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Acute drug-induced spine changes in the nucleus accumbens are dependent on β -adducin

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Abstract

Chronic modifications of dopamine transmission alter striatal dendritic spines. Here, we show that spine density and length are increased in the nucleus accumbens 24 h after a single injection of caffeine or quinpirole, a dopamine D2/D3 dopamine receptors agonist, whereas the dopamine antagonist haloperidol has opposite effects. These effects are absent in mice lacking β -adducin, a protein that stabilizes actin/spectrin cortical cytoskeleton and modulates synaptic plasticity. Phosphorylation of adducin (Ser713 in β -adducin), which disrupts actin/spectrin interaction, is increased by quinpirole, haloperidol, or caffeine. We previously demonstrated that DARPP-32 interacts with β -adducin and facilitates its phosphorylation. Quinpirole increased DARPP-32 phosphorylation at Thr75 and haloperidol at Ser97, two modifications that can have similar consequences on adducin phosphorylation through distinct mechanisms. Experiments in DARPP-32 mutant mice confirmed that the apparently paradoxical similar effects of quinpirole and haloperidol on adducin phosphorylation may result from differential effects of these drugs on DARPP-32 phosphorylation at Thr75 and Ser97. Our data provide novel insights on how a single dose of widely used psychoactive drugs can affect spine plasticity in the nucleus accumbens, a component of the reward system.

1. Introduction

Psychiatric disorders, such as obsessive compulsive disorder (Burguiere et al., 2015), drug addiction (Russo et al., 2010), and major depression (Duman and Duman, 2015) are suggested to be associated with changes in dendritic spines in striatal projection neurons (SPNs), a major target of dopamine neurons. Spines are increased by chronic exposure to drugs that enhance dopamine transmission, such as cocaine, and decreased in the absence of dopamine in Parkinson's disease models (Smith and Villalba, 2013). However, little is known about the rapid effects of drugs acting on striatal neurons. Spines structural plasticity involves many complex signaling pathways and molecular interactions, including remodeling of the cortical cytoskeleton (Nishiyama and Yasuda, 2015). Adducins are actin-capping proteins which stabilize actin filaments and facilitate actin-spectrin network formation (Matsuoka et al., 2000). Adducins are a family of three highly related proteins, α , β , and γ , coded by different genes. α - and γ -adducin are ubiquitous, whereas β -adducin is mostly expressed in brain and red blood cells. These proteins form α/β and α/γ heterotetramers that interact with actin and spectrin. Adducins' carboxy-terminal region is a MARCKS domain (myristoylated alanine-rich C-kinase substrate) which is phosphorylated by PKC on a serine residue (Ser713 in β -adducin) and binds Ca^{2+} /calmodulin (Matsuoka et al., 1996; Matsuoka et al., 1998). Phosphorylation of the MARCKS domain by PKC, as well as its interaction with Ca^{2+} /calmodulin, disrupts adducin interaction with actin and spectrin and favors actin filaments depolymerization (ibid.). Thus, adducins can regulate cortical cytoskeleton stabilization/destabilization. β -adducin has been identified as a regulator of synaptic plasticity in hippocampus (Bednarek, 2011). β -adducin knockout mice have impaired hippocampal long-term potentiation (Rabenstein et al., 2005) and mild alterations in nucleus accumbens spines (Jung et al., 2013). Although β -adducin is not required for the effects of repeated cocaine on spines (Jung et al., 2013), we have recently shown that it is necessary for the rapid spine changes observed in the nucleus accumbens of mice placed for 24 h in a novel enriched environment (NEE) (Engmann et al., 2015). Adducin phosphorylation is increased in striatum by an acute dose of cocaine (Lavaur et al., 2009), by caffeine, and by NEE (Engmann et al., 2015). Dopamine- and cAMP-regulated phosphoprotein, Mr 32,000 (DARPP-32), a protein highly expressed in SPNs of the dorsal striatum and nucleus accumbens, interacts with β -adducin and this interaction appears to mediate the effects of NEE on nucleus accumbens dendritic spines (ibid.).

Since DARPP-32 integrates signaling events from various receptor types via several phosphorylation sites (Yger and Girault, 2011), it could potentially provide a link between multiple pathways and adducin regulation. Here we show that one acute dose of the psychostimulants quinpirole and caffeine as well as the antipsychotic haloperidol can induce changes in nucleus accumbens spine density and morphology. These effects are blocked in the absence of β -adducin. We show that all these drugs increase β -adducin phosphorylation and, using DARPP-32 point mutant mice, that the effects of the various drugs may be mediated by differential regulation of multiple phosphorylation sites in DARPP-32.

