p=.147$, n=12-13/group; Figure 1c,d).

Constitutive Deletion of PKMζ Potentiates Food Self-Administration but does not alter Reinstatement of Food Seeking.

Wildtype controls and PKMζ $KO_{constitutive}$ mice underwent ten days of training to acquire operant responses for food. Both wildtype and PKMζ $KO_{constitutive}$ mice showed a gradual increase in the number of pellets earned and active responses per session over the 10 days of food training (effect of session, pellets= $F(9,342)=7.38$, $p<.0001$; responses= $F(9, 342)=20.53$, $p<.0001$). Additionally, constitutive deletion of PKMζ, led to an increase in responding for food and increase in the number of rewards received (effect of genotype, pellets= $F(1,38)=6.79$, $p=.01$; responses= $F(1,38)=7.15$, $p=.01$; n=22/group; Figure 2a,b). This effect of genotype was seen in both males and females (Males: effect of genotype, pellets= $F(1,25)=6.54$, $p=.02$; responses= $F(1,25)=10.15$, $p=.004$; n=10; Females: effect of genotype, pellets= $F(1,25)=6.75$, $p=.02$; responses= $F(1,25)=8.04$, $p=.009$; n=10). Both wildtype and PKMζ $KO_{constitutive}$ mice showed an

increase in their percent active responding over the 10 sessions but no group differences were seen (effect of session, $F(9,342)=25.68$, $p<.0001$; effect of genotype, $F(1,25)=0.0062$, $p=.94$; data not shown). A subset of mice underwent extinction of food responding and cue-induced reinstatement of food seeking. No differences were seen between the groups in the rate of extinction (days to extinction WT: 3.25 ± 0.25; PKMζ $KO_{constitutive}$: 3.38 ± 0.38; $t(10)=0.22$, $p=.83$). Both wildtype and PKMζ $KO_{constitutive}$ mice exhibited cue-induced reinstatement of food seeking, however no differences were seen between the groups (effect of session, $F(1,10)=12.41$, $p=.006$; effect of genotype, $F(1,10)=0.0007$, $p=.98$; n=6-8/group; Figure 2c).

Constitutive Deletion of PKMζ Potentiates Cocaine Self-Administration and Cue-Induced Reinstatement of Cocaine Seeking.

Following food training, mice received jugular catheterization surgery and underwent ten days of cocaine self-administration. Both wildtype and PKMζ $KO_{constitutive}$ mice showed an increase in cocaine intake over the course of the 10 sessions and no group differences were seen in cocaine intake (effect of session, $F(9,225)=4.93$, $p<.0001$; effect of genotype, $F(1,25)=0.41$, $p=.53$; Figure 3a). However, PKMζ $KO_{constitutive}$ mice exhibited higher rates of responding for cocaine compared to wildtype controls (effect of genotype, $F(1,25)=7.85$, $p=.009$; n=12-15/group; Figure 3b). Following the cocaine self-administration, animals underwent extinction of the cocaine seeking behavior. There was no difference between the two groups in the amount of time taken to reach the extinction criterion ($t(21)=0.30$, $p=0.77$; Figure 3c). Following extinction, mice were exposed to a cue-induced reinstatement session in which active responses resulted in presentation of the cues previously paired with drug administration in the absence of the drug. Disrupting PKMζ function led to increased cue-induced drug seeking compared to wildtype controls (effect of session, $F(1,22)=8.71$, $p=.007$; effect of genotype, $F(1,22)=5.10$, $p=.03$; interaction, $F(1,22)=4.41$, $p=.04$; Sidak's multiple comparison, KO vs. WT RI test, adjusted $p=.0002$; n=8-16/group; Figure 3d).

Site-specific knockdown of PKMζ in the Nucleus Accumbens does not alter Food Seeking or Reinstatement.

To determine if the effects of PKMζ deletion on food and cocaine behavior were due to actions of PKMζ within the nucleus accumbens, we injected floxed PKMζ adult mice with an adeno-associated virus expressing either Cre recombinase or GFP into the accumbens. This led to a significant decrease in PKMζ expression within the nucleus accumbens 6 weeks following viral injection in both males and females [main effect of virus, $F(1,36)=37.49$, $p<.0001$; Figure 4a]. Of note, the western blot analysis revealed a baseline sex difference with both GFP and Cre injected females exhibiting lower levels of PKMζ expression compared to males [main effect of sex, $F(1,36)=7.61$, $p=.009$; Figure 4a]. Similar to what has been shown previously in the PKMζ $KO_{constitutive}$ mice (Volk *et al*, 2013), no changes were seen in the other atypical PKC isozyme, PKCι/λ following PKMζ knockdown [effect of virus, $F(1,36)=2.05$, $p=.16$; effect of sex, $F(1,36)=3.48$, $p=.07$; Figure 4b]. Both wildtype and PKMζ $KO_{accumbens}$ mice showed a gradual increase in the number of pellets earned and active responses per session over the 10 days of food training and no differences were seen between the groups (effect of session, pellets= $F(9,567)=19.85$, $p<.0001$; responses= $F(9,567)=26.26$, $p<.0001$; effect of genotype, pellets= $F(1,63)=0.299$, $p=.59$; responses= $F(1,63)=0.667$, $p=.80$; N=30-35; Figure 5a,b). Furthermore, no differences were seen between the groups in the rate of extinction (days to extinction WT: 3.5 ± 0.5; PKMζ $KO_{accumbens}$: 4.4 ± 0.62; $t(14)=1.007$, $p=.33$). Both wildtype and PKMζ $KO_{accumbens}$ mice exhibited cue-induced reinstatement of food seeking, however no differences were seen between the groups (effect of session, $F(1,14)=21.61$, $p=.0004$; effect of genotype, $F(1,14)=1.24$, $p=.28$; Figure 5c).

