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Inhibition of Protein Kinase Mzeta (PKMζ) in the Mesolimbic System Alters Cocaine Sensitization in Rats

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Abstract Chronic cocaine use produces long-lasting changes in reward circuits that may underlie the transition from casual to compulsive patterns of drug use. Although strong neuroadaptations within the mesocorticolimbic system are known to occur, the specific role of these drug-induced plasticities on sensitization remains to be elucidated. Here we investigate whether PKMζ, a protein involved in maintaining long-term potentiation (LTP), plays a role in these cocaine-induced changes in synaptic strengthening. We performed whole-cell voltage clamp recordings of putative ventral tegmental area (VTA) dopamine (DA) cells 24 hours after 5 days of 15 mg/kg i.p. cocaine or isovolumetric saline injections. We observed that superfusion of 5 μM ZIP (PKMζ inhibitory peptide) decreased AMPA currents and AMPA/NMDA ratios only in cocaine sensitized rats. In vivo ZIP microinfusions (10 nmol) into the VTA after cocaine sensitization decreased locomotor activity on a subsequent cocaine challenge only if ZIP is given before the withdrawal period. On the other hand, ZIP microinfusions into the nucleus accumbens (NAc) core after a 7-day withdrawal period disrupt the expression of locomotor sensitization. The present data provide a potentially relevant region, and time-specific PKMζ-dependent brain mechanism that enables sensitization. Our results support the vision that addiction involves a pathological learning process. They imply that if this synaptic strengthening is reversed, changes in the behavioral response may also be overturned.

Keywords protein kinase Mzeta; VTA; LTP; cocaine sensitization; dopamine; AMPA/NMDA ratio

1. Introduction

Chronic cocaine users undergo a transition from casual to compulsive patterns of drug use [41,52,83] that might be caused by aberrant learning [4,26,51]. Underlying such pathological behavior are strong neuroadaptations within the mesocorticolimbic system (reviewed by [10,30,36,66, 80,87]). The specific role of these drug-induced plasticities in critical components of this circuit, particularly the ventral tegmental area (VTA) and the nucleus accumbens (NAc), remains to be elucidated.

Emerging evidence suggests that glutamatergic neurotransmission plays a central role in the changes that occur in the mesocorticolimbic system regulating the physiological action induced by drugs of abuse. In the VTA, a robust α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPAR)-mediated potentiation of excitatory synapses, typically measured as an increase in AMPA/NMDA ratio, occurs after exposure to cocaine, which can alter reward related behaviors [6,12,62,65, 81]. Similarly, chronic cocaine administration followed by a withdrawal period increases AMPA/NMDA ratio in the NAc [38]. Therefore, cocaine-induced changes in synaptic strengthening may be responsible for the persistent physiological alterations observed during and after discontinuing drug use and could form the basis for maintaining the sensitized response [50,82].

Recently, it has been shown that a variety of long-term memories in different regions of the brain are quickly erased by local inhibition of PKMζ, a persistently active protein kinase that maintains LTP [20,40,45,49,57,63,71, 75,76,84]. The potentiation of synaptic transmission by PKMζ in the hippocampus during LTP may be similar to the change in AMPA/NMDA ratio after cocaine in the VTA in that the plasticity occurs through increases in the AMPAR-mediated synaptic transmission [43,44,49]. Thus, we examined PKMζ's effect on cocaine-related plasticities in the VTA and the NAc.

In our studies, we used a cocaine sensitization model to examine the cellular and molecular changes that occur after chronic drug administration. Behavioral sensitization is characterized by a progressive increase in behavioral response to repeated injections of the same dose of a drug that persists after a withdrawal period [60,69]. We postulate that if drug exposure induces LTP-like changes in reward-related regions, it should be possible to prevent or even reverse behavioral responses with inhibitors of LTP. Here,

we show that infusions of a PKMζ inhibitor alter AMPA-mediated currents and AMPA/NMDA ratio in the VTA and cocaine sensitization, reducing cocaine-induced changes in locomotor activity, first in the VTA, and after a withdrawal period in the NAc.

2. Materials and methods

2.1. Animals

All experimental procedures were performed according to the US Public Health Service publication "Guide for the Care and Use of Laboratory Animals" and were approved by the Animal Care and Use Committee at the University of Puerto Rico Medical Sciences Campus. Sprague-Dawley male rats (150–250 g) were used as the experimental subjects. Animals were housed two per cage and maintained at constant temperature and humidity with a 12-h light/dark cycle. Water and food was provided ad libitum. Rats were randomly assigned to the groups before the study began.

2.2. Whole-cell voltage-clamp recordings

Patch clamp experiments were performed in brain slices from rats (150–250 g) that received a cocaine (15 mg/kg) i.p or an isovolumetric saline injection daily for 5 days. Twenty-four hours after the last injection, rats were anaesthetized by an intraperitoneal injection of chloral hydrate (400 mg/kg) and decapitated. Horizontal slices (thickness: 220 μm) containing the VTA were prepared using a vibratome (VT1000S, Leica, Germany). Slices were cut in ice-cold (2–4 °C) oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 127 NaCl; 2.5 KCl; 1.25 NaH_2PO_4; 25 $NaHCO_3$; 2 $CaCl_2$; 1 $MgCl_2$; 25 D-glucose. The solution was previously saturated with a 95% O_2 and 5% CO_2 gas mixture to pH 7.4. Slices were transferred to an intermediate chamber and incubated at 32 °C in the same ACSF solution for approximately 1 h before transferring them to the recording chamber. In AMPA/NMDA studies, after 1 h in the intermediate chamber half of the slice was placed in a ZIP incubation solution and the other half kept in the intermediate ACSF chamber. Whole-cell voltage-clamp recordings were obtained from visually identified neurons in the VTA area [59] using infrared microscopy with DIC (BX51WI Olympus, Japan) and water-immersion objectives. Putative VTA DA cells were identified by the presence of large I_h current. I_h is present in about 84% VTA DA neurons [65], and VTA GABA cells do not express this conductance [47]. Therefore, the contribution of non-DA cells to our data is likely to be not significant.

The slice was submerged in a 500 μL recording chamber, which was connected to a superfusion system (1–2 mL per minute). The bath solution was the same used for slice preparation, with the chamber temperature maintained at 32 °C. Borosilicate glass patch pipettes (O.D. 1.5 mm, I.D. 1.0 mm; WPI, Sarasota, FL, USA) were pulled to a final resistance of 3–6 MΩ when 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) (Na)GTP and (Mg)ATP were prepared daily. Pharmacologically isolated (100 μM picrotoxin) EPSCs were electrically evoked by placing a stimulating electrode 60–150 μm rostral to the patched cell. Once a stable EPSC was achieved 5 μM ZIP (InVitrogen, CA, USA) was superfused into the slide and EPSCs were recorded for 14 min. Series resistance was monitored during the entire experiment, and data were discarded if changes of more than 15% occurred.