2. Materials and methods

2.1. Drugs, chemicals and antibodies

Mice were injected intraperitoneally (IP) with the following drugs or with their vehicle (9 g/l NaCl): quinpirole hydrochloride (0.2 mg/kg, Sigma, 15 min for biochemistry, 24 h for Golgi-Cox), haloperidol (0.5 mg/kg, Sigma, 1 h for biochemistry, 24 h for Golgi-Cox) and caffeine (7.5 mg/kg, Sigma, 40 min for biochemistry, 24 h for Golgi-Cox). Spine morphology was studied at 24 h because we have previously shown spine changes after 24h NEE. Primary antibodies were for total β -adducin (#AP20545PU-N, 1:500; Santa Cruz, Acris), PKC-phosphorylated adducins (corresponding to pSer713 in β -adducin, #05-587; 1:1,000; Chemicon/Millipore), mouse monoclonal for DARPP-32 (1:5,000, Cell Signaling Technology), rabbit polyclonal for pThr34, pThr75, pSer97 (#12438, #2301, #3401; 1:2,000; Cell Signaling), and β -actin (#A5316; 1:1,000; Sigma). Secondary antibodies were anti-mouse CY3-conjugated antibody (#A10521; 1:400; Molecular Probes) for immunocytochemistry and IRDye800-conjugated anti-mouse and anti-rabbit (#610-132-121, #611-132-122; both 1:4,000; Rockland) antibodies for immunoblotting.

2.2. Animals

Animals were housed in accordance with the ethical guidelines (Declaration of Helsinki and NIH, publication no. 85-23, revised 1985, European Community Guidelines, and French Agriculture and Forestry Ministry guidelines for handling animals, decree 87849, license A 75-05-22) and approved by the local ethical committee. The previously described mutant mice used in this study were DARPP-32 T75A and S97A knock-in mutants (Svenningsson et al., 2003) kindly provided by Paul Greengard (The Rockefeller University, New York) and β -adducin knock-out mice (Gilligan et al., 1999), kindly provided by Marina Picciotto (Yale University, New Haven). In the case of mutant mice, males and females were used

and numbers were balanced between groups. Male C57Bl/6 mice were purchased from Janvier (Le Genest Saint Isle, France).

2.3. Golgi-Cox staining, analysis of spine density and morphology

Golgi-Cox staining was performed as described (Engmann et al., 2015). In brief, mouse brain hemispheres were incubated in the dark for two weeks in a Golgi-dye (1% potassium dichromate, 1% mercury chloride, 0.8% potassium chromate [all weight/vol, filtered]). Next, the tissue was washed 3 x 2 min in H₂O (distilled water) and 30 min in 90% ethanol (vol/vol). Brains were cut in 70% ethanol on a vibratome (Leica) into 200- μ m sections and washed in H₂O for 5 min. They were then reduced in 16% ammonia solution for 1 h, washed in H₂O for 2 min and fixed in 1% sodium-thiosulfate (weight/vol) for 7 min. Following a final wash in H₂O for 2 min, brain sections were mounted on superfrost coverslips. They were gradually dehydrated for 3 min in 50%, 70%, 80% and 100% ethanol. Samples were incubated for 2x 5 min in a 2:1 isopropanol: ethanol mixture, followed by 1x 5 min in pure isopropanol and 2x 5 min in xylol. Finally, the brain sections were mounted with Eukitt mounting medium and left for 24 h to settle. Photography and analysis of spines was conducted exactly as described by Engmann et al. (2015). Z-stacks from 0.2 μ m sections were obtained in bright field at 100x resolution on a DM6000-2 microscope (Leica), and analyzed with the ImageJ software. The total number of spines was obtained using the cell counter tool. At least 40 dendrites per group from at least three mice per group were counted. Between 315 and 345 spines per group were analyzed for major head diameter as well as neck length. For deconvolution the images were inverted and processed with the "2-D deconvolution parallel spectra deconvolution" plug-in (ImageJ).

2.4. Striatal microdisks and immunoblot analysis

Striatal microdisks (punches) were collected as described in Engmann et al. (2015). Essentially, mice were sacrificed by cervical dislocation and heads were frozen in liquid nitrogen. Brains were cut with a Leica cryostat into 200- μ m sections. The striatum and nucleus accumbens were removed with metal punches on dry ice. 1% sodium dodecyl sulfate (weight/vol) was added and the samples were sonicated before placing them at 98°C for 5 min. Then, 2x Laemmli-buffer was added and the samples were heated for another 5 min. For immunoblotting, Bio-rad Mini Protean TGX-gels and the Biorad transfer system were utilized. Secondary antibody binding was detected by Odyssey and analyzed in ImageJ.

2.5. Immunohistochemistry of mouse brain sections

Brains from mice were perfused for 5 min with 4% paraformaldehyde (weight/vol), 50 mM sodium fluoride and post-fixed at 4°C O/N. They were cut on a Leica vibratome into 30 µm sections (Bregma 1.3 mm – 0.6 mm). Immunostaining for DARPP-32 or β -adducin on free-floating sections was performed as described (Engmann et al., 2015). Nuclei were labeled with DAPI-containing Vectashield (Vector). Photos were taken at 63x magnification with a Leica-SP5-confocal microscope. Four or more images were taken per brain area and animal.