Site-specific knockdown of PKMζ in the Nucleus Accumbens Potentiates Cocaine Self-Administration in Males but not Females.

Male and female wildtype and PKMζ $KO_{accumbens}$ mice showed an increase in cocaine intake over the course of the 10 sessions (males: effect of session, $F(9,180)=8.99$, $p<.0001$; females: effect of session, $F(9,243)=13.84$, $p<.0001$; Figure 6a,b). Male PKMζ $KO_{accumbens}$ mice exhibited an increase in cocaine intake and responding for cocaine (effect of genotype, infusions= $F(1,20)=7.25$, $p=.01$; responses= $F(1,20)=4.39$, $p=.049$; Figure 6a,c). Furthermore, Male PKMζ $KO_{accumbens}$ mice also exhibited an increase in cue-induced cocaine seeking (effect of session, $F(1,10)=14.29$, $p=.0036$; effect of viral injection, $F(1,10)=5.76$, $p=.037$; interaction, $F(1,10)=4.60$, $p=.05$; Sidak's multiple comparison, Cre vs. GFP RI test, adjusted $p=.0087$; n=6-7/group; Figure 6e). However, in the female mice, the local PKMζknockout in the nucleus accumbens had no effect on cocaine self-administration (infusions: $F(1,27)= 0.489$, $p=0.490$; responses: $F(1,26)=0.162$, $p=0.691$; n=14-15/group; Figure 6b,c) or reinstatement of cocaine seeking (effect of session, $F(1,9)=13.39$, $p=.0052$; effect of viral injection, $F(1,9)=0.13$, $p=.73$; interaction, $F(1,9)=0.28$, $p=.61$; n=6-8/group; Figure 6f).

Site-specific knockdown of PKMζ in the Nucleus Accumbens Leads to an Increase in GluA1 AMPA Receptor Subunit Expression in Males but not Females.

To determine the effects of accumbal PKMζ knockdown on AMPA receptor subunit expression, we utilized tissue from behaviorally naïve floxed PKMζ adult mice injected with an adeno-associated virus expressing either Cre recombinase or GFP into the accumbens. PKMζ knockdown within the accumbens led to an increase in GluA1 expression in male mice but not females (effect of sex, $F(1,28)=6.30$, $p=.018$; effect of viral injection, $F(1,28)=2.05$, $p=.16$; interaction, $F(1,28)=4.84$, $p=.036$; Sidak's multiple comparison, Cre vs. GFP Males, adjusted $p=.008$; n=6-10/group; Figure 7a). While accumbal PKMζ knockdown did not alter GluA2 subunit expression in either sex, females in both groups exhibited less GluA2 expression compared to males (effect of sex, $F(1,31)=9.82$, $p=.0038$; effect

of viral injection, $F(1,31)=0.810$, $p=.38$; interaction, $F(1,31)=0.521$, $p=.48$; n=7-10/group; Figure 7b).

Discussion

Here, we provide evidence that the atypical PKC, PKMζ, plays a critical role in mediating reward-related behavior. Cocaine self-administration experience led to an increase in PKMζ within the nucleus accumbens at both the transcript and protein levels. This increase appears to be compensatory as we found that constitutive knockout of PKMζ increases cocaine taking and vulnerability to cue-induced cocaine relapse. This increase in cocaine taking and seeking is recapitulated in male mice with a site-specific knockdown of PKMζ in the nucleus accumbens. However, accumbal knockdown of PKMζ did not alter cocaine taking or seeking in females.

The current study is one of two studies to demonstrate a behavioral phenotype in PKMζ knockout mice. Our findings are consistent with previous work demonstrating constitutive PKMζ knockout mice exhibit increased alcohol intake (Lee *et al*, 2014). The data presented here provide support for the idea that PKMζ acts to dampen reward, as cocaine self-administration experience led to an increase in both PKMζ transcript and protein expression within the nucleus accumbens. This is consistent with increases in PKMζ seen following morphine conditioned place preference (Li *et al*, 2011), a single episode of ethanol drinking (Mulligan *et al*, 2011), and experimenter administered cocaine (Ho *et al*, 2012). However, in the absence of PKMζ mice exhibited an increase in cocaine seeking. This suggests that cocaine-induced increases in PKMζ expression are compensatory and act to counter the effects of cocaine.

Within the hippocampus, PKMζ enhances AMPA transmission, an effect mediated by NSF-mediated insertion of GluA2-containing AMPA receptors (Yao *et al*, 2008). Additionally, once these GluA2-containing AMPARs are inserted, PKMζ may also make them harder to move out of the synapse (Yu *et al*, 2017). As the removal of

GluA2-containing AMPARs and corresponding insertion of GluA2-lacking AMPARs plays a critical role in cocaine craving (Briand *et al*, 2016; Conrad *et al*, 2008; Famous *et al*, 2008; McCutcheon *et al*, 2011), this could provide a mechanism by which PKMζ could dampen reward. As none of the studies examining the relationship between PKMζ and GluA2 were done in the nucleus accumbens, more work is needed to determine whether PKMζ is in fact altering AMPAR subunit composition and how it functions following chronic cocaine exposure. However, the results from the current study support the idea that disrupting PKMζ function can lead to alterations in AMPA receptor subunit composition.

The current study was able to recapitulate the increased cocaine seeking seen following constitutive PKMζ knockout with site-specific knockdown of PKMζ within the nucleus accubmens in male mice. Furthermore, PKMζ knockdown within the accumbens led to an increase in GluA1 AMPA subunit expression without any changes in GluA2 expression. This supports our hypothesis that PKMζ could be working to stabilize GluA2-containing AMPA receptors and in its absence, there is an increase in GluA2-lacking AMPA receptors. An increase in GluA2-lacking AMPA receptors could explain the behavioral findings seen in male mice following accumbal PKMζ knockdown. Manipulations increasing GluA2-lacking AMPARs lead to increased cue-driven drug seeking and decreasing the activity of these receptors has the opposite effect (Briand *et al*, 2014; Conrad *et al*, 2008; Lee *et al*, 2013b; Ma *et al*, 2014).

