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aPKC-mediated persistent increase in AMPA/NMDA ratio in the VTA participates in the neuroadaptive signal necessary to induce NAc synaptic plasticity after cocaine administration

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Abstract

Chronic cocaine exposure produces enduring neuroadaptations in the brain's reward system. Persistence of early cocaine evoked neuroadaptations in the ventral tegmental area (VTA) are necessary for later synaptic alterations in the nucleus accumbens (NAc), suggesting a temporal sequence of neuroplastic changes between these two areas. However, the molecular nature of the signal that mediates this sequential event is unknown. Here we used the behavioral sensitization model and the aPKC inhibitor of late-phase LTP maintenance, ZIP, to investigate if a persistent increase in AMPA/NMDA ratio plays a role in the molecular mechanism that allows VTA neuroadaptations to induce changes in the NAc. Results showed that intra-VTA ZIP microinfusion successfully blocked cocaine evoked synaptic enhancement in the VTA and the expected AMPA/NMDA ratio decrease in the NAc following cocaine sensitization. ZIP microinfusions also blocked the expected AMPA/NMDA ratio increase in the NAc following cocaine withdrawal. These results suggest that a persistent increase in AMPA/NMDA ratio, mediated by aPKC's, could be the

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molecular signal that enables the VTA to elicit synaptic alterations in the NAc following cocaine administration.

Keywords

VTA plasticity; NAc plasticity; PKM- ζ ; ZIP; Sprague Dawley

Introduction

Drug addiction is a chronic relapsing disease characterized by enduring alterations in the mesocorticolimbic system's response to rewarding stimuli. These changes drive the persistent habit-learning behavior associated with compulsive drug use (Everitt and Wolf, 2002; Hyman *et al*, 2006). Drugs of abuse induce an increase in dopaminergic and glutamatergic neurotransmission that facilitates neuronal excitability (Di Chiara and Imperato, 1988; Saal *et al*, 2003; Tan *et al*, 2010). Furthermore, drug exposure leads to the formation of long-term potentiation (LTP) (Hyman *et al*, 2006). LTP is considered the cellular substrate for memory formation (Bliss and Lomo, 1973; Lynch, 2004), and a facilitator of the protracted nature of the addictive process (Ungless *et al*, 2001; Saal *et al*, 2003).

Cocaine can evoke an increase in synaptic strength throughout the mesocorticolimbic system, yet the functional significance of this event is not well understood. Several studies suggest that the enhanced plasticity in the ventral tegmental area (VTA) is a temporary event that participates in the early changes that occur in the reward circuit (Ungless *et al*, 2001; Boudreau and Wolf, 2005; Boudreau *et al*, 2007;). In contrast, the NAc develops persistent synaptic enhancement at a later time-point (Kourrich *et al*, 2007). The chronological sequence of synaptic potentiation between these two structures suggests that initial changes in the VTA promote later alterations in the NAc. Recently, it was shown that synaptic potentiation in the VTA is crucial for NAc neuroadaptations to occur (Mameli *et al*, 2009). However, the cellular mechanisms responsible for this hierarchical organization remain to be elucidated.

In vitro experiments demonstrate that the maintenance of cocaine-evoked synaptic enhancement in the VTA is susceptible to the myristoylated atypical PKC pseudosubstrate inhibitory peptide, ZIP (Ho *et al*, 2012; Vélez-Hernández *et al*, 2013), which specifically affects the maintenance of the late-phase LTP (Ling *et al*, 2002) by stimulating the autoinhibitory regulatory subunit of atypical PKCs (Pastalkova *et al*, 2006). Behavioral studies in cocaine-exposed rats revealed that cocaine exposure increases PKM- ζ in the VTA (Ho *et al*, 2012). In addition, ZIP microinfusions in the VTA and NAc disrupt cocaine-related behaviors (Li *et al*, 2011; Shabashov *et al*, 2011; Vélez-Hernández *et al*, 2013). Whether this disruption is long-lasting (Shema *et al*, 2007) or can be naturally regenerated by reward circuit redundancy (Parsons and Davis, 2011), is still contested.

Knowing cocaine evoked behaviors rely on VTA and NAc synaptic potentiation, aPKC-mediated synaptic enhancement could be part of the molecular mechanisms that mediates the sequential generation of plasticity between these two areas. In the present

study, we used the cocaine behavioral sensitization model to investigate the molecular mechanisms that allows for a hierarchical order of cocaine-elicited plasticities between the VTA and NAc. The permanency of these effects was also tested. We hypothesize that persistent increase in AMPA/NMDA ratio in the VTA is one of the cellular events responsible for the neuroadaptations in the NAc.

Experimental Procedures

Animal Housing

Sprague Dawley male rats (Taconic farms, Germantown, N.Y.) were housed in pairs in cages located at the Animal Resource Center (ARC) of our institution. They were exposed to 12hr light/dark cycles. Water and food were available *ad libitum*. Surgical procedures and ZIP microinfusions took place in the ARC, while behavioral experiments were done in our laboratory. All animal procedure protocols were approved by our institutional IACUC committee.