2.6. Statistical analysis

Data were statistically analyzed in GraphPrism. They are expressed as means $\pm$ SEM. For comparison of two groups we used two-tailed Student's *t*-test, for more than two groups, one-way ANOVA was used. Two-way ANOVA was performed when two factors were varied. When ANOVA was significant, we used Tukey's test for one-way and Šidák's test for two-way as post-hoc tests. Log-rank (Mantel-Cox) test was used for cumulative dendritic spine neck length analysis. For western blots on DARPP-32 point mutants, outliers were removed if they deviated more than two standard deviations from the mean. The threshold for significance was $p < 0.05$.

3. Results

3.1. Acute spine density changes induced in nucleus accumbens neurons by quinpirole, haloperidol and caffeine require β -adducin

We examined whether commonly studied pharmacological compounds that target striatal neurons affect nucleus accumbens spines and whether their effects were β -adducin-dependent. We were especially interested in acute effects on dendritic spines after a single dose of drugs, as little is known about acute pharmacological effects on spines. Mice were injected with a single dose of quinpirole (0.2 mg/kg), haloperidol (0.5 mg/kg) or caffeine (7.5 mg/kg), and 24 h later brains were collected to analyze spines in the nucleus accumbens by Golgi-Cox staining (**Fig. 1A-C**). Caffeine acts on multiple targets but it exerts a predominant antagonistic effect on A2_A receptors in the striatum where these receptors are highly expressed in SPNs that also express D2 receptors (Schiffmann et al., 2007; Svenningsson et al., 1999). We observed effects of all these drugs on spine density (**Fig. 1A, B**). Quinpirole and caffeine increased spine density, while haloperidol decreased spine density. Since we had shown previously that effects of 24-h NEE on spine density in nucleus accumbens neurons were blocked in the absence of β -adducin (Engmann et al., 2015), we tested the effects of the three drugs in β -adducin knockout mice. A decreased density

was observed in these mice as compared to wild-type after saline injection, and all the drug effects were prevented (**Fig. 1A, B**). These results showed that a single dose of quinpirole, an agonist of D2/D3 dopamine receptors (D2R/D3R), haloperidol, an antagonist of D2R/D3R, or caffeine altered spine density in nucleus accumbens neurons and that these effects were lost in the absence of β -adducin.

3.2. Role of β -adducin in spine morphological alterations induced by quinpirole, haloperidol and caffeine

Next, we used Golgi-Cox staining to assess how the three drugs of interest may affect spine morphology as indicated in **Fig. 2A**. We observed that quinpirole and caffeine increased spine length, while haloperidol decreased spine length (**Fig. 2B**). These effects were due to a change in neck length (**Fig. 2C**). Changes in spine length and neck length were completely blocked or reversed in the absence of β -adducin. We also observed that quinpirole, but not caffeine or haloperidol, increased spine head width and this effect was also blocked in β -adducin knockout mice (**Fig. 2D**). In agreement with their opposite effects on dopamine receptors, haloperidol and quinpirole had opposite effects on spines. These findings indicated that drugs acting on dopamine D2R/D3R or adenosine receptors altered not only spine density but also spine morphology in nucleus accumbens SPNs and that β -adducin was necessary for these effects regardless of their direction.

3.3. β -adducin phosphorylation is increased by quinpirole and haloperidol

β -adducin effects on dendritic spines are thought to depend on its ability to regulate actin-spectrin interactions and capping the fast-growing ends of actin filaments (Matsuoka, Li & Bennett, 2000). This property is strongly modulated by adducin phosphorylation (Ser713 in β -adducin), which prevents adducin binding to actin and spectrin and promotes filaments depolymerization (ibid). We have previously shown that caffeine injection increases phosphorylation of this residue (Engmann et al., 2015). Using immunoblotting we examined the effects of quinpirole and haloperidol on adducin phosphorylation (**Fig. 3**). Quinpirole (0.2 mg/kg, 15 min) increased phosphorylation of this serine in the striatum (**Fig. 3A**). Surprisingly haloperidol (0.5 mg/kg, 1 h) also increased adducin phosphorylation (**Fig. 3B**), although it exerts opposite effects on D2/D3 receptors. We also examined adducin phosphorylation by immunohistofluorescence (**Fig. 3C and D**), as described previously (Lavaur et al., 2009). Quinpirole and haloperidol increased adducin phosphorylation in the dorsal striatum and in the nucleus accumbens, respectively. These results indicated that haloperidol and quinpirole were able to increase adducin phosphorylation, as previously shown for caffeine.

