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Patrick Curtin 03/31/15

Effects of Cocaine on AMPA Receptor Phosphorylation in the Prefrontal Cortex of Mice

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Abstract

Effects of Cocaine on AMPA Receptor Phosphorylation in the Prefrontal Cortex of Mice

By Patrick Curtin

Cocaine addiction is a serious and persistent problem in the United States. There is currently no FDA approved drug for the treatment of cocaine addiction and there is still much that is not understood about the changes the brain undergoes in response to cocaine. One of the newest areas of cocaine research focuses on cocaine's effect on glutamate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. The goal of this study was to assess cocaine-induced changes in AMPA receptor phosphorylation using a sensitization model, which examines the locomotor activity of mice in response to repeated cocaine exposure.

Although previous experiments have shown that AMPA receptor trafficking is sensitization-dependent, the effects of chronic cocaine exposure on serine 831 phosphorylation levels, a key phosphorylation site in the regulation of AMPA receptor trafficking, have not been investigated. Our goal was to compare the effects of acute and chronic cocaine administration on serine 831 phosphorylation.

Locomotor activity was recorded every day for 5 days, and then again on a "challenge" day 3 days later, in 3 groups of C57BL/6 mice: one group (S/S) received saline every day, one group (S/C) received saline every day and then cocaine (15 mg/kg) on challenge day, and one group (C/C) received cocaine every day. Thirty min after the end of the challenge day session, animals were sacrificed and their

prefrontal cortex and nucleus accumbens were collected and western blots of samples were performed.

Cocaine increased locomotor activity in both the S/C and the C/C groups. However, contrary to what has previously been reported with this paradigm, we did not observe behavioral sensitization in the C/C group, defined as an increase in locomotor response over the days of cocaine treatment. Both the S/C and the C/C groups had similarly higher phosphorylation levels at serine 831 compared to the S/S control group, although the differences did not reach statistical significance. These results suggest that serine 831 phosphorylation is sensitive to acute and chronic cocaine exposure, and will help guide future experiments on cocaine-induced AMPA receptor changes.

Effects of Cocaine Sensitization on AMPA Receptor Phosphorylation in the Prefrontal Cortex of Mice

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Introduction

Cocaine abuse is a serious and persistent health problem within the United States. Currently, there are no FDA approved medications for the treatment of cocaine addiction, and many of cocaine's neurobiological effects are still not understood. Part of the difficulty in treating cocaine addiction is its widespread affects on cellular processes in multiple regions of the brain (reviewed by Schmidt & Weinshenker, 2014).

Cocaine works primarily in the brain by blocking reuptake of norepinephrine, serotonin, and dopamine by binding to and inhibiting their respective plasma membrane transporters. This causes higher concentrations of these neurotransmitters to remain in the synapse for a longer duration of time. Prolonged exposure to these neurotransmitters can cause behavioral changes in animals that approximate some aspects of human addiction (reviewed by Schmidt & Weinshenker, 2014).

One way to mimic the effects of cocaine dependence in an animal model is with daily injections of cocaine in a process called "sensitization". In sensitization studies, locomotor responses in animals exposed to a fixed dose of cocaine increase over time. Although locomotor sensitization is not "addiction" per se, it is a form of neural plasticity that is thought to mimic certain aspects of cocaine dependence such as craving (reviewed here (Robinson & Berridge, 2008)).

The increase in synaptic monoamine levels due to cocaine exposure not only changes the behavior of animal, but it also causes molecular changes to occur within the brain. Several neurochemical and molecular adaptations that may underlie

cocaine-induced behaviors have been identified. Glutamate release in the nucleus accumbens (NAc), which is strongly linked to reward, is altered by cocaine use (Dackis et al, 2003). Research performed by several groups suggests that activating glutamatergic projection neurons from the prefrontal cortex (PFC) to the NAc promotes cocaine seeking in rats (Schmidt and Pierce, 2010). For example, it has been shown that elevated glutamate in the NAc is associated with cocaine-seeking behavior (Anderson et al., 2008), and this increase in glutamate was abolished when neurons in the PFC were inhibited, indicating that the source of glutamate in the NAc was pyramidal projection neurons in the PFC (McFarland et al., 2003). Research has also found increased glutamate release in the NAc core after chronic cocaine injections (Schmidt and Pierce, 2010).