To determine the role of ZIP administration on AMPA/NMDA ratios, slices from rats treated with saline or cocaine for 5 days were cut in half and one portion was incubated in ZIP (cocaine/ZIP and saline/ZIP) and the other in ASCF (cocaine/ASCF and saline/ASCF) for 1 h. NMDA and AMPA receptor traces were constructed by averaging 15 EPSCs elicited at +40 mV. NMDA receptor responses were calculated by subtracting the average response in the presence of 50 μM D-2-amino-5-phosphonovalerate (D-APV) (AMPA receptor-mediated only) from that recorded in its absence. Then the peak of the AMPA receptor-only EPSC was divided by the peak of the NMDA receptor-only EPSC to give an AMPA/NMDA ratio.

Data were 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, USA), and analyzed off line using Clampfit (Molecular Devices) software.

2.3. Placement of guide cannulas for intracranial injections

After 5 days of quarantine, animals (250–300 g) were anesthetized using 50 mg/kg of pentobarbital in 0.9% saline, i.p. A bilateral guide cannula (Plastics One, Roanoke, VA, USA) was placed 1 mm above the area of the VTA or the NAc core. Stereotaxic coordinates for the VTA were −5.3 mm from bregma and −7.0 mm ventral to the cortical surface and 2.3 from bregma, and −5.2 ventral to the cortical surface for the NAc using the Paxino's and Watson Atlas, 1998.

2.4. Sensitization protocol and behavior

Animals were randomly divided into four groups (vehicle/saline, vehicle/cocaine, ZIP/saline, and ZIP/cocaine). Locomotor activity experiments started 1 week after surgery and were performed in an isolated acoustic chamber (Whisper Room Inc. Morristown, TN, USA). As shown in Figure 2(a), 2 days before the beginning of the experiment, each group was habituated (for 1 h) to the infrared photocell box (Accuscan Instruments, Columbus, OH, USA). On experimental day 1, animals were habituated for 15 min after which rats were 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 1 h (Figure 2(a)). This procedure was repeated for 5 days. Before the withdrawal period, on day 5, animals received an intra VTA 10 nmol ZIP microinfusion ($0.5\,\mu$L/side) or isovolumic vehicle after the locomotor assessment. On day 6, all groups received a cocaine challenge (15 mg/kg, i.p.), and locomotion was assessed for an hour. Animals were given a 7-day withdrawal period, and on day 14, all animals received a second cocaine challenge (15 mg/kg, i.p.), and locomotor activity was again recorded for 1 h. To determine the role of VTA-PKMζ on the expression of sensitization, animals received an intra-VTA 10 nmol ZIP microinfusion ($0.5\,\mu$L/side) or isovolumic vehicle on day 7 of the withdrawal period (Figure 2(e)). On day 13, all groups received a cocaine challenge (15 mg/kg, i.p.), and locomotion was assessed for an hour.

For intra NAc microinfusions, animals were injected with 15 mg/kg cocaine once a day for 5 days and locomotor activity was recorded for 1 h (Figure 3(a)). On days 6 and 7 of the withdrawal period (days 11 and 12 of the experiment), animals received either ZIP or vehicle microinfusions, and no locomotor recordings were made. On day 13, all groups received a cocaine challenge (15 mg/kg, i.p.) (no microinjection given), and locomotion was measured for 1 h to assess the expression of cocaine sensitization. Twenty-four hours after the termination of the experiments, animals were euthanized, and cannula placement was determined.

2.5. Drug infusion

ZIP (Myr-SIYRRGARRGARRWRKL-OH, Anaspec) was dissolved in 100 mM Tris-saline solution (vehicle, pH 7.2). Intracranial bilateral infusions of 10 nmol ZIP and 2% Pontamine Sky Blue were made by inserting a 31-gauge injection needle 1 mm below the tip of the guide cannula. Using a $1\,\mu$L syringe (Hamilton, Reno, NV) and a microsyringe drive (Cole Palmer, Chicago, IL), $0.5\,\mu$L/side was delivered at a rate of $0.1\,\mu$L/min. The bilateral cannula was left in place for an additional minute to avoid drug backflow.

2.6. Histological verifications of cannulas' positions

At the end of the experiment, animals were anesthetized with 50 mg/kg of pentobarbital, and the injection site was marked with a pontamine sky blue $0.5\,\mu$L/side (2%) microinfusion. Cannula placement was determined using microscopic examination of Pontamine Sky Blue in coronal brain sections (20 microns, Figures 2(g) and 2(h)). Animals that received microinfusions outside of the region of interest were excluded from data analyses.

2.7. Statistics

Total ambulatory activity, expressed as photocell counts (pcc), between groups were analyzed using one-way or two-way ANOVA followed by bonferroni post test (numbers are presented as mean $\pm$ standard error). A significant statistical difference of $P < .05$ when compared to day 1 was considered a successful sensitization protocol [34]. Two-way ANOVA followed by bonferroni post test was used for statistical analysis of EPSC amplitude. One-way ANOVA followed by Newman-Keuls was used for AMPA/NMDA ratio, and a value of $P < .05$ was considered statistically significant.

3. Results

3.1. ZIP superfusion decreases AMPA-mediated EPSCs in the VTA of cocaine-sensitized rats

PKMζ is thought to enhance synaptic transmission by persistently increasing postsynaptic AMPAR-mediated responses [49,90], thereby maintaining late-LTP [72] in regions like the hippocampus. Excitatory synapses are potentiated after a single or multiple injections of cocaine [81]. Therefore, we examined the effect of the PKMζ inhibitor ZIP on AMPAR current in the VTA after 5 days of cocaine injections. Previous studies on cocaine-induced synaptic plasticity in the VTA were performed 24 h after an in vivo cocaine exposure [3,6,81]. For this reason, we performed whole-cell voltage clamp recordings of putative VTA DA cells 24 h after 5 days of 15 mg/kg cocaine or saline injections (Figure 1(a)). Saline-injected rats show no changes in behavioral response ($1,082 \pm 109$ counts on day 1 and 694 ± 110 counts on day 5, $n = 10$; one-way ANOVA $P < .05$; Figure 1(a)). Cocaine-treated animals show sensitization when day 1 and day 5 locomotor activity are compared ($3,514 \pm 330$ counts on day 1 and $6,880 \pm 587$ counts on day 5, $n = 16$; one-way ANOVA $P < .05$; Figure 1(a)). A time course shows a decrease in AMPAR-mediated EPSCs of cocaine-treated animals when ZIP is added to the bath solution (ANOVA, $F_{32,102} = 6.54$, $P < .05$; Figure 1(c)). Superfusion of $5\,\mu$M ZIP resulted in a significant decrease (81.10 ± 4.58 pA; $n = 9$) in AMPA-mediated EPSCs in cocaine-treated rats when compared with saline-treated (100.32 ± 3.21 pA; $n = 4$) or ACSF-superfused animals (97.48 ± 3.02; $n = 7$; ANOVA $F_{7,44} = 6.91$, $P < .01$; Figures 1(b), 1(c), and 1(d)).

