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Sensitizing exposure to amphetamine increases AMPA receptor phosphorylation without increasing cell surface expression in the rat nucleus accumbens

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Abstract

Exposure to psychostimulants like cocaine or amphetamine leads to long-lasting sensitization of their behavioral and neurochemical effects. Here we characterized changes in AMPA receptor distribution and phosphorylation state in the rat nucleus accumbens (NAcc) weeks after amphetamine exposure to assess their potential contribution to sensitization by this drug. Using protein cross-linking, biochemical, subcellular fractionation, and slice electrophysiological approaches in the NAcc, we found that, unlike cocaine, previous exposure to amphetamine did not increase cell surface levels of either GluA1 or GluA2 AMPA receptor subunits, redistribution of these subunits to the synaptic or perisynaptic cellular membrane domains, protein-protein associations required to support the accumulation and retention of AMPA receptors in the PSD, or the peak amplitude of AMPA receptor mediated mEPSCs recorded in NAcc slices. On the other hand, exposure to amphetamine significantly slowed mEPSC decay times and increased levels in the PSD of PKA and CaMKII as well as phosphorylation by these kinases of the GluA1 S845 and S831 residues selectively in this cellular compartment. As the latter effects are known to respectively regulate channel open probability and duration as well as conductance, they provide a novel mechanism that could contribute to the long-lasting AMPA receptor dependent expression of sensitization by amphetamine. Rather than increase the number of surface and synaptic AMPA receptors as with cocaine, this mechanism could increase NAcc medium spiny neuron reactivity to glutamate afferents by increasing the phosphorylation state of critical regulatory sites in the AMPA receptor GluA1 subunit in the PSD.

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Keywords

CaMKII; GluA1; GluA2; PKA; PSD; receptor trafficking

1. Introduction

Repeated exposure to psychostimulant drugs like amphetamine and cocaine enhances their behavioral and neurochemical effects, a form of plasticity termed sensitization. Rats previously exposed to these drugs show enhanced locomotion as well as enhanced dopamine (DA) and glutamate overflow in the nucleus accumbens (NAcc) when subsequently challenged with the drug (Kalivas and Stewart, 1991; Vanderschuren and Kalivas, 2000; Kim et al., 2005). Importantly, sensitization has been linked to addiction vulnerability in humans and animal models (Leyton and Vezina, 2013, 2014). Psychostimulant exposed rats work more and self-administer more drug (Vezina, 2004), an effect consistent with sensitization of the incentive properties of these drugs and the cues associated with them (Robinson and Berridge, 1993, 2008).

Both DA and glutamate act in the NAcc to promote the behavioral expression of psychostimulant sensitization (Vanderschuren and Kalivas, 2000). D1-like DA receptor initiated signaling in NAcc medium spiny neurons (MSNs) activates cAMP-dependent protein kinase (PKA) and Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) (Anderson et al., 2008) both of which phosphorylate a wide array of downstream targets including residues on the GluA1 subunit of the AMPA receptor. This finding is important because it describes one way in which DA and glutamate can interact in the NAcc to drive the expression of sensitization. The serine (S) 845 PKA and S831 CaMKII residues are important regulatory sites in GluA1 that can increase AMPA receptor function and are critical for the expression of LTD, LTP, and the retention of spatial learning (Lee et al., 2003). Phosphorylation at the S845 residue increases channel open probability (Banke et al., 2000) and open duration (Greengard et al., 1991; Han and Whelan, 2009) while phosphorylation at S831 increases channel conductance (Derkach et al., 1999) and together both contribute to the trafficking and synaptic insertion of AMPA receptors (Gao et al., 2006; Derkach et al., 2007). AMPA receptor blockade prevents the expression of locomotor sensitization by amphetamine (Karler et al., 1991; Tzschenke and Schmidt, 1997; Mead and Stephens, 1998; cf, Li et al., 1997) and cocaine (Pierce et al., 1996; Jackson et al., 1998; Bell et al., 2000; cf, Li et al., 1997) and AMPA receptors are functionally upregulated following exposure to either drug (Bell and Kalivas, 1996; Pierce et al., 1996; Suto et al., 2004). Thus, changes in the phosphorylation of the S845 and S831 GluA1 residues are well positioned to influence the expression of sensitization by either amphetamine or cocaine.

It has been reported that sensitizing exposure to cocaine (Boudreau and Wolf, 2005; Boudreau et al., 2007; Kourrich et al., 2007; Ghasemzadeh et al., 2009), but not amphetamine (Nelson et al., 2009), produces a long-lasting increase in cell surface levels of AMPA receptors in the NAcc. The finding with amphetamine is surprising in light of the above evidence supporting a role for cell surface AMPA receptors in the expression of sensitization by either drug. Thus, the present experiments used biochemical, subcellular

fractionation, and slice electrophysiological approaches to further characterize the state of AMPA receptors in the NAcc weeks following sensitizing exposure to amphetamine. Our findings confirm that exposure to amphetamine does not increase cell surface expression of AMPA receptors but show that levels of PKA and CaMKII are increased in the PSD as is their phosphorylation of the S845 and S831 residues of GluA1 in this subcellular domain. These findings provide a novel mechanism that could contribute to the long-lasting AMPA receptor dependent expression of sensitization by amphetamine.