Bilateral guide cannula placement for intracranial ZIP microinjections

Sprague Dawley male rats (200–230g) were anesthetized with pentobarbital (50mg/kg), administered atropine (0.1mg/kg) and Metacam (1mg/kg). A bilateral cannula (Plastics One, Roanoke, VA) was placed using a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). Stereotaxic coordinates (Paxinos and Watson atlas, 1998) for the VTA were: AP= −5.3 mm caudal from bregma, and −7.0 mm ventral from cortical surface. For the NAc: +2.3 mm rostral from bregma, and −5.2 mm ventral from cortical surface. After cannula placement (1 mm above the desired area) the incision was sutured, and a topical antibiotic was applied to prevent infection. Animals were allowed to recover for 7 days.

Cocaine behavioral sensitization

Cocaine behavioral sensitization is defined as a progressive increase in locomotion following consecutive drug administrations, which persists after an abstinence period (Kalivas and Duffy, 1993). Behavioral procedures were performed as previously described (Vélez-Hernández *et al*, 2013). Animals were randomly assigned to 4 groups (Vehicle/Saline, ZIP/Saline, Vehicle/Cocaine, and ZIP/Cocaine) and subjected to the cocaine sensitization paradigm. Locomotion was assessed inside an isolated acoustic chamber (Whisper Room Inc., Morristown, TN, USA). Two days before the beginning of the experiment, each group was habituated for 1 hour to the infrared photocell box (Accuscan Instruments, Columbus, OH, USA). Locomotion units were assessed as photocell counts (pcc). On experimental day 1 through 5, animals were habituated for 15 minutes and were then treated with either 15 mg/kg i.p. Cocaine (Sigma, St. Louis, MO, USA) or isovolumetric saline injections. Immediately after the injections, locomotion activity was assessed for one hour. On challenge day, all animals received Cocaine. Total locomotion was measured as the total number of times the rat interrupted an infrared beam. Interruption of consecutive beams was considered as ambulatory locomotion. Consecutive interruption of the same beam was counted as stereotypic locomotion.

Daily VTA ZIP or vehicle microinfusions were performed with a microinfusion pump (Cole-Palmer, Chicago IL), six hours after cocaine injection. Vehicle/saline and Vehicle/Cocaine groups received vehicle through a cannula and either saline or cocaine injections (i.p.). ZIP/Saline and ZIP/Cocaine received ZIP through the cannula and either saline or cocaine injections (i.p.), respectively. NAc ZIP microinfused rats were divided into the same groups and sensitized as explained above. They were allowed a 7day withdrawal period followed by bilateral NAc ZIP microinfusions, and allowed another 7day withdrawal period before getting a cocaine challenge.

Averaged data is presented as mean $\pm$ SEM and analyzed using Two-way repeated measures ANOVA followed by Sidak's and Tukey's post-hoc test. A $p < 0.05$ between day 5 and day 1 of the sensitization protocol is considered a successful sensitization (Kalivas and Stewart, 1991).

Drug Infusion

ZIP (Myr-SIYRRGARRGARRWRKL-OH, Anaspec, CA) was dissolved in a 100 mM Tris-saline solution (pH 7.3). Rats were microinfused bilaterally with 10 nmol ZIP (0.5 μ l/side, 0.1 μ l/min) using a 10 μ l syringe (Hamilton, Reno, NV). The bilateral cannula was left in place for an additional minute to avoid drug backflow.

Electrophysiological studies

Rats were sacrificed at different time points of the behavioral experiments: on day 5, after a 7-day withdrawal period, and 24 hours following a cocaine challenge. Animals were anesthetized with chloral hydrate (400 mg/Kg) and decapitated. Brains were removed and placed in cold (2–4°C) and oxygenated (95% O₂ and 5% CO₂ gas mixture to pH = 7.4) artificial cerebrospinal fluid (ACSF) containing (in mM): 127 NaCl; 2.5 KCl; 1.25 NaH₂PO₄; 25 NaHCO₃; 2 CaCl₂; 1 MgCl₂; 25 D-glucose. NAc coronal and horizontal VTA sections were taken using a Vibratome (VT1000S, Leica, Germany). Slices were transferred to an incubation chamber containing oxygenated ACSF at 32°C for 1 hr and subsequently placed in a recording chamber superfused with oxygenated ACSF (25°F) at 2 mL/min. ACSF was supplemented with Picrotoxin (100 μ M) to block GABA_A receptor mediated IPSC's. Whole-cell voltage clamp recordings were obtained from putative dopaminergic (DA) cells of the VTA and medium spiny neurons (MSN) of the NAc core. They were identified as previously described (Velez-Hernandez *et al*, 2013; Velazquez-Martinez *et al*, 2015), using infrared microscopy with DIC (BX51WI Olympus, Japan). Recordings from putative VTA DA were done in the region lateral to the fasciculus retroflexus and medial to the medial terminal nucleus of the accessory optic tract. Recordings from putative NAc (MSN) were done in the region lateral to the NAc shell and medial to the anterior commissure (Paxinos and Watson, 1998). The NAc core was chosen for these experiments since it has been demonstrated that it contributes to drug associated, cue-induced cocaine seeking and to the incentive value of reward-conditioned stimuli (Hollander and Carelli, 2007; Goto and Grace, 2008; Owesson-White *et al*, 2009). Moreover, the NAc core undergoes synaptic strengthening after repeated exposure to cocaine followed by a withdrawal period (Korrich *et al*, 2007; Ferrario *et al*, 2010). Recent studies suggest that the NAc core is the key structure where DA release attributes incentive salience and