However, accumbal knockdown of PKMζ did not alter cocaine taking or seeking in female mice. Furthermore, the upregulation in GluA1 expression seen following PKMζ knockdown in males was absent in females. Of note, wildtype female mice exhibited lower levels of PKMζ expression compared to males. Additionally, we detected lower levels of GluA2 receptor expression in females. Lower GluA2 subunit expression in the absence of lower GluA1 expression might suggest an increase in CP-AMPAR expression in females. Lower levels of GluA2-containing

receptors in female mice may limit any further plasticity and prevent the effects of PKMζ knockdown. Although no work has examined this in the nucleus accumbens, there is evidence for enhanced AMPA receptor mediated synaptic responses in other brain regions of male rodents (Monfort *et al*, 2015) compared to female rodents, an effect that could be mediated by CP-AMPAR expression.

Ceftriaxone, an antibiotic that normalizes basal extrasynaptic glutamate levels, attenuates reinstatement in males and non-estrus females. However, females in the estrus phase are unaffected by ceftriaxone, suggesting an interaction between the estrus cycle and glutamateric system following cocaine self-administration (Bechard *et al*, 2018). As the site-specific PKMζ knockdown was a chronic manipulation, we did not examine the role of the estrus cycle in the current study. However, this suggests that the estrus cycle could interact with any manipulation of the glutamate system.

There is some evidence that PKMζ has sex-specific effects in other behaviors as well as the cocaine effects reported here. Constitutive deletion of PKMζ leads to decreased anxiety-like behavior in male mice but not females (Lee *et al*, 2013a). However, as anxiolytic medications can decrease cocaine addictive phenotypes (Augier *et al*, 2012; Barbosa-Mendez *et al*, 2017), it is unlikely that our behavioral effects are mediated by this decreased anxiety. PKMζ deletion also increases muscle pain threshold in male mice but not female mice (Nasir *et al*, 2016). Together with the current findings, these studies support a greater role for PKMζ in males. However, as we do see effects of constitutive deletion of PKMζ in females, further work is needed to determine the sex-specific roles of PKMζ in these behaviors.

Constitutive PKMζ deletion led to an increase in food self-administration as well along with the increased cocaine taking. This suggests that PKMζ plays a more general role in reward learning and reward consumption. However, systemic administration of a competitive AMPA antagonist decreases sucrose self-

administration (Stephens and Brown, 1999) and application of PKMζ in the slice appears to increase AMPAR transmission (Yao *et al*, 2008). In contrast, AMPA antagonist administration into the nucleus accumbens facilitates feeding behavior (Maldonado-Irizarry *et al*, 1995). However, the lack of increase in food seeking following site-specific knockdown of PKMζ suggests that this effect is not accumbally mediated. Thus, PKMζ must play a critical role in food seeking in another brain region. Changes in feeding behaviors have been observed following glutamate manipulations in the ventral tegmental area (D'Souza & Markou, 2011), the dorsal striatum (Alsio et al., 2011) and the posterodorsal medial amygdala (Rosa, Goularte, Trindada, De Oliveira & Rasia-Filho). Further work will need to be done to determine if the effects seen in the constitutive knockout mice are due to the role of PKMζ in these regions. Despite the increase in food seeking in constitutive knockout mice, they do not demonstrate any alterations in cue-induced reinstatement of food seeking. As many studies manipulating AMPA receptors have seen specific alterations in cocaine seeking without corresponding changes in food seeking (Anderson *et al*, 2008; Briand *et al*, 2016; Briand *et al*, 2014; Famous *et al*, 2008), this supports our hypothesis that PKMζ knockout may be altering accumbal AMPA receptors.

Conclusion

In the current study, we have shown that constitutive deletion of PKMζ enhances cocaine seeking and taking, while only increasing intake but not seeking of a natural reward. This can be explained by PKMζ's ability to regulate GluA2 expression. We propose that PKMζ increases the amount of GluA2 in the synapse, which simultaneously reduces the amount of CP-AMPAR and inhibits reinstatement. In males, deletion of PKMζ only in the nucleus accumbens is sufficient to enhance cocaine seeking and taking, but has no effect in females. It's possible that the sex differences observed in this study are also due to sex differences in the glutamatergic system following cocaine exposure. While further studies are needed to clarify the relationship between PKMζ and GluA2, these data

suggest that PKMζ works to prevent the development of addictive behaviors and has potential as a novel therapeutic agent for the treatment of addiction.

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Figure 1. Cocaine self-administration leads to an increase in PKMζ in the nucleus accumbens. After 14 days of abstinence, chronic cocaine self-administration (10 days, 0.5mg/kg/inf) leads to an upregulation of PKMζ mRNA (a; *p<.01; n=5-9/group) and protein levels (b; *p<.05; n=12-13/group) within the nucleus accumbens but not the hippocampus (c-d).

Figure 2. Constitutive PKMζ knockout potentiates food self-administration without altering cue-induced food seeking. PKMζ $KO_{CONSTITUTIVE}$ mice exhibit significantly greater responding (a) and earn more pellets (b) than wildtype mice. While both wildtype and PKMζ $KO_{CONSTITUTIVE}$ mice exhibit a significant increase in responding during reinstatement (c), there were no differences between the groups. *p<.05, effect of genotype (a,b; n=22/group); **p<.01, effect of session (c; n=6-8/group).