3.2. AMPA/NMDA ratio

Slices from animals treated with saline or cocaine i.p. injections for 5 days were incubated in ACSF or ZIP for 1 h before recording. There was a significant increase in AMPA/NMDA ratio in cocaine-treated (1.17 ± 0.12, $n = 18$) compared to saline-injected animals (0.66 ± 0.10, $n = 10$) after incubation in regular ACSF (Figures 1(e) and 1(f)). However, incubation with $5\,\mu$M ZIP resulted in a decrease of AMPA/NMDA ratio only in cells from cocaine-treated rats (0.82 ± 0.11, $n = 16$; one-way ANOVA $P < .05$). ZIP incubation had no effect on putative VTA
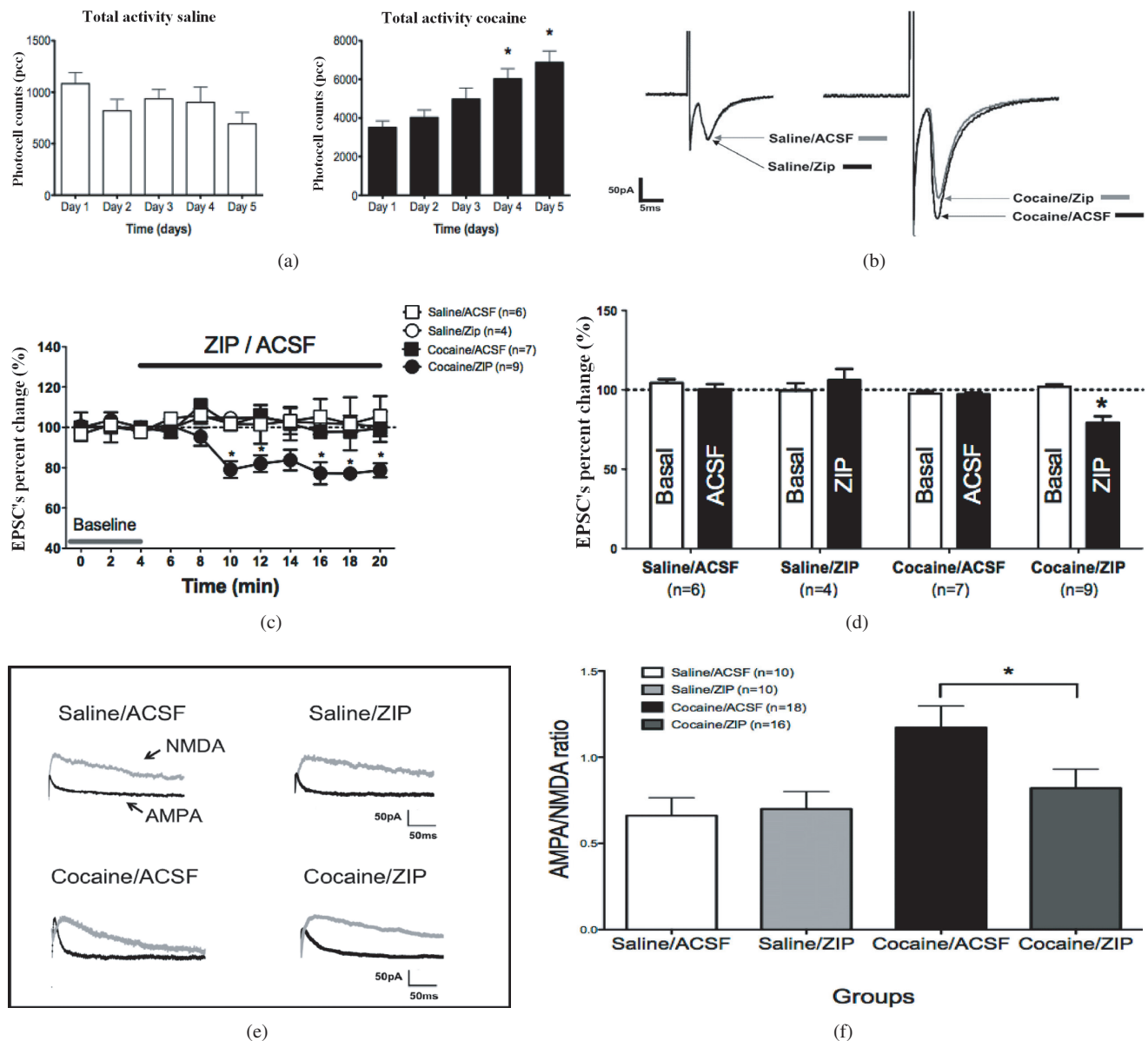
(a)

(b)

(c)

(d)

(e)

(f)

Figure 1: *ZIP decreases AMPA currents and AMPA/NMDA ratio in cocaine-sensitized rats.* (a) Mean total ambulatory activity (pcc/60 min, $\pm$SEM) was recorded each day for saline (white) ($n = 10$) and cocaine (black) ($n = 16$) injected animals. Asterisks ($*$) denote group significance when compared to day 1. (b) Sample traces show AMPA EPSCs reduction upon ZIP superfusion (5 μM). Zip had no effect on saline-treated animals (left). Notice the potentiated EPSC in cocaine-treated rats, which was diminished by ZIP application (right). (c) Time course showing that ZIP superfusion significantly decreases AMPA EPSC amplitudes in slices taken from cocaine-treated animals (cocaine/ZIP) ($*P < .05$) and has no effect on saline-treated animals (saline/ZIP) ($P > .05$; two-way ANOVA, followed by bonferroni post test). ACSF superfusion has no effect on EPSC amplitude in control experiments (saline/ACSF and cocaine/ACSF). (d) Bar graph shows AMPA EPSCs reduction as percentage of change from basal (EPSC amplitude at 4 min). Continuous ZIP application was able to significantly reduce AMPA-mediated transmission by $\sim 20\%$ ($*P < .01$ Student t-test) in cocaine-treated animals when compared to basal but failed to do so in saline-injected rats. ACSF superfusion had no effect in AMPA-mediated currents in cocaine- or saline-injected animals. Data are percent changes $\pm$SEM. (e) Representative traces showing AMPA (black)/NMDA (gray) ratio from brain slices incubated in ZIP (5 μM) or ACSF from saline- or cocaine-treated animals (scale: 50 pA, 50 ms). (f) Bar graph showing AMPA/NMDA ratios of saline- and cocaine-treated animals. The AMPA/NMDA ratio increased with cocaine injections. ZIP incubation significantly reduced the AMPA/NMDA ratio from cocaine-treated rats but failed to do so in saline-injected animals ($*P < .05$ one-way ANOVA Newman-Keuls, ratios $\pm$SEM).