motivational properties to reward cues which are of the utmost importance in the cocaine sensitization model used here (Saunders and Robinson, 2012). These findings have been currently supported using optogenetic techniques that investigated DA circuit-specific functions in Pavlovian reward learning (Saunders *et al*, 2018).

Borosilicate glass patch pipettes were pulled to a final resistance of 3–6 M Ω and filled with (in mM): 120 cesium methanesulfonate, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 2.5 MgATP and 0.25 NaGTP, pH 7.2–7.3 (270–285 mOsm). A bipolar stimulating electrode was placed 50–150 μ m rostral to the recording electrode to stimulate at 100 ms intervals. AMPA/NMDA ratios were measured as described by Thomas *et al*, 2001. Briefly, cells were clamped at +40mV and the NMDAR inhibitor D-2-amino-5phosphonovalerate (D-APV) (50 μ M) was applied to the superfusion medium. NMDAR responses were calculated by subtracting the average responses in the presence of 50 μ M D-APV (AMPA-mediated only) from that recorded in its absence. Experiments with a series resistance change of more than 20% were discarded. Averaged data are presented as a mean $\pm$ SEM and analyzed using Two-way ANOVA followed by Sidak's post-hoc test.

Data was collected with PClamp 9 software through a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, Ca), filtered at 10 kHz through a Bessel filter, digitized at 5 kHz using Digidata 1322A (Axon Instruments, Foster City, CA), and analyzed off line using Clampfit (Molecular Devices) software.

Histological verification of cannula placement

Following behavioral experiments, animals were anesthetized with 50mg/kg of pentobarbital and the microinfusion site was marked with Pontamine Sky Blue (0.5 μ l/side). Cannula placement was determined using microscopic examination of Pontamine Sky Blue in brain sections. Animals that received microinfusions outside of the region of interest were excluded from data analyses.

Statistical analyses and figures were done using Graph Pad PRISM software.

Results:

Daily ZIP microinjections into the VTA alter AMPA/NMDA ratios in the VTA and NAc

ZIP inhibition of aPKC's has been shown to disrupt AMPA receptor trafficking to the membrane, decreasing AMPA/NMDA ratios. AMPA/NMDA ratios are an electrophysiological signature of LTP expression (Lu *et al*, 2001). As a normalization procedure, it allows the comparison of the strength of synaptic potentiation of excitatory synapses between slices, by correcting for electrode placement and density of fibers activated. Recording electrode and stimulating electrode placement illustrated in Fig. 1C and Fig. 1F represent the configurations used in all AMPA/NMDA ratio experiments (Fig. 1, 2, and 3).

First, we assessed if *in vivo* ZIP microinfusions in the VTA could effectively block AMPA/NMDA ratio increase following a single cocaine exposure (Fig. 1A). Additionally, we examined AMPA/NMDA ratios in MSN of the NAc core to see if this disruption had any

effect on NAc synaptic plasticity. Twenty-four hours following a responsive single cocaine injection (ANOVA $F(1,28)=0.1239$, $p=0.72$, Tukey's multiple comparisons test significant $p=0.001$ (Vehicle/Saline vs Vehicle/Cocaine), $p=0.001$ (Vehicle/Saline vs ZIP/Cocaine), $p=0.001$ (ZIP/Saline vs Vehicle/Cocaine), and $p=0.001$ (ZIP/Saline vs ZIP/Cocaine)) (Fig. 1.B) and a ZIP microinfusion, there is a statistical significant difference in AMPA/NMDA ratios in VTA DA neurons between Vehicle/Cocaine and ZIP/Cocaine groups (ANOVA $F(1,22)=21.72$, $p=0.0001$). The increased AMPA/NMDA ratio of Vehicle/Cocaine animals (1.12 ± 0.06) is not observed in ZIP/Cocaine rats (0.47 ± 0.02) (Fig. 1E). In the NAc core, there was no statistical significant difference between the NAc AMPA/NMDA ratios of Vehicle/Cocaine group and ZIP/Cocaine group (Fig. 1H). This reveals that AMPA/NMDA ratio enhancement in the VTA can be effectively blocked after a single microinfusion.