Since cocaine does not act directly on glutamate receptors, it is thought that cocaine-induced increases in one or more of the monoamine neurotransmitters triggers the activity of PFC neurons that release glutamate in the NAc. One such candidate is norepinephrine, a neurotransmitter that is directly increased by cocaine. This leads to activation of adrenergic receptors in animals exposed to cocaine (reviewed by Schmidt & Weinshenker, 2014). Alpha-1 adrenergic receptor activation in the PFC is required for cocaine-induced locomotion and sensitization. When prazosin, an alpha-1 adrenergic receptor antagonist, is administered systemically or directly into the PFC, cocaine-induced locomotion and sensitization is reduced (Drouin et al., 2002; Darracq et al., 1998).

The alpha-1 adrenergic receptor is a G-protein coupled receptor that activates phospholipase C (PLC), which then cleaves PIP2 into PIP3 and DAG. DAG then

activates protein kinase C (PKC), which phosphorylates various targets. The target of interest for my research is the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. Alpha-1 adrenergic receptor activation by an alpha-1 agonist in the PFC enhances miniature excitatory postsynaptic currents (mEPSCs) (Luo et al., 2014). It was found that this pathway is linked to AMPA receptors in the PFC. When AMPA receptors in the PFC were blocked, the enhancement of mEPSCs disappeared (Luo et al., 2014). This process also appears to be mediated through a PKC-dependent pathway, as seen by a loss of alpha-1-mediated enhancement of mEPSCs when cells are exposed to PKC inhibitor (Luo et al., 2014). A hypothetical pathway linking cocaine-induced alpha-1 receptor activation to AMPA receptor phosphorylation, and therefore AMPA receptor trafficking, is shown in Figure 1. Although other kinases such as CaMKII may play a role in the phosphorylation of AMPA receptors, the decrease in mEPSCs when exposed to PKC inhibitor is a strong indicator that PKC is the major kinase within this pathway.

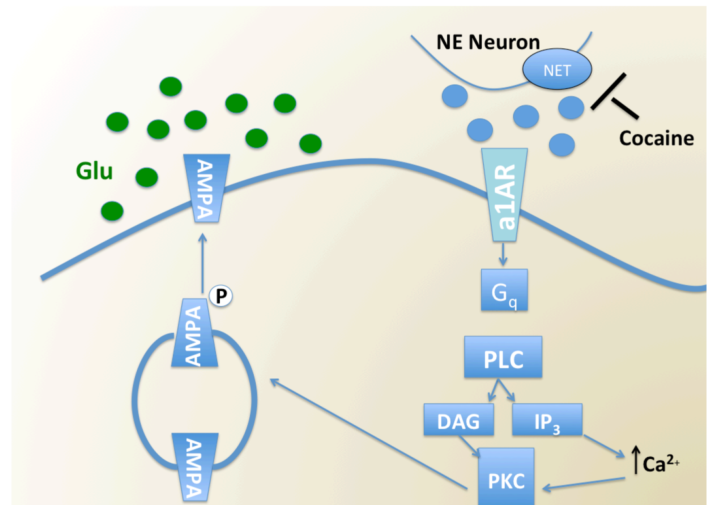

Figure 1. Proposed pathway of cocaine's effect on adrenergic signaling in the PFC. Cocaine prevents the reuptake of norepinephrine, causing an increase in alpha-1 adrenergic receptor activation. This should lead to an increase in downstream effects like PKC activation and subsequent AMPA receptor phosphorylation.

AMPA receptors are comprised of 4 subunits and are typically heterotetrameric. AMPA receptors open when activated by glutamate, allowing sodium to enter the neuron and increasing the probability of a depolarization event. The AMPA

receptors being studied in this experiment in the PFC and NAc are typically comprised of GluR1 and GluR2 subunits. Phosphorylation at serine 831 on GluR1 and serine 880 on GluR2 on AMPA receptors is known to affect synaptic localization (Terashima, A. *et al.,* 2004, Wang, J. Q. *et al.*, 2014) and channel conductance (Boehm, J. *et al.*, 2006, Jenkins, M. A. & Traynelis, S. F., 2012), respectively. Phosphorylation at serine 831 increases AMPA receptor trafficking to the neuronal surface. Phosphorylation at serine 880 causes a removal of GluR2 subunits, allowing calcium ions to enter the neuron and further enhancing channel conductance as well as downstream signaling.

Plasma membrane-bound AMPA receptors mediate synaptic transmission when they are activated by glutamate. Cocaine causes an increase in the trafficking of AMPA receptors to neuronal cell surfaces in the NAc in cocaine sensitizated animals (Kalivas et al., 2009). A major goal of the current study was to determine whether cocaine also increases AMPA receptor phosphorylation in the PFC, which would suggest that cocaine sensitization alters AMPA receptor trafficking and excitability in this critical brain region as well.