DA cells from saline-treated animals (0.69 ± 0.10, $n = 10$; one-way ANOVA $P > .05$; Figures 1(e) and 1(f)).

3.3. Intra-VTA ZIP microinfusions before a withdrawal period decrease behavioral sensitization

To determine if PKMζ inhibition has behavioral consequences in vivo, we examined the effect of intra-VTA ZIP microinfusions on behavioral sensitization to cocaine. Rats were chronically injected with 15 mg/kg cocaine or saline i.p. over five consecutive days to induce behavioral sensitization [28, 32, 60, 69]. Cocaine-injected animals showed a significant increase in locomotor activity on day 5 ($6,830 \pm 698$ counts for vehicle/cocaine, $n = 13$ and $7,443 \pm 625$ counts for ZIP/cocaine, $n = 11$), compared to day 1 ($3,615 \pm 318$ counts for vehicle/cocaine and , 3378 ± 649 counts for ZIP/cocaine; ANOVA $F_{27,287} = 23.06$, $P < .01$; Figure 2(b)). To determine whether inhibition of PKMζ affects the already established sensitization, 10 nmol ZIP was infused into the VTA after 1 h of locomotion assessment on day 5. On day 6, a single cocaine challenge (15 mg/kg) was given to all animals, and locomotion activity was assessed for 1 h. This day, ZIP/cocaine animals (i.e., animals treated with ZIP the day

before the cocaine challenge) showed a significant decrease in locomotion ($4,095 \pm 724$ counts; $n = 11$) compared to vehicle/cocaine-treated animals ($6,575 \pm 652$ counts; $n = 13$; ANOVA $F_{27,287} = 23.06$, $P < .01$; Figure 2(b)). ZIP microinfusion on day 5 did not alter cocaine's acute response since locomotor activity of ZIP/cocaine animals ($4,095 \pm 724$ counts; $n = 11$) was not significantly different from those that received cocaine for the first time on day 6 ($3,545 \pm 348$ counts, $n = 12$, for ZIP/saline and $3,200 \pm 221$ counts, $n = 10$ for vehicle/saline; ANOVA $F_{27,287} = 23.06$, $P > .05$; Figure 2(b)).

Time course of locomotor responses on days 1 and 5 (Figure 2(c)) compares locomotor activity after 1 (gray) and 5 (black) days of cocaine (15 mg/kg, i.p. filled) or saline (unfilled). Cocaine sensitization was manifested as an increase in locomotor activity primarily during the first 40 min after cocaine injection (ANOVA $F_{71,774} = 22.97$, $P < .05$). Figure 2(d) shows a decrease in locomotor activity primarily in the first 30 min after cocaine injection in ZIP/cocaine animals when compared to vehicle/cocaine rats on day 6 (*$P < .05$) and ZIP/cocaine on day 5 (ANOVA $F_{71,774} = 18.89$, #$P < .05$). There is no statistical differences on any time point between ZIP/cocaine group and animals
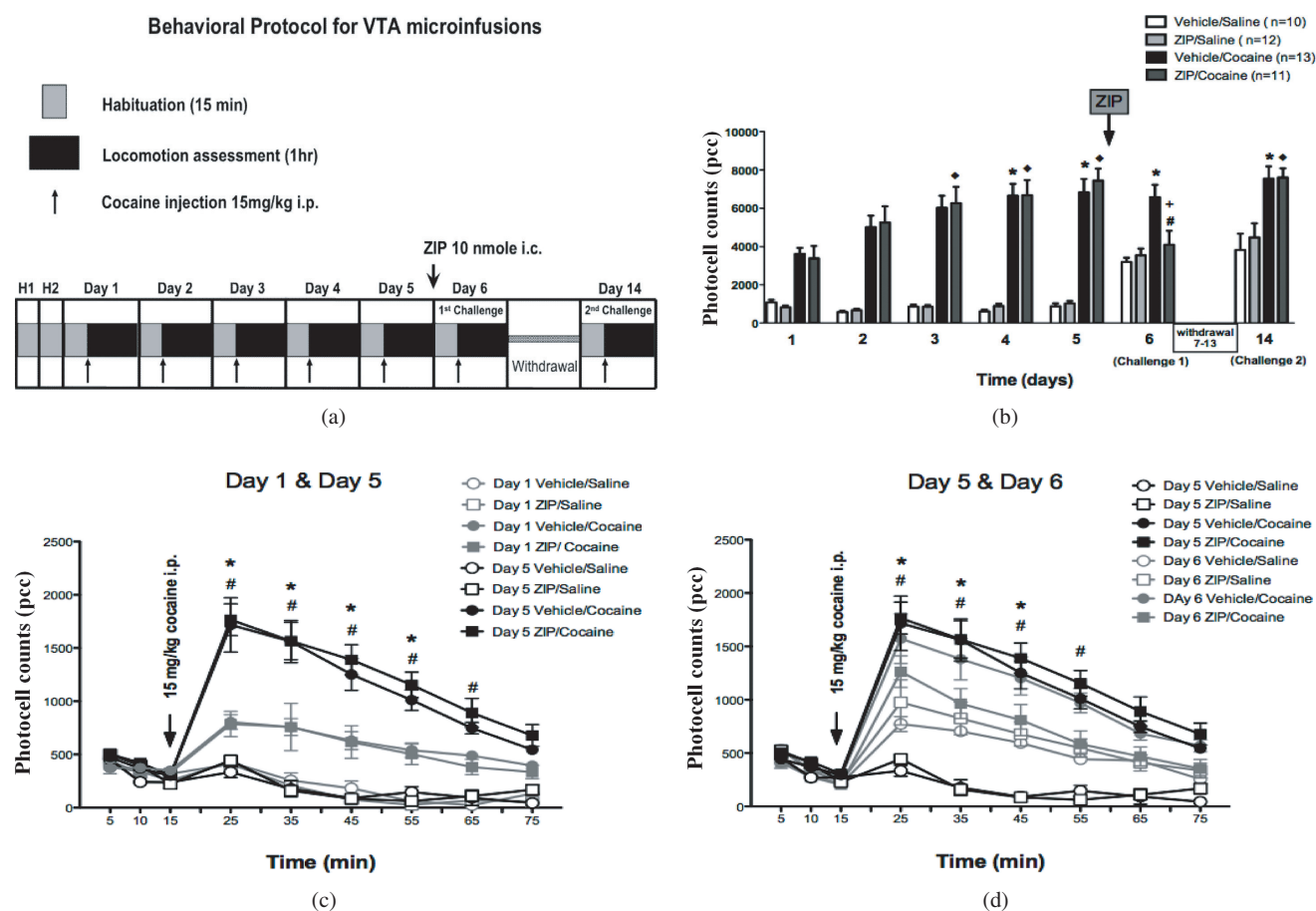

