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Constitutive knockout of the membrane cytoskeleton protein beta adducin decreases mushroom spine density in the nucleus accumbens but does not prevent spine remodeling in response to cocaine

Yonwoo Jung[1,2,4], **Patrick J. Mulholland**[3,4], **Shari L. Wiseman**[1,2], **L. Judson Chandler**[3], and **Marina R. Picciotto**[1]

[1]Division of Molecular Psychiatry, Yale University School of Medicine, New Haven, CT, 06508

[2]Interdepartmental Neuroscience Program, Yale University School of Medicine, New Haven, CT, 06508

[3]Deparement of Neurosciences, Medical University of South Carolina, Charleston, SC 29425

Abstract

The adducin family of proteins associates with the actin cytoskeleton in a calcium-dependent manner. Beta adducin (βAdd) is involved in synaptic plasticity in the hippocampus; however, the role of βAdd in synaptic plasticity in other brain areas is unknown. Using diolistic labeling with the lipophilic dye DiI, we found that the density of mature mushroom-shaped spines was significantly decreased in the nucleus accumbens (NAc) in brain slices from βAdd KO mice as compared to their wildtype (WT) siblings. The effect of 10 days of daily cocaine (15 mg/kg) administration on NAc spine number and locomotor behavior was also measured in β Add WT and knockout (KO) mice. As expected, there was a significant increase in overall spine density in NAc slices from cocaine-treated WT mice at this time-point, however, there was a greater increase in the density of mushroom spines in β Add KO animals following chronic cocaine administration compared to WT. In addition, β Add KO mice showed elevated locomotor activity in response to cocaine treatment compared to WT siblings. These results indicate that β Add is required for stabilizing mature spines under basal conditions in the NAc, but that lack of this protein does not prevent synaptic remodeling following repeated cocaine administration. In addition, these data are consistent with previous studies suggesting that β Add may normally be involved in stabilizing spines once drug- or experience-dependent remodeling has occurred.

Keywords

dendritic spines; diolistic labeling; behavioral sensitization; synaptic plasticity; cocaine; adducin; medium spiny neurons; nucleus accumbens; DiI

Introduction

Several studies have shown that repeated administration of cocaine results in increases in spine density in the nucleus accumbens (NAc) (Pulipparacharuvil et al. 2008; Shen et al.

Corresponding Author: Marina R. Picciotto 34 Park Street New Haven, CT 06508 Phone: 203-737-2041 Fax: 203-737-2043 marina.picciotto@yale.edu.

[4]These authors contributed equally

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2009). Synaptic remodeling is known to require alterations of the cytoskeleton, but the molecular mechanisms underlying those alterations induced by cocaine are not well understood. Adducins are cytoskeleton-associated proteins that are phosphorylated in the NAc in response to cocaine administration (Toda et al, 2006; Lavaur et al, 2009). Adducins can cap and bundle actin filaments and can promote spectrin binding (Gardner and Bennett, 1987; Mische et al., 1987; Kuhlman et al., 1996). Calmodulin binding and phosphorylation by protein kinase A and protein kinase C cause adducin to dissociate from the actin cytoskeleton, allowing alterations in actin polymerization to occur (Matsuoka et al., 1996, 1998). Thus, adducins may contribute to activity-dependent cytoskeletal remodeling because they could provide a link between intracellular signaling pathways induced by neuronal activity and structural changes in the cytoskeleton.

Three genes (α, β and γ) encode mammalian adducin proteins and several splice variants have been identified (Suriyapperuma et al. 2000). Functional adducin is composed of α/β or α/γ heterodimers or heterotetramers (Dong et al. 1995; Hughes and Bennett 1995). Interestingly, α and γ adducin are expressed ubiquitously whereas the β subunit of adducin is enriched in brain and erythrocytes (Gilligan et al. 1999). Late-stage long-term potentiation is impaired in the CA1 field of the hippocampus in knockout (KO) mice lacking βadducin (β Add) despite relatively normal development of synaptic plasticity in the first hour after high-frequency stimulation (Rabenstein et al. 2005). This study suggests that adducin is important for the establishment of long-term morphological changes related to synaptic plasticity following early-stage biochemical changes.

In the current study, we analyzed spine density in the NAc of cocaine-naiveβAdd KO mice and their WT siblings. Spine morphology was examined using diolistic labeling with the lipophilic dye DiI followed by quantitation using Neuron Studio and IMARIS software. Changes in spine density in the NAc have been reported following chronic administration of a number of drugs of abuse including cocaine (Robinson and Kolb 1999; Robinson et al. 2001). Since the adducins are cytoskeleton-associated proteins that may be involved in alterations in actin dynamics underlying spine formation, we hypothesized that the ability of adducins to contribute to actin dynamics might suggest a role for β Add in cocaine-dependent spine remodeling, using a regimen that has been used previously to induce spine changes in the NAc of C57BL/6J mice (Lee et al. 2006). Finally, to determine whether the spine density changes in βAdd KO mice might be correlated with alterations in a behavior thought to relate to changes in synaptic strength in the NAc, we evaluated cocaine-induced locomotor sensitization in βAdd KO mice and their WT siblings. The data presented here show that β Add may be important for regulating mature dendritic spine density at baseline, but the protein is not required for spine remodeling in response to repeated cocaine administration.