3.4. Quinpirole, haloperidol, and caffeine have differential effects on DARPP-32 phosphorylation and cytonuclear localization

Although quinpirole and haloperidol have opposing actions on D2R/D3R, they both increased adducin phosphorylation (see above). This prompted us to investigate the molecular pathways that regulate β -adducin phosphorylation under these conditions. We focused our attention on the potential role of DARPP-32, whose function depends on the phosphorylation state of several phosphorylated residues, including Thr34, Thr75, and Ser97, and which is an integrator of various signaling events in MSNs, including dopamine- and A2_A-receptors signaling (Yger and Girault, 2011). We previously identified DARPP-32 as a binding partner of β -adducin and showed that the interaction between the two proteins is enhanced by phosphorylation of DARPP-32 on Ser97 (Engmann et al., 2015). We also observed that DARPP-32 facilitates adducin phosphorylation through regulation of protein phosphatase 2A (PP2A) (ibid.). More precisely, when phosphorylated on Thr75, DARPP-32 inhibits PKA (Bibb et al., 1999) and thereby appears to prevent the activation of a PKA-dependent form of PP2A active on Ser713 (Engmann et al., 2015). Indeed Thr75 mutation to alanine in DARPP-32 prevented the increase in Ser713 phosphorylation observed after NEE or caffeine administration (ibid.). Quinpirole, haloperidol, and caffeine have been previously shown to differentially alter DARPP-32 phosphorylation in a cell-type specific manner (Bateup et al., 2008). We examined the overall effects of these three drugs in our experimental conditions. Quinpirole (0.2 mg/kg, 15 min) decreased Thr34 and Ser97 phosphorylation, whereas it enhanced Thr75 phosphorylation (**Fig. 4A**). Haloperidol (0.5 mg/kg, 1 h) predominantly enhanced Ser97 phosphorylation (**Fig. 4B**). Finally, as previously demonstrated (Lindskog et al., 2002), caffeine (7.5 mg/kg, 40 min) increased Thr75 (**Fig. 4C**). Thus, our results confirmed previous findings on the regulation of Thr34 and Thr75 by quinpirole (Bateup et al., 2008) and of Thr75 by caffeine (Lindskog et al., 2002) and expanded them, showing that quinpirole decreased Ser97 phosphorylation while haloperidol increased it.

Ser97 phosphorylation has been shown to facilitate nuclear export of DARPP-32 (Stipanovich et al., 2008). Since quinpirole and haloperidol had opposite effects on Ser97 phosphorylation we examined whether these drugs modified DARPP-32 cytonuclear localization. Quinpirole injection decreased the percentage of cells with predominantly cytoplasmic DARPP-32 was observed (**Fig. 5A**) whereas this proportion was increased following haloperidol injection (**Fig. 5B**). This result was in agreement with the observed changes in Ser97 phosphorylation and the proposed role of this residue in regulating DARPP-32

intracellular localization. Accordingly, caffeine, which did not alter Ser97 phosphorylation, had no effect on DARPP-32 distribution in these experiments (data not shown). Altogether these experiments showed that quinpirole and haloperidol altered Ser97 phosphorylation and DARPP-32 cytonuclear distribution in opposite directions.

3.5. Differential effects of quinpirole and haloperidol on DARPP-32 phosphorylation may account for their convergent effects on adducin phosphorylation

We reasoned that given the role of both Thr75 and Ser97 in the regulation of β -adducin by DARPP-32, the different effects of quinpirole and haloperidol on DARPP-32 phosphorylation on these two residues might provide an explanation to the apparently paradoxical enhancement of Ser713 phosphorylation by an agonist and an antagonist of D2/D3 receptors. We have previously shown that caffeine effects on phospho-adducin were blocked in Thr75Ala-DARPP-32 mutant mice (Engmann et al., 2015). We therefore hypothesized that Thr75 phosphorylation, which is enhanced by quinpirole might also contribute to the increased adducin phosphorylation induced by quinpirole. To test this hypothesis we compared the effects of quinpirole on adducin phosphorylation in Thr75Ala DARPP-32 mutant mice and in their wild-type littermates (**Fig. 6A**). The stimulation of adducin phosphorylation by quinpirole was not observed in Thr75Ala mutant mice (**Fig. 6A**), supporting our hypothesis.

In contrast, the effects of haloperidol could not be accounted for by an increased phosphorylation of Thr75, since haloperidol did not alter Thr75 phosphorylation in our conditions (see **Fig. 4B**) or in a previous cell-specific study (Bateup et al., 2008). However we noticed that haloperidol increased DARPP-32 phosphorylation on Ser97 (see **Fig. 4B**). Since phosphorylation of Ser97 enhances the interaction between DARPP-32 and β -adducin (Engmann et al., 2015), we hypothesized that this increased interaction, while phospho-Thr75 remained constant, might account for the haloperidol-stimulated phosphorylation of adducin. To test this hypothesis we compared haloperidol effects on adducin phosphorylation in wild-type and DARPP-32 Ser97Ala mutant mice (**Fig. 6B**). As observed above (see **Fig. 3B**), haloperidol increased adducin phosphorylation in wild-type mice, whereas it had no effect in Ser97Ala mutant mice (**Fig. 6B**). These findings strongly indicated that regulation of DARPP-32 phosphorylation on Ser97 could account for the effect of haloperidol on adducin phosphorylation. Altogether these results showed that the differential regulation of Thr75 by quinpirole and of Ser97 by haloperidol can explain the paradoxical similar effects of a D2/D3 agonist and a D2/D3 antagonist on adducin phosphorylation.