2. Materials and Methods

2.1. Animals

Male Sprague-Dawley rats weighing 250–275 g on arrival were obtained from Harlan Sprague-Dawley (Madison, WI) and housed individually in a reverse cycle room (12-h light/12-h dark) with food and water freely available. All procedures were conducted during the dark cycle and according to approved Institutional Animal Care and Use Committee and Institutional Biosafety Committee protocols.

2.2. Exposure to amphetamine and tissue harvest

Rats were administered five injections of amphetamine (1.5 mg/kg, i.p.) or saline (1.0 ml/kg, i.p.), one injection every other day. In all cases, rats were transported in their housing cages to a distinctive dimly lit experimental room, administered their respective injections, placed back in their housing cages, and returned to the colony room 2 h later. After the last exposure injection, rats were afforded a 2–3 week procedure- and drug-free period after which they were transported to a procedure room, killed by decapitation, their brains removed rapidly, and brain sections and NAcc tissues obtained as described below for cross-linking, subcellular fractionation, immunoprecipitation, immunoblotting, and whole-cell voltage clamp recordings.

2.3. Surface receptor cross-linking

Surface (S) and intracellular (I) levels of the AMPA receptor GluA1 and GluA2 subunits were assessed in the NAcc using the protein cross-linking reagent bis(sulfosuccinimidyl)suberate (BS³; Pierce Biotechnology) as described previously (Boudreau and Wolf, 2005). Coronal sections (1 mm thick) extending 1.0–3.0 mm from bregma (Paxinos and Watson, 2005) were obtained on ice with a brain matrix. Because similar effects were previously reported in both core and shell subregions of the NAcc (Nelson et al., 2009), in this assay, the entire NAcc (containing both subregions) was bilaterally hand dissected on ice as illustrated in Singer et al. (2009) and chopped into 400 µm slices using a McIlwain tissue chopper (Mickle Laboratory Engineering, Surrey, England). For each animal, all slices were combined, immediately added to an Eppendorf tube containing ice-cold artificial CSF spiked with 2 mM BS³, and incubated with gentle agitation for 30 minutes at 4°C. Cross-linking was terminated by quenching the reaction with 100 mM glycine for 10 minutes at 4°C. The slices were then subjected to 2 minutes of centrifugation at 16000 g, the supernatant discarded, and the pellets re-suspended in ice-cold lysis buffer containing protease and phosphatase inhibitors [(in mM unless stated otherwise) 25 HEPES (pH 7.4); 500 NaCl; 2 EDTA; 1 dithiothreitol; 1 phenylmethanesulfonyl fluoride;

20 NaF; 1 Na orthovanadate; 10 Na pyrophosphate; 1 μ M microcystin-LF; 1 μ M okadaic acid; 1 $\times$ protease inhibitor cocktail (EMD Biosciences, San Diego, CA, USA); 0.1% Nonidet P-40 (v/v) (Fluka, Buchs, Switzerland)] and sonicated twice each time for 5 seconds. Samples were then centrifuged for 5 min at 16000 g and the supernatant fractions aliquoted and stored at -80°C for subsequent western blot analysis.

2.4. Subcellular Fractionation

For all remaining assays, the shell subnucleus of the NAcc was targeted as neurons in this site are known to process the psychomotor activating and incentive motivational properties of psychostimulant drugs (Everitt and Robbins, 2005). Again, 1 mm thick coronal sections extending 1.0–3.0 mm from bregma were cut on ice with a brain matrix and NAcc tissues consisting of the medial and ventral shell were obtained bilaterally with a 2 mm diameter skewed crescent donut punch. All tissues were pooled for each subject and stored at -80°C until use. Typically, 4 NAcc shell punches were obtained per subject (~5 mg total wet tissue). Subcellular fractions were subsequently prepared as outlined in Figure 1A. For tissue homogenization and subsequent fractionations, all solutions contained 10 mM Tris (pH=7.4) as well as protease and phosphatase inhibitors as described above. All centrifugations took place at 4°C . Briefly, the tissues were transferred to a glass conical tissue grinder and homogenate prepared in 0.2 mL of an ice-cold 320 mM hyperosmotic sucrose buffer containing (in mM) 5 NaF, 1 EDTA (pH=8.0), 1 PMSF, and 2 Na_3VO_4 . After centrifuging the homogenates at 1000 g for 10 minutes, the supernatant was transferred to a new Eppendorf tube and centrifuged at 10,000 g for 15 min to obtain the cytoplasm fraction and a crude membrane fraction pellet. The latter was resuspended in buffer containing (in mM) 0.5% Triton X-100, 5 NaF, 1 EDTA (pH=8.0), 1 PMSF, 2 Na_3VO_4 and centrifuged at 10,000 g for 30 minutes to produce the non-PSD membrane fraction (supernatant) and the pellet. The latter was resuspended in buffer containing (in mM) 1% NP40, 0.1% SDS, 0.5% SDC, 5 NaF, 1 EDTA, and 2 Na_3VO_4 and centrifuged again at 10,000 g for 30 minutes. The resulting supernatant provided the PSD fraction. Total PSD fraction protein yield ranged from 50–100 μg . The cytoplasm, non-PSD membrane, and PSD membrane fractions were stored at -80°C for subsequent western blot analysis. The purity of the PSD (PSD95-containing) and non-PSD (syntaxin-containing) membrane fractions was verified as illustrated in Figure 1B.

