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Stimuli associated with the presence or absence of amphetamine regulate cytoskeletal signaling and behavior

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Abbreviations:

AMPAR = α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor
Arp2/3 = actin-related protein 2/3
CS = conditioned stimulus
DA = dopamine
IP = intraperitoneal
LIMK = LIM-domain-containing protein kinase
MSN = medium spiny neuron
NAcc = nucleus accumbens
PKC = protein kinase C
PSD = post-synaptic density

Abstract

Drug-paired stimuli rapidly enlarge dendritic spines in the nucleus accumbens (NAcc). While increases in spine size and shape are supported by rearrangement of the actin cytoskeleton and facilitate the synaptic expression of AMPA-type glutamate receptors, it remains unclear whether drug-related stimuli can influence signaling pathways known to regulate these changes in spine morphology. These pathways were studied in rats trained on a discrimination learning paradigm using subcellular fractionation and protein immunoblotting to isolate proteins within dendritic spine compartments in the NAcc shell. An open field chamber was repeatedly associated with amphetamine in one group (Paired) and explicitly unpaired with amphetamine in another (Unpaired). Rats in a third group were exposed to the open field but never administered amphetamine (Control). When administered saline and returned to the open field one week later, Paired rats as expected displayed a conditioned locomotor response relative to rats in the other two groups. NAcc shell tissues were harvested immediately after this 30-minute test. Re-exposing Paired rats to the drug-paired excitatory context significantly decreased p-GluA2(S880), an effect consistent with reduced internalization of this subunit and increased spine proliferation in these rats. In contrast, re-exposing Unpaired rats to the drug-unpaired context, capable of inhibiting conditioned responding in these animals, significantly decreased levels of both actin binding protein Arp2/3 and p-cofilin, consistent with spine volatility, shrinkage, and inhibition of spine proliferation in these rats. These findings show that contextual stimuli previously associated with either the presence or absence of amphetamine differentially regulate cytoskeletal signaling pathways in the NAcc.

Highlights

- Amphetamine-paired, but not unpaired, contextual cues elicit conditioned locomotion.
- Drug-paired cues reduce phosphorylation of GluA2-containing NAcc shell AMPARs.
- Drug-unpaired cues decrease synaptic Arp2/3 and p-cofilin levels in the NAcc shell.
- Drug associated contextual cues can bidirectionally regulate cytoskeletal signaling.

Introduction

Rapid increases in dendritic spine size and shape are elicited by drug-paired stimuli in nucleus accumbens (NAcc) medium spiny neurons (MSNs), suggesting a role for these morphological effects in the expression of conditioned excitatory behavioral responding (Gipson et al., 2013; Singer et al., 2016). Recent studies have reported that rearrangement of the actin cytoskeleton regulates spine dynamics following psychostimulant administration (Shen et al., 2009), but it remains largely unknown whether conditioned stimuli can initiate actin cycling in the absence of drug. Conversely, stimuli explicitly unpaired with drug can inhibit the expression of conditioned responding (Vezina and Leyton, 2009), but again, it remains unknown what effects if any these stimuli may have on cytoskeletal signaling.

With the help of various binding proteins, actin cycles between monomeric (globular, "G") and polymerized (filamentous, "F") states. In the spine, a complex involving actin-related protein 2/3 (Arp2/3), cofilin, and cortactin is necessary to promote F-actin branching (Hotulainen and Hoogenraad, 2010; Ichetovkin et al., 2002). Inhibiting Arp2/3 reduces actin polymerization and results in smaller spines (Nakamura et al., 2011). Cofilin also binds and removes G-actin to disassemble F-actin, again resulting in smaller spines. Phosphorylation of cofilin by LIM-domain-containing protein kinase (LIMK) prevents this effect thereby stabilizing F-actin to maintain spine length (Van Troys et al., 2008).

The lengthening and branching of F-actin in the post-synaptic density (PSD) provides structural support for changes in synaptic plasticity (Hotulainen and Hoogenraad, 2010; Matsuo et al., 2008; Shen et al., 2009), including psychostimulant-induced increases in the surface expression and function of glutamatergic α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPARs) in the NAcc (Boudreau et al., 2007; Loweth et al., 2010). Interestingly, the AMPAR GluA2 subunit also contributes to spine formation (Passafaro et al., 2003) via interactions between its extracellular N-terminal domain and presynaptic processes (Saglietti et al., 2007). Phosphorylation by protein kinase C (PKC) of membrane-bound GluA2 at S880 results in the rapid internalization of this subunit (Chung et al., 2000), an event that, in addition to inducing long-term depression (Chung et al., 2003), would significantly curtail the contribution of GluA2 to spine proliferation (Passafaro et al., 2003).