Material and Methods

Animals

βAdd KO mice aged 8-12 months were generated as reported previously (Gilligan et al., 1999). The βAdd mouse line was backcrossed onto the C57BL/6J background (The Jackson Laboratory, Bar Harbor, ME) for at least 13 generations. Male littermate controls were used for all experiments. Mice were group-housed with no more than 5 animals per cage and kept in a colony room at 22°C on a 12 h light/dark cycle (lights on at 7:00 A.M.). Food and water were available *ad libitum* . All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Yale Animal Care and Use Committee.

Confocal imaging and quantitation of dendritic spines

Mice were administered saline (wildtype (WT): n = 11, KO: n = 18) or cocaine (15 mg/kg, i.p.; WT: n = 12, KO: n = 16) every day for 10 days, locomotion was measured as described below, and mice were then left undisturbed in their home cages for 2 weeks. Two weeks after the last cocaine injection, the same animals used for behavioral analysis were challenged with a single intraperitoneal (i.p.) administration of cocaine (15 mg/kg) and 15 min later, mice were perfused (5 ml/min) with 300 ml of 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were removed and postfixed in the same fixative for 1 h, then were coronally sectioned (150 μm thick) using a vibratome at room temperature. Data were obtained from animals with the researcher blind to condition.

Diolistic labeling

Tungsten particles (1.3 μm diameter, Bio-Rad, Berkeley, CA) were coated with the lipophilic carbocyanine dye DiI (Invitrogen, Calrsbad, CA). DiI-coated particles were delivered diolistically into tissue at 120 psi using a Helios Gene Gun system (Bio-Rad, Berkeley, CA) fitted with a polycarbonate filter (3.0 μm pore size; BD Biosciences, Franklin Lakes, NJ). DiI was allowed to diffuse along neuronal dendrites and axons in PBS for 16 - 24 h at 4°C, and then labeled sections were fixed again in 4% paraformaldehyde for 1 h. After brief washing in PBS, sections were mounted with Fluoromount G mounting media (Electron Microscopy Sciences, Hatfield, PA) onto glass slides (Thermo Scientific, Waltham, MA).

Confocal imaging and three-dimensional reconstructions

Medium spiny neurons were imaged using an UltraVIEW VoX spinning disc confocal microscope with a 60x CFI Plan Apo objective and equipped with a Hamamatsu C9100-50 camera, with excitation at 543 nm. Z-stacks were collapsed into projection images and 2- 3 neurons/brain were manually analyzed per section.

Measurement of spine density and size

For initial quantitative analysis, collected data were processed for reconstruction with NeuronStudio software as described in the manufacturer's manual (CNIC, Mt. Sinai School of Medicine, New York, NY; http://research.mssm.edu/cnic/help/ns/index.html). The entire dendritic branch was analyzed, with lengths of 120-130 uM. Maximum and minimum spine height was set at < 3.5 um and > 0.5 um respectively. Minimum stubby spine size was set at > 22 voxels. After automatic detection of spines by the NeuronStudio software, spots that were not on the quantified dendrite were removed manually. Medium spiny neurons in nucleus accumbens were imaged across core and shell.

For alternative automated spine analysis, a three-dimensional perspective was rendered by the Surpass module of IMARIS software package (Version 5.5, Bitplane), as has been described in detail previously (Shen et al., 2008, 2009; Kroener et al., 2012). To increase the accuracy of identification of the surfaces of dendrite shaft and spines, images were deconvolved to reduce point-spread functions using the adaptive blind 3D deconvolution method (AutoQuant X, Version X2.1.3, Media Cybermedics, Inc., Bethesda, MD) prior to analysis. Using the Imaris XT (Bitplane AG, version 6.2.1, Zurich, Switzerland) module, a filament of the dendritic shaft and spines was created from ~50 μm length sections starting 30-50 μm from the soma, following previously reported methods (Shen et al., 2008, 2009; Kroener et al., 2012). Dendritic spines were then classified into 4 categories (i.e., mushroom, thin, stubby) based on their neck and head morphology, where L is spine length, W_H is spine head width, and W_N is spine neck width. Following these criteria, long spines were identified as having a mean $W_H \geq$ mean W_N, mushroom spines were identified as

having a $L < 3$ and maximum $W_H >$ mean $W_N * 2$, and stubby spines were identified as having a $L < 0.5$ μm. The remaining spines were identified as filopodia, and were typically observed to be long, thin protuberances stemming from the dendritic shaft.

Locomotor activity measurements

Mice (WT treated with saline: n=11, WT treated with cocaine: n=18, KO treated with saline: n=12, KO treated with cocaine: n=16) were placed in 45 × 24 × 20 cm clear plastic cages between two rows of photocells (8 total, 4 cm apart) for 30 min. This time interval for measuring locomotor activity was chosen because previous studies have shown that locomotor activity plateaus after 30 min. Locomotor activity was assessed as beam breaks per min using personalized Windows-compatible software. Beam breaks were compiled into four 5 min bins for analysis. Statistical analysis was performed using ANOVA with Tukey's-HSD *post-hoc* tests when relevant.