Figure 3. Constitutive PKMζ knockout potentiates cocaine self-administration and cue-induced cocaine seeking. Although the groups did not differ in the number of cocaine infusions (a), PKMζ $KO_{CONSTITUTIVE}$ mice exhibited significantly greater responding during both cocaine self-administration (b) and cue-induced reinstatement (d). No differences were seen in the time it took for the groups to extinguish the cocaine seeking behavior (c). **p<.01, effect of genotype (b; n=12-15/group); pairwise comparison WT vs. KO (c; n=8-16/group).

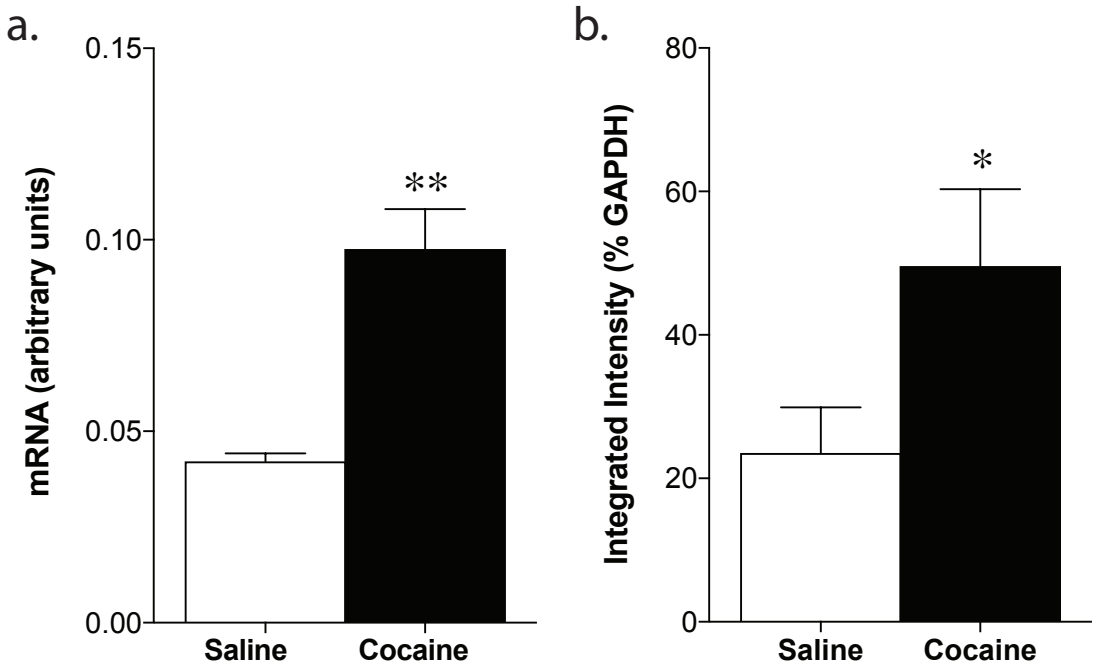
Figure 4. Cre recombinase injection into the nucleus accumbens leads to a significant decrease in PKMζ protein levels without altering PKCι/λ. Quantification of western blot showing a significant decrease in PKMζ protein in the nucleus accumbens following AAV-Cre injection in both male and female mice, as normalized to glyceraldehyde 3-phosphate dehydrogenase (a,c; ***p<.0001 main effect of virus; **p<.01 main effect of sex; n=9-13/group). PKMζ knockdown did not alter the levels of PKCι/λ within the accumbens (b,c).

Figure 5. Site-specific knockdown of PKMζ within the nucleus accumbens does not affect food self-administration or reinstatement. No differences were seen between the groups in the number of pellets earned or the number of active responses during the self-administration phase (a, b; n=30-36). During the cue-induced reinstatement session both groups exhibited a significant increase in responding compared to their extinction responding but not differences were seen between the groups (c; **p<.01 effect of session, n=6-10).

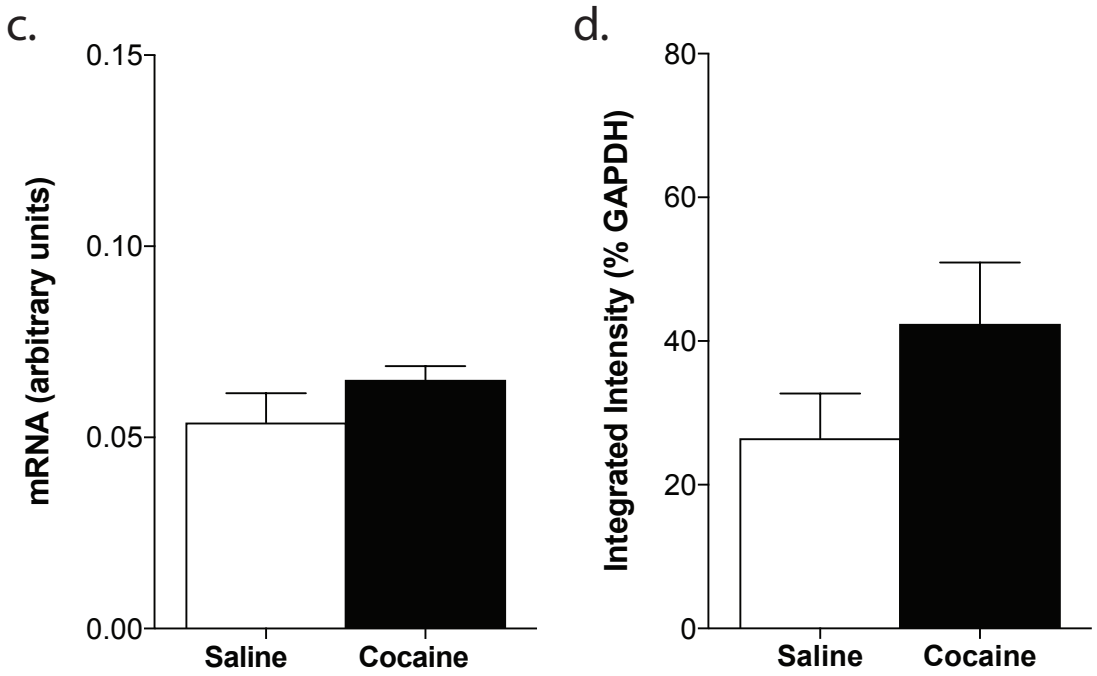
Figure 6. Site-specific knockdown of PKMζ within the nucleus accumbens enhances cocaine self-administration in male mice. Male PKMζ $KO_{accumbens}$ mice exhibited a significant increase in cocaine taking, as indicated by an increase in cocaine infusions earned (a) and active responses completed (c) compared to GFP-injected control males (*p<.05, effect of genotype, n=11/group). Further, male PKMζ $KO_{accumbens}$ mice exhibit greater cocaine seeking during cue-induced reinstatement (e; **p<.01 pairwise comparison GFP vs. Cre on RI day, n=6-7/group). Female PKMζ $KO_{accumbens}$ mice did not differ from GFP injected controls during self-administration (b,d;n=14-15/group) or reinstatement (f, n=6-8/group).