Measuring AMPA receptor phosphorylation in the PFC is a way of indirectly measuring the downstream effects of cocaine on alpha-1 adrenergic receptor signaling pathways. If higher relative phosphorylation of glutamate receptors is measured, it is an indicator that cocaine promotes alpha-1 adrenergic receptor hyperactivation and downstream effects on PKC in the PFC. This increase of PKC activity, among other actions, would cause an increase in AMPA receptor phosphorylation.

Previous cocaine sensitization studies have focused on AMPA receptor trafficking. This was typically measured by comparing the relative abundance of cell surface and intracellular AMPA receptors after chronic cocaine exposure, followed by several days of withdrawal and then a cocaine challenge. Tissue was then collected between 30 minutes to a full day after the final challenge (Ferrario et al., 2011). Due to the previous success of these studies, we used a very similar approach to achieve cocaine sensitization. We administered cocaine for 5 consecutive days, had a 2-day withdrawal period, and then collected brain tissue 30 minutes after a cocaine challenge. The 30-minute time point was chosen because phosphorylation evens are typically short-lived, and our goal was to examine receptor phosphorylation as opposed to trafficking.

Methods

Behavioral Testing and Tissue Harvesting

Sixteen C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were used in this study. Mice were placed in locomotor chambers for 30 min/d for 3 days before the start of the experiment to habituate them to the test environment, and locomotor activity was measured by infrared beam breaks. Mice were then divided into three groups: one group received cocaine injections on testing days and challenge day ("C/C", n=6), one group received saline injections on testing days and cocaine on challenge day ("S/C", n=6), and a third group received saline injections on both testing days and challenge day ("S/S", n=4). On testing days, mice were placed in the chambers for 30 min and were then injected with saline or cocaine (15 mg/kg, i.p.).

Locomotor activity was then measured for an additional 2 hours following injection. Mice were then given mock injections in their home cages for 2 "withdrawal" days, during which locomotor activity was not collected. The next day, mice were given a saline or cocaine challenge (15 mg/kg, i.p.), and locomotor activity was measured for 30 min. Animals were then euthanized, brains were removed, and PFC and NAc tissue were collected. Tissue was sonicated for 10 seconds, centrifuged for 10 minutes at 14,000 xG at 4˚C, and then processed for western blots. In between the last habituation day and challenge day, one of the C/C mice became ill and was euthanized, so was not included in the analyses.

Bicinchoninic Acid (BCA) Assay of Tissue Protein Levels

BCA of all tissue samples were performed so that the concentrations of protein were known for each tissue sample. BCA was performed against standards so as to accurately measure mean absorbance of sample tissues. This was than used to determine the protein concentration of our samples. Reagent A and Reagent B were mixed at a ratio of A:B = 1:20 and then added to each well. The wellplate was allowed to incubate for 30 min at 37˚C. Samples were then analyzed immediately after in a plate reader at 562nm.

Western Blots

Western blots of samples were performed. The concentrations determined from the BCA of the tissues were used to calculate the volume of sample necessary to achieve 20.0 μg of protein for each well. In order to measure the relative levels of receptors and phosphorylation, membranes were blotted mouse anti-rabbit

antibodies against total GluR1 (Thermo Scientific, Rockford, IL) and Ser831 (Santa Cruz Biotechnology, Santa Cruz, CA). Concentrations of antibody used during the western blot procedure were determined from pilot experiments. An iBind system (Life Technologies, Grand Island, NY) was used to stain membranes with antibodies. Once the procedure was optimized, the gels were run with the experimental samples under the optimal conditions of 1:1000 primary antibody, and 1:4000 secondary antibody. Bands were then quantified with fluorescence imaging (LiCor, Lincoln, NE). The relative amount of subunit phosphorylation was determined by dividing the density of Ser831 by the density of total GluR1 for each mouse and each brain region.

Statistical Analysis

Data were analyzed by 1-way and 2-way ANOVA followed by Tukey posthoc tests, where appropriate, using Prism 6 software (Graphpad). Statistical power was also calculated for some data in order to determine if non-significant results could be explained by a lack of power. Power was computed on G* Power software. Results were considered significant at $p < 0.05$.

