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Suppressing calcium/calmodulin-dependent protein kinase II activity in the ventral tegmental area enhances the acute behavioural response to cocaine but attenuates the initiation of cocaine-induced behavioural sensitization in rats

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Abstract

In the present experiments we administered an α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor antagonist (CNQX), *N*-methyl-D-aspartate (NMDA) receptor antagonist (AP-5), or L-type calcium channel blocker (diltiazem) directly into the ventral tegmental area (VTA) before each of four daily systemic cocaine injections in order to assess their influence on the initiation phase of behavioural sensitization. Results indicated that pretreatment with CNQX or AP-5 impaired the initiation of cocaine-induced behavioural sensitization. Intra-VTA administration of diltiazem significantly increased the behavioural activation induced by an acute cocaine injection, but impaired the development of cocaine-induced behavioural sensitization. Because AMPA and NMDA receptors, as well as L-type calcium channels are calcium permeable, we also investigated the role of the calcium-activated second messenger calcium/calmodulin-dependent protein kinase II (CaM-KII). Similar to the results obtained with diltiazem, administration of the CaM-KII inhibitor KN-93 into the VTA enhanced the acute behavioural response to cocaine but prevented the augmentation of cocaine-induced behavioural hyperactivity following repeated injections. Consistent with this finding, the behavioural hyperactivity produced by cocaine was markedly enhanced among homozygous α-CaM-KII knockout mice but the initiation of behavioural sensitization to cocaine was attenuated relative to wild-type mice. Separate experiments performed in rats demonstrated an increase in total protein levels of CaM-KII in the VTA 24 h after the last of seven daily injections of cocaine. Taken together, these results indicate that blocking L-type calcium channels or impairing CaM-KII activity in the VTA augments the acute behavioural hyperactivity induced by cocaine. The present findings also suggest that increased calcium influx through AMPA receptors, NMDA receptors and L-type calcium channels on dopaminergic neurons in the VTA contributes significantly to the initiation of behavioural sensitization by amplifying calcium signalling through CaM-KII.

Introduction

Repeated exposure to cocaine results in behavioural sensitization, a phenomenon characterized by augmentation of the locomotor activating effect of this psychostimulant in rats. The neuroadaptations mediating the sensitized response to psychostimulants also could underlie some of the behavioural changes associated with chronic psychostimulant abuse (Robinson & Berridge, 1993; Bartlett *et al*., 1997), making it desirable to elucidate those neuroadaptations in order to better understand the mechanisms underlying drug craving and addiction.

A growing literature indicates that ionotropic glutamate receptors and L-type calcium channels, all of which are calcium permeable, are involved in the sequelae of cellular and molecular events that lead to psychostimulant-induced behavioural sensitization. Systemic injections of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) or *N*-methyl-D-aspartate (NMDA) receptor antagonists (Karler *et al*., 1989; Stewart & Druhan, 1993; Wolf & Jeziorski, 1993; Li *et al*., 1997) as well as L-type calcium channel blockers (Karler *et al*., 1991; Reimer & Martin-Iverson, 1994) prevent the initiation of behavioural sensitization to cocaine or amphetamine. Moreover, microinjections of an NMDA antagonist directly into the VTA attenuates the development of both amphetamine- (Vezina & Queen, 2000) and cocaine-induced behavioural sensitization (Kalivas & Alesdatter, 1993), while repeated administration of an L-type calcium channel agonist directly into the ventral tegmental area (VTA) cross-sensitizes to a subsequent challenge injection of cocaine (Licata *et al*., 2000). Collectively, these data suggest that ionotropic glutamate receptors and L-type calcium channels located on dopaminergic cells in the VTA play critical roles in the initiation of behavioural sensitization to psychostimulants. To investigate this hypothesis further, the present studies assessed the effect of intra-VTA administration of an AMPA receptor antagonist (CNQX), NMDA receptor antagonist (AP-5), or an inhibitor of L-type calcium channels (diltiazem) on the initiation of behavioural sensitization to cocaine in rats.