Next, we set out to explore if aPKC-mediated persistent increase in AMPA/NMDA ratio is the molecular mechanism underlying the hierarchical nature of plasticity between the VTA and the NAc. For this, we made daily intra-VTA ZIP microinfusions during the initiation of cocaine behavioral sensitization and evaluated AMPA/NMDA ratios in the VTA at different time-points of the behavioral sensitization model: on day 5 of daily cocaine and zip microinfusions, following a 7-day cocaine withdrawal, and 24 hours after a cocaine challenge. This last timepoint would allow us to see if ZIP disruption of plasticity in the VTA would preclude the formation of new cocaine-evoked synaptic potentiation. We also measured AMPA/NMDA ratios in the MSN of the NAc core to reveal if disruption of VTA AMPA/NMDA ratio had an effect in NAc synaptic plasticity.

After 5 days of cocaine injections and ZIP microinfusions (Fig. 2A), there is a statistically significant difference in AMPA/NMDA ratios in VTA DA neurons between Vehicle/Cocaine and ZIP/Cocaine groups (ANOVA $F(1,22)=20.88$, $p=0.0002$). The increased AMPA/NMDA ratio of Vehicle/Cocaine animals (1.24 ± 0.1) is not observed in ZIP/Cocaine animals (0.53 ± 0.02) (Fig. 2D). In the NAc core, Vehicle/Cocaine animals showed a statistically significant decrease in AMPA/NMDA ratio (0.99 ± 0.06), which was not present in ZIP/Cocaine animals (1.37 ± 0.07) (ANOVA $F(1,44)=4.585$, $p=0.0378$) (Fig. 2G). Therefore, intra-VTA ZIP microinfusions blocked the decrease in AMPA/NMDA ratio in the NAc after consecutive cocaine injections.

Following a 7-day withdrawal period (Fig. 3A), VTA DA cells of Vehicle/Cocaine animals continued to exhibit a statistically significant increased AMPA/NMDA ratio (1.22 ± 0.06) when compared with ZIP/Cocaine animals (0.55 ± 0.015) (ANOVA $F(1,24)=87.33$, $p<0.0001$) (Fig. 3C). NAc core MSN revealed an increase AMPA/NMDA ratio in Vehicle/Cocaine animals (1.61 ± 0.07) when compared with ZIP/Cocaine animals (0.132 ± 0.04) (ANOVA $F(1,33)=6.526$, $p=0.0154$) (Fig. 3E). This implies daily intra-VTA ZIP microinfusions also blocked an increase in AMPA/NMDA ratio in the NAc core following a cocaine withdrawal period.

Finally, 24 hours after a cocaine challenge (Fig. 4A), there was no statistical significant difference between the VTA and NAc AMPA/NMDA ratios of Vehicle/Cocaine group and ZIP/Cocaine group (Fig. 4C, E).

Daily ZIP microinjections into the VTA attenuate initiation but not the expression of cocaine sensitization

Previously, our laboratory showed that a single ZIP microinjection in the VTA on day 5 of a cocaine behavioral sensitization protocol blocked initiation of sensitization but not the expression (Velez-Hernandez *et al.*, 2013). We hypothesized that uninterrupted neural signaling from the VTA to the NAc for 5 days before inhibiting VTA plasticity with ZIP, triggered the necessary plastic changes in the NAc for the expression of cocaine sensitization. Here we examine if daily interruption of cocaine-induced VTA plasticity can alter cocaine sensitization initiation and expression (Fig. 5A). Rats received cocaine or saline i.p. injections for 5 consecutive days, to induce cocaine sensitization. Six hours after each cocaine injection, they received bilateral intra-VTA ZIP or Vehicle microinfusions (Fig. 5B). To determine ZIP's effects on the initiation of cocaine sensitization, we examined locomotion changes on day 5 of the protocol. Vehicle/Cocaine and ZIP/cocaine rats demonstrated cocaine sensitization on day 5 (9738.2 ± 1036.6 counts and 6851.5 ± 930.1 counts, respectively), compared to day 1 (3722 ± 356.45 counts and $3,022 \pm 405.56$ counts respectively; ANOVA $F(4,124)=1.475$, $p=0.2139$, Tukey's multiple comparison test significant $p=0.0001$). Total locomotor sensitization of ZIP/Cocaine animals was significantly reduced on day 5, when compared to Vehicle/Cocaine animals (ANOVA $F(4,124)=1.475$, $p=0.2139$, Sidak's multiple comparison test significant $p=0.01$) (Fig. 5C). Ambulatory movement of Vehicle/Cocaine and ZIP/Cocaine groups were significantly increased by day 5 (2857.3 ± 355.12 counts for, and 2825.7 ± 340.95 counts, respectively), compared to day 1 (1908.4 ± 206.04 counts and 1544.4 ± 192.0 counts respectively; ANOVA $F(4,124)=0.310$, $p=0.87$, Tukey's multiple comparison test significant $p=0.001$). No significant differences were observed between Vehicle/Cocaine and ZIP/Cocaine groups (Fig. 5D). Stereotypic movement of Vehicle/Cocaine and ZIP/Cocaine was significantly increased by day 5 (6202.3 ± 1186.5 counts, and 4026.3 ± 646.9 counts, respectively), compared to day 1 (1691.0 ± 229.1 counts and 1478.0 ± 244.5 counts respectively; ANOVA $F(4,124)=2.509$, $p=0.0453$, Tukey's multiple comparison test significant $p=0.001$). ZIP/Cocaine group stereotypic movement on day 5 was significantly reduced when compared to the Vehicle/Cocaine group (ANOVA $F(4,124)=2.509$, $p=0.0453$, Sidak's multiple comparison test significant $p=0.001$) (Fig. 5E).

