Rapid synaptogenesis in the Nucleus Accumbens is induced by a single cocaine administration and stabilized by MAP Kinase interacting kinase 1 activity

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ABSTRACT

Background
Repeated cocaine exposure produces new spine formation in Striatal Projection Neurons (SPNs) of the Nucleus Accumbens (NAc). However, an acute exposure to cocaine can trigger long-lasting synaptic plasticity in SPN leading to behavioral alterations. This raises the intriguing question as to whether acute cocaine could modify enduringly striatal connectivity.

Methods
A 3D morphometric analysis of presynaptic glutamatergic boutons and dendritic spines was performed on SPN one hour and one week after a single cocaine administration. Time-lapse two-photon microscopy in adult slices was used to determine the precise molecular events sequence responsible for the rapid spine formation.

Results
A single injection triggered a rapid synaptogenesis and persistent increase in glutamatergic connectivity in SPN from the shell part of the NAc, specifically. Synapse formation occurred through clustered growth of active spines contacting pre-existing axonal boutons. Spine growth required ERK activation, while spine stabilization involved transcription-independent protein synthesis driven by MAP kinase interacting kinase-1 (MNK-1), downstream from ERK. The maintenance of new spines driven by MNK-1 was essential for long-term connectivity changes induced by cocaine in vivo.

Conclusions
Our study originally demonstrates that an acute administration of cocaine is able to induce stable synaptic rewiring in the NAc, which will likely influence responses to subsequent drug exposure. It also unravels a new functional role for cocaine-induced ERK pathway
independently of nuclear targets. Finally, it reveals that MNK-1 has a pivotal role in cocaine-induced connectivity.
INTRODUCTION

Cocaine administration induces long-lasting changes in behaviors that result from synaptic plasticity and morphological changes in neurons of the brain’s reward circuitry (1,2). Cocaine increases dopamine (DA) in the Nucleus Accumbens (NAc) (3), with dendritic spines from striatal projection neurons (SPN) receiving glutamate inputs informative of contextual information, together with DA inputs that code for reward signals (4, 5) and modulate the efficacy of glutamatergic synapses (6, 7). Most studies assessing structural changes in the striatum have focused on the observation of dendritic spine density changes induced by chronic cocaine in the adult striatum. However, in-depth studies of synaptogenesis and connectivity changes, which require the analysis of both pre and postsynaptic elements (8,9), remained largely unexplored until recently. To assess this issue we have set up a method that allows for 3D automated morphological analysis of synaptic connections onto SPN (10). Using VGLUT1\textsuperscript{Venus} mice, which express the vesicular glutamate transporter 1 (VGLUT1), a presynaptic protein present in afferents from cortices (neocortex, hippocampus and amygdala) fused to the fluorescent protein Venus (11), we showed that new spines formed after chronic exposure to cocaine were indeed connected to presynaptic cortical terminals (10). How and when these new glutamatergic synapses are formed within the striatum still remains a key issue, which has to be addressed to disentangle the cellular bases responsible for the initiation of neuronal plasticity mediated by cocaine. It was recently shown that DA gated glutamate driven spine enlargement in the NAc in a narrow time window, thereby providing a mechanism for reward-driven learning at the level of the dendritic spine(12). Furthermore, the synergistic interaction between D1R and glutamate NMDA receptor induces activation of the MAPkinase/ERK (extracellular-signal regulated kinase) pathway (13-15), which plays a key role in cocaine-induced neuronal and behavioral adaptations (16-19). The ERK pathway also regulates spine formation induced by chronic cocaine administration onto SPN expressing the
DA receptor of type 1 (D1-SPN) (20, 21). Classically, these long-term adaptations are thought to rely on gene transcription and epigenetic regulation (22). However, it was recently demonstrated that a single injection of cocaine was sufficient to cause an ERK-dependent synaptic plasticity and subsequent behavioral adaptations induced by a second administration of cocaine (23, 24). We therefore hypothesized that a single injection of cocaine was able to induce a rapid and stable modification of striatal connectivity. To address these issues, we investigated the changes in glutamatergic connectivity, along with the rules of synapse formation, as well as the associated molecular mechanisms. We bring evidence that an acute administration of cocaine is able to induce stable synaptic rewiring in the NAc and demonstrate that the ERK pathway plays a dual role in the growth and stabilization of new spines in D1-SPN.
METHODS AND MATERIALS

Animal care.

Animal care of wild-type C57BL/6 and VGLUT1-venus knock-in was conducted in accordance with standard ethical guidelines (NIH publication no. 85-23, revised 1985 and European Committee Guidelines on the Care and Use of Laboratory Animals 86/609/EEC), and the experiments were approved by the local ethic committee.

In vivo treatments, tissue preparation.

Intraperitoneal injection of cocaine was performed. When indicated anisomycin (100 mg/kg) or SL327 (50 mg/kg) were injected intraperitoneally 30 min and 60 min, respectively, prior to cocaine. For CGP57380 treatment, cannulae were pre-implanted in the NAc shell in anesthetized 7-8 weeks-old mice. One week later, unilateral injections of 10 mM CGP57380 were performed, along with contralateral vehicle injection, followed by cocaine administration 15 min later. Mice brains were fixed in situ after intracardiac perfusion of paraformaldehyde (PFA) and coronal sections were cut with a vibratome. Dendrites were labeled with DiI using diolistic method.