Data analysis

The spine data were statistically analyzed using a one- or two-way ANOVA. For comparing spine density the data were calculated as percentage change from saline values and compared using a two-tailed Student's *t* test. *Post hoc* tests were conducted using Tukey's-HSD test for repeated measures ANOVA

Results

Naïve βAdd knockout mice have a lower density of dendritic spines in the NAc

Dendritic spines on medium spiny neurons in the NAc were visualized with the lipophilic dye DiI and spine density was measured using NeuronStudio software for 3-D image analysis. Spines were measured, and classified as thin, mushroom or stubby subtypes. There was a significant difference in the number of mushroom and thin spine subtypes on NAc neurons from βAdd WT and KO mice (Fig. 1. B; $F_{1, 47} = 8.723$, $p = 0.005$, Fig. 1. C; $F_{1, 47} = 5.116$, $p = 0.028$), with a significant decrease in the density of both mushroom and thin spines at baseline in βAdd KO mice as compared to WT mice.

Constitutive knockout of βAdd does not prevent spine remodeling in response to cocaine treatment

We measured spine density in the NAc of βAdd KO mice and their WT siblings following a single challenge dose of cocaine (15 mg/kg, i.p.) 2 weeks after repeated cocaine administration (15 mg/kg, i.p.; 10 days). Dendritic spines were classified as thin, mushroom, and stubby spines based on the ratio of head and neck diameter. Spine density (spine number/μm) was used as the primary outcome measure for analysis (Fig. 2) and was also measured as percentage change from basal spine density (Fig. 3). Cocaine treatment resulted in a significant increase in the density of spines in both WT and KO mice (Fig. 2A; $F_{1, 121} = 6.875$, $p < 0.01$, Fig. 2B; $F_{1, 121} = 12.624$, $p < 0.001$). Despite the similar increase in overall spine density, there was a much greater percent increase in mushroom spines in the NAc following cocaine treatment in βAdd KO mice as compared to neurons from WT βAdd mice (Fig. 3B; $t_{31} = 4.795$, $p = 1.4 \times 10^{-5}$).

Similar pattern of spine density changes observed with an automated analysis procedure

Dendritic spines can be classified into individual subtypes including stubby, mushroom, thin and branched, but each software program uses different spine detection algorithms. We therefore used an independent method to analyze spine density and spine morphology to verify the findings obtained using NeuronStudio. As a result, we re-analyzed the images obtained as described above using IMARIS software with 3-dimensional image

reconstruction (Fig. 4). The IMARIS software classifies spines into filopodia, thin, mushroom, and stubby spines. The IMARIS software had more stringent requirements for images that could be analyzed, so some images were excluded and the "n" size for these measurements was therefore somewhat lower than the analysis using NeuronStudio. Consistent with the findings obtained using NeuronStudio, there was a significant interaction between genotype and spine number, and a significant decrease in mushroom spines at baseline in the NAc of βAdd KO mice as compared to WT mice (Fig. 5B: $F_{1,11}$ = 26.087, p=0.001, Fig. 5C; $F_{1,11}$ = 3.754, p=0.085). Cocaine treatment also similarly resulted in a significant increase in spine density in both WT and βAdd KO mice (Fig. 6A; $F_{1, 32}$ = 3.603, p = 0.024, Fig. 6B; $F_{1, 32}$ = 10.956, $p < 0.01$). In addition, repeated cocaine administration significantly increased the number of mushroom spines in the NAc of βAdd KO mice (Fig. 7B; t_{10} = 4.933, p=0.001, Fig. 7C; t_{10} = 1.188, p=0.088).

Enhanced locomotor sensitization in βAdd KO mice

We evaluated cocaine-induced locomotor sensitization in βAdd KO mice and their WT siblings to determine whether spine density changes might be correlated behavior. Mice were treated with saline for three days to habituate the mice to injections and to the novel environment. There were no significant differences in locomotor activity between βAdd WT and βAdd KO mice over the three days of habituation (Fig. 8A). Following habituation, cocaine (15 mg/kg, i.p.) was administered once a day for 10 days, and locomotor activity was monitored for 30 min after cocaine administration. Both WT and βAdd KO mice showed significantly enhanced locomotor activity following acute cocaine administration (Fig. 8B). In addition, both groups of animals showed a sensitized locomotor response after 10 days of cocaine administration. Analysis of locomotor activity over daily sessions identified a significant interaction between genotype and treatment (Fig. 8B; Treatment × Genotype, $F_{1,34}$ = 7.691, p=0.009). βAdd KO mice showed enhanced locomotor activity compared to WT mice across all 9 days of testing (Fig. 8B; * $p \leq 0.05$ for *post hoc* comparison between and cocaine-treated WT and KO mice, Tukey's-HSD).

Discussion

Behavioral changes due to learning or drug exposure are thought to be the result of synaptic plasticity and neuronal adaptations that alter circuit level activity in the brain (Nestler, 2001). As a result, drug-induced structural changes in neuronal connections are mechanisms that likely underlie behavioral responses related to drug addiction (Robinson and Kolb 1999). Although the relationship between structural changes in neurons of the NAc and behaviors related to drug addiction has been investigated extensively, it is not clear how changes in spine density following repeated drug exposure contribute to behavioral responses to drugs of abuse (Robinson and Kolb 2004; Shen et al. 2009). Further, the molecular mechanisms contributing to spine remodeling following repeated drug exposure are still not well understood. In this study, we hypothesized that the cytoskeleton interacting protein βAdd might be important for spine remodeling in response to cocaine administration. In erythrocytes, βAdd contributes to membrane-cytoskeleton remodeling in a phosphorylation-dependent manner (Bennet et al, 1988). In the NAc, βAdd is phosphorylated following cocaine administration (Toda et al, 2006; Lavaur et al., 2009) suggesting that cocaine-dependent synaptic remodeling might depend on adducin signaling. We therefore used βAdd KO mice to investigate whether βAdd contributes to changes in spine density following repeated cocaine exposure.