Results

During the first 3 habituation days, locomotor activity in all 3 groups decreased similarly over time, indicating that mice were habituating to the chambers and that there were no differences prior to cocaine exposure (Figure 2). 2-way ANOVA

revealed a main effect of day ($F_{2, 24}$ = 11.91, p<0.01) but not treatment ($F_{2, 12}$ = 0.20, p=0.82).

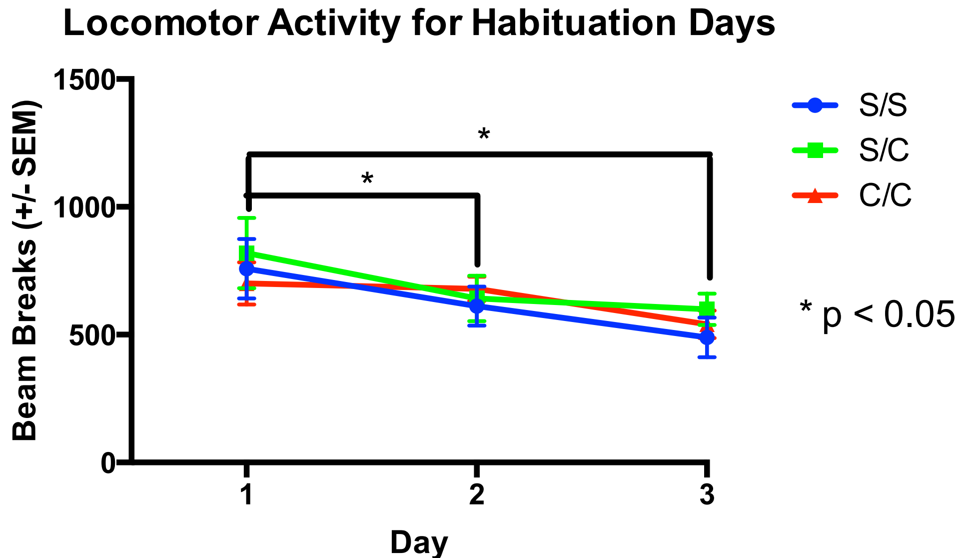

Figure 2. Locomotor activity for habituation days. This graph shows the mean ± SEM locomotor activity during the first 20 min of the 30-min sessions for each treatment group over the initial three habituation days.

Mice then received either saline or cocaine for 5 consecutive days and then on challenge day following a 2-day withdrawal period. Shown in Figure 3 is the total locomotor activity for the 20 min following injection for each treatment group on each injection day and challenge day. Overall, cocaine increased locomotor activity in the C/C and S/C groups compared to the S/S group. There were no statistically significant changes in beam breaks observed over injection days in the C/C group, indicating that behavioral sensitization did not occur. 2-way ANOVA revealed a main effect of treatment ($F_{2, 12}$ = 19.93, p<0.001) but not day ($F_{5, 60}$ = 1.75, p=0.14) or treatment x day interaction ($F_{10,60}$ = 1.00, p=0.46). Follow-up 1-way ANOVAs revealed that only the locomotor activity of the S/C group changed over time ($F_{5,30}$ = 4.36, p<0.01). Post hoc tests showed that there was a significant increase in

locomotor activity on challenge day compared to days 3 (p<0.05), 4 (p<0.05), and 5 (P<0.01) for the S/C group.