To determine if persistent inhibition of VTA cocaine-evoked plasticity blocks the expression of sensitization, animals were allowed a 7-day withdrawal period followed by a cocaine challenge (Fig 5A). On challenge day, ZIP/Cocaine pre-treated animals showed no statistical difference in total, ambulatory or stereotypic locomotion, compared to Vehicle/Cocaine rats (Fig. 5C, F, and I).

Discussion

How memories are maintained in the brain has been a fundamentally unanswered question in science. Currently, LTP is the most commonly studied physiological model of memory. Inhibition of the late-phase of LTP by ZIP has been shown to erase spatial, instrumental, and classically conditioned long-term memories (Pastalkova *et al.*, 2006; Shema *et al.*, 2007; 2009). Surprisingly, this advance has been scarcely tested for addiction-related plasticities in

the mesolimbic DA system (Li *et al*, 2011; Ho *et al*, 2012; Vélez-Hernández *et al*, 2013). Recently it was proposed that the neuroplastic changes between the VTA and the NAc follows a pattern; persistence of VTA plasticity is needed for NAc neuroadaptations (Mameli *et al*, 2009). In the present study, we explored if the hierarchical nature of this plasticity relies on an aPKC-mediated persistent increase in AMPA/NMDA ratio. We observed that *in vivo* ZIP administration in the VTA modified *in vitro* AMPA/NMDA ratios in both the VTA and NAc core at different time points of the behavioral sensitization paradigm.

ZIP is a myristoylated aPKC pseudosubstrate inhibitory peptide that simulates the autoinhibitory regulatory subunit of atypical PKCs (Pastalkova *et al*, 2006). aPKC phosphorylation enhances AMPA receptor neurotransmission during the late-phase of LTP by increasing and maintaining AMPA receptors in synapses (Ling *et al*, 2002; Serrano *et al*, 2005). aPKC inhibition disrupts AMPA receptor incorporation at potentiated synapses and it is associated with retrograde amnesia of long-term memories (Pastalkova *et al*, 2006; Shema *et al*, 2009). The identity of the aPKC that mediates these effects has been heavily disputed (Lee *et al*, 2013; Volk *et al*, 2013). Recent evidence indicates that ZIP's effect in wild-type mice is likely due to specific inhibition of PKM- ζ , whereas its ability to reverse LTP and erase memory in PKM- ζ -knockout mice is due to its effect on another upregulated atypical PKC, PKC ν/λ (Tsokas *et al*, 2016). Regardless of the specific aPKC ZIP disrupts, there is a consensus that this drug is a unique tool to study the maintenance of LTP. In our experiments, we used ZIP as a molecular tool to disrupt cocaine-evoked plasticity and evaluate if the hierarchical pattern of plastic changes between the VTA and NAc relies on ZIP-sensitive mechanism.

It is known that a single cocaine exposure is sufficient to induce LTP in the VTA (Ungless *et al*, 2001). Here, a single *in vivo* ZIP microinfusion into the VTA proved sufficient to block this cocaine-elicited enhancement of AMPA/NMDA ratio. Hence, ZIP microinfusion in the next set of experiments continuously and effectively blocked cocaine-evoked increase in AMPA/NMDA ratios. Daily ZIP administration into the VTA prevented an AMPA/NMDA ratio increase in putative DA cells of the VTA, as expected (Ho *et al*, 2012; Vélez-Hernández *et al*, 2013). Interestingly, NAc core putative MSN also presented a disruption of plasticity. Unlike the VTA, one cocaine injection does not elicit plastic changes in the NAc (Thomas *et al*, 2001; Kourrich *et al*, 2007). It has been shown that repeated cocaine exposure causes an initial depression of synaptic strength and neuronal excitability in the NAc (Zhang *et al*, 1998; Thomas *et al*, 2001; Hu *et al*, 2004; Dong *et al*, 2006; Kourrich *et al*, 2007; Mameli *et al*, 2009;). In our hands, this reduction in synaptic strength was not present in the NAc core of animals treated with ZIP in the VTA. This suggests that the presence of a persistent increase in AMPA/NMDA ratio in the VTA participates in the decrease of AMPA/NMDA ratio in the NAc core after cocaine administration.

Synaptic potentiation in the VTA can persist for several days after the last cocaine exposure (Ungless *et al*, 2001; Borgland *et al*, 2004). In our study, ZIP-treated animals did not present an increased synaptic strength in the VTA after a withdrawal period. Therefore, the disruption of cocaine-evoked increase in AMPA/NMDA ratio in the VTA can be long-lasting. In addition, in the NAc it has been demonstrated that a withdrawal period enables a transition from a depressed state to increased synaptic potentiation, together with an elevated

AMPA receptor surface expression (Boudreau and Wolf, 2005; Boudreau *et al*, 2007; Kourrich *et al*, 2007; Conrad *et al*, 2008). Our results do not show this enhanced synaptic potentiation after ZIP treatment. Together, this reveals that NAc core neuroadaptations, both in the forms of depressed and increased AMPA/NMDA ratio, seem to be mediated in part by the occurrence of a persistent increase in AMPA/NMDA ratio in the VTA.