Dendritic spine analysis, segmentation of presynaptic boutons and detection of synaptic contacts from confocal images.

Dendritic spine detection was performed using the Neuronstudio software and data were imported into ImageJ for custom 3D automated spine heads segmentation. Segmentation of VGLUT1 objects and detection of synaptic contacts were performed in 3D as described previously (10). Border to border distance between each spine head and all VGLUT1 objects was computed and the synaptic contact was decided for a distance of zero, meaning that the two objects overlap.

Viral infections and preparation of slices for two-photon microscopy.
The AAVrh9-PPTA-Cre viral construct contains an expression cassette consisting of the Cre recombinase driven by the promoter of PPTA (precursor of substance P) gene, which is specifically expressed in SPN expressing D1R (12). The virus was injected in the NAc of 4-week-old mice in combination with the AAVrh9-pCAG-flex-tdTomato-WPRE virus and when stated AAV1-hSyn-flex-GCaMP6S-WPRE virus. Coronal slices of 200-250 µm thickness of brains from 6 to 8 week-old mice were cut on a vibratome and maintained in equilibrated ACSF. Slices were stimulated with glutamate 3 µM and (R)-(+)SKF-38393 10 µM in ACSF for 20 min, followed by 40 min in ACSF alone. Pharmacological inhibitors were applied 30 min before co-stimulation and washed 30 min after the end of the co-stimulation. Slices were either imaged in time-lapse or fixed with PFA 4% at RT for 60 min, processed for DiI dendrite labelling or immunofluorescence. For time lapse studies, slice imaging was performed in ACSF with two-photon upright microscope (SP5 MPII, Leica) equipped with water-immersion objective (25x, NA 0.95, Leica) and images were acquired with hybrid detector (Leica). Image stacks were sampled with voxels size of 100 nm in xy axis and z-step of 500 nm.

See Supplemental information for fully detailed experimental procedures
RESULTS

A single cocaine exposure induces a rapid and stable connectivity change in the NAc shell

We investigated spine formation and potential changes of striatal connectivity induced by a single administration of cocaine. Dendritic spine density of SPNs from the NAc shell was increased one hour after a single injection of cocaine (Figure 1A-C). A similar increase was observed at a lower dose (10 mg/kg, Supplemental Figure S1A), while no change was observed either in the NAc core, or in the dorsal striatum at higher doses (Supplemental Figure S1B, C). Upon cocaine administration, activated D1-SPNs express immediate early genes, including c-Fos (25). Spine density increase in SPNs expressing c-Fos was greater when compared to randomly picked SPN (Figure 1A-C), thus indicating that cocaine-induced spine formation occurred in activated SPNs. VGLUT1-positive boutons from glutamatergic axons were segmented, and synaptic contacts were identified by measurement of the 3D contacts between spine heads and boutons. About 60% of spines were connected to VGLUT1-positive boutons in saline conditions (Figure 1A, E), a result in agreement with electron microscopic studies (26). One hour after cocaine administration the number of spines in contact with a VGLUT1 bouton was increased (Figure 1A, D), indicating that acute cocaine rapidly increases glutamatergic connectivity in the NAc. Of note, the percentage of spines connected to VGLUT1 boutons remained unchanged, indicating that the increased connectivity did not modify the stoichiometry of VGLUT1 connections onto SPN (Figure 1E). We next assessed whether this rapid increase in connectivity was stable over time. One week after a single injection of cocaine, the increase in spine density and synaptic contacts were still present, while the percentage of VGLUT1-positive synapses remained stable (Figure 1F-I). An increased spine density was also found 28 days after a single administration of cocaine (Supplemental Figure S1D). Following a custom 3D procedure for spine heads segmentation (Figure 2A), the measurements of spine head volume and spine length showed
an increase one hour after cocaine administration, which was better revealed in c-Fos positive neurons (Figures 2B, C). One week after cocaine administration, spine head volume, but not spine length, was still larger in cocaine-treated mice as compared to saline controls (Figures 2D, E). The increased volume of spine heads corresponded to an increase in mushroom-shaped spine density that was significantly increased one hour and one week after a single cocaine administration (Figures 2F, G), indicating a rapid maturation of the newly-generated spines.

**Synapse formation occurs in clusters with spines connecting pre-existing presynaptic boutons**

The total density of VGLUT1-positive boutons in the NAc-shell was not increased either one hour or one week after cocaine administration (Figures 3A, B, left panels). Since synaptogenesis occurred, this indicates that new spines contact pre-existing presynaptic boutons. Accordingly, the occurrence of spines connected to a common VGLUT1-positive bouton—an observation reported in electron microscopy study (26)—was increased one hour and one week after cocaine injection (Figure 3A, B, right panels). The rules of synapse formation were further studied using live imaging in acute adult striatal slices. In order to recapitulate the effects of cocaine on SPN, we applied a co-stimulation with a low dose of glutamate together with a D1R agonist, as previously shown (13, 14). As expected, the co-stimulation, but not the application of each agonist independently, induced ERK activation and increased spine density and synaptic contacts in the NAc-shell (Supplemental Figure S2).