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Downstream from these calcium-permeable receptors and channels are calcium-mediated second messengers such as the calcium/calmodulin (CaM)-dependent kinases. Of the CaM kinases, CaM-KII in particular is highly abundant in the brain comprising close to 2% of the brain's total protein (Sola *et al*., 1999). CaM-KII is a holoenzyme composed of alpha, beta, or a combination of both subunits; however, alpha is the predominant isoform in the rat brain (Sola *et al*., 1999). CaM-KII has been proposed as a candidate molecule for the long-term storage of information because of its ability to remain phosphorylated in the absence of CaM (Lisman, 1994; Lisman *et al*., 2002). In order to evaluate the role of CaM-KII in cocaine-induced plasticity, we administered a CaM-KII inhibitor (KN-93) directly into the VTA during a sensitizing regimen of daily cocaine injections. In addition, the extent to which α-CaM-KII knockout mice sensitize to the locomotor activating effects of cocaine was assessed. Finally, the effect of acute or repeated cocaine administration on total protein levels of CaM-KII in the VTA was investigated.

Materials and methods

Animals

Male Sprague–Dawley rats weighing 250–275 g were obtained from Taconic Farms (Germantown, NY, USA). Rats were individually housed with food and water available *ad libitum*. Male homozygote α-CaM-KII knockout mice, bred in the C57BL/6 background strain, weighed 17–23 g and were obtained from The Jackson Laboratories (Bar Harbor, ME, USA). Control mice were the C57BL/6 strain. All mice were housed four per cage. A 12 : 12 h light : darkness cycle was used with lights on at 06.00 h. All experimental procedures were performed during the animals' light cycle. All experimental protocols were consistent with guidelines issued by the National Institutes of Health and were approved by Boston University School of Medicine's Institutional Animal Care and Use Committee.

Surgery

Before surgery, the rats were anaesthetized with 80 mg/kg ketamine and 12 mg/kg xylazine and mounted in a stereotaxic apparatus. Stainless steel guide cannulae (14 mm, 24-gauge) were implanted bilaterally 1 mm dorsal to the VTA (−5.5 A/P, ±0.5 M/L, −7.0 D/V relative to bregma; Paxinos & Watson, 1997) and cemented in place by affixing dental acrylic to three stainless steel screws secured in the skull. Following surgery the rats were allowed to recover for 3–5 days before the start of the microinjection regimen.

Apparatus

Locomotor behaviour was measured in 40 × 40 × 40 cm photocell-based activity monitors (AccuScan Instruments, Columbus, OH, USA). All behaviour monitors were illuminated with a 15 W light bulb, and were housed in separate sound-attenuation chambers.

Behaviour experiments

The day before the first of the four daily microinjections, the rats were habituated to the activity monitors for 3 h. On each treatment day the rats were habituated to the photocell boxes for 1 h. Following habituation, injectors (33-gauge stainless steel) were lowered into the VTA 1 mm below the tips of the guide cannulae in order to administer the microinjections.

In the antagonist/inhibitor experiments in rats, on each of four consecutive days animals received bilateral microinjections of either the AMPA receptor antagonist CNQX (0.3 μg/0.5 μL), the NMDA receptor antagonist AP-5 (30 μg/0.5 μL), the L-type calcium channel blocker diltiazem (0.1 μg or 1.0 μg/0.5 μL), the CaM-KII inhibitor KN-93 (0.6 μg or 6.0 μg/0.5 μL) or vehicle (0.9% saline) over 2 min in a volume of 0.5 μL/side. The injectors were left in place for an additional minute to allow the compound to diffuse from the site of injection and were then removed. The animals were returned to the behaviour chamber immediately following microinjections. Ten minutes after antagonist or vehicle administration rats received an injection of cocaine (15 mg/kg, i.p.) or saline and their behaviour was monitored for 2 h postinjection.