(a)

(b)

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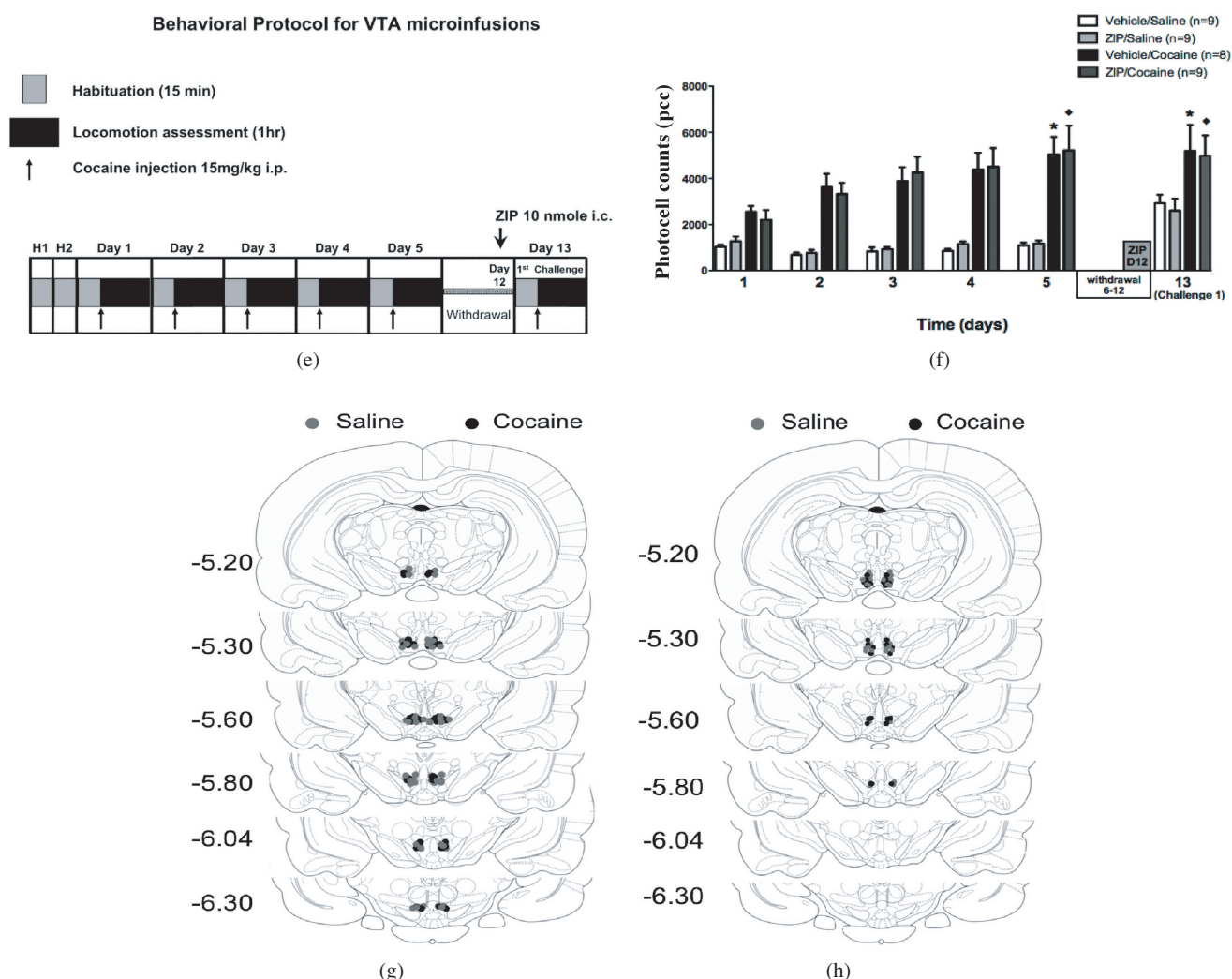**Figure 2:** To be continued.

(e)

(f)

(g)

(h)