Time-lapse experiments were performed on D1-SPN since cocaine induces ERK activation (25) and dendritic spine increase (21, 27) in this subpopulation. To this end, D1-SPN were sparsely labeled by injecting in the NAc from adult mice a combination of an adeno-associated virus (AAV) carrying tdTomato sequence flanked by loxP sites and a virus expressing Cre recombinase under the promoter of preprotachykinin A (PPTA), which is specifically expressed in D1-SPN (12), as confirmed by immunodetection of the D1R
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Striatal slices were performed two weeks after viral-infections, and time-lapse imaging of D1-SPN was performed by two-photon microscopy. Dendritic spine growth occurred as soon as 25 minutes after the beginning of the co-stimulation, and reached a plateau at 60 min (Figure 3C, D) and was absent when the selective D1R antagonist SCH23390 was applied (Supplemental Figure S3C). The amplitude of spine density increase at one hour was +21% (Fig. 3E).

A single cocaine administration occludes synaptic potentiation induced in striatal slices (23). We next tested whether previous cocaine administration could alleviate the spine growth induced by the co-stimulation in striatal slices. The spine density increase induced by cocaine in vivo was preserved in the slices before co-stimulation (Supplemental Figures S4A, B). A partial occlusion was observed in slices from mice acutely administrated with cocaine, since co-stimulation induced spine growth but the percentage of increase was not significant. On the other hand, chronic administration of cocaine led to complete occlusion (Supplemental Figures S4C-D). Those results are consistent with observation in the prefrontal cortex in vivo of progressive decline of spine density increase upon repeated injection of cocaine (28).

The connectivity and functionality of spines observed in time-lapse were then explored. In acute slices from VGLUT1VENUS mice 62.8% of spines induced by the co-stimulation were in contact with a VGLUT1-positive bouton (5 mice, n = 35 spines). Newly-generated spines entered in contact with a VGLUT1-positive bouton that was in all cases present before spine growth (5 mice, n = 22 spines) (Figure 3F), which is consistent with our in vivo observation of increased density of synaptic contacts along with constant density of VGLUT1 boutons upon acute cocaine. In order to assess the functionality of the newly formed spines, the viruses AAV-PPTA::Cre, AAV-FLEX-tdTomato and AAV-FLEX-GCaMP6S were injected in the NAc. Spine growth and calcium events were analyzed in D1-SPN from striatal slices (Fig. 3G, left panel). Following co-stimulation, 6 out of 10 newly formed spines in the NAc
responded by an increase in calcium content. These functional spines had a bigger head diameter than the newly formed spines that did not respond to the stimulation (Figure 3G). This kinetics of maturation is in accordance with previous studies from other neuronal types (29-32). The fact that new spines contact axonal boutons already in place raised the question as to whether synaptogenesis could be driven by a stochastic spine growth. We generated an \textit{in silico} simulation of synaptogenesis from random positions on the dendricle, and found that it did not reproduce the experimental data (Supplemental Figures S5A, B). Moreover, the analysis of spine distribution \textit{in vivo} revealed a bimodal distribution with a high proportion of adjacent spine heads, which was increased after cocaine exposure (Supplemental Figures S5C, D). Hence, spine growth does not occur randomly but at locations where synapse clusters are generated. Taken together, our results indicate that new spines grow in clusters, are rapidly functional and contact pre-existing VGLUT1 boutons.

\textbf{A single administration of cocaine induces the formation of thalamo-striatal synaptic contacts}

Since a single administration of cocaine induced an increase in VGLUT1-positive synaptic contacts while keeping the ratio of spines connected to VGLUT1 afferents (see Figure 1), it was plausible that the unconnected spines would correspond to thalamo-striatal synapses, which are VGLUT2-positive (33). D1-SPN were labeled by \textit{in vivo} viral infections (AAV-PPTA::Cre and AAV-FLEX-tdTomato, and the mice were treated with a single administration of cocaine. VGLUT2-positive boutons were immunolabeled and thalamo-striatal synaptic contacts were analysed (Figure 4). The density of VGLUT2-connected spines significantly increased upon cocaine administration, thus indicating that acute cocaine also induces the formation of thalamo-striatal synapses.

\textbf{Dendritic spine growth and stabilization are regulated by ERK and MNK-1}
We next investigated the signaling pathways involved in spine formation induced in striatal slices one hour after the co-stimulation. A pretreatment with U0126, a selective inhibitor of the ERK pathway, abolished the spine density increase induced by the co-stimulation (Figure 5A), revealing that the ERK pathway is necessary for spinogenesis. Similarly, rapamycin an inhibitor of the mammalian target of rapamycin (mTOR) pathway completely blocked the increase in spine density induced by co-stimulation in slices (Figure 5B). Since the ERK and mTOR pathways converge on the control of protein synthesis via the eIF complex, we investigated the possible implication of MNK-1, a cytoplasmic substrate of ERK, which activates the eIF complex to initiate protein translation (34)(Figure 5C). An increase in phosphorylated MNK-1 (pMNK-1) positive cells was measured by immunodetection in the NAc twenty minutes after co-stimulation (Figure 5D). Slices were then pre-treated with CGP57380, a specific inhibitor of MNK-1 activation, which totally inhibited the increase in spine density induced by the co-stimulation (Figure 5E).