The current set of experiments demonstrates that the density of mushroom spines is decreased at baseline in βAdd KO mice compared to their WT siblings. This decrease in spine density did not appear to disrupt behavioral sensitization to cocaine. Indeed, there was a statistically significant increase in the initial locomotor response to cocaine in βAdd KO

mice that was maintained following repeated cocaine exposure. Although the current data are correlational, one possibility is that the decrease in spine density in the βAdd KO mice at baseline disinhibited the initial locomotor response to cocaine. This would be consistent with previous studies demonstrating that decreased expression of the transcription factor MEF2 decreases spine density in the NAc, prevents spine remodeling in response to cocaine, and significantly increases the locomotor response to repeated cocaine administration (Pulipparacharuvil et al. 2008). Similarly, inhibition of CDK5 by roscovitine decreases spine density in the NAc, prevents cocaine-induced increases in NAc spine density (Norrholm et al. 2003) and increases the locomotor response to repeated cocaine (Taylor et al. 2007). In contrast, knockout of the cytoskeletal protein spinophilin decreases spine remodeling overall (Feng et al. 2000), prevents cocaine-induced changes in excitatory signaling and increases the locomotor response to cocaine (Allen et al. 2006, Wu et al. 2008). Thus, it seems possible that decreased spine density in the NAc could disinhibit the locomotor response to cocaine, supporting the suggestion that spine remodeling in the NAc is a counter-adaptive homeostatic response that opposes sensitization to cocaine (Chandler and Kalivas, 2008; Pulipparacharuvil et al. 2008; Kiraly et al. 2010).

In contrast to our prediction, knockout of βAdd did not prevent the cocaine-induced increase in mushroom spine density in the NAc following repeated cocaine administration. Indeed, there was a greater increase in mushroom spines in response to cocaine in βAdd KO mice as compared to in βAdd WT mice. This suggests that βAdd may normally stabilize spines, and that loss of βAdd is permissive for greater remodeling. A recent study showed that environmental enrichment increases spine density in the hippocampus in both WT and βAdd KO mice, but that synapse stability was greatly altered in the absence of β Add (Bednarek et al 2011). β Add KO mice showed enhanced spine turn over, implying that loss of β Add increases spine lability. This partially explains that increase of mushroom spine density under cocaine challenge. Alternatively, it is also possible that α/β Add heterodimers replace α/β Add function in the NAc following constitutive knockout of the β Add protein. Future studies that manipulate all three adducin family members will be required to resolve this question.

We used two independent methods to analyze spine density in this study. Spines can be classified into categories based on spine morphology (Garcia-Lopez et al. 2006, Jedynak et al. 2007.); however, there is some disagreement on how to classify spines and consistent parameters for spine classification have not yet been established. We therefore used both NeuronStudio and IMARIS software, which use different algorithms to classify spine types, to identify common changes in spine density in the NAc of βAdd KO mice. Importantly, the two complementary approaches identified very similar patterns of spine density changes. Both methods detected a decrease in mushroom spine density at baseline in βAdd KO compared to βAdd WT mice. Overall, both methods of analysis detected consistent proportions of mushroom and long spines, and identified a very similar level and pattern of change following cocaine exposure.

In conclusion, the current study suggests that the cytoskeleton-interacting protein β Add is important for regulating mature mushroom-shaped spines under basal conditions and stabilizing cocaine-induced spines on medium spiny neurons in the NAc. These mice also showed a greater locomotor response to cocaine in βAdd KO mice as compared to their WT siblings, supporting the idea that decreased spine number at baseline could result in a greater behavioral response to cocaine challenge. Finally, in the absence of βAdd, repeated cocaine administration can still increase dendritic spine number in the NAc, although other adducin family members (α and β) may replace βAdd following constitutive knockout. Taken together, these data support a potential role for βAdd in stabilization of dendritic spines in the NAc.