Here we show that contextual stimuli previously associated with either the presence or absence of amphetamine differentially regulate these signaling pathways in the NAcc, implicating them in the conditioned regulation of spine dynamics and the elicitation of conditioned locomotor responding by amphetamine-associated cues. Protein expression was examined specifically in the NAcc shell because this site is known to process contextual, as

opposed to discrete, stimulus information (Bossert et al., 2013, 2007; Everitt and Robbins, 2005; Singer et al., 2014a, 2014b). Contextual cues also elicit rapid changes in dendritic spine morphology in the NAcc shell (Singer et al., 2016).

Experimental Procedures

Subjects

Male Sprague-Dawley rats (Harlan Sprague-Dawley; Madison, WI) weighing 250-275g on arrival were individually housed with food and water available *ad libitum* in a reverse cycle room (12- h light/12-h dark, lights on at 8pm). Rats were given 4-5 days to acclimate to housing conditions. All procedures were performed during the dark phase of the lighting cycle and conducted according to an approved Institution of Animal Care and Use Committee (IACUC) protocol.

Behavioral Conditioning

Behavioral training and testing were conducted as previously described (Singer et al., 2016, 2014a, 2014b). Briefly, rats were conditioned using a discrimination learning paradigm over the course of five consecutive 3-day blocks. On the first day of each block, rats were transported to an experimental room, administered either amphetamine (Paired rats; 1 mg/kg, IP) or saline (Unpaired and Control rats; 1 ml/kg, IP), and placed in Med Associates open fields for 2-hours. On the second day, rats were transported to another room in which Unpaired rats were administered amphetamine (1 mg/kg, IP) and Paired and Control rats administered saline. Following these injections, rats were immediately returned to their home cages and transported back to the colony room. Thus, the open field contextual stimuli were associated with the presence of amphetamine and the development of excitatory conditioning in Paired rats. Conversely, these contextual stimuli were associated with the absence of amphetamine and the development of conditioned inhibition in Unpaired rats. This discrimination learning paradigm is known to establish stimuli explicitly unpaired with the unconditioned stimulus as conditioned inhibitors. When used in a summation procedure, these stimuli reduce responding to a drug unconditioned stimulus (Vezina and Leyton, 2009). Importantly, procedures known to extinguish conditioned inhibition in this paradigm selectively disinhibit the expression of locomotor sensitization by amphetamine to reveal sensitized responding in Unpaired rats (Stewart and Vezina, 1991). No procedures took place on the third day. After the fifth 3-day block, rats were left undisturbed for 1-week. On the subsequent test for conditioning, all rats were administered saline and their locomotor responding in the open fields was recorded for 30-minutes. Rats used

for subcellular fractionation and immunoblotting were then immediately sacrificed without anesthesia, their brains rapidly harvested, and bilateral NAcc shell dissections obtained and immediately stored at -80^{o}C.

Subcellular Fractionation and Immunoblotting

For tissue homogenization and subsequent fractionation, all solutions contained 10mM Tris (pH=7.4) as well as protease and phosphatase inhibitors (Sigma). All centrifugations took place at 4^{o}C. Tissue was first homogenized using a 320mM hyper-osmotic sucrose buffer containing 5mM NaF and 1mM EDTA (pH=8.0). After 10 minutes centrifugation (1,000 x *g*), the supernatant was collected (S1) and the pellet (P1) was stored at -80^{o}C for future use. The S1 fraction was then spun at 9,200 x *g* for 15 minutes, after which the supernatant (S2) was collected and frozen. The pellet (P2) was subsequently processed using a 35.6mM sucrose buffer (hypo-osmotic) containing 5mM NaF, 1mM EDTA (pH=8.0), 1mM Na_3VO_4 (with SDC). After rocking on ice for 30 minutes, the processed tissue underwent centrifugation at 25,000 x *g* for 20 minutes. The resulting supernatant (LS1) was frozen and the pellet was re-suspended in a buffer containing 0.5% Triton and 100mM NaCl and rocked for 15 minutes on ice. Samples then underwent centrifugation for 20 minutes at 10,000 x *g*. The resulting supernatant containing synaptosomal proteins within the extrasynaptic space [cofilin, p-cofilin, GluA2, pGluA2(S880), synaptophysin] was frozen. The pellet was re-suspended in a solution containing 0.15 mM NaCl, 1% SDS, 1% Triton, 1% SDC, 1mM DTT, 5mM NaF, and 1mM Na_3VO_4 (with SDC). Samples were packed on ice and gently rotated for 1 hour before undergoing centrifugation for 20 minutes at 10,000 x *g*. The final resulting supernatant contained the actin-rich PSD fraction (actin, Arp2, GluA2, pGluA2(S880), PSD-95). See inset, Figure 2.