2.5. Immunoprecipitation in PSD fractions

After washing 3 times with binding buffer (in mM; 50 Tris, 1 EDTA, 0.1% Triton), 40 μL of a protein-A agarose bead slurry was transferred to an Eppendorf tube and 3 μg of antibody was added (CaMKII α , Sigma-Aldrich; PSD95, NR2b, Cell Signaling; SAP97, AKAP150, Santa Cruz). The tubes were placed in a rack and rocked at 4°C for 4 hours. The antibody coated beads were then washed 3 times with the binding buffer and 20 μg of PSD fraction protein samples were added to each tube. After rocking overnight at 4°C , the beads were washed again 3 times and then immediately subjected to western-blot analysis. To assess the proportion of total CaMKII α /pCaMKII α (T286) associated with PSD95 as well as selective CaMKII α / β association in the PSD, 20 μg of PSD fraction protein previously found to be unbound to PSD95 was probed for CaMKII α /pCaMKII α (T286) or again subjected to

immunoprecipitation with 3 μ g of CaMKII α antibody and probed with an antibody for CaMKII β as outlined below.

2.6. Immunoblotting

Protein levels were measured by the Bradford method. For the cross-linking experiment, 20 μ g of protein from each rat was loaded in each lane of a 4–15% Tris-HCl polyacrylamide gradient gel (Bio-Rad) for electrophoresis. For analysis of the PSD fractions, 5 μ g of protein was loaded in each lane of a 10% Tris-HCl polyacrylamide gel. Following transfer, membranes were incubated in blocking solution [5% non-fat milk in Tris buffered saline containing Tween 20 (TBS-T buffer)] for 1 hour at room temperature. Membranes were then incubated overnight at 4°C in primary antibody [GluA1, 1:500; GluA2, 1:5000; pGluA1(S831), 1:500; pGluA1(S845), 1:1000; TH, 1:2000; Millipore; β -actin, 1:10000; CaMKII α , 1:5000; Syntaxin, 1:5000; Sigma-Aldrich; pCaMKII α (T286), 1:1000; NR2b, 1:1000; PSD95, 1:2000; Stargazin, 1:1000; Cell Signaling; CaMKII β , 1:1000; Invitrogen; PKA, 1:500; AKAP150, 1:5000; Santa Cruz]. After extensive washing in TBS-T buffer, membranes were then incubated in an HRP-conjugated secondary antibody (anti-rabbit, 1:10000; anti-mouse, 1:10000; Upstate) for 60 minutes followed by further extensive washing. The protein bands were visualized using a chemiluminescence detection system (ECL Advanced). Blots were digitally imaged using the GeneSnap Bio Imaging System and quantified using Genetool software (Syngene).

2.7. Whole-cell voltage clamp recordings

Rat brains were removed into ice cold sucrose-artificial CSF (aCSF) with 1 mM ascorbic acid (in mM: 252 sucrose, 2.5 KCl, 1 MgCl₂, 2.5 CaCl₂, 20 glucose, and 25 NaHCO₃). Sagittal slices (250 μ m) were taken at the level of the NAcc (3–4 slices per animal) and transferred to a perfusion chamber held at 32°C and perfused at 20ml/min with normal aCSF (sucrose replaced with 125 mM NaCl) containing 1 mM ascorbic acid. Slices were equilibrated for at least 30 min prior to recording. Cells were visualized with a fixed-stage upright microscope (Zeiss, Oberkochen, Germany) using infrared-differential interference contrast microscopy. During recording, slices were perfused with normal aCSF (in mM: 125 NaCl, 2.5 KCl, 1MgCl₂, 2.5 CaCl₂, 20 glucose, 1 NaH₂PO₄, and 25 NaHCO₃) saturated with 95% O₂/5% CO₂ at a flow rate of 3–5 ml/min. GABA_A receptors were blocked with bicuculline (20 μ M) in all experiments. Tetrodotoxin (1 μ M) was included in all external solutions to block action potential activity and isolate miniature excitatory postsynaptic currents (mEPSCs). MSNs in the NAcc shell were identified by their small soma diameters (<20 μ m), hyperpolarized resting membrane potential (–75 to –85 mV), and lack of spontaneous action potential activity. Whole-cell voltage clamp recordings were made using borosilicate electrodes (4–7M Ω) containing (in mM) 117.5 potassium gluconate, 17.5 potassium methylsulfate, 8 NaCl, 10 HEPES, 0.2 EGTA, 5 Mg-ATP, and 0.2 GTP with pH=7.4 and osmolarity=280–290 mOsm. Data were acquired using an Axopatch 200B amplifier (2kHz low-pass Bessel filter) and DigiData 1200 digitizer (10kHz sampling rate) with pClamp 8.2 software (Molecular Devices). In all experiments, series resistance (< 25 M Ω) was monitored. Cells with variation in series or input resistance of more than 10% were excluded from the analysis. Amplitude, frequency, and decay characteristics of recorded mEPSCs were analyzed using Minianalysis software. Average mEPSCs were generated by