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Abbreviations

βAdd	β adducin
KO	knockout
NAc	nucleus accumbens
WT	wildtype

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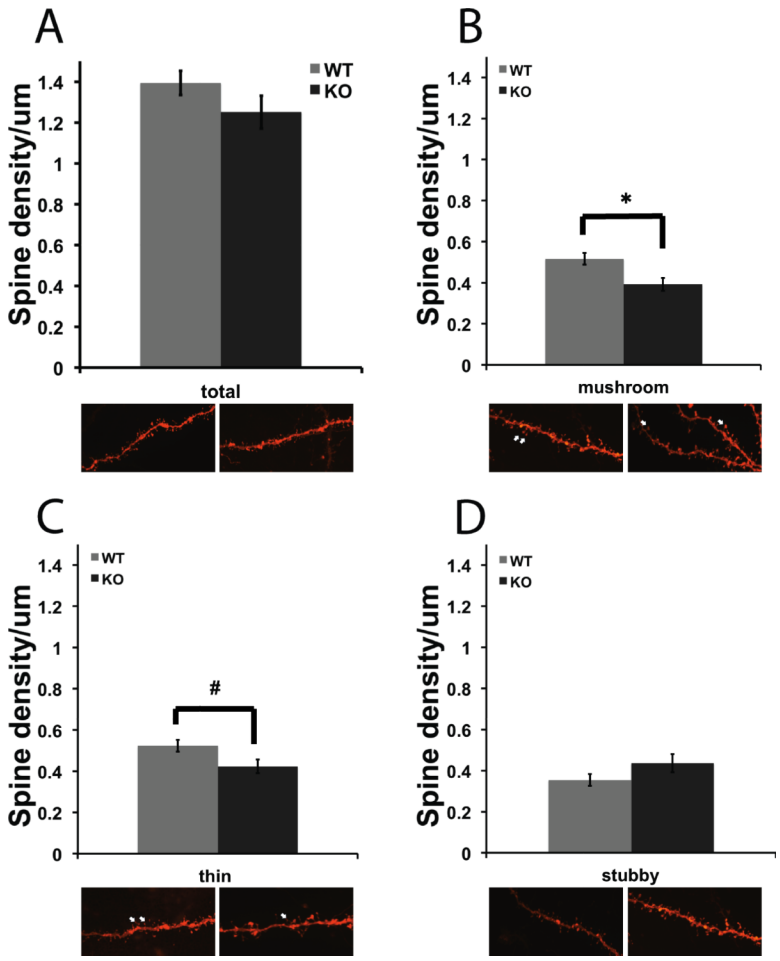

Figure 1. βAdd KO mice show decreased mushroom spine density at baseline

Dendritic spines on medium spiny neurons in the NAc were labeled with DiI and analyzed using NeuronStudio software. Spines were classified as A) thin, B) mushroom or C) stubby. There was a significant decrease in the number of mushroom spines (B; * $p \leq 0.05$, *post hoc* analysis, Tukey's-HSD). There was a significant decrease in density of thin spines in the NAc of βAdd KO mice as compared to WT mice (n=28 neurons from 11 WT saline treated mice, 43 neurons from 12 WT cocaine treated mice, 21 neurons from 18 KO saline treated mice, 29 neurons from 16 cocaine treated KO mice; $p \leq 0.05$, *post hoc* analysis, Tukey's-HSD).

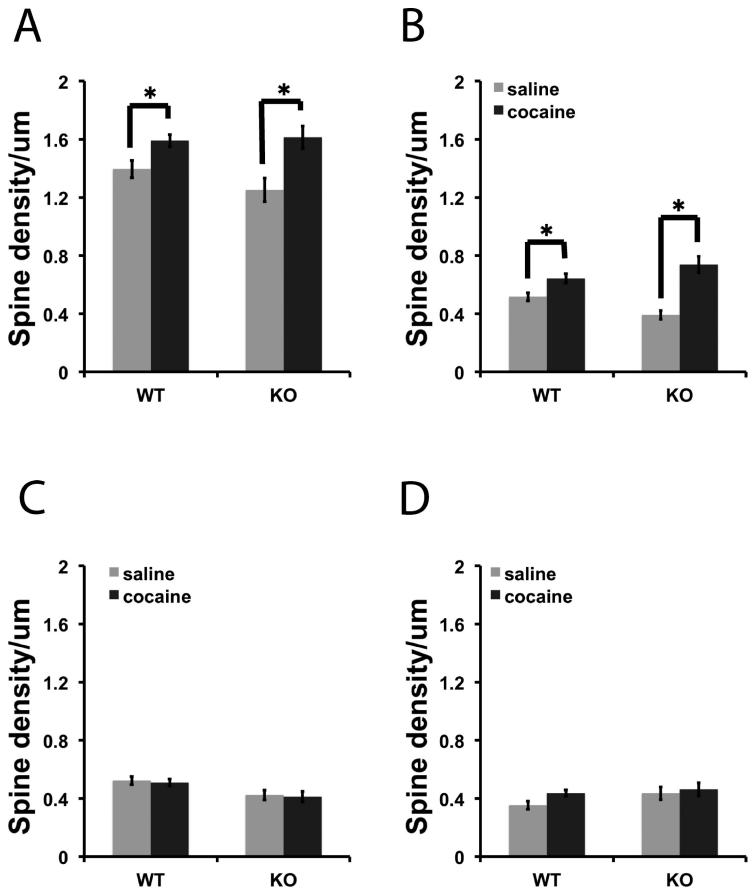

Figure 2. Cocaine treatment significantly increases overall spine density in βAdd KO and WT mice

Following repeated cocaine administration (15 mg/kg, , i.p.; 10 days), spine density was measured in the NAc of βAdd KO mice and their WT siblings. Density (spine number/um) of A) Total, B) mushroom, C) thin, and D) stubby spines was measured using NeuronStudio. There was a significant increase in the number of mushroom and thin spines in the NAc of βAdd WT and KO mice following cocaine treatment (*, $p \leq 0.05$ *post hoc* analysis, Tukey's-HSD).