In the mouse experiments, both homozygote α-CaM-KII knockout mice and C57BL/6 control mice were habituated initially to the photocell chambers for a 1-h period. All mice then received an injection of saline, were returned to the photocell chambers and behaviour was monitored for the following 2 h. On each of the next four consecutive days the mice were habituated to the behaviour boxes for 1 h, received cocaine (20 mg/kg, i.p.), and were returned to the boxes for behavioural monitoring for a 2-h period. Seven days after the last cocaine injection (i.e. day 11) all mice were habituated to the photocell chambers for 1 h before receiving a challenge injection of cocaine (20 mg/kg, i.p.). The behavioural activity was subsequently monitored for 2 h. At the completion of the experiment the mice were lightly anaesthetized with isofluorane, decapitated and the brains were removed and homogenized for Western blot analysis to confirm phenotype.

Acute or repeated cocaine administration for Western blotting experiments

Rats received either a single injection or seven daily injections of cocaine (15 mg/kg, i.p.) or an equal volume of 0.9% saline in the home cage. Twenty-four hours postinjection the rats were killed by decapitation under light anaesthesia with isofluorane.

Western blotting

Brains from the knockout and C57BL/6 mice were removed and frozen on dry ice. Brains from the acute- or repeated cocaine-treated rats were removed and the VTA was dissected and frozen on dry ice. The brains and VTA samples were subsequently thawed and homogenized in lysis buffer (30 μL 1 M Hepes, pH 7.85; 30 μL Igepal (Sigma, St. Louis, MO, USA); 300 μL glycerol (Sigma); 6 μL 100 mM EGTA; 12 μL 0.5 M EDTA; 30 μL 1 M KCl; 15 μL 100 mM PMSF (American Bioanalytical, Natick, MA, USA); 12 μL 1 M DTT (American Bioanalytical); 20 μL aprotinin (Sigma); 30 μL leupeptin (1 mg/mL; American Bioanalytical); and 2.47 mL distilled water in a total of 3 mL) by sonication. Samples were shaken at 4 °C for 25 min then centrifuged (10 000 ***g***) for 10 min. The supernatant was assayed subsequently for protein concentration using the Bradford method.

Sample buffer (Novex sample buffer; Invitrogen; Carlsbad, CA, USA) was added to the preparations in a 1 : 1 ratio. Samples were incubated at 90 °C for 5 min and aliquots containing equal amounts of protein were separated using 10% Tris-glycine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Invitrogen). Proteins were transferred to a nitrocellulose membrane (nitrocellulose membrane filter paper sandwich; Invitrogen) electrophoretically and blocked with 5% nonfat dry milk in buffer containing phosphate-buffered saline (PBS) and 0.2% Tween 20. Proteins were immunolabelled with the CaM-KII primary antibody, diluted 1 : 220 (for VTA) or 1 : 1000 (for brain homogenate) from the initial concentration of 200 μg/mL (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA). The primary antibody was detected with horseradish peroxidase-protein A (Zymed Laboratories, Inc.; San Francisco, CA, USA; diluted 1 : 3000), enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech; Piscataway, NJ, USA) and autoradiography. Bands on the autoradiograms were quantified by densitometry using Image Quant v1.2 (Amersham Pharmacia Biotech).

Statistical analyses

Time course data were analysed with mixed factor analyses of variance (ANOVA). The between-subjects measure was drug treatment, whereas the within-subjects measure was time. The total distance travelled recorded during the testing session was analysed with paired *t*-tests. In all cases, pairwise comparisons were made using Fisher's least squares difference (significant at $P < 0.05$). The Western blotting data represent the percentage change from saline as measured by densitometric band volume and were analysed with separate unpaired *t*-tests.

Histology

Following the completion of the microinjection experiments, rats were given an overdose of sodium pentobarbital (> 100 mg/kg, i.p.) and perfused intracardially with 0.9% saline followed by 10% formalin. The brain was removed and coronal sections (100 μm) were taken at the level of the VTA with a vibratome (Technical Products International; St. Louis, MO, USA). The sections were mounted on gelatin-coated slides and stained with Cresyl violet. An individual unaware of the rats' behavioural response determined cannulae placements as well as cannulation- or drug-induced cell death and gliosis.