aligning the rising phase of each event. The amplitude and decay time constant (τ) were determined for each event by the software and averaged for each recorded neuron. The values for each recorded cell were then averaged for the exposure groups (n=13 and 12 cells for the saline and amphetamine exposure groups, respectively). NAcc slices were taken from 4 animals in each treatment group.

2.8. Data analysis

All comparisons between group means were conducted with one- or independent-samples t-tests using the SPSS Statistics 22 module. Because no data were subjected to more than one statistical analysis or non-orthogonal comparisons, no corrections for multiple t-tests were applied.

3. Results

3.1. Previous exposure to amphetamine does not increase cell surface or intracellular expression of GluA1 and GluA2 in the NAcc

Unlike cocaine, cell surface levels of AMPA receptors in the NAcc have been reported not to be increased weeks following exposure to amphetamine (Nelson et al., 2009). We thus started by using the same BS³ cross-linking approach used in this report to reassess this effect of amphetamine exposure in the NAcc. High molecular weight aggregate S and unmodified I levels of the GluA1 and GluA2 AMPA receptor subunits were determined as well as the S/I ratios and total protein levels (S+I). As illustrated in Figure 2 and consistent with Nelson et al. (2009), exposure to amphetamine three weeks earlier produced no significant effects on any of these measures (S, I, S+I, S/I) for either the GluA1 ($t_{13}=0.17-0.51$, ns) or GluA2 ($t_{13}=0.02-0.33$, ns) subunits.

3.2. Previous exposure to amphetamine does not alter the subcellular distribution of GluA1 and GluA2 in the NAcc

Sensitizing exposure to cocaine leads to increases in the NAcc not only of cell surface (Boudreau and Wolf, 2005; Boudreau et al., 2007) but also of synaptic (Kourrich et al., 2007; Ghasemzadeh et al., 2009) levels of AMPA receptors. We thus used subcellular fractionation to determine the effects of exposure to amphetamine on the expression of the GluA1 and GluA2 subunits in PSD (M PSD) and non-PSD (M non-PSD) membrane as well as cytoplasm (C) cellular compartments in the NAcc. Extending the BS³ findings above and those of Nelson et al. (2009), exposure to amphetamine three weeks earlier produced no significant effects (Figure 3) on any of these measures (M PSD, M non-PSD, C) for either GluA1 ($t_{14}=0.20-0.75$, ns) or GluA2 ($t_{6-14}=0.49-1.62$, ns). Total protein levels (M PSD + M non-PSD + C) were also not significantly affected either for GluA1 ($t_{14}=0.023$, ns) or GluA2 ($t_6=0.97$, ns).

3.3. Protein-protein associations supporting the accumulation of AMPA receptors in the PSD are not altered by prior exposure to amphetamine

The trafficking of AMPA receptors to the PSD is associated with a number of protein-protein interactions that stabilize the receptor at the synapse. A number of proteins contribute in this way by forming AMPA receptor scaffolding assemblies anchored to

PSD95. These include the NR2b subunit of the NMDA receptor (Strack and Colbran, 1998; Bayer et al., 2001), CaMKII, SAP97 (Lisman and Zhabotinsky, 2001), and stargazin (Tomita et al., 2005; Opazo et al., 2010). As an increase in the number of synaptic AMPA receptors would require a corresponding increase in the aggregate association of these proteins, we used immunoprecipitation with different antibodies (IP) and probed for associated proteins in the PSD compartment to assess this possibility in rats previously exposed to amphetamine. Consistent with the above results showing no change in PSD expression of AMPA receptors, we found no change three weeks after amphetamine exposure (Figure 4) in the association of NR2b with CaMKII α ($t_6=0.98$, ns), CaMKII α with PSD95 ($t_{10}=1.25$, ns) or SAP97 ($t_6=0.45$, ns), PSD95 with NR2b ($t_{14}=1.40$, ns), or stargazin with PSD95 ($t_{10}=0.64$, ns).