In order to gain further insights into the specific role of ERK and MNK-1 in spine growth and maintenance, we used time-lapse imaging from striatal slices (Figure 6A, B). D1-SPN dendrites were labeled in vivo after intra-NAc infection with AAV PPTA::Cre and AAV-FLEX-tdTomato. Spine growth was absent in the presence of U0126, the inhibitor of the ERK pathway (Figure 6C), indicating that ERK activation is mandatory for the formation of new spines. In contrast, CGP57380 did not modify growth rate, clearly indicating that MNK-1 is not required for spine growth (Figure 6B, C). However, it strongly impacted the maintenance of the newly formed spines, with a loss of 59.1% and 91% of newly-formed spines, 30 minutes and 90 minutes after growth, respectively (Figure 6B, D). At this later time point, 67% of newly-formed spines remained stable in control conditions, as previously reported (27)(Figure 6B, D). Noteworthy, the inhibition of MNK-1 activation did not affect the stability of spines present before stimulation (Supplemental Figure S6). Hence these data
show that MNK-1 is mandatory for stabilization but not for spine growth. Since MNK-1 regulates protein synthesis, we interrogated the respective role of global transcription and translation for spine growth and maintenance. Inhibition of protein synthesis with anisomycin did not modify spine growth, but reduced spine stabilization, with identical kinetics and amplitude to that observed with the MNK-1 inhibitor (Figure 6B, D). By contrast, bath application of the transcription inhibitor actinomycin D did not alter either growth or stabilization of new spines (Figure 6B, D). Therefore, time-lapse analysis unraveled sequential molecular events involved in spine growth and stabilization. While ERK is essential for both steps of spine formation, MNK-1 activation and protein synthesis selectively control spine stabilization independently of transcription.

**MNK-1 is involved in structural plasticity in vivo**

We analyzed the impact of MNK-1 activation on long-lasting spinogenesis in vivo. An increase in pMNK-1-positive cells was observed in the NAc 10 minutes after a single cocaine administration, and the inhibition of ERK activation by SL327 completely abolished this increase (Figure 7A, B), thus confirming that cocaine-induced MNK-1 phosphorylation is strictly dependent on ERK activation. The MNK-1 phosphorylation state was transient, with return to basal levels 30 minutes after cocaine administration (Figure 7B). The dendritic localization of pMNK-1 in D1-SPN was shown by immunodetection of pMNK-1 in dendrites labeled with the PPTA::Cre and FLEX-tdTomato AAV. pMNK-1 positive punctates were detected in the dendritic shaft and in dendritic spines from D1-SPN (Figure 7C), further demonstrating the local activation of MNK-1. In agreement with the results from acute slices, inhibition of ERK or protein synthesis abolished spine formation one hour after cocaine administration in vivo (Figure 7D). The activation of MNK-1 was inhibited locally by intracranial injection of CGP57380 in the shell part of the NAc prior to either saline or
cocaine single administration. The local inhibition of MNK-1 did not affect basal spine density of SPN, but abolished the spine density increase observed 1 and 24 hours after cocaine administration (Figure 7E). Taken together, these results identify MNK-1 as a cytoplasmic target downstream from ERK activated by cocaine and posit MNK-1 and protein synthesis as essential for striatal structural plasticity \textit{in vivo}. 
DISCUSSION

Our data show that a single administration of cocaine is sufficient to trigger long-lasting increase in glutamatergic connectivity in the NAc, with new spines growing in clusters and contacting pre-existing presynaptic boutons. We unravel the sequence of signaling events specifically involved in spine growth and stabilization. Spine growth was transcription and translation-independent, thus supporting a key role of ERK-induced phosphorylation of pre-existing proteins in this process. By contrast, spine stabilization required an ERK-mediated proteins synthesis via MNK-1, independently of transcription. Finally, we identify MNK-1 as essential for long-term connectivity changes induced by cocaine (Figure 8).

It has been long thought that persistent structural plasticity/morphology changes were selectively engaged following repeated administration of cocaine (10, 21, 28, 35, 36). Here we provide evidence that a single administration is sufficient to induce a rapid and persistent change in glutamatergic connectivity in the NAc. The rapid formation of mushroom-shaped spines with increased spine head volumes in vivo, and our calcium imaging in adult slices, both showed a rapid functional maturation of nascent spine. These observations fit with electrophysiological recordings performed after acute exposure to cocaine, showing an increase in the frequency of miniature currents in c-Fos positive SPN (37), and synaptic potentiation (23). Interestingly, repeated cocaine regimen leads to an increased spine density along with unchanged (10, 21) or decreased spine head size (36, 38), and the presence of immature spines is coherent with the observation of LTD (39) and generation of silent synapses (40, 41), which are not induced by acute cocaine injection (37). The shift from potentiated mature spines to silent immature spines is an active process, since chronic regimen recruits specific molecular pathways involved in dendritic spine remodeling, such as deltaFosB accumulation (42) and inactivation of Rac1 (43), which are not observed after...
single cocaine injection. In this context, it is noteworthy that dendritic spine maturation and unsilencing of synapses are observed after a 3 weeks period of cocaine withdrawal (38, 44) and following a challenge injection (35, 39). Hence, the morphology of spines is dynamically regulated during the different stages of cocaine administration and addiction development (45), through the combined involvement of metaplasticity and homeostatic processes (46, 47).