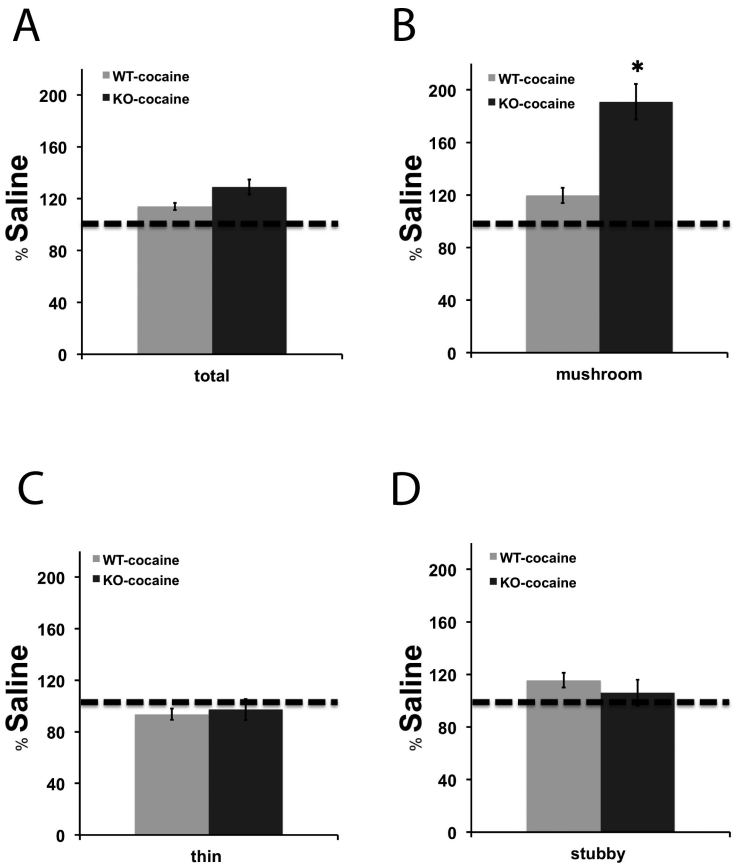

Figure 3. Mushroom spine density is greatly increased in βAdd KO mice following cocaine treatment

The percentage change in density of A) total, B) mushroom, C) thin, and D) stubby spines over saline-treatment was measured following repeated cocaine administration (15 mg/kg, , i.p.; 10 days), spine density was measured in the NAc of βAdd KO mice and their WT siblings using NeuronStudio. There was a significant increase in the density of mushroom spines in the NAc of βAdd KO mice following cocaine treatment (*, $p \leq 0.05$).

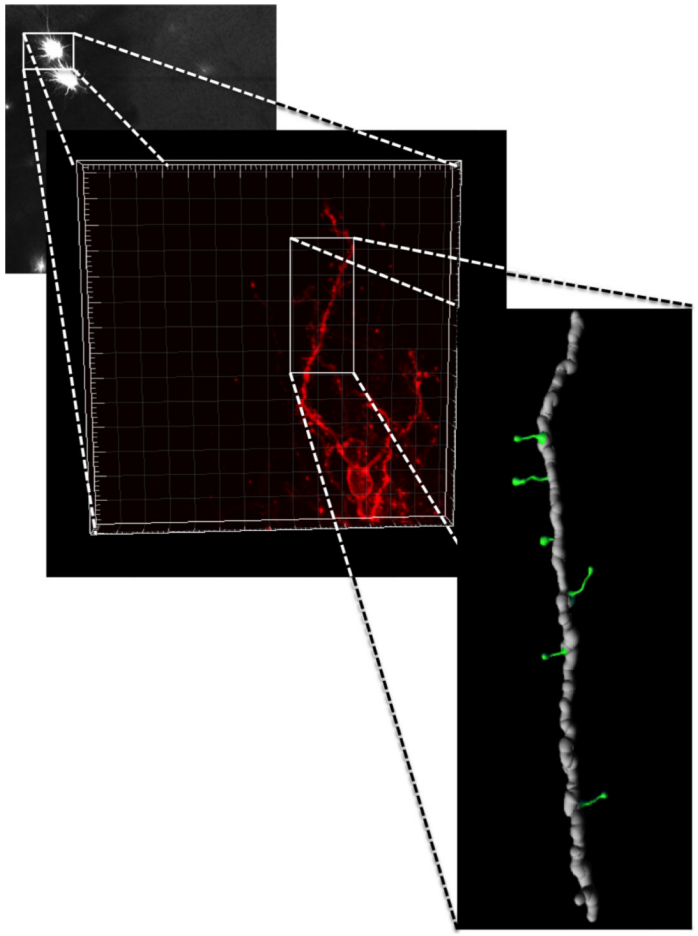

Figure 4. Three-dimensional reconstruction of confocal images

DiI labeled medium spiny neurons in NAc were imaged and DiI was excited using the Helium/Neon 543 nm laser line. Each neuron was scanned at 0.15μm intervals along the z-axis (maximum 90 planes, depending on the depth of whole neuron and objective WD), and the morphology of the dendritic tree was reconstructed in 3-D for analysis with IMARIS software.