4. Discussion

Spine alterations in the dorsal and ventral striatum have been reported following prolonged treatments that alter dopamine transmission [see (Villalba and Smith, 2013)]. Here we provide evidence that spine alterations can also be detected in the nucleus accumbens 24 h after a single injection of drugs that alter dopamine or adenosine transmission. Quinpirole, a D2R/D3R agonist, increased spine density whereas haloperidol a D2R/D3R antagonist had opposite effects, decreasing the number of spines. Interestingly, chronic treatment with haloperidol has been previously shown to decrease SPN spine density (Kelley et al., 1997), similarly to the chronic lack of dopamine in models of Parkinson's disease (Villalba and Smith, 2013). In contrast, a stimulatory effect of quinpirole on spines has been reported in hippocampal neurons in culture (Yoon et al., 2015) but the effects of this drug on SPNs were not known. In the present study we found that caffeine, a drug predominantly acting as an A_{2A} antagonist in the striatum (Schiffmann et al., 2007; Svenningsson et al., 1999), also increased spine density. Chronic caffeine has been reported to increase spine density in hippocampus (Vila-Luna et al., 2012), but its effects on SPNs were not investigated. Morphological analysis of spines revealed partly different effects of quinpirole and caffeine. Quinpirole increased spine length and head volume, whereas caffeine only increased spine length, suggesting that they had, at least in part, different mechanisms of actions. Both quinpirole and caffeine are expected to decrease cAMP production in D2-SPNs, quinpirole by stimulating D2R that inhibits adenylyl cyclase and caffeine by antagonizing adenosine effects on A_{2A} receptors that stimulate adenylyl cyclase. The partial difference in their morphological effects on spines suggests that additional signaling mechanisms contribute to their effects on spines.

We investigated the role of β -adducin in spine regulation. β -adducin can be knocked out without severe general consequences and was shown to facilitate synaptic plasticity and to allow its maintenance (Rabenstein et al., 2005). *Drosophila* single adducin homolog, encoded by the *hu-li tai shao* (hts) gene, is critical for several processes associated with synapse remodeling (Pielage et al., 2011). In mice, β -adducin is required for the effects of prolonged enriched environment on hippocampal synapses, preventing the formation of new synapses (Bednarek, 2011). In the striatum cocaine increases adducin phosphorylation (Lavaur et al., 2009) and β -adducin knockout mice have altered spine morphology (Jung et al., 2013). β -adducin is also necessary for the rapid effects of novel enriched environment on nucleus accumbens spines (Engmann et al., 2015).

Because of this large body of evidence implicating β -adducin in plasticity we investigated the role of β -adducin in drug-induced spine alterations in the nucleus accumbens. Our results showed that in the absence of β -adducin not only the effects of caffeine and quinpirole were lost, but also those of haloperidol, although the former increased spine density and the latter decreased spine density. We have previously shown that caffeine enhanced adducin phosphorylation (Engmann et al., 2015). Here we provide evidence that both quinpirole and haloperidol have a similar action on adducin phosphorylation. These results suggest that enhanced adducin phosphorylation after drug injection is an important step in the morphological effects of drugs on spines observed at 24 h. It is therefore tempting to speculate that adducin phosphorylation allows a transient destabilization of the dendritic cortical cytoskeleton that is necessary for spine retraction as well as elongation. Interestingly the lack of β -adducin did not appear to prevent spine increases induced in the nucleus accumbens by chronic cocaine administration (Jung et al., 2013) or in the hippocampus by prolonged environmental enrichment (Bednarek, 2011), whereas it appeared sufficient to prevent spines alterations 24 h after NEE (Engmann et al., 2015) or a single drug administration (this study). This suggests that compensatory mechanisms can overcome the absence of β -adducin following repeated or prolonged stimuli.

One apparently paradoxical finding of our study was that both quinpirole and haloperidol had similar effects on adducin phosphorylation, whereas they have opposite pharmacological actions on D2/D3 receptors and on spine density. Since we have previously shown that DARPP-32 is associated with β -adducin in SPNs and regulates its phosphorylation in response to NEE (Engmann et al., 2015), we investigated whether it played a role in mediating drug effects on adducin phosphorylation. We found that caffeine, quinpirole, and haloperidol had distinct effects on DARPP-32 multiple phosphorylation sites, confirming and extending previous findings. In particular caffeine and quinpirole increased DARPP-32 phosphorylation on Thr75, whereas haloperidol had no effect on this site, in agreement with previous reports (Bateup et al., 2008; Lindskog et al., 2002). In contrast, haloperidol increased Ser97 phosphorylation whereas quinpirole decreased this phosphorylation and caffeine had no effect. Quinpirole and haloperidol also modified the pattern of DARPP-32 cytonuclear distribution in opposite directions, whereas caffeine had no effect, in agreement with previous studies indicating a role of Ser97 phosphorylation in nuclear export of DARPP-32 (Stipanovich 2008). The differential regulation of DARPP-32 phosphorylation sites provided a potential mechanism to account for the regulation of adducin phosphorylation. Caffeine and quinpirole can facilitate adducin phosphorylation by preventing PKA-induced activation of PP2A, through inhibition of PKA by phospho-Thr75-DARPP-32, as previously