Whether VGLUT1 afferences arising from hippocampus, cortex or amygdala are similarly engaged onto new synapses remain to be established. On the other hand, we reveal that thalamic, VGLUT2-positive, synaptic contacts are generated after a single administration of cocaine. Along with previous work showing a plasticity of thalamo-striatal synapses upon chronic cocaine treatments (48, 49), our results pave the way for future study on the role of thalamic inputs on D1-SPN in long term adaptation induced by cocaine. In line with our results, it was previously observed that acute cocaine induces an increase in spine density in dopaminergic neurons (50) and in the prefrontal cortex (28), although those studies did not assess the connectivity of dendritic spines. Our results bring evidence that acute cocaine is a first essential step that primes the meso-cortico-limbic connectivity, which implies that subsequent cocaine action will operate on a modified network.

Our results also unravel the rules of synapse formation that leads to this connectivity change. Previous studies showed that new spines could make contact with pre-existing boutons (30, 51-54) and our in vivo as well as time-lapse observations in slices extend this mode of formation to striatal neurons (Figure 8). This mode of formation might imply a constraint on the location of spine growth. Accordingly, our modeling of synaptogenesis revealed the occurrence of clustered spines along the dendrite, indicating that the position of spine growth along the dendrite is actively regulated. The formation of dendritic spines in clusters has been studied in other brain structures (55-57) where they have implication on the integrative properties of the dendrite (58). Our study reinforces these observations, and further
show that newly-formed spines rapidly form synapses with pre-existing glutamatergic boutons. Moreover, we observed in vivo spine clusters in contact with a single bouton, which implies a temporal and spatial synchrony of the glutamatergic inputs on these spines. In conclusion, our observations of both pre and postsynaptic elements combined with a model of spinogenesis, which includes the constraint of synaptic contact formation, allows a better understanding of the rules of connectivity changes in the adult striatum.

It has been shown that dopamine gates glutamate-mediated spine enlargement in SPN via D1R (12). Along those lines, we have previously demonstrated that cocaine effects result from D1R gated NMDAR-mediated ERK activation (13-15). Here we observed that both a D1R agonist and Glu are required for ERK-dependent synaptogenesis in SPN downstream from D1R. Accordingly, it was recently shown that a single administration of cocaine can produce an ERK-dependent occlusion of synaptic potentiation on SPN of the NAc, and optogenetic inhibition of cocaine-induced plasticity prevented the behavioral adaptations induced by cocaine (23). Altogether, these data support that ERK-induced rapid synaptogenesis might be a critical cellular mechanism involved in the induction of long-term striatal plasticity and in the development of behavioral alterations induced by cocaine. We further demonstrate that synaptogenesis can be divided in different phases, with at least two distinct molecular mechanisms downstream the ERK pathway (Figure 8). We reveal a transcription and translation-independent role of ERK in the initiation of spine formation. While a previous study has shown that ERK activity is essential for spine morphogenesis in developing cortical neurons (32), we make the first demonstration of a functional role of cocaine-induced ERK activation independently of nuclear targets. It may likely imply the phosphorylation of pre-existing proteins, such as spinophilin, which upon phosphorylation by ERK, can increase spine protrusion density in hippocampal neurons (59).
We further show that spine stabilization requires MNK-1 activation and local protein synthesis independently of transcription. It is now clearly established that, downstream from ERK, MNK-1 phosphorylates initiation factors essential for the onset of local translation required for long-term plasticity (60, 61). Moreover, cocaine administration can induce synaptic plasticity via a mechanism that involves translation independently of transcription (62). Of interest, local synaptic translation also involves the mTOR signaling pathway, which is activated downstream Rap1b, a small GTPase essential for spine formation upon chronic cocaine administration (38). Several messengers are translated in dendrites (63) and could be involved in the stability of newly formed spines. The local translation of Arc is controlled by MNK-1 (64) and Arc proteins regulate spine density and morphology (65). Furthermore, the expression of Arc is induced in dendrites of D1R-SPN upon acute cocaine administration (66). PSD-95 is another candidate locally translated in response to intra-NAc injection of a D1R agonist (67), and involved in spine stabilization (68, 69). Importantly, we show that cocaine induces a rapid ERK-mediated phosphorylation of MNK-1 in dendrites of SPNs in the NAc, and its pharmacological inhibition leads to a loss of spine density increase observed one hour and 24 hours after a single administration of cocaine. Hence stabilization of newly-formed spines via MNK-1 is essential for long-term changes in glutamatergic striatal connectivity in the NAc.
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REFERENCES


FIGURE LEGENDS

Figure 1. A single administration of cocaine induces the rapid formation of stable glutamatergic synapses in the NAc

(A) Upper panel: time-course of the experiment. Following three days of habituation, VGLUT1venus mice were injected with either saline or cocaine (ip, 20mg/kg) and perfused one hour later. Lower panel: 3D surface rendering of dendritic segments labeled by DiI (red) with the VGLUT1 boutons (green) in contact with spines. Spine heads were segmented and the associated boutons automatically selected using a 3D custom procedure implemented in ImageJ.

(B) Immunostaining for c-Fos following diolistic labeling of striatal neurons allowed the identification of SPN that responded to cocaine.

(C) Spine density of SPN from the NAc was measured one hour after a single exposure to saline or cocaine. Spine density was measured in randomly chosen SPN from saline and cocaine-treated mice and in c-Fos positive SPN from cocaine-treated mice.