Protein levels were measured by the Bradford method and 5µg of protein in homogenate was loaded in each lane of an SDS-PAGE gel. After transfer, membranes were incubated in blocking solution (5% milk in tris buffered saline containing 0.1% Tween; TBS-T) and then exposed to primary [Arp2 (1:500), Synaptophysin (1:5K), Cell Signaling; Cofilin (1:500), BD Bioscience; p-cofilin(S3) (1:500), PSD-95 (1:2K), GluA2 (1:1K), Millipore/Upstate; p-GluA2(S880) (1:2K), PhosphoSolutions] followed by a secondary HRP-conjugated antibody (Jackson ImmunoResearch). Membranes were then washed and developed to visualize bands using an ECL detection system (ECL Advanced, GE Healthcare). p-GluA2(S880) was expressed as p-GluA2(S880)/GluA2 as no group differences in total GluA2 were observed ($F_{2,15}$=1.11, ns).

Results

Conditioned Locomotion

Repeated exposure to amphetamine in a specific environment allows for the formation of associative memories linking drug effects to the contextual environmental cues (Muñoz-Cuevas *et al* 2013; Singer *et al.* 2009, 2014a,b). Upon re-exposure to the drug-paired context, rats display excitatory conditioned locomotor activity; they approach and engage with environmental cues previously paired with the drug (Vezina and Leyton, 2009). As expected, in the present study, Paired rats previously administered amphetamine in the open fields displayed greater locomotor activity compared to Control rats that were never administered the drug or Unpaired rats that were administered the same number of amphetamine injections but unpaired with the open field (Figure 1; 2-way ANOVA, Group effect, $F_{2,26}$=7.74, p<0.01). This effect was observed throughout testing as activity decreased with time in all rats (Time effect, $F_{2,26}$=63.12, p<0.001; Group X Time interaction, $F_{4,26}$=0.88, ns). Unpaired and Control rats did not differ significantly.

Conditioned Regulation of Cytoskeletal Signaling

Recently, conditioned excitatory behavioral responding was shown to be associated with rapid increases in dendritic spine size and shape in NAcc MSNs (Gipson et al., 2013; Singer et al., 2016). Using subcellular fractionation in Paired, Unpaired, and Control rats, we investigated changes in cytoskeletal signaling pathways regulating spine dynamics that may contribute to the observed changes in spine morphology.

Assessment of PKC phosphorylation of GluA2 in the PSD fraction with one-way ANOVA and post-hoc LSD tests revealed a significant decrease in p-GluA2(S880) in Paired rats (Figure 2A; $F_{2,15}$=3.85, p<0.05; p<0.05, relative to both Unpaired and Control rats). No effect was found in the extrasynaptic fraction (data not shown). Given that the AMPAR GluA2 subunit contributes to spine formation via direct extracellular protein-protein interactions and that phosphorylation at S880 leads to its rapid internalization, the present finding is consistent with less internalization of this subunit and thus enhanced spine proliferation in Paired rats produced by exposure to the drug-paired context.

Arp2 was enriched in the PSD fraction and not quantifiable in the extrasynaptic space. In the PSD, Arp2 expression was decreased in Unpaired rats (Figure 2B; $F_{2,20}$=2.47, p=0.11; p<0.05, relative to paired and control rats together). In contrast, no significant group differences in F-actin expression were observed ($F_{2,16}$=0.617, ns). It is thus feasible that, in Unpaired rats, this reduction in Arp2/3 may initiate a re-arrangement of F-actin that promotes spine shrinkage

thereby opposing spine proliferation without affecting total F-actin levels (Nakamura et al., 2011).