Investigation indicates that a single cocaine exposure can elicit a maximally potentiated LTP in VTA DA cells (Ungless *et al*, 2001; Borgland *et al*, 2004;). Here we observed that 24 hours after a cocaine challenge, all groups presented an increase in synaptic strength, reflecting cocaine's acute effects. Similar to previous findings (Shema *et al*, 2009), the increase in synaptic potentiation in ZIP treated animals implies that this drug does not preclude the formation of new synaptic potentiation in the VTA. It has been shown that a cocaine challenge reverts synaptic potentiation and triggers AMPA receptor internalization in the NAc (Thomas *et al*, 2001; Boudreau *et al*, 2007; Kourrich *et al*, 2007). This effect was not observed in ZIP-treated animals, probably because they did not have an increased AMPA/NMDA ratio after the withdrawal period. Previous findings show that one single cocaine exposure does not increase AMPA receptor expression in the NAc, which could also explain the absence of any alterations in AMPA/NMDA ratio at this time point (Boudreau and Wolf, 2005).

Taken together our electrophysiological results reveal that NAc core neuroadaptations during cocaine sensitization, both in the forms of depressed and increased synaptic strength, are dependent on the persistence of an aPKC-mediated persistent increase in AMPA/NMDA ratio in the VTA.

We also evaluated how inhibition of cocaine-evoked enhancement of AMPA/NMDA ratio affects the expression of cocaine related behaviors in the animals used in the previous experiments. We observed that daily inhibition of synaptic potentiation in the VTA attenuates the initiation of sensitization. The results parallel previous findings from our laboratory, in which a single ZIP microinfusion disrupted cocaine sensitization (Vélez-Hernández *et al*, 2013). Similar to our experiment, others have shown that daily ZIP microinfusions into the ventricle of mice, which reached several brain structures, progressively blocked cocaine sensitization (Howell *et al*, 2014). However, our study provides evidence that ZIP-induced behavioral effects are specifically due to an inhibition of AMPA/NMDA ratio increase in the VTA.

The expression of cocaine sensitization remained unaffected by the persistent inhibition of synaptic potentiation in the VTA. This suggests that NAc neuroadaptations mediating the maintenance of sensitization can develop independently of VTA LTP. There is a consensus that the expression of cocaine sensitization is mediated by the NAc (Kalivas, 1995; Wang *et al*, 1997). Previous experiments have shown that disruption of NAc plasticity by inhibition of AMPA receptors and aPKCs, block the maintenance of sensitization and other cocaine conditioned reward memories (Kalivas and McFarland, 2003; Li *et al*, 2011; Shabashov *et al*, 2011; Vélez-Hernández *et al*, 2013). Therefore, NAc neuroplasticity seems necessary for the expression of cocaine-evoked behaviors. Unlike these experiments, in our examination of the effect of ZIP in VTA, our subjects were not microinfused with this plasticity disruptor

into the NAc. Hence, the expression of cocaine behavioral sensitization could have been mediated by direct cocaine-mediated plasticity changes in the NAc or other cellular processes that allow for the expression of sensitization (Borgland *et al*, 2006; Valjent *et al*, 2006). An alternative explanation could be that plasticity changes evoked by afferents from other brain areas unaffected by ZIP administration are the ones responsible for the maintenance of behavioral sensitization (Goto and Grace, 2005).

It did not escape our attention that some of the behavioral results observed did not reflect our electrophysiological findings. Considering that daily ZIP administration into the VTA only attenuated behavioral sensitization, this result adds to the literature indicating that there might be a temporal discrepancy between drug-adaptive behaviors and drug-evoked plasticity (Luscher & Malenka 2011; Luscher *et al* 2013; Zhang *et al* 2016). For example, it was demonstrated that locomotor sensitization precedes the reduction of GABA inhibition in dopaminergic neurons, an event that leads to enhanced synaptic signaling (Liu *et al*, 2005). In our case, it could be that increases in VTA AMPA/NMDA ratio becomes more relevant for cocaine-related behaviors following consecutive drug exposures.

The unaltered expression of cocaine sensitization was surprising considering the disruption of NAc core synaptic plasticity following daily ZIP microinfusions into the VTA. This could imply that cocaine elicited behaviors can develop independently of some NAc core synaptic potentiation. Another explanation for the observed maintenance of sensitization is that synaptic enhancement of non-VTA synapses in the NAc could mediate the expression of cocaine sensitization. Previous research shows that ZIP microinfusions into the NAc reverts cocaine behaviors (Li *et al*, 2011; Shabashov *et al*, 2011; Vélez-Hernández *et al*, 2013). In our experiments ZIP was microinfused in the VTA, leaving non-VTA synapses unaltered. It could be presumed that non-VTA synapses in the NAc play a more vital role in the maintenance of cocaine sensitization such as the connection between the prefrontal cortex and NAc core (Pierce et al., 1998).