reported (Engmann et al., 2015). This mechanism was supported by the absence of effect of caffeine (ibid.) and quinpirole (this study) on adducin phosphorylation in Thr75Ala DARPP-32 mutant mice. The mechanism of action of haloperidol is likely to be different since it did not alter Thr75 phosphorylation. In contrast, haloperidol increased DARPP-32 phosphorylation at Ser97, which enhances DARPP-32 interaction with β -adducin (Engmann et al., 2015). We hypothesized that increased Ser97 phosphorylation facilitated the recruitment of preexisting phosphoThr75-DARPP-32 to β -adducin, thereby resulting in local PKA and PP2A inhibition. This hypothesis was supported by the lack of effect of haloperidol on β -adducin Ser713 phosphorylation in Ser97Ala mutant mice.

In addition to the pathways investigated in the current study, regulation of PKC, which is the kinase phosphorylating Ser713 in β -adducin, may also be involved in the effects reported here. Our study did not distinguish between SPNs of the direct and indirect pathways which express different receptors and display different signaling pathways regulations (Valjent et al., 2009). Since the drugs investigated in this study act on either D2 or A2_A receptors that are both located in SPNs of the indirect pathway, the simplest hypothesis is that observed effects on spines take place in these neurons. However, we cannot rule out non cell-autonomous effects through short or long distance crosstalk between these pathways and other cells. Finally, it should be emphasized that multiple molecular pathways are involved in spine control (Nishiyama and Yasuda, 2015). DARPP-32-mediated regulation of β -adducin is probably only a permissive factor among the large array of molecular mechanisms involved in spine plasticity. The interest of our study is to provide evidence for the functional importance of this permissive role.

In conclusion, our study shows that a single injection of three pharmacologically distinct psychoactive drugs can modify spines in the nucleus accumbens within a day. We show that β -adducin is necessary for these effects, presumably through regulation of its phosphorylation at Ser713. We also provide evidence that fine regulation of DARPP-32 phosphorylation is a major factor in mediating the final common effects on β -adducin of various drugs with distinct or even opposite modes of action. Since adducin phosphorylation is altered by a variety of psychoactive drugs and appears necessary for spine alterations, it may be a relevant therapeutic target in conditions that are known to affect spines.

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Figure legends**Figure 1. Quinpirole, haloperidol, and caffeine alter spine density in a β -adducin-dependent manner.**

A) Golgi-Cox staining was performed on male C57Bl/6 mice sacrificed 24 h after a single i.p. injection of vehicle (Saline), quinpirole (0.2 mg/kg), haloperidol (0.5 mg/kg) or caffeine (15 mg/kg). Two-D deconvoluted representative images obtained from Z-stacks are shown as negatives. Scale bar: 2 μ m. **B)** Spine density was determined using ImageJ software. Fifty to 63 nucleus accumbens dendrites were analyzed from 3-4 mice per group. Data are means + s.e.m. Saline, S, quinpirole, Q, haloperidol, H, caffeine, C. Two-way ANOVA: genotype effect: $F_{(1,424)} = 95.26$, $p < 0.0001$; drug effect: $F_{(3,424)} = 12.75$, $p < 0.0001$; interaction of drugs and genotype: $F_{(3,424)} = 21.35$, $p < 0.0001$; Sidak's multiple comparisons tests for each drug vs saline: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0001$, for wt vs KO, °, $p < 0.05$, °°, $p < 0.001$. **C)** Coronal section of mouse brain showing an overview of the analyzed region (rectangle) in the nucleus accumbens.

Figure 2. Spine changes induced by quinpirole, haloperidol and caffeine are β -adducin-dependent.