3.4. Effects of prior exposure to amphetamine on AMPA receptor mediated synaptic transmission in the NAcc

To further assess the effects of amphetamine exposure on the state of AMPA receptors in NAcc MSNs, whole cell voltage clamp recordings were conducted in sagittal brain slices in the presence of bicuculline and tetrodotoxin to isolate AMPA receptor mediated mEPSCs (Figure 5). Again consistent with the above results showing no change in PSD expression of AMPA receptors in the NAcc, the average peak amplitude of recorded mEPSCs was similarly unaffected following exposure to amphetamine two weeks earlier ($t_{23}=1.12$, ns; Figure 5C). The time course of these synaptic events was significantly altered, however, as indicated by the slower decay times observed in slices from amphetamine relative to saline exposed rats ($t_{23}=2.69$, $p<0.01$; Figure 5D). No increase in mEPSC frequency was detected (not shown). Together with the above findings, these results, consistent with longer channel open times, prompted a closer examination of PKA and CaMKII in the PSD and particularly their phosphorylation of the GluA1 S845 and S831 residues in this cellular compartment as they are known respectively to regulate channel open duration (Greengard et al., 1991; Han and Whelan, 2009) and conductance (Derkach et al., 1999).

3.5. Previous exposure to amphetamine increases PKA and pGluA1(S845) in the PSD

As illustrated in Figure 6A, immunoblotting of NAcc PSD fractions revealed a significant increase in levels of PKA ($t_6=2.22$, $p<0.05$) in rats previously exposed to amphetamine relative to saline three weeks earlier. This provides a mechanism for the long-lasting significant increase in pGluA1(S845) that was also observed in the PSD ($t_{10}=2.18$, $p<0.05$; Figure 6B), a finding itself consistent with the significantly slower mEPSC decay times observed above in amphetamine exposed rats (Figure 5D). The increase in pGluA1(S845) was specific to the PSD as no group differences were detected in the non-PSD membrane ($t_{10}=0.95$, ns) and cytoplasm fractions ($t_{10}=0.50$, ns). Thus, increased PKA phosphorylation may not have been detected by Nelson et al. (2009) following amphetamine exposure because they assayed tissues as whole cell lysates.

The lack of an increase in pGluA1(S845) in non-PSD membrane fractions in particular is consistent with the above results showing no change in PSD expression of AMPA receptors. The latter is thought to require phosphorylation by PKA of GluA1(S845) to stabilize AMPA receptors in perisynaptic membrane pools (Esteban et al., 2003; Oh et al., 2006) prior to

their insertion and trapping at the synapse by CaMKII (Hayashi et al., 2000; Gao et al., 2006). CaMKII achieves these effects by phosphorylating AMPA receptor associated stargazin (Opazo et al., 2010). However, because the association of stargazin with PSD95 was not altered following amphetamine exposure (Figure 4E), it is possible that enhanced synaptic insertion of AMPA receptors was not achieved even with elevated levels of PSD CaMKII (see below).

As the localization of PKA to postsynaptic spines and the synaptic fractions of these spines in the rat is critically dependent on the A-kinase anchoring protein AKAP150 (Smith et al., 2006; Weisenhaus et al., 2010; Sanderson and Dell'Acqua, 2011), this protein was also assessed in the PSD fractions. No evidence for an effect of amphetamine exposure on levels of AKAP150 was observed ($t_6=0.54$, ns; Figure 6C). However, the association of PKA with AKAP150, determined by immunoprecipitation with AKAP150 and probing for PKA, was significantly increased in rats exposed to amphetamine three weeks earlier ($t_6=2.53$, $p<0.05$; Figure 6D), consistent with a critical role for AKAP150 in the delivery of increased PKA levels to the PSD.

3.6. Previous exposure to amphetamine increases CaMKII α and pGluA1(S831) in the PSD

As with PKA, a significant increase in the levels of CaMKII α ($t_{14}=2.193$, $p<0.05$) and pGluA1(S831) ($t_{10}=2.318$, $p<0.05$) was also observed in the NAcc PSD fractions of rats previously exposed to amphetamine three weeks earlier (Figure 7A–B). This increase in CaMKII α resembles the accumulation of CaMKII α observed in the PSD following LTP (Otmakhov et al., 2004) and provides a mechanism for the long-lasting increase in PSD pGluA1(S831) observed here and reported previously in the NAcc shell long after exposure to amphetamine (Loweth et al., 2010, 2013). In light of these findings, it was at first surprising that mEPSC amplitudes were not also enhanced following exposure to amphetamine (Figure 5B; Malenka and Bear, 2004). pGluA1(S831) has been reported to increase conductance in GluA1 homomers (Oh and Derkach, 2005) as well as GluA1-GluA2 heteromers when the latter are associated with stargazin (Kristensen et al., 2011). Thus, the lack of an increase in mEPSC amplitudes, a finding consistent with the lack of an increase in channel conductance, can be interpreted to suggest that exposure to amphetamine may not have made a sufficient number of either GluA1 homomers or stargazin-associated GluA1-GluA2 heteromeric AMPA receptors available in the PSD.

How psychostimulant exposure might influence the association of AMPA receptors with stargazin remains unknown. Considering that neither the subcellular distribution of GluA1 and GluA2 (Figure 3) nor the overall association of stargazin with PSD95 (Figure 4E) were altered in the present experiments, it is possible that exposure to some sensitizing drug regimens might produce more subtle effects on TARP-AMPA receptor associations capable of influencing receptor function without affecting overall receptor number in the synapse. Notably, only a minor portion (~30%) of AMPA receptors in rat brain have been shown to be associated with stargazin and the closely related TARP γ -3 (Schwenk et al., 2009) and these receptors are capable of rapid dissociation from stargazin to influence receptor mobility (Constals et al., 2015). It will be important to better understand these associations and how

they might be affected by different forms of plasticity (see Kato et al., 2010; Ferrario et al., 2011).