Drugs

Cocaine was obtained from the National Institute on Drug Abuse (Rockville, MD, USA) and was dissolved in 0.9% saline. CNQX (Sigma RBI; St. Louis, MO, USA), AP-5 (Sigma RBI), diltiazem (Calbiochem; La Jolla, CA, USA) and KN-93 (Sigma RBI) were prepared in 0.9% saline. All drug doses were chosen on the basis of microinjection experiments designed to assessed the modulation of psychostimulant-induced behaviours by glutamate receptor antagonists (Pierce *et al*., 1996; Cornish *et al*., 2001), diltiazem (Pierce *et al*., 1998) or KN-93 (Pierce *et al*., 1998).

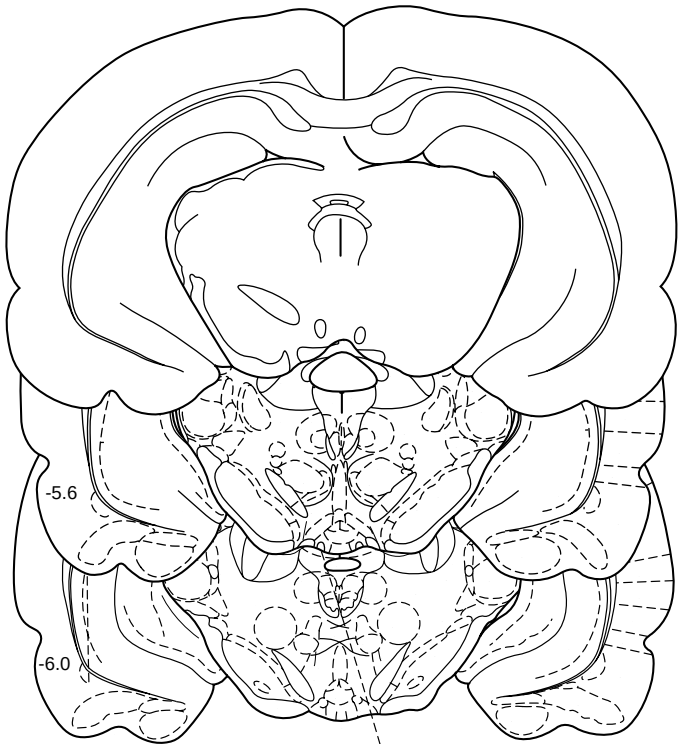
Results

Location of microinjection sites in the VTA

The schematic coronal brain sections depicted in Fig. 1 are from the atlas of Paxinos & Watson (1997). The filled circles represent the placement of the tips of the microinjection cannulae in the VTA from the experiments graphed in Figs 2–5. In some cases the cannula placements bordered nearby structures (i.e. the medial substantia nigra, ventral medial lemniscus and ventral red nucleus). A total of 170 rats were used in these experiments; the data obtained from 64 of these animals were removed from subsequent data analysis because of faulty cannula placements. All sections were checked closely for microinjection-induced neurotoxicity. In no case was cell death or gliosis noted other than what is typically observed following cannula placement.

Repeated intra-VTA administration of the AMPA antagonist CNQX or the NMDA antagonist AP-5 prevented the initiation of behavioural sensitization to cocaine

Figure 2 summarizes the total distance travelled by animals pretreated with CNQX, AP-5 or saline. These data were analysed with separate paired *t*-tests, which were planned comparisons between days 1 and 4 within treatment groups. These analyses revealed a significant difference between days 1 and 4 for the saline-treated animals ($t_{14} = 3.25$, $P < 0.006$), but not for those treated with either CNQX ($t_8 = 1.56$, $P < 0.157$) or AP-5 ($t_9 = 1.44$, $P < 0.184$). In other words, behavioural sensitization was observed in the saline but not the CNQX or AP-5 groups. There were 9–15 rats per group for these experiments.