A) Schematic representation of a dendritic spines indicating the measurement of spine length (left panel) and spine head width and neck length (right panel). **B)** The total spine length was measured in Golgi-Cox stained nucleus accumbens sections from wild-type mice (left panels) and β -adducin knockout littermates (right panels) that had been injected with saline, quinpirole (Quin, 0.2 mg/kg), haloperidol (Halo, 0.5 mg/kg), or caffeine (Caff, 7.5 mg/kg) 24h before. Results are plotted as cumulative frequency. In wild-type mice quinpirole and caffeine increased spine length and haloperidol reduced spine length. These results were blocked (Quin, Caff) or reversed (Halo) in β -adducin KO mice. Log-rank (Mantel-Cox, DF = 1 in all cases) saline vs drug: wild-type, Quin, $\chi^2 = 9.75$, $p < 0.01$, Halo, $\chi^2 = 5.32$, $p < 0.05$, Caff, $\chi^2 = 16.02$, $p < 0.0001$; β -adducin KO, Quin, $\chi^2 = 0.80$, $p > 0.05$, Halo, $\chi^2 = 8.57$, $p < 0.01$; Caff, $\chi^2 = 1.98$, $p > 0.05$. **C)** The neck length of spines was measured as in **A)**. Quinpirole and caffeine increased spine neck length, while haloperidol had an opposite effect. These changes were prevented (Quin, Caff) or reversed (Halo) in β -adducin knockout mice. Log-rank (Mantel-Cox, DF = 1 in all cases) saline vs drug: wild-type, Quin, $\chi^2 = 4.50$, $p < 0.05$, Halo, $\chi^2 = 7.85$, $p < 0.01$, Caff, $\chi^2 = 20.03$, $p < 0.0001$; BADD KO, Quin, $\chi^2 = 0.51$, $p > 0.05$, Halo, $\chi^2 = 8.23$, $p < 0.01$, Caff, $\chi^2 = 2.30$, $p > 0.05$. **D)** Spine head width was assessed as in **A)**. Only quinpirole resulted in wider spine heads, while haloperidol and caffeine did not alter spine head width. β -adducin knockout prevented the effect of quinpirole. Log-rank (Mantel-Cox, DF = 1 in all cases) saline vs drug: wild-type, Quin, $\chi^2 = 7.70$, $p < 0.01$, Halo, $\chi^2 = 2.58$, $p > 0.05$, Caff, $\chi^2 = 1.00$, $p > 0.05$; β -

adducin KO, Quin, $\chi^2 = 0.43$, $p > 0.05$, Halo, $\chi^2 = 7.57$, $p < 0.01$, Caff, $\chi^2 = 0.08$, $p > 0.05$.

Figure 3. β -adducin phosphorylation is increased by quinpirole and haloperidol.

A) C57BL/6 mice were injected with 0.2 mg/kg quinpirole (Quin) and brains were dissected 15 min later. Punches from dorsal striatum were analyzed by immunoblotting with antibodies for phospho-adducin, total β -adducin, and actin as loading control (top panel) and quantified (bottom panel). Student's t test, $t_{18} = 2.13$, $p = 0.04$ ($n = 10$ mice per group). **B)** Mice were injected with haloperidol (Halo, 0.5 mg/kg, 1 h) and samples studied by immunoblotting (upper panel) and quantified (lower panel), as in A. Student's t test, $t_{16} = 3.70$ ($n = 10$ mice per group). **C, D)** Mice were treated as in A and B, respectively, brain sections of dorsal striatum (DS, bottom panels) and nucleus accumbens (NAc) were incubated with the phospho-adducin antibody, and immunofluorescence was quantified (bottom panels). Student's t test: **C)** Quinpirole: DS, $t_{18} = 2.53$, $p = 0.02$, NAc, $t_{18} = 2.08$, $p = 0.05$; **D)** Haloperidol: DS, $t_{18} = 2.03$, $p = 0.057$, NAc, $t_{18} = 3.17$, $p = 0.005$. **(A-D)** Data are expressed as % of controls (saline) average and are means + s.e.m., *, $p < 0.05$, **, $p < 0.01$. In each experiment, $n = 10$ mice per group.

Figure 4. Quinpirole, haloperidol, and caffeine differentially alter DARPP-32 phosphorylation.

A) Striatal samples were prepared 15 min after quinpirole injection (Quin, 0.2 mg/kg) and immunoblotted with antibodies for phosphoThr34 (pT34), phosphoThr75 (pT75) or phosphoSer97 (pS97), total DARPP-32, and actin as a loading control (left panel). Immunoreactive bands were quantified (right panel). Statistical analysis, Student's t test with Holm-Sidak method for multiple comparisons. pT34, $t_{32} = 4.91$, $p = 2.54 \times 10^{-6}$, pT75, $t_{18} = 2.27$, $p = 0.03$, pS97, $t_{31} = 2.56$, $p = 0.015$ ($n = 10-17$ mice per group). **B)** Samples were obtained 60 min after haloperidol injection (Halo, 0.5 mg/kg), analyzed by immunoblotting (left panel) and quantified (right panel) as in A. pT34, $t_{29} = 1.68$, ns, pT75, $t_{18} = 0.08$, ns, pS97, $t_{32} = 2.09$, $p = 0.04$ ($n = 10-17$ mice per group). **C)** Mice were sacrificed 40 min after caffeine injection (Caff, 7.5 mg/kg) and proteins were analyzed by immunoblotting (left panel) and quantified (right panel) as in A. pT34, $t_{12} = 0.06$, ns, pT75, $t_{12} = 4.02$, $p = 0.0017$, pS97, $t_{12} = 0.10$, ns ($n = 7$ per group). **(A-C)** Data are expressed as % of control average (saline) and are means + s.e.m. Drug vs Saline *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Figure 5. Quinpirole and haloperidol differentially alter DARPP-32 cytonuclear localization.