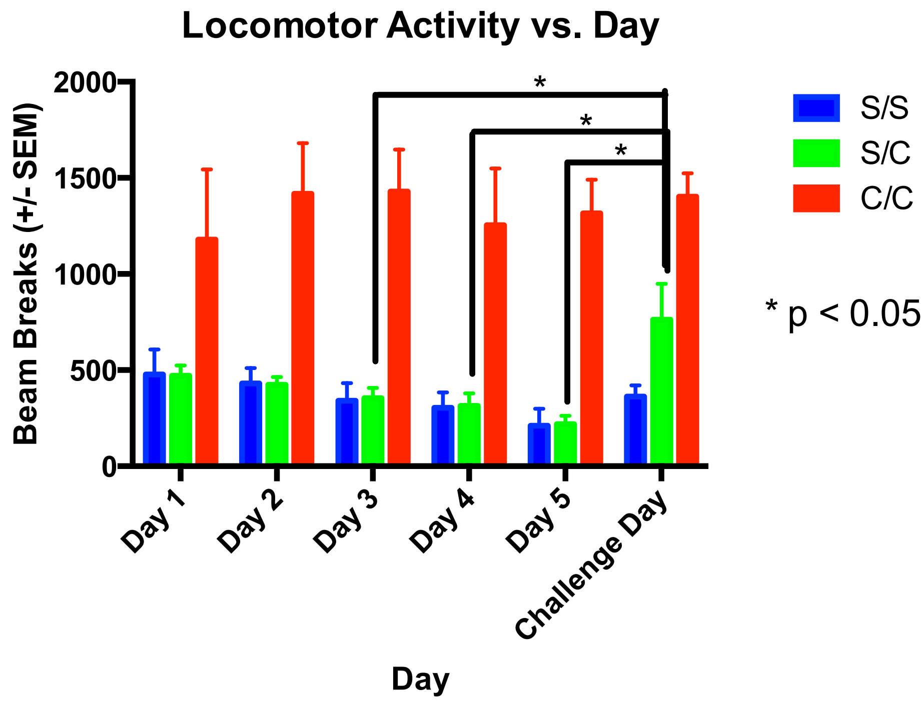

Figure 3. Locomotor Activity vs. Day. This graph shows the total mean ± SEM locomotor activity 20 min after injection of all three treatment groups for all injection days. *p<0.05.

AMPA Receptor Phosphorylation

Tissue was collected 30 minutes after the final injection on challenge day. Samples underwent BCA in order to determine protein concentration and were then subjected to western blotting. Relative expression of GluR1 and Ser831 were measured by average densitometry of bands. Representative blots from each of the brain regions are shown in Figure 4

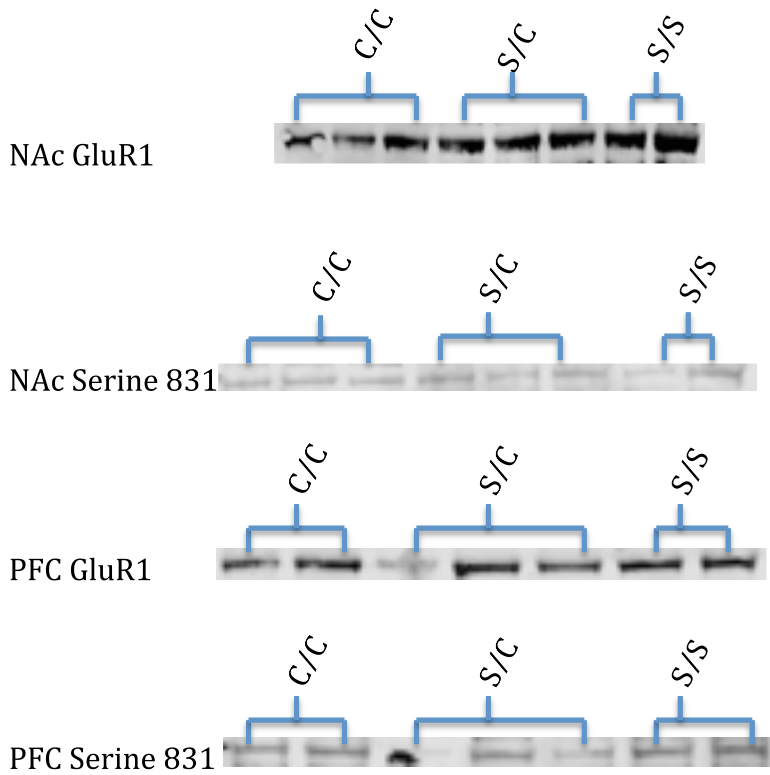

Figure 4. Representative western results. Representative bands for GluR1 and serine 831 for both PFC and NAc brain regions.

As shown in Figure 5, there was no significant difference in total GluR1 levels in the PFC between treatment groups (one-way ANOVA: $F_{2, 12} = 0.24$, $p=0.79$). Similar results were observed for total GluR1 levels in the NAc (one-way ANOVA: $F_{2, 12} = 0.23$, $p=0.79$) (Figure 6).