For cofilin and p-cofilin, both enriched in the extrasynaptic fraction, one-way ANOVA found no significant group differences in total cofilin expression ($F_{2,20}$=0.969, ns) but a significant effect of conditioning on p-cofilin levels (Figure 2B; $F_{2,18}$=5.63, p<0.05). Unpaired rats showed significantly lower p-cofilin levels than both Paired and Control rats (p<0.05-0.01). As a reduction in p-cofilin would enable cofilin to disassemble F-actin more freely, this finding, together with the reduction in Arp2 levels above, suggests that stimuli explicitly unpaired with amphetamine can promote dendritic spine shrinkage to oppose spine proliferation in these rats. These proteins may thus participate selectively in Unpaired rats to inhibit processes underlying conditioned responding.

Discussion

As drug-paired stimuli have been shown to elicit rapid increases in dendritic spine size and shape in NAcc MSNs (Gipson et al., 2013; Singer et al., 2016), we assessed how conditioned stimuli might affect signaling in pathways known to regulate the actin cytoskeleton. Using subcellular fractionation and protein immunoblotting to isolate proteins within dendritic spine compartments in the NAcc shell, we found that contextual stimuli previously paired with amphetamine and that elicit a conditioned excitatory response, decrease p-GluA2(S880). We also found that contextual stimuli previously explicitly unpaired with amphetamine decrease levels of Arp2 and p-cofilin, presumably by acting as a conditioned inhibitor.

Manipulations known to perturb the cytoskeletal regulation of dendritic spine dynamics, such as inhibiting the proline-directed serine/threonine kinase cyclin-dependent kinase 5 and its phosphorylation target the guanine-nucleotide exchange factor kalirin-7 (Penzes and Jones, 2008; Xin et al., 2008), prevent the proliferation of spines in the NAcc (Kiraly et al., 2010; Norrholm et al., 2003; Wang et al., 2013) as well as the accrual of conditioning with psychoactive drugs (Kiraly et al., 2010; Singer et al., 2014b). The present results identify three proteins known to participate in the control of spine dynamics that are altered by drug-associated contextual stimuli and thus are likely to contribute to the expression of conditioned responses.

The observed decrease in p-GluA2(S880) is consistent with enhanced spine proliferation in Paired rats by the drug-paired context. By reducing internalization of the AMPAR GluA2 subunit (Chung et al., 2000), decreased p-GluA2(S880) would increase its contribution to spine formation (Passafaro et al., 2003). Because spine number and volume are proportional to the

area of the postsynaptic density and to the synaptic content of AMPA receptors (Holtmaat and Svoboda, 2009), this effect would enable an increase in the number of excitatory synapses on MSNs in the NAcc shell to support increased behavioral output (Loweth et al., 2010). Surprisingly, while GluA2 (and GluA1) AMPAR subunits in Paired rats showed small corresponding increases in the PSD (3.4-13.5%) relative to Unpaired or Control rats, these did not achieve statistical significance. Notably, these small increases are in the range reported for decreased GluA2 surface expression following PKC activation and robustly increased pGluA2(S880) (14.9%; Chung et al., 2000), suggesting that this measure may not provide a sufficiently sensitive index of AMPAR subunit surface expression for the conditioning experiments described here. Rather, future experiments will need to focus on relevant consequences of GluA2 trafficking and determine, for example, whether decreased pGluA2(S880) leads to increased interactions between the GluA2 N-terminal domain and extracellular proteins like N-cadherin (Saglietti et al., 2007) that are known to promote spine formation. The time course of such effects will also need to be determined as greater (and more easily detectable) effects may be observed in the period soon after exposure to drug-associated cues when locomotor conditioning is also more pronounced (Figure 1).

Conversely, inhibiting Arp2/3 reduces actin polymerization (Nakamura et al., 2011) while decreased p-cofilin enables F-actin disassembly (Van Troys et al., 2008), both effects promoting smaller spines. The decrease in Arp2 and p-cofilin observed selectively in Unpaired rats thus demonstrates how a context explicitly unpaired with amphetamine can promote spine shrinkage, an effect that by preventing an increase in the number of available excitatory synapses on MSNs in the NAcc shell could prevent conditioned responding in these animals. This finding is consistent with the expression of conditioned inhibition in Unpaired rats, ultimately preventing drug-seeking behavior in a context that predicts the absence of amphetamine (Cortright et al., 2012; Vezina and Leyton, 2009). While Paired rats may be expected to show the opposite effects (increased Arp2/3 and p-cofilin levels), such an increase in these rats does not necessarily follow. Indeed, the present results suggest that separate biochemical pathways mediate the expression of excitatory conditioning and conditioned inhibition.