Intra-VTA administration of the L-type calcium channel antagonist diltiazem enhanced the acute behavioural response to cocaine but impaired the initiation of behavioural sensitization to cocaine

The distance traveled following cocaine for animals pretreated with diltiazem or saline are summarized in Fig. 3. For animals pretreated with saline, 0.1 μg diltiazem or 1.0 μg diltiazem, the time course of the behavioural response following cocaine on days 1 and 4 is presented in the left panels, whereas the total distance travelled on these treatment

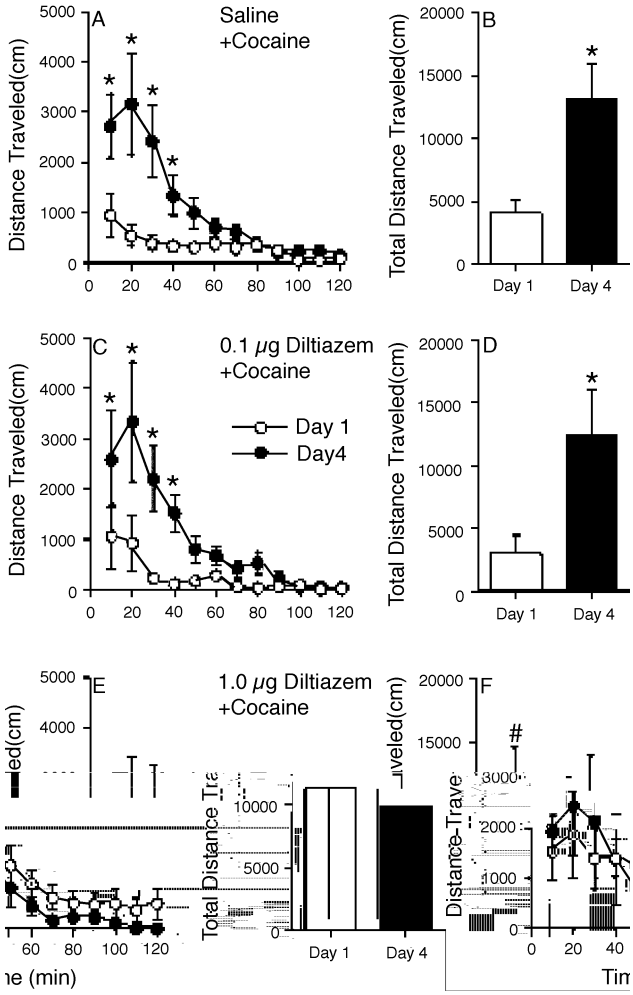


FIG. 3. Repeated microinjections of diltiazem directly into the VTA dose-dependently prevented the initiation of behavioural sensitization to cocaine. On days 1–4 rats received bilateral microinjections of saline or diltiazem (0.1 or 1.0 μg/0.5 μL) into the VTA. Ten minutes later they received a systemic injection of cocaine (15 mg/kg, i.p.) and their behaviour was monitored for the following 2 h. The data shown in the left panels (A, C and E) depict the time course of the distance travelled (mean ± SEM) following cocaine administration. The asterisks represent significant differences from day 1 at that time point. The data summarized in the right panels (B, D and F) represent the total distance traveled (mean ± SEM) over the 120 min following cocaine administration on days 1 and 4. The asterisks represent significant differences in total distance travelled compared to day 1 for that treatment group (Fisher's LSD, $P < 0.05$). #Significant difference in total distance travelled relative to saline on day 1 (Fisher's LSD, $P < 0.05$). There were 5–9 rats per group.