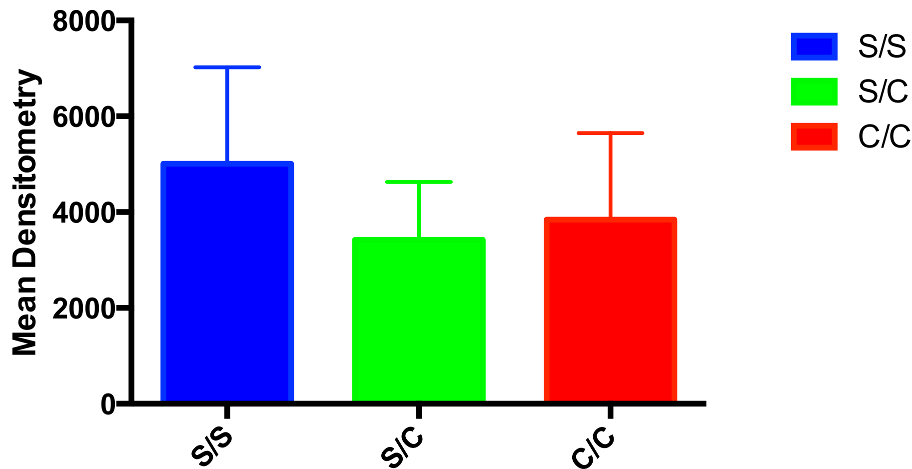

Figure 5. Average GluR1 in PFC. Shown are mean ± SEM densitometry readings for GluR1 levels in the PFC for all three treatment groups.

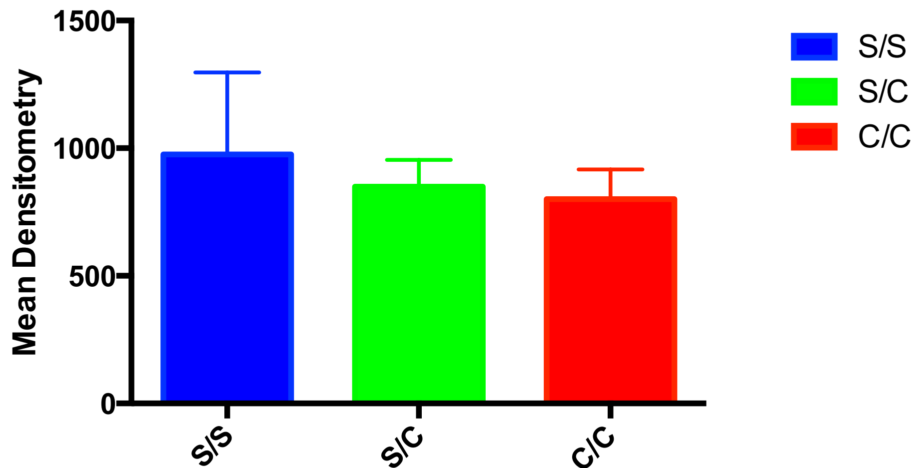

Figure 6. Average GluR1 in NAc. Shown are mean ± SEM densitometry readings for GluR1 levels in the NAc for all three treatment groups.

Percent phosphorylation was calculated by dividing the density of Ser831 bands by the density of GluR1 bands. For both the PFC (Figure 7) and the NAc (Figure 8), there was a trend towards increased GluR1 phosphorylation at Ser831 in both the S/C and the C/C groups compared to the S/S group, but one-way ANOVA revealed

that differences did not reach statistical significance (PFC: $F_{2, 10} = 1.10$, p=0.37; NAc: $F_{2, 9} = 1.74$, p=0.23). Further analyses revealed to achieve > 90% power, the sample sizes would need to be increased to 8-10 for each treatment group.

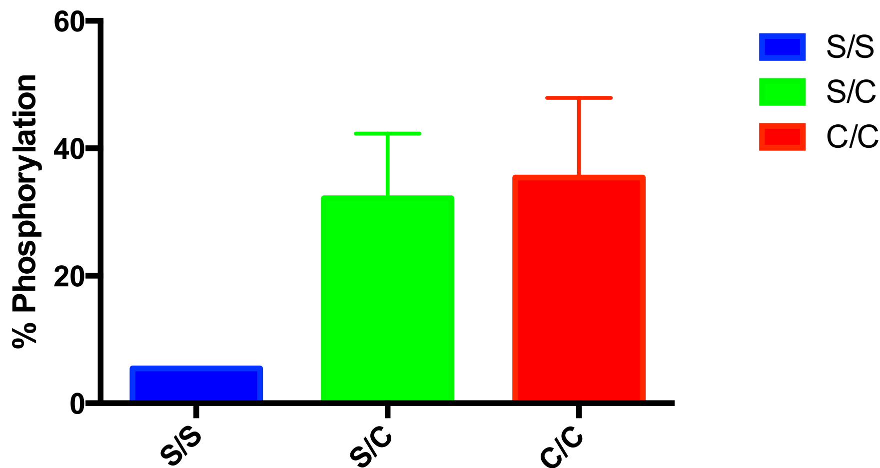

Figure 7. PFC GluR1 phosphorylation at serine 831. Shown are mean ± SEM percent phosphorylation of AMPA receptors at Ser831 in the PFC for each treatment group.