(D) Synapse density, defined as the density of spines in contact with VGLUT1 boutons, is increased by acute cocaine.

(E) Percentage of spines connected to a VGLUT1 bouton is not altered by cocaine.

(F) Spine density from saline and cocaine-treated mice was measured one week after a single cocaine injection preceded by three days of habituation. Images illustrate the result of the 3D automated analysis of synaptic contacts.

(G) Measurements of spine density one week after a single exposure to cocaine.

(H) Density of VGLUT1-positive synapses was measured one week after a single injection of cocaine. Note the persistent connectivity change induced by cocaine.
The percentage of VGLUT1-positive synapses remains constant one week after cocaine administration.

Scale bar in A and F = 5 microns, B = 10 microns. Histograms represent mean ± SEM with n = number of neurons analyzed from N = 4-5 mice in each condition. C-E: One-way ANOVA and Bonferroni post-test. G-I: T-test. *: p < 0.05, ***: p < 0.001. See supplemental figure S1 and S2.

**Figure 2. Effect of a single cocaine exposure on spine morphology of SPN from NAc**

(A) Results of the custom 3D segmentation of spine heads implemented in ImageJ software. A1: 3D volume rendering of a dendrite. A2: 3D volume rendering of the spine heads extracted with a custom procedure implemented in ImageJ, merged with 3D surface rendering of the dendrite. A3: 3D volume rendering of segmented head spine merged with 3D surface rendering of the original dendrite image.

(B-C) Cumulative frequency distribution of head spine volumes (B) and length (C) measured in neurons from mice perfused one hour after treatment with either saline or cocaine. When indicated (Cocaine c-Fos+) spines were quantified from neurons identified as SPN expressing c-Fos. Note that one hour after cocaine injection spines have a bigger head (B) and are longer (C) than in saline controls.

(D-E) Cumulative frequency distribution of head spine volumes (D) and length (E) measured in neurons from mice perfused one week after single injection of either saline or cocaine. One week after cocaine the spine heads volume (D) remained increased upon cocaine but spine length (E) was identical to saline controls.

(F-G) Dendritic spines were classified as thin or mushroom based on the diameter of the spine head. An increase in mushroom-shaped spine density is observed both one hour (F) and one week (G) after a single cocaine administration.
3900 to 6200 spines analyzed in n = 29 to 59 neurons from N = 4 mice in each condition. B-E: Kolmogorov-Smirnov test of distribution, F: One-way ANOVA and Bonferroni post-test, G: T-test. *: p < 0.05; **: p < 0.01, ***: p < 0.001.

**Figure 3. Synapse formation through contact with pre-existing presynaptic boutons**

(A-B) Density of VGLUT1\textsuperscript{Venus} boutons in the NAc, in a volume corresponding to a cube of 10 microns side. A single plane from confocal image stacks is shown (left panels). The density is similar in saline and cocaine-treated mice one hour A), and one week B) after a single cocaine injection (Mean ± SEM, t-test). Right panels: Percentage of spines connected to a common VGLUT1 bouton (Median ± Interquartiles, Mann Whitney test). N mice and n neurons are identical to figure 1.

(C) Maximum intensity projection of a dendritic segment imaged from tdTomato-labeled D1-SPN in acute striatal slice with two-photon microscopy. D1-SPN were labeled by in vivo co-infection with an AAV9 expressing Cre recombinase under PPTA promoter and an AAV9 carrying FLEX-tdTomato. Two weeks later, slices are stimulated with SKF38393 (10 µM) and glutamate (3 µM) for 20 minutes then washed with control ACSF. Green arrows indicate locations of newly-formed spines.

(D) Cumulative curve of spine growth represents the percentage of new spines using the number of spines present before the co-stimulation as reference. The increase in spine growth becomes significant between control and stimulated D1-SPN 25 minutes after the beginning of the stimulation. Two-way ANOVA and Bonferroni post-test.

(E) Comparison of spine density between 0 and 60 minutes time points confirms an increase in stimulated condition similar to \textit{in vivo} conditions (see fig. 1). Data are expressed as mean ± SEM, n = 6 neurons from N = 4 mice for each condition, T-test.
(F) Time-lapse analysis of synaptogenesis shows that new spines contact pre-existing VGLUT1-positive boutons. Single plane from 3D two-photon microscopy images.

(G) left panel: Nascent spines in D1-SPN are identified with tdTomato labeling and their synaptic activity is visualized using GCaMP6S calcium sensor during co-stimulation. To avoid fluorescence fluctuations independent of calcium variations, GCaMP6S signal is normalized using tdTomato signal. Nascent spines are considered as active if they present GCaMP6 fluorescent peaks (ΔF/F) exceeding 0.3, compared to baseline. Maximum projections show active (green arrow) and silent (red arrow) nascent spines. The spine head diameter was measured from non-active and active spines (Fig. 3G, right panel). Data show values and mean ± SEM, n = 10 spines from 3 independent neurons and N = 3 mice, T-test.

*: p < 0.05, **: p < 0.01, ***: p < 0.001. See supplemental figures S3 and S4. Scale bar in A, B = 5 microns, C = 4 microns, F and G = 1 micron.

Figure 4. A single administration of cocaine induces the rapid formation of thalamo-striatal synaptic contacts in the NAc

(A) D1-SPN were labelled through intra-NAc viral infection with viruses AAV-PPTA::Cre, AAV-FLEX-tdTomato (orange) and thalamic boutons were immunolabeled for VGLUT2 (cyan).