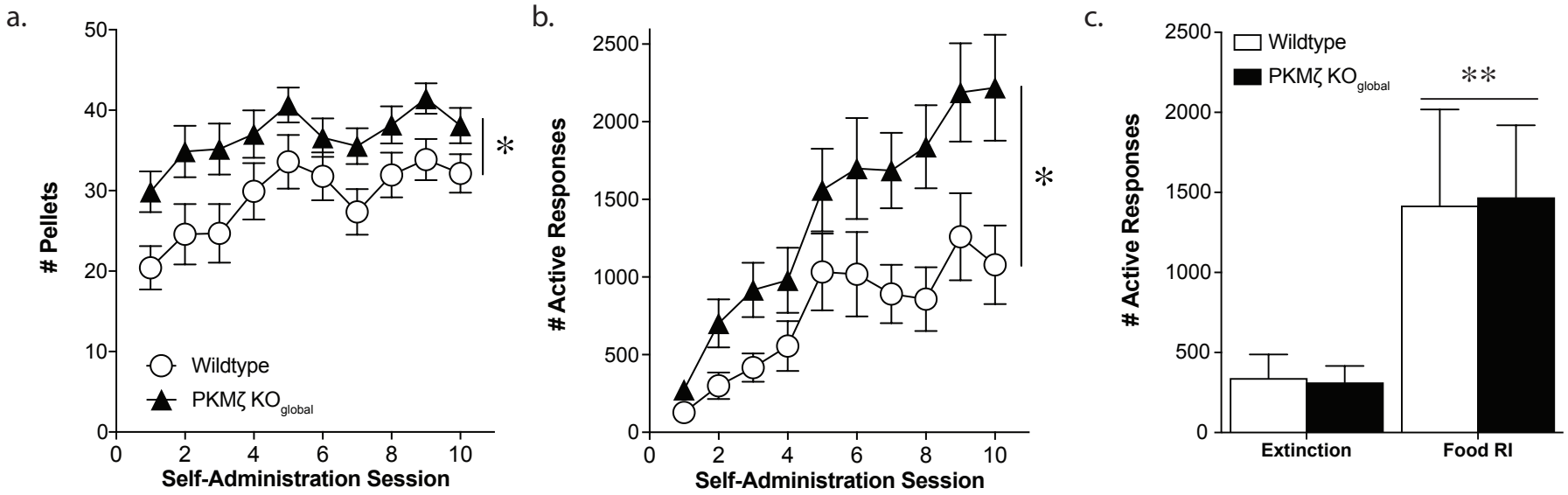
Figure 7. Site-specific knockdown of PKMζ within the nucleus accumbens increases GluA1 expression in male mice. Male PKMζ $KO_{accumbens}$ mice exhibited a significant increase in GluA1 protein expression that is not seen in female PKMζ $KO_{accumbens}$ mice (a; **p<.01 pairwise comparison GFP vs. Cre males, n=6-10/group). Females exhibited lower levels of GluA2 expression regardless of PKMζ knockdown (b, **p<.01 effect of sex, n=7-10/group).