Figure 2: *Intra-VTA ZIP microinfusions's effect on behavioral sensitization.* (a) Diagram showing the cocaine sensitization protocol with the time point for intra VTA ZIP microinfusion. (b) Mean total ambulatory activity (pcc/60 min, ±SEM) was recorded each day for every group. Asterisks (∗) and diamonds (♦) denote group significance when compared to day 1 for their respective group. One day after ZIP treatment (day 6), ZIP/cocaine-treated animals showed a significant decrease (#) in locomotor activity compared to day 5 ($P < .01$). Locomotion was also significantly different (+) from that of vehicle/cocaine animals on day 6. On day 14, ZIP/cocaine pretreated animals showed a sensitized response. No statistical differences between ZIP/cocaine- and vehicle/cocaine-treated animals were observed on day 14 ($P > .05$). There was no change between ZIP/saline and vehicle/saline controls throughout the experiment ($P > .05$). (c) Time course of locomotor responses after 1 (gray) and 5 (black) days of cocaine (15 mg/kg, i.p.) (filled) or saline (unfilled). Cocaine sensitization was manifested as an increase in locomotor activity primarily during the first 10–40 min (25–55 min) after cocaine injection. Significant differences ($P < .05$) are indicated by asterisks (∗) for vehicle/cocaine and numeral (#) for ZIP/cocaine. Each time point represents the mean ±SEM. (d) Time course for the locomotor response on days 5 (black) and 6 (gray). ZIP/cocaine-injected animals (filled gray squares) show decreased locomotor activity ($^{\#}P < .05$) compared to day 5. Asterisks (∗) denote significance between ZIP/cocaine and vehicle/cocaine animals on day 6. Each point represents the mean ±SEM. (e) Diagram showing the cocaine sensitization protocol with the time point for intra VTA ZIP microinfusion after a withdrawal period. (f) On day 5, cocaine-treated animals were sensitized to cocaine. One day after ZIP treatment (day 12), ZIP/cocaine-treated animals showed no significant change in locomotor activity compared to day 5 or to vehicle/cocaine animals on day 12 ($P > .05$). There was no change between ZIP/saline and vehicle/saline controls throughout the experiment ($P > .05$, two-way ANOVA, followed by bonferroni post test for all comparisons). (g) and (h) Injection sites within the VTA for saline- (gray circles) and cocaine- (black circles) treated animals. (g) corresponds to Figure 2(a) protocol and (h) corresponds to Figure 2(e) protocol.
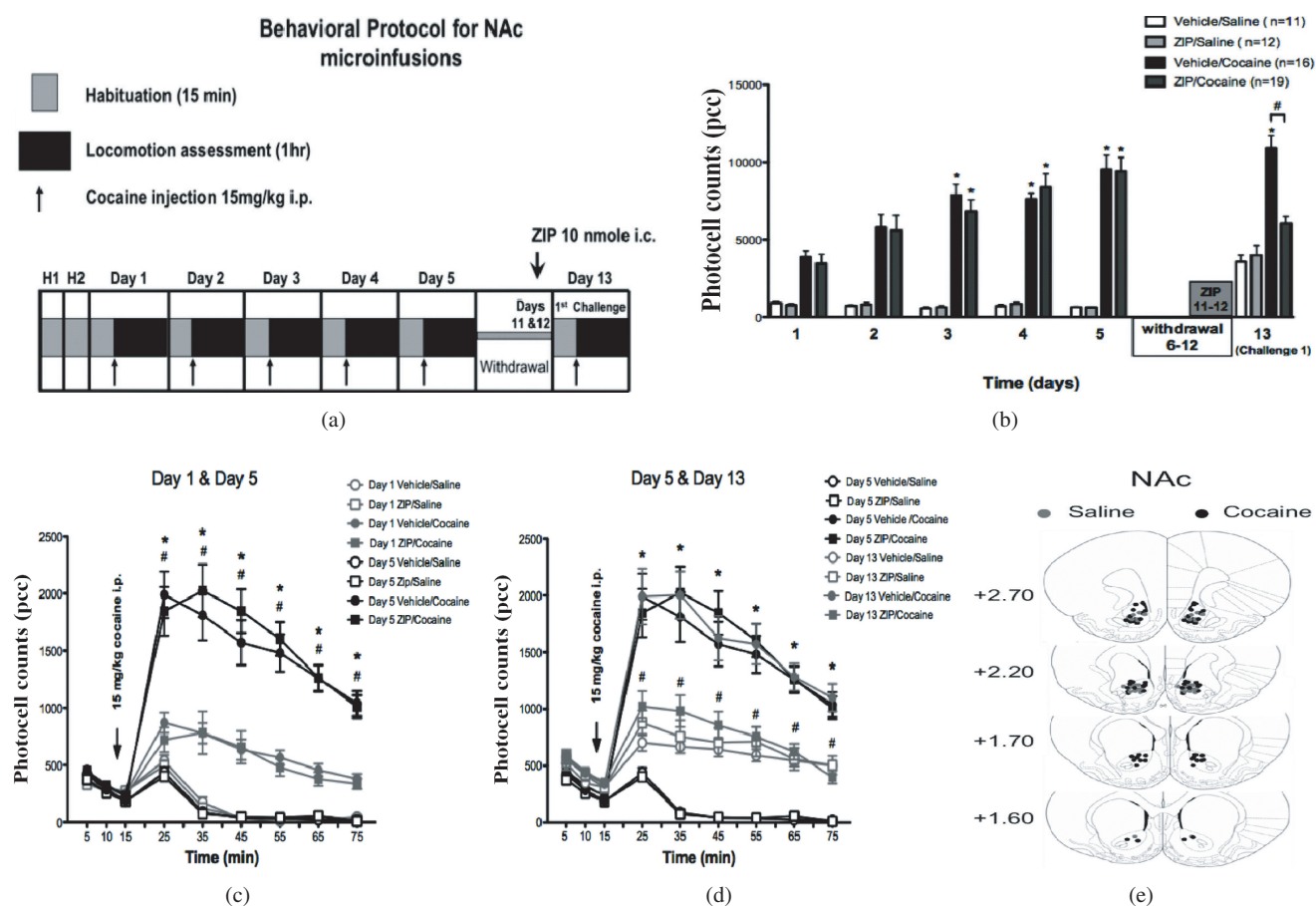
Figure 3: *Intra NAc ZIP microinfusions decrease the expression of cocaine sensitization.* (a) Diagram shows cocaine sensitization protocol with time points for intra NAc ZIP microinfusions. (b) Mean total ambulatory activity (pcc/60 min, ±SEM) was recorded for each group. Asterisks (∗) denote significance of the group compared to corresponding day 1 ($P < .01$). Numeral (#) denotes statistical significance between groups on day 13. On day 5, cocaine-treated animals show sensitization to cocaine. On day 13, ZIP/cocaine-treated animals did not express sensitization ($P > .05$). Instead, they showed a significant decrease (#) (day 13) in locomotor activity when compared to vehicle/cocaine animals ($P < .05$). There was no significance between ZIP/saline and vehicle/saline controls throughout the experiment ($P > .05$). (c) Time course of locomotor responses after 1 (gray) and 5 (black) days of cocaine (15 mg/kg, i.p.) (filled) or saline (unfilled) administration. Cocaine sensitization was manifested as an increase in locomotor activity in all time points. Significant differences between day 1 and day 5 ($P < .05$) are indicated by asterisk (∗) for vehicle/cocaine and # for ZIP/cocaine. Each time point represents the mean ±SEM. (d) Time course for the locomotor response on days 5 (black) and 13 (gray). ZIP/cocaine-injected animals (filled gray squares) show decreased locomotor activity ($^*P < .05$) compared to day 5 (#). Asterisks (∗) denote significance between ZIP/cocaine and vehicle/cocaine animals on day 13. Each point represents the mean ±SEM. (Two-way ANOVA, followed by bonferroni post test for all comparisons). (e) Injection sites within the NAc for saline- (gray circles) and cocaine- (black circles) treated animals.

that received cocaine for the first time (vehicle/saline and ZIP/saline). Also, ZIP pre-treatment has no effect on the acute response to cocaine since there is no difference at any time point between vehicle/saline and ZIP/saline on day 6.

To determine whether ZIP's effect on locomotion on day 6 (decreased locomotion) alters the expression of sensitization after withdrawal, we then tested the effect of a second challenge of cocaine (15 mg/kg i.p.) after a 7-day drug-free period. On day 14, ZIP/cocaine-pretreated animals showed no significant difference in locomotion ($7,542 \pm 640$ counts; $n = 13$) compared to vehicle/cocaine pretreated rats ($7,608 \pm 475$ counts; $n = 11$; ANOVA $F_{27,287} = 23.06$, $P > .05$; Figure 2(b)). These results suggest that either intra-VTA ZIP's effect on sensitization is temporary (short lived) or that a different mechanism independent of VTA's PKMζ is active on day 5 that sustains expression of sensitization after a withdrawal period. Because previous studies have suggested a time-limited role

for the VTA in cocaine-sensitization, which is later taken over by the NAc [86], we investigated whether inhibition of PKMζ in the VTA or the NAc, after a withdrawal period, modifies the expression of previously established sensitization. Animals that received microinfusions outside of the VTA were excluded from data analyses (Figure 2(g)).