days is shown in the right panels. The data presented in the left panels (3A, C and E) were analysed with mixed factors ANOVAs, with repeated measures over time. The data shown in the right panels (3B, D and F) were analysed with paired *t*-tests. The data from animals pretreated with saline and then administered cocaine are summarized in 3A and 3B. The results of the analysis of the data depicted in panel A revealed significant main effects of treatment day ($F_{1,16} = 9.702$, $P < 0.0067$) and time ($F_{11,176} = 9.077$, $P < 0.0001$) as well as a significant interaction between these factors ($F_{11,176} = 4.497$, $P < 0.0001$). Subsequent pairwise comparisons (Fisher's LSD) showed that the hyperactive behavioural response to cocaine was increased significantly during the first 40 min following cocaine administration on day 4 relative to day 1. The data from panel B were analysed with a paired *t*-test, which revealed a significant difference between the behavioural responses on days 1 and 4 ($t_8 = 3.372$, $P < 0.0098$). The data from animals administered 0.1 μg diltiazem before the daily cocaine injections are summarized in panels C and D. The analysis of the data from panel C revealed significant main effects of treatment day ($F_{1,8} = 6.09$, $P < 0.0388$) and time ($F_{11,88} = 8.571$, $P < 0.0001$) as well as a significant interaction between these factors ($F_{11,88} = 2.885$, $P < 0.0028$). Subsequent pairwise comparisons (Fisher's LSD) showed that the hyperactive behavioural response to cocaine was significantly increased during the first 40 min following cocaine administration on day 4 relative to day 1. The paired *t*-test performed on the data from panel D revealed a significant difference between the behavioural responses on days 1 and 4 ($t_4 = 3.117$, $P < 0.0356$). Panels E and F depict the data from rats pretreated with 1.0 μg diltiazem followed by cocaine. The analysis of the data from panel E revealed a significant main effect of time ($F_{11,154} = 6.543$, $P < 0.0001$) but not treatment day ($F_{1,14} = 0.075$, $P < 0.7882$) and no significant interaction between these factors ($F_{11,154} = 0.653$, $P < 0.7807$). Analysis of the data presented in panel F revealed no significant difference between days 1 and 4 ($t_7 = 0.279$, $P < 0.7885$). Collectively, these data indicate that behavioural sensitization to cocaine was observed in the saline and 0.1 μg diltiazem groups but not the 1.0 μg diltiazem group. In addition, there was a significantly increased behavioural response to cocaine on day 1 in the 1.0 μg diltiazem group relative to the saline group ($t_{15} = 2.191$, $P < 0.0447$), which indicates that intra-VTA diltiazem enhances the behavioural hyperactivity induced by an acute injection of cocaine (see day 1 in panels B and F). There were 5–9 rats per group for these experiments.

Intra-VTA administration of the CaM-KII inhibitor KN-93 enhanced the acute behavioural response to cocaine but impaired the initiation of behavioural sensitization to cocaine

Figure 4 summarizes data for animals pretreated with KN-93 or saline. These data are presented in the same format as those in Fig. 3. The analyses are also the same as used for the data shown in Fig. 3. The data from animals pretreated with saline and then administered cocaine are summarized in panels A and B. The results of the analysis of the data depicted in panel A revealed significant main effects of treatment day ($F_{1,12} = 11.457$, $P < 0.0054$) and time ($F_{11,132} = 15.511$, $P < 0.0001$) as well as a significant interaction between these factors ($F_{11,132} = 4.369$, $P < 0.0001$). Subsequent pairwise comparisons (Fisher's LSD) showed that the hyperactive behavioural response to cocaine was significantly increased during the first 50 min following cocaine administration on day 4 relative to day 1. Analysis of the data from panel B revealed a significant difference between the behavioural responses on days 1 and 4 ($t_6 = 3.146$, $P < 0.0199$). The data from animals administered 0.6 μg KN-93 before daily cocaine injections are summarized in panels C and D. The analysis of the data from panel C revealed no significant main effect of treatment day ($F_{1,14} = 2.193$, $P < 0.1608$) but did show a significant main effect of time ($F_{11,154} = 8.622$, $P < 0.0001$) as well as a significant interaction between treatment day and time ($F_{11,154} = 2.446$, $P < 0.0077$). Subsequent pairwise comparisons (Fisher's LSD) showed that the hyperactive behavioural response to cocaine was increased significantly during the first 40 min following cocaine administration on day 4 relative to day 1. The lack of a main effect of treatment day also was reflected in the analysis of the data from panel D, which revealed no significant difference between the behavioural responses on days 1 and 4 ($t_7 = 1.423$, $P < 0.1977$). Nonetheless, there is a clearly a difference between the behavioural responses between days 1 and 4 in this group, which is reflected in the significant interaction from the analysis of the time course data presented in panel C. Panels E and F show the data from rats pretreated with 6.0 μg KN-93 followed by cocaine. The