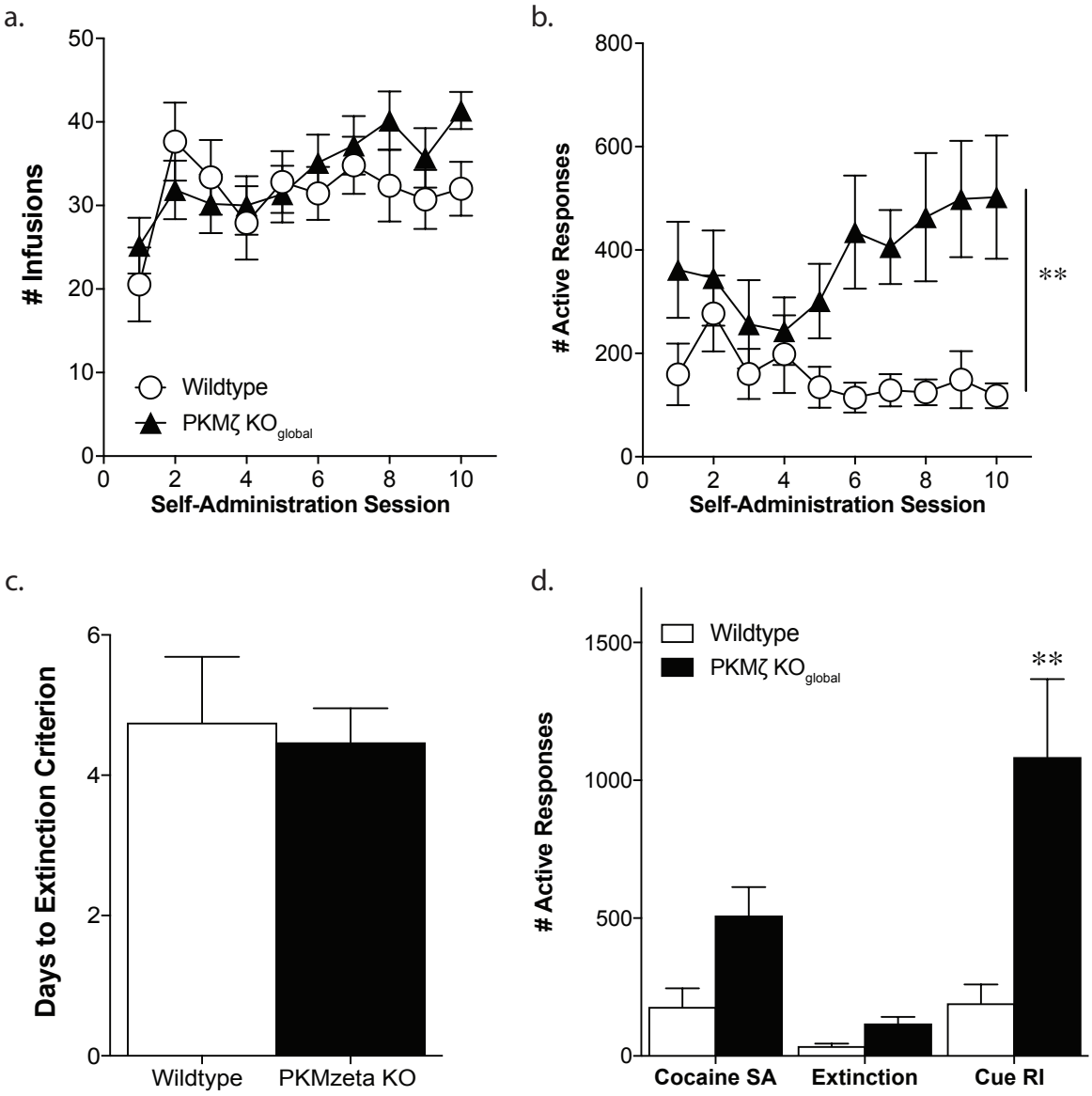
Nucleus accumbens

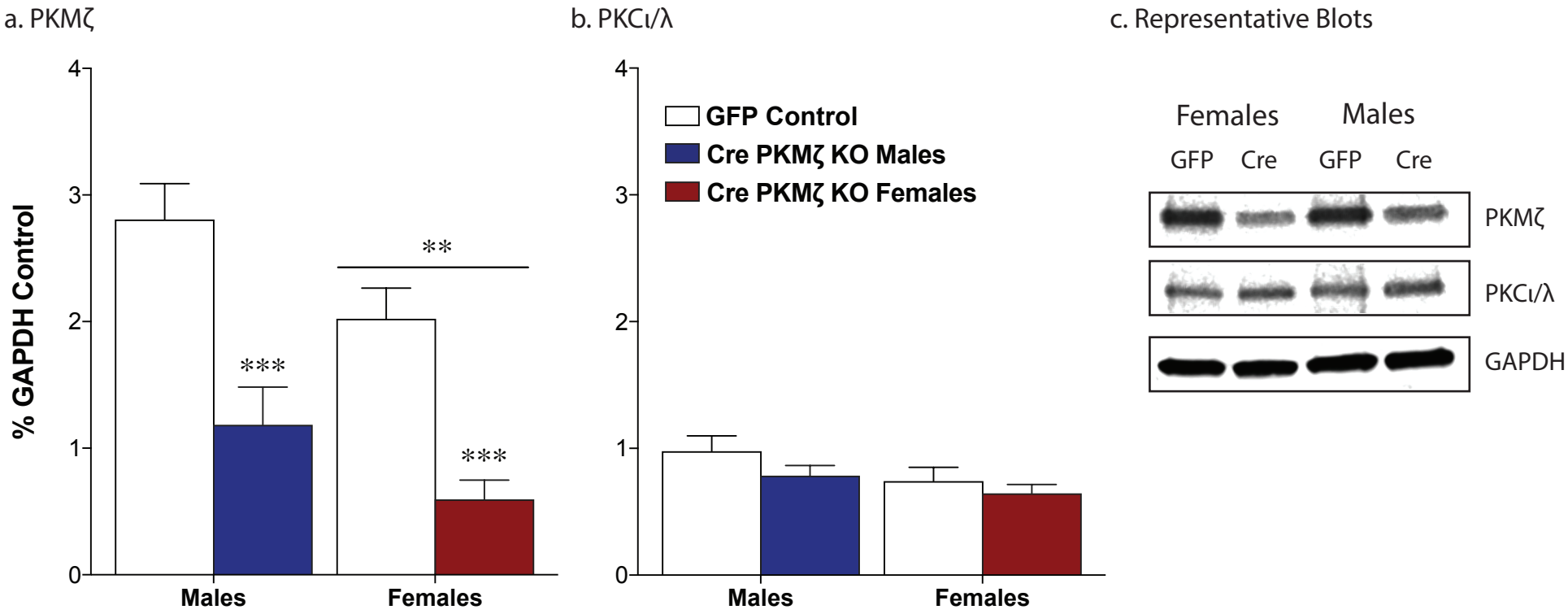


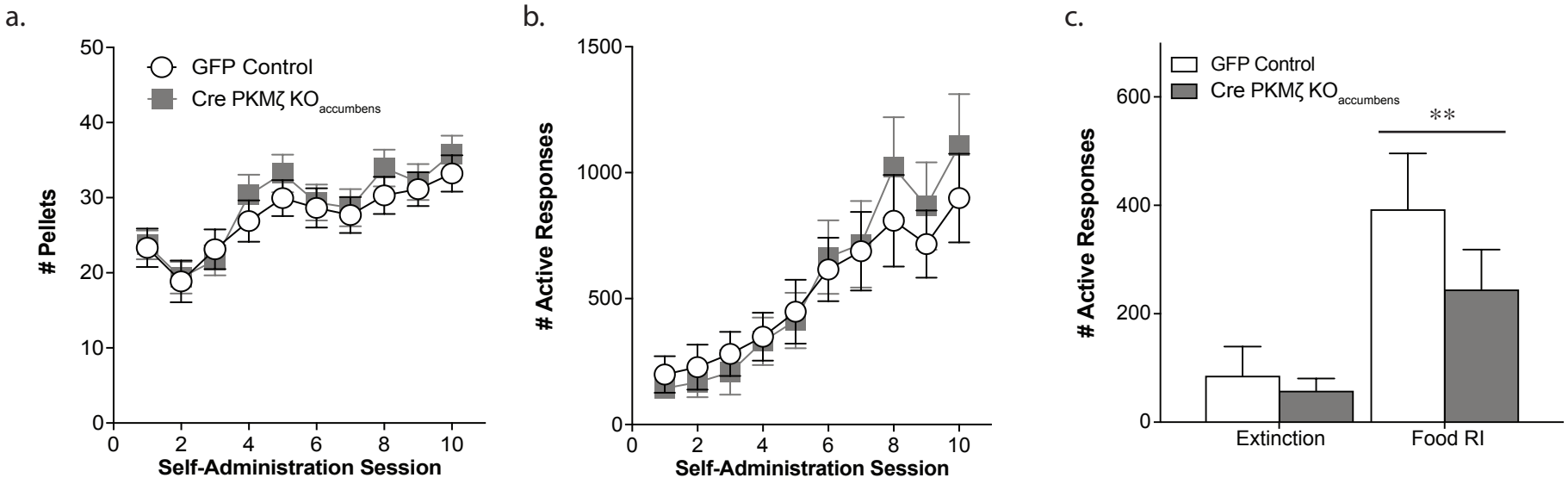
Hippocampus

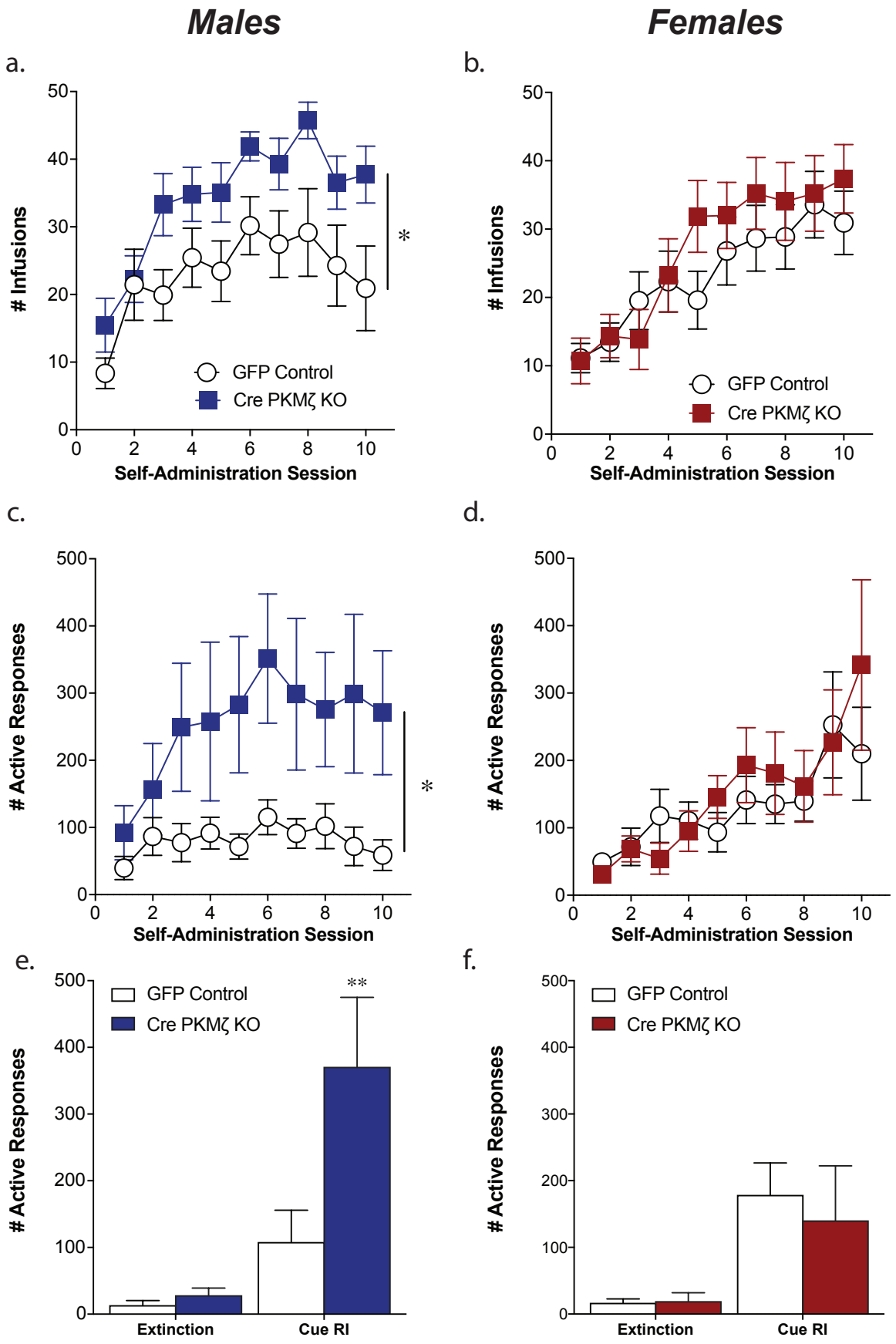


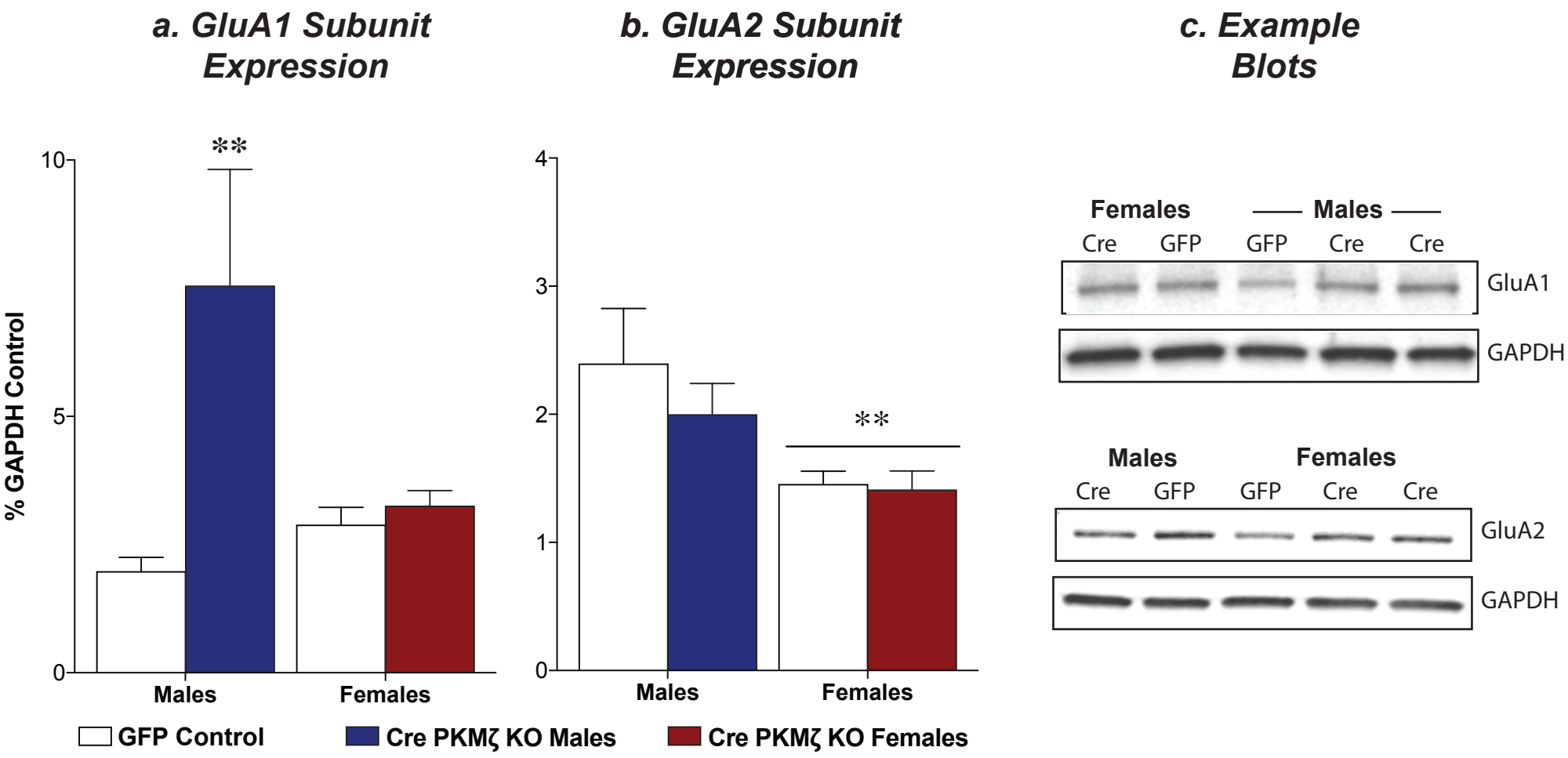

a.
Pellets
Self-Administration Session
Wildtype
PKMζ KO$_{global}$
*
b.
Active Responses
Self-Administration Session
*
c.
Active Responses
Wildtype
PKMζ KO$_{global}$
**
Extinction
Food RI


a.
Infusions
Self-Administration Session
Wildtype
PKMζ KO global
b.