3.4. Intra-VTA ZIP microinfusions after a withdrawal period do not alter the expression of behavioral sensitization

Rats were chronically injected with cocaine (15 mg/kg) or saline i.p. over five consecutive days to induce behavioral sensitization. Cocaine-injected animals showed a significant increase in locomotor activity on day 5 ($5,043 \pm 762$ counts, $n = 9$ for vehicle/cocaine and $5,217 \pm 1079$ counts, $n = 8$ for ZIP/cocaine) compared with day 1 ($2,553 \pm 256$ counts for $n = 9$ vehicle/cocaine and $2,203 \pm 423$ counts for ZIP/cocaine, ANOVA $F_{23,190} = 9.89$, $P < .05$; Figure 2(f)). ZIP (10 nmol) was microinfused into the VTA on the last day of the withdrawal period (day 12, Figure 2(e)). A cocaine challenge (15 mg/kg i.p.) was given 24 h later. On day 13, ZIP/cocaine-pretreated animals did not present any change in locomotion ($4,991 \pm 878$ counts, $n = 8$) compared with vehicle/cocaine-pretreated rats (5193 ± 1133 counts, $n = 9$, ANOVA $F_{23,190} = 9.89$, $P > .05$; Figure 2(f)). This figure also shows that ZIP pre-treatment has no effect on the acute response to cocaine since there is no difference between ZIP/saline ($n = 9$) and vehicle/saline animals ($n = 9$) on day 13. Animals that received microinfusions outside of the VTA were excluded from data analyses (Figure 2(h)).

3.5. Intra-NAc ZIP microinfusions after a withdrawal period decrease the expression of behavioral sensitization

We performed microinjections of PKMζ inhibitor ZIP directly into the NAc at the end of a withdrawal period following a cocaine sensitization scheme. Rats were chronically injected with 15 mg/kg cocaine or saline i.p. over five consecutive days to induce behavioral sensitization. Cocaine-injected animals showed a significant increase in locomotor activity on day 5 ($9,438 \pm 871$ counts, $n = 19$ for ZIP/cocaine and $9,535 \pm 914$ counts, $n = 16$ for vehicle/cocaine) compared with day 1 ($3,456 \pm 574$ counts for ZIP/cocaine and $3,865 \pm 391$ counts for vehicle/cocaine; ANOVA $F_{23,319} = 25.43$, $P < .01$; Figure 3(b)). ZIP (10 nmol) was microinfused into the NAc on the last two days of the withdrawal period (days 11 and 12, Figure 3(a)). A cocaine challenge (15 mg/kg i.p.) was given on day 13. On day 13, ZIP/cocaine-pretreated animals presented a significant decrease in locomotion ($6,027 \pm 464$ counts) compared with vehicle/cocaine-pretreated rats ($10,906 \pm 811$ counts; ANOVA $F_{23,319} = 25.43$, $P < .05$; Figure 3(b)). Time course of locomotor responses on days 1 and 5 (Figure 3(c)) compares locomotor activity after 1

(gray) and 5 (black) days of cocaine (15 mg/kg, i.p.) (filled) or saline (unfilled). Cocaine sensitization was manifested as an increase in locomotor activity for ZIP/cocaine ($^{\#}P < .05$) and vehicle/cocaine ($^{*}P < .05$) animals during all time points after cocaine injection (ANOVA $F_{71,1026} = 29.99$, $P < .05$; Figure 3(c)). Figure 3(d) shows that there is a decrease in locomotor activity of ZIP/cocaine animals in every time point on day 13 when compared to day 13 vehicle/cocaine ($^{*}P < .05$) animals and when compared to ZIP/cocaine animals on day 5 (ANOVA $F_{71,1026} = 23.34$, $^{\#}P < .05$). This figure also shows that ZIP pre-treatment has no effect on the acute response to cocaine since there is no difference between ZIP/saline ($n = 12$) and vehicle/saline ($n = 11$) animals. Histological analysis (Figure 3(e)) confirmed cannula placements, and animals that received microinfusions outside of the NAc were excluded from data analyses. These results suggest that PKMζ may help maintain the expression of cocaine sensitization in NAc.

4. Discussion

In this study, we demonstrated that ZIP decreased AMPA-mediated currents and AMPA/NMDA ratios in VTA-DA cells from cocaine-treated animals. Moreover, VTA-ZIP infusions before a withdrawal period decrease locomotor behavioral sensitization. On the other hand, intra VTA ZIP infusions after a withdrawal period, had no effect on the expression of cocaine sensitization. Administration of the PKMζ inhibitor into the NAc following a withdrawal decreases the expression of cocaine sensitization. These results indicate that the persistent activity of PKMζ in the VTA is critical for the maintenance of cocaine-induced behavioral sensitization only before a withdrawal period. They also suggest that the there is a need of a persistently active PKMζ in the NAc for the expression of behavioral sensitization after a withdrawal period.

Synaptic plasticity that occurs in the reward system as a consequence of addictive drugs is thought to contribute to addiction-related behaviors [10,31,36,61,66,79,87]. In particular, excitatory synaptic transmission in the mesolimbic DA system has been shown to support some of the behavioral long-lasting drug effects [29,54,85]. However, to our knowledge, evidence demonstrating that selectively reversing these late LTP-like neuroadaptations after chronic cocaine treatment alters the enhanced behavioral response characteristic of sensitization is sorely missing.

One of the immediate molecular effects that underlies single and multiple injections of cocaine are long-term changes in synaptic plasticity in the VTA [6,67,81]. This area is implicated in the development of drug addiction [27, 35,70] and sensitization [33,89]. In addition, the NAc core, which contributes to drug-associated, cue-induced cocaine seeking and mediates the incentive value of reward-conditioned stimuli [1,19,24,25,55], also undergoes

synaptic strengthening after repeated exposure to cocaine followed by a period of abstinence [8, 9, 15, 38]. The specific molecular mechanisms that sustain these changes have been unknown but may be related to those implicated in LTP.