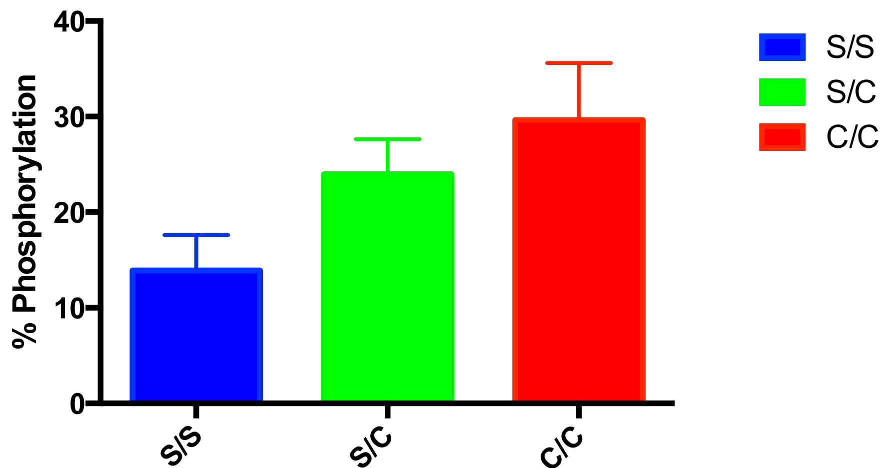

Figure 8. NAc GluR1 phosphorylation at serine 831. Shown are mean ± SEM percent phosphorylation of AMPA receptors at Ser831 in the NAc for each treatment group.

We next ran correlation analyses to determine whether there was a relationship between GluR1 phosphorylation and cocaine-induced locomotor activity. As shown in Figure 9 and Figure 10, there was no correlation between AMPA receptor phosphorylation in the PFC or NAc and locomotor activity.

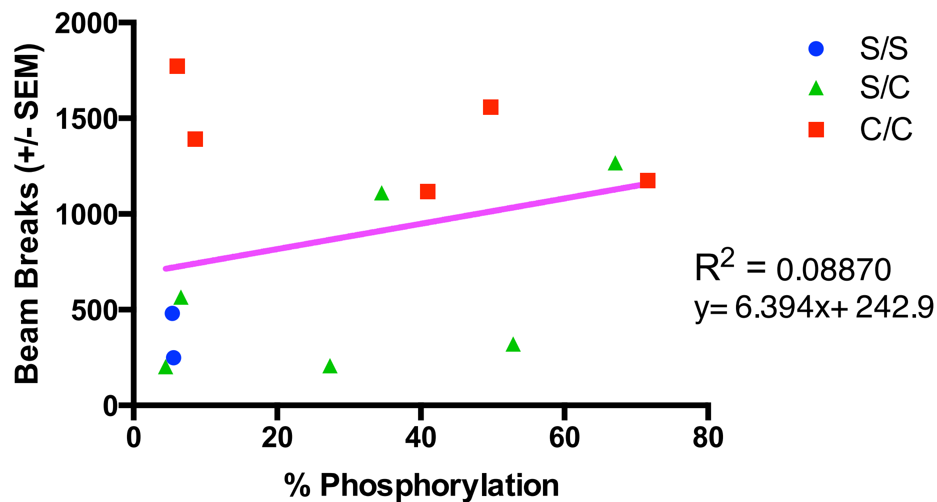

Figure 9. PFC percent serine 831 phosphorylation vs locomotor activity. Shown is the percent of Ser831 phosphorylation (x-axis) and locomotor activity on challenge day (y-axis).