A) C57BL/6 mice were injected with vehicle (Saline) or 5 mg/kg quinpirole and perfused 15 min later. DARPP-32 immunostaining was studied in dorsal striatum (DS, left panel picture) and nucleus accumbens

(NAC). The percentage of cells with predominantly cytoplasmic DARPP-32 was quantified (right panel). Student's t test: DS, $t_{27} = 2.57$, $p = 0.016$, NAC, $t_{27} = 2.45$, $p = 0.02$. **B)** Mice were injected with vehicle or 0.5 mg/kg haloperidol and perfused 1 hour later. DARPP-32 immunostaining is shown in the DS (left panel) and the percentage of cells with predominantly cytoplasmic DARPP-32 quantified (right panel). Student's t test DS, $t_{26} = 4.71$, $p < 0.0001$, NAC, $t_{16} = 2.4$, $p = 0.029$. In **A)** and **B)** there were 14-15 mice per group, data are means + s.e.m., * $p < 0.055$, *** $p < 0.0001$. Scale bar: 20 μm .

Figure 6. β -adducin phosphorylation after quinpirole and haloperidol treatment depends on DARPP-32 phosphorylation of Thr75 and Ser97, respectively.

A) Thr75Ala (T75A) mutation of DARPP-32 prevents quinpirole-induced β -adducin phosphorylation. Wild-type (WT) or T75A mutant mice were injected with saline (Sal) or quinpirole (Quin, 0.2 mg/kg) as in Fig. 3 and brains dissected 15 min later. Punches from dorsal striatum were analyzed by immunoblotting with antibodies for phospho-adducin (pSer713 in β -adducin, pAdducin), total β -adducin, and actin as loading control (left panels) and quantified (right panel). $n = 18$ -25 mice per group, in 7 independent experiments. Two-way ANOVA: Quin effect, $F_{(1,80)} = 2.79$, $p = 0.09$; genotype effect, $F_{(1,80)} = 0.12$ ns; interaction, $F_{(1,80)} = 6.95$, $p = 0.01$; Sidak's post-hoc test Sal vs. Quin, * $p < 0.05$. **B)** Ser97Ala (S97A) mutation prevents haloperidol-induced β -adducin phosphorylation. Wild-type or S97A mutant mice were injected with haloperidol (0.5 mg/kg) as in Fig. 3 and brains were dissected 60 min later. Analysis as in A. $n = 16$ -18 mice per group, in 4 independent experiments Two-way ANOVA: Halo effect, $F_{(1,65)} = 3.05$, $p = 0.08$; genotype effect, $F_{(1,65)} = 0.85$, ns; interaction, $F_{(1,65)} = 3.37$, $p = 0.07$. Sidak's post-hoc test, Sal vs Halo, * $p < 0.05$. (A-B) Data are means + s.e.m.

Fig. 7. Schematic diagram summary of the effects of drugs on striatal spines.

A) Schematic summary of drug-induced spine changes in wild-type mice. A single injection of quinpirole increased spine head size and neck length. Haloperidol reduced neck length. Caffeine increased spine length without affecting head width. Knockout of β -adducin prevented, and in some instances reversed, all drug effects independently of their direction (not shown in summary). This indicated that β -adducin was critical for drug effects on spines. **B)** A key factor in adducin regulation is its phosphorylation in the MARCKS domain (at Ser713 in β -adducin), which disrupts its interaction with actin and spectrin, facilitates actin depolymerization and cortical cytoskeleton dynamics (Matsuoka et al., 2000). DARPP-32 facilitates adducin phosphorylation in striatal neurons (Engmann et al., 2015). DARPP-32 interacts with adducin and this interaction is facilitated phosphorylation by phosphorylation of DARPP-32 at Ser97 by

CK2. When DARPP-32 is phosphorylated at Thr75 by CDK5 it inhibits PKA. Since phosphorylation of certain PP2A subunits by PKA enhances its phosphatase activity, the net result of DARPP-32 phosphorylation at Thr75 is to facilitate adducin phosphorylation. Quinpirole and caffeine increase Thr75 phosphorylation, while haloperidol increases Ser97. These effects indirectly favor adducin phosphorylation. Note that quinpirole was also found to slightly decrease Ser97 phosphorylation, but this effect was apparently not important for adducin regulation.

Engmann et al. Fig. 1

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Engmann et al. Highlights

- Acute caffeine or quinpirole increases spine density & length in nucleus accumbens
- A single injection of haloperidol has opposite effects
- The effects of the 3 drugs are absent in mice lacking β -adducin
- Phosphorylation of adducin is increased by quinpirole, haloperidol, and caffeine
- DARPP-32 phosphorylation mediates the effects of the 3 drugs on adducin