Active Responses
Self-Administration Session
**
c.
Days to Extinction Criterion
Wildtype
PKMzeta KO
d.
Active Responses
Wildtype
PKMζ KO global
**
Cocaine SA
Extinction
Cue RI


a. PKMζ
% GAPDH Control
4
3
2
1
0
***
**
***
Males
Females
b. PKCι/λ
GFP Control
Cre PKMζ KO Males
Cre PKMζ KO Females
4
3
2
1
0
Males
Females
c. Representative Blots
Females
Males
GFP
Cre
GFP
Cre
PKMζ
PKCι/λ
GAPDH


a.
GFP Control
Cre PKMζ KO accumbens
Pellets
Self-Administration Session
b.
Active Responses
Self-Administration Session
c.
GFP Control
Cre PKMζ KO accumbens
Active Responses
**
Extinction
Food RI


Males
Females
a.
b.
c.
d.
e.
f.
Infusions
Active Responses
Self-Administration Session
GFP Control
Cre PKMζ KO
*
**
Extinction
Cue RI


a. GluA1 Subunit Expression
b. GluA2 Subunit Expression
c. Example Blots
% GAPDH Control
10
5
0
**
Males
Females
4
3
2
1
0
**
Males
Females
GFP Control
Cre PKMζ KO Males
Cre PKMζ KO Females
Females
Males
Cre
GFP
GFP
Cre
Cre
GluA1
GAPDH
Males
Females
Cre
GFP
GFP
Cre
Cre
GluA2
GAPDH