Finally, in our behavioral experiments, ZIP mediated changes in locomotion were due to a specific reduction in stereotypic movement. During locomotor sensitization, rats transition from ambulatory movement to an enhanced repetitive behavior, termed stereotypy (Wood *et al*, 1998), which includes intensive head bobbing, sniffing, and teeth clenching (Rebec, 2006). This type of behavior has been associated with the development of addiction (Henry and White, 1995; McClung and Hirsh, 1998). Therefore, ZIP-mediated disruption of this type of movement in the VTA indicates that stereotypy might rely on a cellular memory component.

In sum, our study proposes aPKC-mediated synaptic potentiation to be one of the “key signals” that elicits sequential plasticity formation between the VTA and the NAc during behavioral cocaine sensitization. Furthermore, the susceptibility of this potentiation to ZIP implies that this VTA-NAc sequential neuroadaptive signal relies on aPKC’s, possibly PKM- ζ (Sacktor and Hell, 2017; Tsokas *et al*, 2016). Future experiments will require the use of contingent behavioral models to better assess the role of LTP in drug-associated behaviors. Having a better understanding of the cellular substrates that allow addiction

memory formation, such as the aPKC isoforms, can support their exploration and relationship to drug addiction susceptibility in humans.

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Highlights

1. Increased AMPA/NMDA is key in mesolimbic cocaine evoked sequential plasticity
2. aPKC's mediate cocaine-evoked synaptic potentiation in the VTA
3. Disruption of aPKC-mediated increase AMPA/NMDA decrease cocaine-evoked behavior

Figure 1. A single ZIP microinfusion into the VTA after acute cocaine exposure effectively disrupts VTA LTP

(A) Diagram of the behavioral sensitization and ZIP microinfusion protocol with the sacrifice and whole-cell recording time point. (B) Mean total locomotor activity (**pcc/60min**, $\pm$ S.E.M.). **Plus (+) and numeral (#) signs denote group significance when compared to vehicle saline (*) and ZIP/Saline (#) groups.** (C) Image illustrating the placement of the recording electrode and the bipolar stimulating electrode in the VTA. (D) Sample traces display AMPA/NMDA ratio of VTA DA cells of all groups (scale: 200pA, 20ms). (E) Mean AMPA/NMDA ratios are presented in bar graphs (ratio + S.E.M.). a single ZIP microinfusions in the VTA of ZIP/Cocaine group blunted an AMPA/NMDA ratio increase of VTA DA cells when compared with Vehicle/Cocaine group (****, $p < 0.0001$ Two-way ANOVA, followed by Sidak's multiple comparisons post-hoc test) (Vehicle/Saline: $n=6/6$, ZIP/Saline: $n=6/6$, Vehicle/Cocaine: $n=8/7$, ZIP/Cocaine: $n=6/6$). (F) Image illustrating the placement of the recording electrode and the bipolar stimulating electrode in the NAc Core. (G) Sample traces display AMPA/NMDA ratio of NAc core MSNs of all groups (scale: 100pA, 20ms). (H) In the NAc core, no significant difference was observed between all groups (Vehicle/Saline: $n=6/6$, ZIP/Saline: $n=6/6$, Vehicle/Cocaine: $n=5/5$, ZIP/Cocaine: $n=6/6$) ($p > 0.05$, Two-way ANOVA). n = number of cells / number of animals.

Figure 2. Daily ZIP microinfusions in the VTA blocks VTA LTP and disrupts NAc core plasticity by day 5 of the cocaine sensitization protocol.

(A) Diagram of the behavioral sensitization and ZIP microinfusion protocol with the sacrifice and whole-cell recording time point. **(B)** Sample traces display AMPA/NMDA ratio of VTA DA cells of naïve animals and all groups (scale: 200pA, 20ms). **(C)** Mean AMPA/NMDA ratios are presented in bar graphs (ratio $\pm$ S.E.M.). Daily ZIP microinfusions in the VTA of ZIP/Cocaine group blunted an AMPA/NMDA ratio increase of VTA DA cells when compared with Vehicle/Cocaine group (****, $p < 0.0001$, Two-way ANOVA, followed by Sidak's multiple comparisons post-hoc test) (Naïve: $n=6/6$, Vehicle/Saline: $n=5/5$, ZIP/Saline: $n=6/6$, Vehicle/Cocaine: $n=8/6$, ZIP/Cocaine: $n=7/6$). **(D)** Image illustrating the placement of the recording electrode and the bipolar stimulating electrode in the NAc Core. **(E)** Sample traces display AMPA/NMDA ratio of NAc core MSNs of naïve animals and all groups (scale: 100pA, 20ms). **(F)** In the NAc core, a decrease in AMPA/NMDA ratio of ZIP/Cocaine group was not observed when compared with Vehicle/Cocaine group (***, $p < 0.001$, Two-way ANOVA, followed by Sidak's multiple comparisons post-hoc test). No significant difference was observed in Naïve, Vehicle/Saline and ZIP/Saline control groups throughout the experiment (Naïve: $n=8/7$, Vehicle/Saline: $n=7/7$, ZIP/Saline: $n=6/6$, Vehicle/Cocaine: $n=17/9$, ZIP/Cocaine: $n=18/9$) ($p > 0.05$, Two-way ANOVA). n = number of cells / number of animals.