The effects observed here were also specific to the PSD as no group differences in CaMKII α ($t_{10}=0.29-0.39$, ns) and pGluA1(S831) ($t_{10}=0.43-0.91$, ns) were detected in the non-PSD membrane and cytoplasm fractions, providing a likely explanation for the failure of whole cell lysate preparations to detect long-lasting increases in NAcc CaMKII α weeks after exposure to amphetamine (Nelson et al., 2009; Loweth et al., 2010, 2013).

PSD CaMKII α was further characterized to assess potential effects of amphetamine exposure on its autophosphorylation state [pCaMKII α (T286)] as well as its association with CaMKII β , which can act as a targeting module to localize CaMKII α to the synapse (Shen et al., 1998). Using immunoprecipitation with a PSD95 antibody and probing for bound and unbound CaMKII α and pCaMKII α (T286), we found that approximately 35% of CaMKII α in the PSD was associated with PSD95 ($t_{15}=10.53$, $p<0.001$) but that this PSD95 associated CaMKII α was almost twice as likely to be in the autophosphorylated state ($t_{15}=12.85$, $p<0.001$; Figure 7C–D). The remaining 65% was almost half as likely to be in the autophosphorylated state and existed as free enzyme, was associated with other proteins, or formed heterooligomers with CaMKII β . Importantly, previous exposure to amphetamine did not change the proportion of PSD CaMKII α associated with PSD95 ($t_6=0.05$, ns; Figure 7C; consistent with the lack of effect on overall association with PSD95; Figure 4B) or the proportion of PSD CaMKII α in the autophosphorylated state ($t_6=0.37$, ns; Figure 7D), suggesting a minimal role for pCaMKII α (T286) in amphetamine sensitization (see also Loweth et al., 2010). Previous exposure to amphetamine also did not change the association between the α and β isoforms of CaMKII as determined by subjecting PSD95-unbound protein to immunoprecipitation with a CaMKII α antibody and probing for CaMKII β (Figure 7G). Thus, it is unlikely that the increase in PSD CaMKII α observed here was due to CaMKII β enabled translocation. Given this finding, the lack of association of CaMKII β with PSD95 and SAP97 (Figure 7E–F), and the absence of an increase in the association of CaMKII α with PSD95 associated proteins following exposure to amphetamine (Figure 4; Figure 7C), it is possible that the enhanced CaMKII α levels observed here existed at least in part as free enzyme in the PSD.

4. Discussion

In the present experiments, previous exposure to amphetamine did not increase cell surface expression of AMPA receptors in the NAcc. Rather, here we show that sensitizing exposure to amphetamine increases levels of PKA and CaMKII α in NAcc PSD fractions as well as their phosphorylation of the S845 and S831 residues of GluA1 selectively in this subcellular domain. These effects, observed two to three weeks following exposure to the drug, provide a novel mechanism that could contribute to the long-lasting AMPA receptor dependent expression of sensitization by amphetamine.

The ability of sensitizing cocaine exposure to increase cell surface levels of AMPA receptors in the NAcc has been proposed to explain the gradual increase in responsiveness of NAcc MSNs to excitatory cortical inputs and the long-lasting increase in behavioral responsiveness

sensitized animals exhibit (Boudreau and Wolf, 2005; Boudreau et al., 2007; Kourrich et al., 2007). Because the expression of both cocaine and amphetamine sensitization requires cell surface AMPA receptors (see Introduction), the increased phosphorylation of these receptors observed here indicates that a similar outcome can be obtained with amphetamine without increasing AMPA receptor cell surface expression. Indeed, unlike cocaine, previous exposure to amphetamine did not increase cell surface levels of either GluA1 or GluA2 AMPA receptor subunits in the NAcc (Figure 2; Nelson et al., 2009), redistribution of these subunits to the synaptic or perisynaptic membrane domains (Figure 3), or protein-protein associations required to support the accumulation and retention of AMPA receptors in the PSD (Figure 4). On the other hand, the increased synaptic levels of GluA1(S845) and (S831) phosphorylation observed following exposure to amphetamine (Figures 6B and 7B) would be expected under the right circumstances to respectively increase AMPA receptor channel open probability (Banke et al., 2000) and open duration (Figure 5; Greengard et al., 1991; Han and Whelan, 2009) as well as channel conductance (Derkach et al., 1999) in this subcellular domain, leading to enhanced MSN and behavioral activation upon excitation by cortical glutamate afferents. Together, these findings highlight the potential importance of increased NAcc MSN reactivity in the expression of sensitization whether it is achieved by increasing the number (cocaine) or phosphorylation state (amphetamine) of surface AMPA receptors. Why and how amphetamine and cocaine might arrive at this common end via different neuroadaptations remains unknown. Importantly, either adaptation could function as a molecular memory ensuring enhanced AMPA receptor mediated excitation of NAcc MSNs and the maintenance of sensitized responding to and for psychostimulant drugs like amphetamine and cocaine.