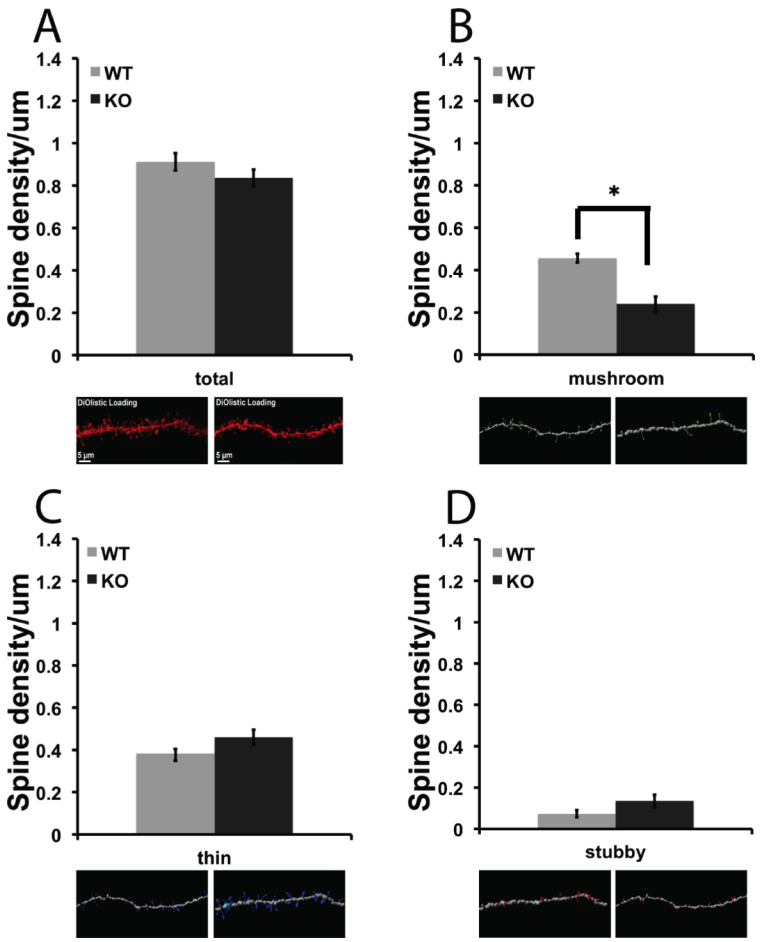

Figure 5. Analysis using IMARIS software confirms a significant baseline decrease in NAc spine number in βAdd KO mice

Using IMARIS software as an independent method of spine analysis, the density (spine number/μm) of A) total, B) mushroom, C) thin, and D) stubby spines was measured. There was a decrease in mushroom spine number in βAdd KO mice as compared to their WT siblings at baseline (*, $p \leq 0.05$ *post hoc* analysis, Tukey's-HSD).

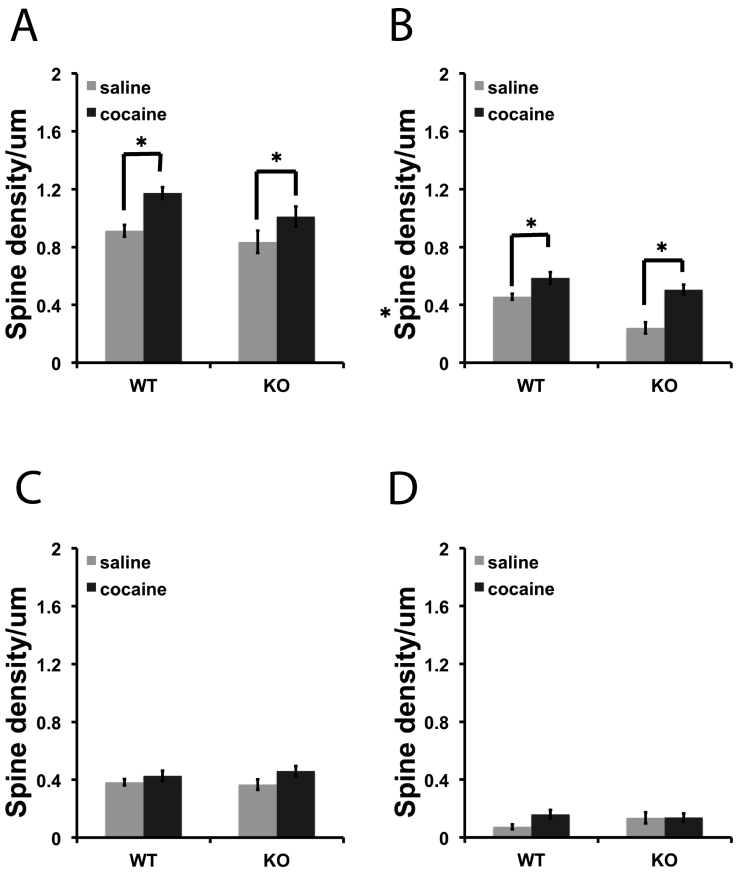

Figure 6. Cocaine treatment significantly increases spine density in βAdd KO and WT mice as analyzed using IMARIS software

Following repeated cocaine administration (15 mg/kg, , i.p.; 10 days), spine density was measured in the NAc of βAdd KO mice and their WT siblings using IMARIS software. Density (spine number/um) of A) Total, B) mushroom, C) thin, and D) stubby spines was measured. There was a significant increase in the number of mushroom and thin spines in the NAc of βAdd WT and KO mice following cocaine treatment (*, $p \leq 0.05$ for *post hoc* comparison between cocaine treatment and saline treatment in WT and KO mice, Tukey's-HSD).