(B) One hour after a single administration of cocaine, the spine density is increased.

(C) Acute cocaine induces the rapid formation of VGLUT2-positive, thalamo-striatal synaptic contacts.

(D) Percentage of spines connected to a VGLUT2 bouton is not altered by cocaine. Note that the percentage is lower than for VGLUT1, owing to the difference in the efficiency of the labeling (knock-in mouse versus immunolabeling).
Scale bar = 2 microns. Histograms represent mean ± SEM with n=number of neurons analyzed from N = 4 mice in each condition. B-D: T-test. **: p < 0.01, ***: p < 0.001.

**Figure 5. Molecular mechanisms involved in spine density increase**

Measurements of spine density from SPN labeled with DiI in striatal slices co-stimulated with low doses of glutamate and D1R agonist. The co-stimulation induces an increase in spine density.

(A) Inhibition of ERK pathway by U0126 (10 µM) abolishes the spine density increase.  
(B) Blockade of mTOR pathway with rapamycin (25 nM) in acute slices totally inhibited the increase in spine density elicited by co-stimulation.  
(C) Scheme of the involved pathways showing the inhibitors used in the study. Both MNK-1 and mTOR activation are essential for the onset of translation.  
(D) Measurement of the density of pMNK-1 positive cells in acute slice after co-stimulation.  
(E) The inhibitor of MNK-1 activation, CGP57380 (10 µM), cancels the increase in spine density induced by co-stimulation of SPN from striatal slices.

Histograms show mean ± SEM with n=number of neurons analyzed from N = 3-5 mice for each condition. A-D and G: Two-way ANOVA and Bonferroni post-test; F: T-test; *: p < 0.05, **: p < 0.01, ***: p < 0.001. Scale bar in F = 30 microns, G= 10 microns.

**Figure 6. Time lapse imaging and molecular dissociation of spine growth and stabilization**

(A) Schematic representation of the experiment. D1-SPN were labelled through intra-NAc viral infection with viruses AAV-PPTA::Cre, AAV-FLEX-tdTomato ; spine growth and stabilization were analysed in 3D in time-lapse images from striatal slices. Green arrows show
new spines forming during the hour following the co-stimulation, the red arrow indicates when these new spines are not stabilized.

(B) Maximum intensity projection of two-photon image stacks from D1-SPN in NAc acute slices. Time-lapse observations reveal spine growth and stabilization of the new spines upon co-stimulation. Also shown are slices pre-treated with the inhibitor of MNK-1 (CGP 57380), protein synthesis (anisomycin) and transcription (actinomycin D).

(C) Cumulative curves of spine growth following co-stimulation. In the presence of the ERK inhibitor (U0126, 10 µM), the spine growth is absent, remaining at levels identical to vehicle condition. Note that spine growth kinetics and amplitude induced by co-stimulation are not altered by CGP 57380 (10 µM), anisomycin (25 µM) or actinomycin D (25 µM). Two-way ANOVA and Bonferroni post-test.

(D) Survival curve showing the maintenance of newly formed spines through time. Spines formed in the presence of CGP 57380 or anisomycin show a marked loss of stability. Actinomycin D does not alter spine maintenance in this time window. Log rank test.

n = 5-7 neurons from N = 3-4 mice, with 27-44 spines formed in each condition. *: p < 0.05, **: p < 0.01, ***: p < 0.001. Scale bar = 2 microns.

Figure 7. MNK-1 is activated after acute cocaine injection and is necessary for structural plasticity in vivo

(A) Immunolabeling for pMNK-1 in the shell of the NAc from mice perfused ten minutes after either saline or cocaine injection. Single planes of confocal image stacks from the shell part of the NAc. The density increase in pMNK-1 positive cells induced by a single administration of cocaine is abolished by ERK inhibitor SL327 injected in vivo prior to cocaine. Mean ± SEM, N = 3 mice for each condition. One-way ANOVA and Bonferroni post-test.
(B) Analysis of the density in P-MNK-1 positive cells in the shell of the NAc reveals a rapid and transient phosphorylation following a single cocaine administration. Two-way ANOVA and Bonferroni post-test, Drug effect: *, Time effect: °.

(C) Immunostaining for pMNK-1 in D1-SPN labeled with tdTomato reveal dendritic localization of pMNK-1. Upper panel shows single planes from confocal stack with arrows indicating pMNK-1 spots in the dendrite. Lower panel shows 3D volume rendering of the co-localization of pMNK-1 in a dendrite.

(D) The MNK-1 inhibitor CGP 57380 was injected into the NAc prior cocaine administration.

(E) *In vivo* analysis of dendritic spines labeled with DiI following injection in the NAc of the inhibitor CGP 57380 prior a single administration of cocaine. 3D surface rendering of confocal images from different conditions. The inhibition of MNK-1 blocks the increase in spine density induced by cocaine. Histograms show mean ± SEM with n = number of neurons analyzed from N = 3-5 mice for each condition. Two-way ANOVA and Bonferroni post-test.

Scale bar in A = 50 microns, C-E = 1 micron. *: p < 0.05, ** or °°: p < 0.01, *** or °°°: p < 0.001.