Together, these results offer insight into some of the molecular signaling events that are capable of supporting conditioned changes in spine morphology and behavior and provide potential neuropsychological targets for the treatment of addiction and the prevention of context-evoked relapse to drug seeking and taking. They clearly show that contextual stimuli associated with the presence or absence of amphetamine can affect cytoskeletal signaling pathways to

regulate spine morphology and behavior (Figure 3). They are consistent with behavioral models indicating that conditioned locomotion can be elicited by a drug-paired context and inhibited by a context explicitly unpaired with the drug (Vezina and Leyton, 2009). In the latter case, stimuli explicitly unpaired with amphetamine can suppress the actin polymerization that supports the spine growth observed during the expression of conditioned responding (Singer et al., 2016).

In light of the rapid and transient increase in dendritic spine size and shape elicited by drug associated stimuli in NAcc MSNs (Gipson et al., 2013; Singer et al., 2016), the observed lack of a change in F-actin likely indicates its rapid re-arrangement by Arp2 and cofilin during the conditioned response rather than the need for additional F-actin formation in the PSD. However, because synaptic excitability is not always temporally coupled with cofilin signaling or remodeling of the actin cytoskeleton in the PSD (Bosch et al., 2014; Gu et al., 2010), more detailed temporal characterization is warranted as it remains possible that F-actin is differentially regulated at various times following exposure to conditioned stimuli. Indeed, it is likely that additional signaling pathways are evoked by drug associated conditioned stimuli. As the NAcc is positioned at a sensory-motor interface in the basal ganglia (Mogenson et al., 1980), future experiments will need to delineate the circuitry and the nature of the conditioned excitatory and inhibitory sensory information it delivers to induce the changes in cytoskeletal signaling observed in the present experiments.

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Figure Legends

Figure 1. Conditioned Locomotion

On the test for conditioning, conducted one week after the last conditioning day, all rats were injected with saline and placed in the open fields for 30-minutes. Paired rats previously administered amphetamine in the open fields showed significantly greater locomotor activity than rats in the other two groups. Bar graph shows group mean (+SEM) 30-minute total locomotor counts. **Inset** shows the time course of the effect as means (±SEM). **, $p<0.01$, Paired compared to Unpaired and Control. n=4-6/group.

Figure 2. Conditioned regulation of cytoskeletal signaling

One week after the last conditioning day, rats were re-exposed to the open field and sacrificed 30-minutes later to assess changes in cytoskeletal and AMPAR GluA2 signaling in the NAcc shell. **A.** Relative to Unpaired and Control rats, Paired rats exposed to the drug-paired context showed a significant reduction in the phosphorylation of GluA2(S880) by PKC in the PSD. This is consistent with less internalization of the subunit and enhanced spine proliferation in these rats. **B.** Relative to Paired and Control rats, Unpaired rats exposed to the drug-unpaired context showed a significant decrease in Arp2 and p-Cofilin levels promoting spine shrinkage and opposing spine proliferation in these rats. Data are shown as mean (+SEM). OD, optical density. Representative western blots are shown below the bar graphs. **Inset** shows western blots characterizing the purity of PSD (PSD-95-containing) and extrasynaptic (synaptophysin-containing) subcellular fractions. *, $p<0.05$, relative to two other groups. n=5-7/group.

Figure 3. Regulation of cytoskeletal signaling and spine dynamics by drug associated stimuli
Re-exposure to a drug-paired contextual stimulus (in Paired rats) decreases p-GluA2(S880), decreases internalization of this subunit, and promotes spine proliferation. Re-exposure to a drug-unpaired contextual stimulus (in Unpaired rats) decreases Arp2/3 and p-cofilin, thereby promoting smaller spines that oppose spine proliferation. These effects are consistent with the ability of a drug-paired contextual stimulus to rapidly increase spine size and enable conditioned excitatory behavioral output (Singer et al., 2016), as well as the ability of a drug-unpaired contextual stimulus to prevent these effects via conditioned inhibition (Vezina and Leyton, 2009).

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Conflicts of Interest

There are no perceived conflicts of interest to report.

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