Several protein kinases have been shown to be important for LTP. Recent studies have focused on the activity of enzymes critical within 30 min after tetanic stimulation, but not afterwards. Therefore, these proteins are thought to be important for the induction but not the maintenance of LTP. During LTP, a single PKC isozyme, PKMζ [53, 64] is activated by a mechanism fundamentally different from that of the kinases important for LTP induction [22, 53]. PKMζ is a brain-specific protein kinase C isoform [22] that lacks inhibition from a regulatory domain and, thus, is persistently active [64]. ZIP is a myristoylated PKMζ pseudosubstrate inhibitory peptide that specifically mimics the autoregulatory domain of PKCζ therefore blocks PKMζ. For this reason, ZIP infusions produce retrograde amnesia of several long-term memories [14, 16, 20, 21, 39, 40, 45, 49, 58, 63, 71, 74, 75, 76, 84]. Hence, PKMζ is the first enzyme known to sustain memory by maintaining LTP in the brain.

Cocaine administration has been recently shown to increase PKMζ in the VTA [23]. Phosphorylation by PKMζ contributes to the late-phase of LTP, by increasing the number of AMPA receptors that are expressed at synapses, enhancing AMPAR-mediated synaptic transmission [43, 72, 90]. Thus PKMζ inhibition reverses additional AMPAR responses that contribute to potentiated synapses, but has no effect on baseline AMPAR responses, either in vitro [43, 72, 90] or in vivo [57]. For this reason, we investigated whether ZIP selectively decreases AMPA currents from putative VTA-DA neurons of cocaine-sensitized animals and has no effect on AMPA currents of saline-injected rats (Figure 1). The results confirmed that only potentiated synapses are altered by PKMζ inhibition and that the potentiation observed after cocaine administration is in part mediated by PKMζ. Since the basal or potentiated strength of excitatory synapses is difficult to compare between slices, we calculated the ratio between AMPA and NMDA currents as a normalization procedure [78]. We found an increase in AMPA/NMDA ratio in cocaine-treated rats (Figure 1). This increase in synaptic strength is a key feature of LTP production in animals with repeated cocaine injections [2, 5, 6, 65, 81]. ZIP incubation decreased AMPA/NMDA ratio only in cells from cocaine-sensitized rats (Figure 1). This results are in agreement with recent evidence showing that inhibition of PKMζ blunts cocaine-enhanced AMPAR/NMDAR ratio [23].

In our studies, intra-VTA ZIP infusion on day 5 altered the established cocaine sensitization (Figure 2), indicating a modification in the mechanism that maintains the long-lasting effects after chronic cocaine exposure [29]. Since ZIP is thought to reverse already established LTP, these findings imply that LTP occurring in the VTA is PKMζ-dependent and critical for the maintenance of behavioral sensitization prior to a withdrawal period.

ZIP's effect on behavioral sensitization, AMPA currents and AMPA/NMDA ratios could be due to removal of AMPA receptors from postsynaptic sites [43, 72, 90]. Potentiation in the VTA after cocaine administration is thought to be accompanied by an exchange of GluA2-containing for GluA2-lacking AMPA receptors [3, 11, 18]. However, recent studies in the NAc show that GluA2-lacking receptors do not seem to mediate expression of cocaine sensitization since animals that have received non-contingent cocaine administration do not show the change from GluA2-containing to GluA2-lacking AMPA receptors, but still show sensitization to cocaine [9, 15, 17, 38]. Therefore, it might be possible that decreasing the number of GluA2-containing AMPA receptors left at the synapse can modify the synaptic strengthening in these regions and therefore alter sensitization. To confirm this hypothesis, and therefore find the specific mechanism of ZIP's effect, further experiments exploring the presence or absence of GluA2 containing receptors before and after ZIP treatment should be performed.

Here, the expression of sensitization measured after a 7-day withdrawal period was not altered by ZIP infusions into the VTA on day 5 (Figure 2). A simple explanation to this result might be that ZIP's effect on sensitization is just temporary, as was shown in fear conditioning experiments (see [16, 56]). It is also possible that other drug-induced VTA neuroadaptations, independent of PKMζ, may exist and allow the expression after a withdrawal period. On the other hand, numerous studies have shown that AMPA receptor-mediated activation in the VTA may be a key process responsible for the development of addiction [36, 37, 39, 82, 88], whereas the NAc mainly supports the mechanisms for expression [50, 82]. This change in anatomical location is thought to reveal a "transfer" of sensitization from the VTA to the NAc as one progress from initiation to expression [86]. We believe that synaptic potentiation in the VTA, which eventually induces alterations in the NAc before the withdrawal period [13, 38, 46], had already occurred by the time ZIP was infused into the VTA. For this reason, it might be too late for intra-VTA ZIP infusions to alter expression after withdrawal. The data suggest that elimination of PKMζ-dependent drug-evoked synaptic plasticity within the VTA is not necessary for the maintenance of cocaine-induced synaptic changes in the NAc. It is consistent with previous observations that, following cocaine exposure, long-lasting neuroadaptations take place in the NAc even before withdrawal periods [38, 77].

To confirm that PKMζ-dependant potentiation in the VTA is not important for the expression of sensitization, ZIP was microinfused into the VTA after a 7-day withdrawal

period. There was no change in locomotor activity after ZIP infusion. Therefore, PKMζ-dependent potentiation in the VTA seems to not play a role in the expression of sensitization. This is also supported by previous studies that say that the synaptic potentiation in the VTA decays during the withdrawal period [2].

Finally, we tested whether synaptic plasticities occurring in the NAc were also maintained by PKMζ. ZIP was infused into the NAc of sensitized rats on the last two days of the withdrawal period, immediately prior to a challenge dose of cocaine. This manipulation prevented the increase in locomotor activity normally elicited by a subsequent cocaine challenge (Figure 3). Our NAc results are consistent with two recently published articles that show a role of PKMζ in maintaining addiction memories [42, 73]. In addition, an increase in AMPAR has been shown to occur in the NAc at different time points of the withdrawal period [8,9]. This increase is thought to be responsible for the drug-evoked synaptic potentiation observed in the NAc [7,8,9,15,17,38,68]. Furthermore, GluA2-containing receptors are present in the NAc after a withdrawal period in non-contingent cocaine administration [48]. Thus, in our studies, PKMζ inhibition may be decreasing the number of GluA2-containing AMPA receptors at the NAc synapse. While further experiments should test the latter hypothesis, our results are in agreement with recent findings showing that NAc postsynaptic GluA2-containing receptors are relevant for the maintenance of memories for drug cues via PKMζ [42].

Taken together, the present data provide a potentially relevant region and time-specific brain mechanism, dependant of PKMζ that enables sensitization. It suggests that PKMζ may maintain a VTA-dependent mechanism that underlies sensitization prior to a withdrawal period but that a different neuroadaptation, VTA-PKMζ insensitive, must come on-line after a withdrawal period, which mediates expression. We postulate that this VTA-PKMζ insensitive mechanism may be a PKMζ-dependent NAc synaptic plasticity. Our results confirm the vision that addiction involves a pathological learning process. They imply that if this learning process is reversed changes in the behavioral response may also be at least temporary overturned.

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