Figure 8. Proposed model for synaptogenesis in the adult striatum

Dopamine, which level is elevated under cocaine condition, facilitates NMDAR functions and triggers ERK activation and spinogenesis. Synapse formation occurs through clustered spine growth and contact with pre-existing presynaptic boutons. ERK pathway regulates distinct steps of spine formation through different signaling modules. Firstly, spine growth is controlled by ERK, probably through phosphorylation of pre-existing cytoplasmic targets. Secondly, stabilization of the spine is controlled by ERK-induced MNK-1 dependent protein synthesis in a transcription independent manner. Importantly, MNK-1 and protein synthesis
are not involved in spine growth, so spine growth and stabilization involve different signaling molecules downstream ERK.
Saline

Cocaine

COCAINE 1h

Random neuron

cFos+ neuron

SALINE

COCAIN 1 wk

Random neuron

cFos positive neuron

Day -3
day -2
day -1
day 0

coc perf PFA

Spine density (per 10 µm)

% of spines connected to VGLUT1

n=52

n=59

n=48

n=54

Spine density (per 10 µm)

Density of spines connected to VGLUT1 (per 10 µm)

Saline

Cocaine

Day -3
day -2
day -1
day 0

coc perf PFA

Spine density (per 10 µm)

% of spines connected to VGLUT1

n=54

n=51

n=29

n=53

Spine density (per 10 µm)

Density of spines connected to VGLUT1 (per 10 µm)

Saline

Cocaine

Day -3
day -2
day -1
day 0

coc perf PFA

Spine density (per 10 µm)

% of spines connected to VGLUT1

n=45

n=53

n=29

n=54
Spine density

**Density of spines connected to VGLUT2**

- **Saline**
- **Cocaine**

**Day**
- day -3
- day -2
- day -1
- day 0 + 1h

**Saline**
- Saline
- Cocaine

**Bar Graph**

- Spine density
- Density of spines connected to VGLUT2
- % of spines connected to VGLUT2

**Statistical Significance**
- ***
- **
- ns (not significant)
**A**

Control: [Column Graph]

Co-stimulation: [Column Graph]

**B**

Control: [Column Graph]

Co-stimulation: [Column Graph]

**C**

U0126

ERK

Rapamycin

mTOR

SKF38393 (10 µM)

Glutamate (3 µM)

Protein synthesis

**D**

Control 20'

Co-stimulation 20'

pMNK-1 positive cells per 0.15 mm²

**E**

Vehicle

Co-stim

Control

Co-stimulation

Spine density

**Statistical Significance**

* p < 0.05

*** p < 0.001

Sample Size:

- Control: n=25
- Co-stimulation: n=20
- U0126: n=19
- Rapamycin: n=21

- Control: n=42
- Co-stimulation: n=37
- U0126: n=31
- Rapamycin: n=33

- Control: n=14
- Co-stimulation: n=33
- CGP 57380: n=33
A. Diagram illustrating the relationship between growth phase and maintenance. New spine formation and survivorship are shown over time.

B. Time-lapse images of spine dynamics under various conditions:
- Co-stimulation + CGP 57380
- Co-stimulation + U0126
- Co-stimulation + actinomycin D

C. Graph showing new spine formation (%) over time after stimulation.

D. Graph showing new spine survivorship (%) over lifetime (minutes) for different conditions:
- Vehicle (DMSO 0.1%)
- Co-stimulation + vehicle
- Co-stimulation + CGP 57380
- Co-stimulation + actinomycin D
- Co-stimulation + U0126
- Co-stimulation + anisomycin
**A**

Saline + Vehicle  Cocaine + Vehicle  Cocaine + Vehicle

**B**

![Graph showing spine density over time](image)

**C**

3D rendering of tdTomato and pMNK-1 positive cells per mm²

**D**

- Vehicle
- Anisomycin

**E**

- Vehicle Saline  Cocaine
- CGP 57380 Saline  Cocaine

- Vehicle Saline  Cocaine
- SL327 Saline  Cocaine

**Time after injection (minutes)**

- 0 min
- 15 min
- 1 hour or 24 hours

**Spine density**

- 0
- 5
- 10
- 15
- 20

**Vehicle CGP 57380 Cocaine Saline Cocaine Saline**

**pMNK-1 positive cells per mm²**

- 0
- 36
- 51
- 76
- 59

- 38
- 37
- 47
- 46

**Vehicle Saline Cocaine Saline Cocaine SL327 Cocaine**

**pMNK-1 positive cells per mm²**

- 0
- 2
- 4
- 6

**SL327 Vehicle Cocaine**

**Cocaine + Vehicle**

**Cocaine + SL327**

- significance levels
  - *p < 0.05*
  - **p < 0.01**
  - ***p < 0.001***
  - ns: not significant

**n=17 n=32 n=53 n=53 n=29 n=35 n=32 n=31**

- SL327 and cocaine treatment interactions
Cocaine

Clustered spines

Spine stabilization

Protein synthesis

ERK

MNK-1

Time

0 min

60 min

VGLUT1 positive bouton

Striatal projection neuron

VGLUT2 positive bouton

Cocaine

Dopamine

Glutamate

Protein synthesis

Spine stabilization

MNK-1 inhibitor or anisomycin

Striatal projection neuron

Striatal projection neuron

Protein synthesis

Spine stabilization

MNK-1 inhibitor or anisomycin