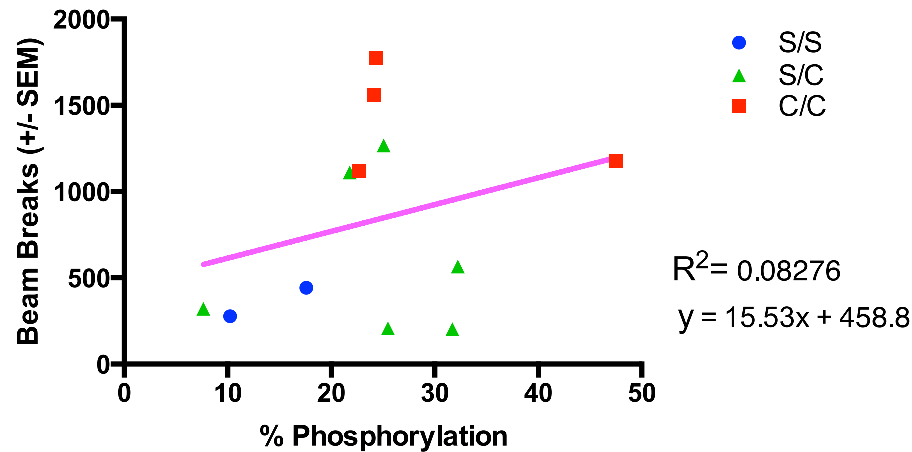

Figure 10. NAc percent serine 831 phosphorylation vs locomotor activity. Shown is the percent of Ser831 phosphorylation (x-axis) and locomotor activity on challenge day (y-axis).

Discussion

The purpose of this study was to determine the effect of cocaine on GluR1 phosphorylation in the PFC and NAc. The main results obtained were that either acute or chronic cocaine exposure tended to increase Ser831 phosphorylation in the PFC and NAc, although none of these results achieved statistical significance. In addition, the Ser831 phosphorylation levels in the S/C and C/C groups were similar, which went against our hypothesis that the increase would be larger in cocaine-sensitized mice.

It is important to note that the mice that received chronic cocaine did not appear to display behavioral sensitization, as their cocaine-induced locomotor activity did not increase over time. Given that we used a fairly standard sensitization paradigm, this was a surprising and disappointing result. One possibility is that the dose we used was too high, and a "ceiling effect" prevented behavioral sensitization. This

could be corrected for by using a lower dose of cocaine. Alternatively, it is possible that the mice did sensitize but engaged in stereotypic behaviors that were not detected by the photobeam breaks, which can only detect horizontal locomotor activity. Anecdotally, a few of the mice in the C/C group were observed to exhibit stereotypic behaviors such as excessive grooming and rearing/jumping following cocaine administration. This could be tested by videotaping the mice and scoring their behavior for stereotypy. Large individual variability as well as a small sample size could also explain the lack of clear locomotor sensitization. Given our data, we can only conclude that both acute and chronic cocaine exposure similarly increase Ser831 phosphorylation. The fact that the increase in Ser831 phosphorylation did not reach statistical significance likely reflects our relatively small sample size and large individual variability within treatment groups. Based on power analysis, this experiment should be repeated with approximately 10 mice per treatment group.

To determine whether activation of alpha-1 receptors mediates the increase in Ser831 phosphorylation, we could repeat this experiment and include a group of mice that are pretreated with an α1 antagonist prior to each saline or cocaine injection. If cocaine were acting on AMPA receptors through our proposed mechanism, this would prevent the increased activation of α1-adrenergic receptors and downstream increase in PKC activity and subsequent AMPA receptor phosphorylation. Another way of testing the validity of our model would be to use a PKC inhibitor pretreatment, which should also prevent the increase in phosphorylation of AMPA receptors if it is in fact regulated primarily by PKC.

If neither of these experimental methods yielded a decrease in cocaine-induced phosphorylation of AMPA receptors, then norepinephrine may not be the primary monoamine effecting AMPA receptor phosphorylation. Dopamine and serotonin levels are also increased due to reuptake inhibition by cocaine. These neurotransmitters act on different types of G-coupled receptors and kinases. For example, some evidence suggests that CAMKII activation following D1 dopamine receptor stimulation may be playing a large role in AMPA receptor phosphorylation at serine 831 in the NAc (Qin, Y. *et al*, 2005). By blocking these different receptors or kinases, it may be possible to determine which neurotransmitter and signaling pathway is critical for cocaine-induced GluR1 phosphorylation. It would also be interesting to have examined GluR2 and Ser880. While we did not have enough tissue to do so in the current study, this could also be the focus of future experiments.

In summary, exposure to cocaine did not cause a significant increase in AMPA receptor phosphorylation at Ser831 in C57BL/6 mice in this experiment. However, there was a trend, so repeating this experiment with more mice is a priority. To increase the chances of achieving behavioral sensitization, I would recommend lowering the cocaine dose to 5 or 10 mg/kg. It would then be interesting to see whether sensitized mice had an even greater increase in Ser831 phosphorylation. Past experiments suggest that norepinephrine and its G_q-coupled receptor pathway are the major players in cocaine-induced AMPA receptor changes via hyper α1-adrenergic receptor activation. Other monoamines like dopamine and serotonin however may play a significant role in this pathway. Future experiments will need

to be performed in order to determine if the proposed pathway is indeed the major cause of this change in phosphorylation.

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