Because the AKAP150 scaffolding protein is known to bind to PSD associated scaffolding proteins such as PSD95 and SAP97 (Colledge et al., 2000; Robertson et al., 2009), the increased association of PKA with AKAP150 observed here in the PSD following exposure to amphetamine is consistent with a critical role for AKAP150 in the delivery of increased PKA levels to this subcellular domain (Smith et al., 2006; Weisenhaus et al., 2010; Sanderson and Dell'Acqua, 2011). The resulting proximity of this enzyme to synaptic AMPA receptors could have resulted in increased phosphorylation of the S845 residue of GluA1 (Figure 6), a possibility consistent with the slower decay times observed (Figure 5) and that, with expected increases in channel open probability, could lead to enhanced AMPA receptor excitation by glutamate (Figure 8).

The accumulation of CaMKII observed in the PSD following exposure to amphetamine (Figure 7) likely resulted from simple diffusion of the enzyme from the cytoplasm following activation during exposure as described by Lisman et al. (2012) as there was no evidence of increased association between the α and β isoforms of CaMKII, the latter of which can target CaMKII α to the synapse (Shen et al., 1998). Amphetamine exposure also did not increase the association of CaMKII α with NR2b, PSD95, or SAP97, indicating that the resulting increase in phosphorylation of the S831 residue of GluA1 by CaMKII α in the PSD was achieved independent of these PSD associated proteins likely by increased levels of free CaMKII α enzyme. It remains unknown how increased levels of CaMKII α are maintained in the PSD where it may alternate between free and bound as well as un- and autophosphorylated states (Figure 8).

Unlike PKA, the increased levels of PSD pGluA1(S831) detected following amphetamine exposure were not paralleled by a corresponding change (increased current amplitudes) in the mEPSCs measured in the whole cell voltage clamp recordings (Figure 5; Malenka and Bear, 2004). As outlined above, this finding, consistent with a lack of increase in channel conductance, may reflect the failure of the sensitizing amphetamine exposure regimen to sufficiently increase insertion into the PSD of GluA1 homomers (Oh and Derkach, 2005) or stargazin-associated GluA1-GluA2 heteromers (Kristensen et al., 2011). Both of these AMPA receptor subtypes show increased conductance when phosphorylated by CaMKII α at S831. While requiring extended energy expenditure, maintaining enhanced CaMKII α and pGluA1(S831) levels in the PSD would notably and effectively prime NAcc MSNs to respond readily to the arrival of PKA phosphorylated AMPA receptors in the perisynaptic domain, ensuring their rapid insertion, retention, phosphorylation, and activation in the PSD, ultimately leading to the rapid expression of a sensitized cellular and behavioral response.

5. Conclusions

The ability of sensitizing cocaine exposure to increase cell surface and synaptic levels of AMPA receptors in the NAcc has been proposed to explain the gradual increase in responsiveness of NAcc MSNs to excitatory glutamate inputs and the long-lasting increase in behavioral responsiveness sensitized animals exhibit. Using a number of biochemical and cellular approaches, we show here that unlike cocaine, amphetamine exposure does not increase cell surface and synaptic levels of AMPA receptors but rather increases the phosphorylation state of these receptors selectively in the synapse. This finding provides a new potential mechanism by which amphetamine exposure might similarly lead to a long-lasting increase in responsiveness of NAcc MSNs to glutamate and enhanced behavioral reactivity in sensitized rats. Future studies will need to address a number of interesting questions that require clarification. For example, there is a need to better understand whether and how the association of AMPA receptors with stargazin and other TARPs, regulatory proteins well positioned to influence AMPA receptor function, can be altered by different forms of plasticity (Kato et al., 2010) including psychostimulant sensitization. In addition, it will be important to determine the potentially different effects on signaling of different drug exposure regimens (e.g., non-contingent versus self-administered; extended versus limited versus intermittent access self-administration; see Ferrario et al., 2011; Allain et al., 2015). Finally, because the expression of sensitization requires a drug challenge, it will be necessary to assess the effect of this challenge on the different neuroadaptations initially produced by exposure to the drug.

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Highlights

- Amphetamine exposure does not increase cell surface levels of AMPA receptors
- Amphetamine exposure increases phosphorylation of these receptors in the synapse
- The latter may contribute to the enduring expression of amphetamine sensitization

Figure 1. Procedures used to prepare subcellular fractions from NAcc shell tissues. **A.** Schema outlining the different steps used to prepare the cytoplasm, non-PSD membrane, and PSD membrane fractions. See text for more details. **B.** Immunoblots illustrating the relative purity of the PSD (PSD95-containing) and non-PSD (syntaxin-containing) membrane fractions obtained.

Figure 2.