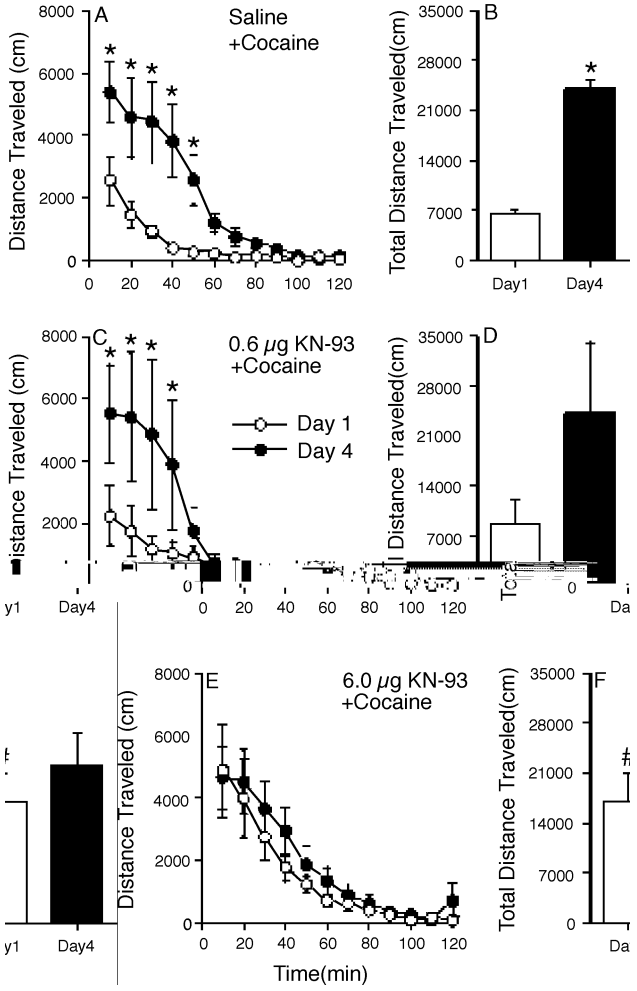


FIG. 4. Repeated microinjections of KN-93 directly into the VTA prevented cocaine-induced behavioural sensitization. On days 1–4 rats received bilateral microinjections of saline or KN-93 (0.6 or 6.0 μg/0.5 μL) into the VTA. Ten minutes later they received a systemic injection of cocaine (15 mg/kg, i.p.) and their behaviour was monitored for the following 2 h. The data depicted in the left panels (A, C and E) reflect the time course of the distance travelled (mean ± SEM) following cocaine administration. The asterisks represent significant differences from day 1 at that time point. The data summarized in the right panels (B, D and F) represent the total distance travelled (mean ± SEM) over the 120 min following cocaine administration on days 1 and 4. The asterisk represents a significant differences in total distance travelled compared to day 1 in the saline + cocaine group (Fisher's LSD, $P < 0.05$). #Significant difference in total distance travelled relative to saline on day 1 (Fisher's LSD, $P < 0.05$). There were 7–10 rats per group.

analysis of the data from panel E revealed no significant main effect of treatment day ($F_{1,18} = 0.710$, $P < 0.4106$) but did show a significant main effect of time ($F_{11,198} = 18.307$, $P < 0.0001$); there was no significant interaction between these factors ($F_{11,198} = 0.278$, $P < 0.9894$). Analysis of the data presented in panel F revealed no significant difference between days 1 and 4 ($t_9 = 1.448$, $P < 0.1815$). Collectively, these data indicate that behavioural sensitization to cocaine was observed in the saline and 0.6 μg KN-93 groups but not the 6.0 μg KN-93 group. In addition, an unpaired *t*-test ($t_{15} = 2.234$, $P < 0.0412$) revealed that the behavioural response to cocaine on day 1 with 6.0 μg KN-93 was significantly greater than saline (see day 1 in panels B and F). There were 7–10 rats per group for these experiments.