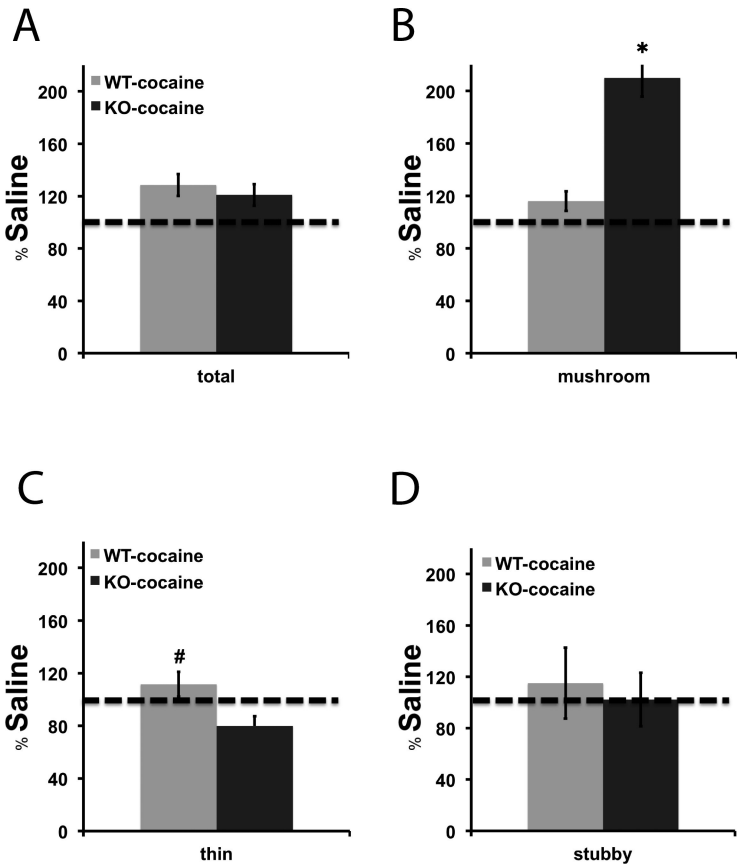

Figure 7. An independent analysis method confirms that mushroom spine density is selectively increased in βAdd KO mice

The percentage change in density of A) total, B) mushroom, C) thin, and D) stubby spines over saline-treatment was measured following repeated cocaine administration (15 mg/kg, , i.p.; 10 days), spine density was measured in the NAc of βAdd KO mice and their WT siblings using IMARIS software. There was a significant increase in the density of mushroom spines in the NAc of βAdd KO mice (*, $p \leq 0.05$).

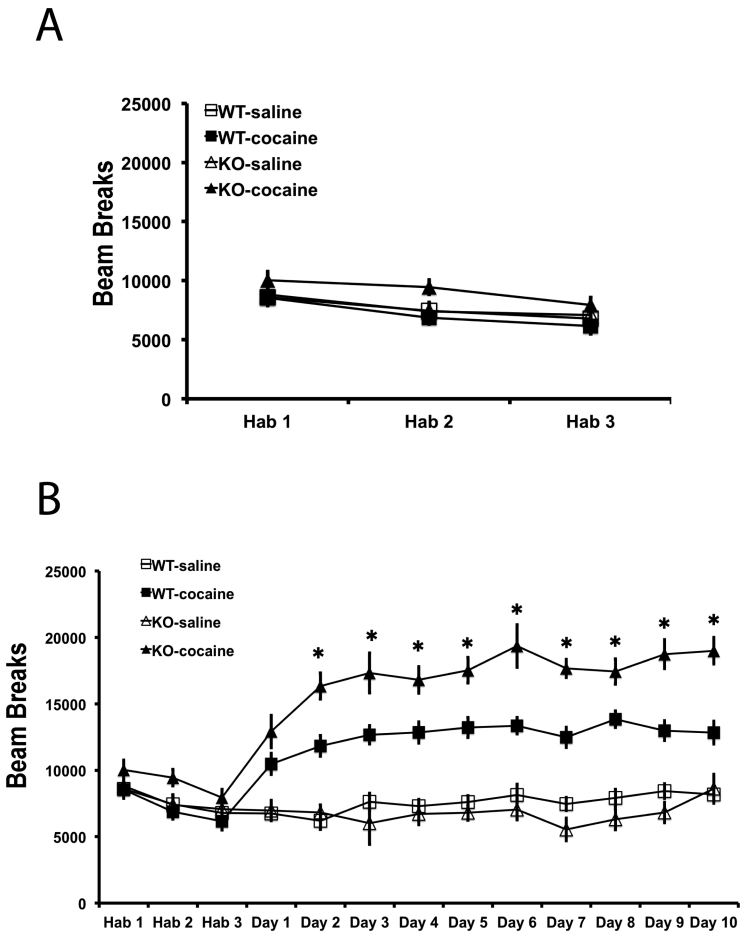

Figure 8. βAdd KO mice show an increase in cocaine-induced locomotor activity

βAdd WT and KO mice were A) habituated to the locomotor apparatus for 3 days and then B) administered cocaine (15 mg/kg, i.p.) once a day for 10 days (WT-Sal: n=11, WT-Coc: n=18, KO-Sal: n=12, KO-Coc: n=16). Locomotor activity was monitored for 30 min after saline or cocaine administration. A) There were no significant differences in locomotor activity between groups during the habituation period. B) Locomotor activity was significantly increased in βAdd KO mice compared to their WT siblings following repeated cocaine administration (*, $p \leq 0.05$ for *post hoc* comparison between cocaine-treated WT and KO mice, Tukey's-HSD).