Surface and intracellular expression of GluA1 and GluA2 in the NAcc is not altered by amphetamine exposure. Three weeks after exposure to amphetamine (AMPH) or saline (SAL), a BS³ crosslinking assay was used to assess cell surface and intracellular expression of the AMPA receptor GluA1 (**A**) and GluA2 (**B**) subunits. Data are shown as group mean (+SEM) percent of saline controls. S, surface protein; I, intracellular protein; S+I, total protein; S/I, surface to intracellular protein ratio. **C.** Verification of BS³ crosslinking. As BS³ does not cross the cell membrane, SDS-PAGE can distinguish between the cross-linked S higher molecular weight protein aggregate and the I protein appearing at the expected molecular weight band. As expected, detection of proteins only expressed in the intracellular compartments (right) is not affected by BS³. n=7–8/group.

Figure 3.

Subcellular distribution of GluA1 and GluA2 in the NAcc is not altered by amphetamine exposure. Three weeks after exposure to amphetamine (AMPH) or saline (SAL), subcellular fractionation was used to assess levels of GluA1 (A) and GluA2 (B) in the PSD and non-PSD membrane as well as cytoplasm cellular compartments. Data are shown as group mean (+SEM) percent of saline controls. M, membrane; C, cytoplasm; T, total protein. n=4-8/group.

Figure 4.

Protein-protein associations supporting AMPA receptor accumulation in the PSD are not altered by amphetamine exposure in the NAcc. Three weeks after exposure to amphetamine (AMPH) or saline (SAL), immunoprecipitation was used in PSD fractions to assess the association of proteins known to promote AMPA receptor trafficking and stabilization in the PSD. **A–E**, different protein association pairs. IP, indicates the protein targeted by the immunoprecipitation bead coating antibody. Data are shown as group mean (+SEM) percent of saline controls. n=4–8/group.

Figure 5.

Exposure to amphetamine prolongs AMPA receptor miniature excitatory synaptic currents in the NAcc. Two weeks after exposure to amphetamine (AMPH) or saline (SAL), whole cell voltage clamp recordings were conducted in sagittal NAcc slices to assess effects on mEPSCs. **A.** Raw traces from representative recordings from AMPH and SAL exposed rats. **B.** Averaged mEPSC traces from AMPH and SAL exposed rats. Each trace is the average of 1100–1500 mEPSCs recorded from 12–13 cells/group obtained from 4 rats in each exposure condition. **C.** No effect of exposure was detected on average peak amplitudes of recorded mEPSCs. **D.** Exposure to AMPH significantly prolongs mEPSC decay time constants relative to SAL exposed rats. **, $p < 0.01$.

Figure 6.

Exposure to amphetamine increases PKA and its phosphorylation of GluA1(S845) in the PSD. Three weeks after exposure to amphetamine (AMPH) or saline (SAL), immunoblotting of NAcc MSN PSD fractions was performed to assess effects on PKA and its phosphorylation of GluA1. Exposure to AMPH significantly increases levels of PKA (A), pGluA1(S845) (B), and the association of PKA with AKAP150 (D) in the PSD. The latter was determined by IP with AKAP150 and probing for PKA. Levels of the anchoring protein AKAP150 were not significantly altered (C). $n=4-6/\text{group}$. *, $p<0.05$.

Figure 7.

Exposure to amphetamine increases CaMKIIα and its phosphorylation of GluA1(S831) in the PSD. Three weeks after exposure to amphetamine (AMPH) or saline (SAL), immunoblotting of NAcc MSN PSD fractions was performed to assess effects on CaMKIIα and its phosphorylation of GluA1. Exposure to AMPH significantly increases levels of CaMKIIα (**A**) and pGluA1(S831) (**B**) in the PSD. The proportions of CaMKIIα (**C**) or CaMKIIα in the autophosphorylated state (**D**) associated with PSD95 were not altered. Association was determined by IP with a PSD95 antibody and probing for bound (B) and unbound (U) CaMKIIα and pCaMKIIα(T286). Association of CaMKIIα with CaMKIIβ,

which can act as a PSD targeting module, was equally unaltered (**G**). Association here was determined by subjecting PSD95-unbound protein to IP with a CaMKII α antibody and probing for CaMKII β . CaMKII β is not associated with the PSD scaffolding proteins PSD95 (**E**) and SAP97 (**F**) suggesting different pools of CaMKII α in this subcellular compartment. n=6–8/group. *, $p<0.05$.

Figure 8.

Schematic diagram summarizing the protein-protein interactions observed in the PSD following exposure to amphetamine. Increased association with AKAP150 enhances delivery of PKA to the PSD, placing it in close proximity to local GluA1 subunits thereby permitting increased pGluA1(S845). The association of CaMKIIα with CaMKIIβ or with the PSD95, SAP97 and NR2b scaffolding proteins was not altered, suggesting that CaMKIIα accumulated in the PSD by simple diffusion where it could alternate between free and associated as well as un- and autophosphorylated states and increase pGluA1(S831). Red arrows indicate phosphorylation sites for PKA and CaMKIIα.