Figure 3. ZIP inhibition of VTA plasticity is long lasting and blunts NAc core synaptic potentiation after a withdrawal period.

(A) Illustration of the behavioral protocol and whole-cell recording time point. (B) Sample traces display AMPA/NMDA ratio of VTA DA cells of all groups (scale: 200pA, 20ms). (C) AMPA/NMDA ratios are presented in bar graphs (ratio $\pm$ S.E.M.). After a withdrawal period, AMPA/NMDA ratio of ZIP/Cocaine treated animals is significantly reduced when compared with Vehicle/Cocaine group (****, $p < 0.0001$, Two-way ANOVA, followed by Sidak's multiple comparisons post-hoc test), and is not significantly different from control groups (Vehicle/Saline: $n=6/6$, ZIP/Saline: $n=7/6$, Vehicle/Cocaine: $n=6/6$, ZIP/Cocaine: $n=9/7$). (D) Sample traces and (E) mean data display AMPA/NMDA ratio of NAc core MSNs of naïve animals and all groups (scale: 100pA, 20ms). After a withdrawal period, AMPA/NMDA ratios of NAc cells of ZIP/Cocaine animals do not show a significant increase as observed in the Vehicle/Cocaine group (**, $p < 0.001$) (Vehicle/Saline: $n=7/7$, ZIP/Saline: $n=5/5$, Vehicle/Cocaine: $n=11/8$, ZIP/Cocaine: $n=14/8$). No significant difference was observed in Naïve, Vehicle/Saline and ZIP/Saline control groups throughout the experiment ($p > 0.05$, Two-way ANOVA). n = number of cells / number of animals.

Figure 4. VTA plasticity can be generated again after ZIP inhibition and does not participate in NAc core plasticity after a cocaine challenge.

(A) Behavioral sensitization protocol and whole-cell recording time point. **(B) Sample traces display AMPA/NMDA ratios of VTA DA cells of all groups (scale: 200pA, 20ms).** **(C) AMPA/NMDA ratios were presented in bar graphs (ratio $\pm$ S.E.M.). After a cocaine challenge, Vehicle/Saline, ZIP/Saline and ZIP/Cocaine groups present an increase in AMPA/NMDA ratios (see fig. 2D). There is no significant difference between all groups ($p > 0.05$, Two-way ANOVA) (Vehicle/Saline: $n=6/6$, ZIP/Saline: $n=8/6$, Vehicle/Cocaine: $n=4/4$, ZIP/Cocaine: $n=5/5$). **(D, E) Sample traces and mean data display AMPA/NMDA ratios of NAc core MSNs of naïve animals and all experimental groups (scale: 100 pA, 20ms).** Following a cocaine challenge, there is no significant difference between the AMPA/NMDA ratios of the NAc core MSN of all groups (Vehicle/Saline: $n=6$, ZIP/Saline: $n=7$, Vehicle/Cocaine: $n=11$, ZIP/Cocaine: $n=9$) ($p > 0.05$, Two-way ANOVA). n = number of cells / number of animals.**

Figure 5. Daily ZIP microinfusions in the VTA attenuate cocaine sensitization. (A) The cocaine behavioral sensitization protocol. (B) Histological localization of infusion cannula within the VTA. (C, E, I) Mean total locomotor activity recorded each day for all groups (pcc/60min, $\pm$ S.E.M.). Plus (+) and numeral (#) signs denote significance in locomotor activity within the same group when compared to Day 1 (Two-way ANOVA, followed by Tukey's multiple comparisons post-hoc test). (C) **Daily Intra-VTA ZIP microinfusions attenuated total locomotor sensitization of ZIP/Cocaine animals, evident by Day 5 (*, $p=0.01$, Two-way ANOVA, followed by Sidak's multiple comparisons post-hoc test).** (D) **No significant difference in ambulatory activity was observed ($p>0.05$, Two-way ANOVA).** (E) **Stereotypic activity of ZIP/Cocaine animals was significantly reduced on day 5 when compared to Vehicle/Cocaine animals (**, $p=0.001$, Two-way ANOVA, followed by Sidak's multiple comparisons post-hoc test).** No significant difference was observed in Vehicle/Saline and ZIP/Saline control groups throughout the experiment ($p > 0.05$, Two-way ANOVA). (Vehicle/Saline n23/12, ZIP/Saline n19/12, Vehicle/Cocaine 13/7, ZIP/Cocaine 20/14) n=number of animals up to day 5/number of animals on challenge day. Difference in numbers is due to use of these animals for the assessment of AMPA/NMDA ratios in Figures 1, 2 and 3.