TABLE 1. The effect of repeated microinjections of CNQX, AP-5, diltiazem, or KN-93 directly into the VTA on locomotor activity

Treatment	Day 1	Day 2	Day 3	Day 4
Saline	1137 ± 422	1034 ± 361	1200 ± 340	1867 ± 291
CNQX	1930 ± 359	1766 ± 893	1825 ± 164	3081 ± 732
AP-5	1190 ± 333	1950 ± 418	1537 ± 231	1952 ± 684
Diltiazem	2087 ± 311	1957 ± 501	1105 ± 301	1747 ± 362
KN-93	3361 ± 399	2651 ± 508	2144 ± 509	2101 ± 205

On days 1–4 rats received bilateral microinjections of either saline, CNQX (0.3 μg/0.5 μL), AP-5 (3.0 μg/0.5 μL), diltiazem (1.0 μg/0.5 μL), or KN-93 (6.0 μg/0.5 μL) into the VTA. Ten minutes later they received a systemic injection of saline and their behaviour was monitored for the following 2 h. The data represent the total distance travelled in centimetres (mean ± SEM) over the 120 min following the saline injection on days 1–4. There were 4–7 rats per group.

Effect of antagonist administration alone on locomotor activity

Table 1 summarizes the control experiments designed to determine if the antagonists elicited a behavioural effect of their own. The animals were pretreated with saline, CNQX, AP-5, diltiazem or KN-93 before a systemic injection of saline rather than cocaine. These data were analysed with a two-way mixed factors ANOVA, with repeated measures over days. The analysis of the data in Table 1 revealed no significant main effect of drug treatment ($F_{4,21} = 1.849$, $P < 0.16$), no significant main effect of day ($F_{3,63} = 1.569$, $P < 0.21$), and no significant drug treatment × day interaction ($F_{3,63} = 1.389$, $P < 0.19$). Nonetheless, there was a marked trend toward an increase in behavioural activation following the first administration of KN-93, which is likely to have contributed to the augmentation of acute cocaine-induced behavioural hyperactivity induced by KN-93 described above.

Effects of acute and repeated cocaine administration on behavioural activity in homozygous α-CaM-KII knockout and wild-type mice

The mice lacking the gene encoding the alpha subunit of CaM-KII were first described by Silva and colleagues and were found to be deficient in their ability to produce long-term potentiation; these knockout mice also display impaired spatial learning (Silva *et al.*, 1992). Further characterization of α-CaM-KII knockout mice revealed abnormal fear responses and excessively aggressive behaviour (Chen *et al.*, 1994).

In the present experiments, before the first day of repeated cocaine administration, all mice were habituated to the activity chambers and administered saline. The distance travelled following the saline injection did not differ between the knockout and wild-type mice ($t_{11} = 0.148$, $P < 0.89$; data not shown).

Figure 5A and B depict the time course and totals, respectively, for the distance travelled by wild-type mice following cocaine administration on days 1 and 4. Figure 5A shows the time course of the distance travelled following cocaine administration in wild-type mice. These data were analysed with a mixed factors ANOVA, with repeated measures over days. This analysis revealed significant main effects of treatment day ($F_{1,14} = 22.387$, $P < 0.0003$), time ($F_{11,154} = 38.057$, $P < 0.0001$) and a significant interaction between these variables ($F_{11,154} = 20.944$, $P < 0.0001$). *Post hoc* analyses (Fisher's LSD) revealed a significant difference between the hyperactive behavioural responses to cocaine on days 1 and 4 over the first 50 min after cocaine administration. The total distance traveled in the wild-type mice on days 1 and 4 of cocaine treatment is depicted in panel B. These data were analysed with a paired *t*-test, the results of which were significant ($t_7 = 8.491$, $P < 0.0001$). The time course data from the α-CaM-KII

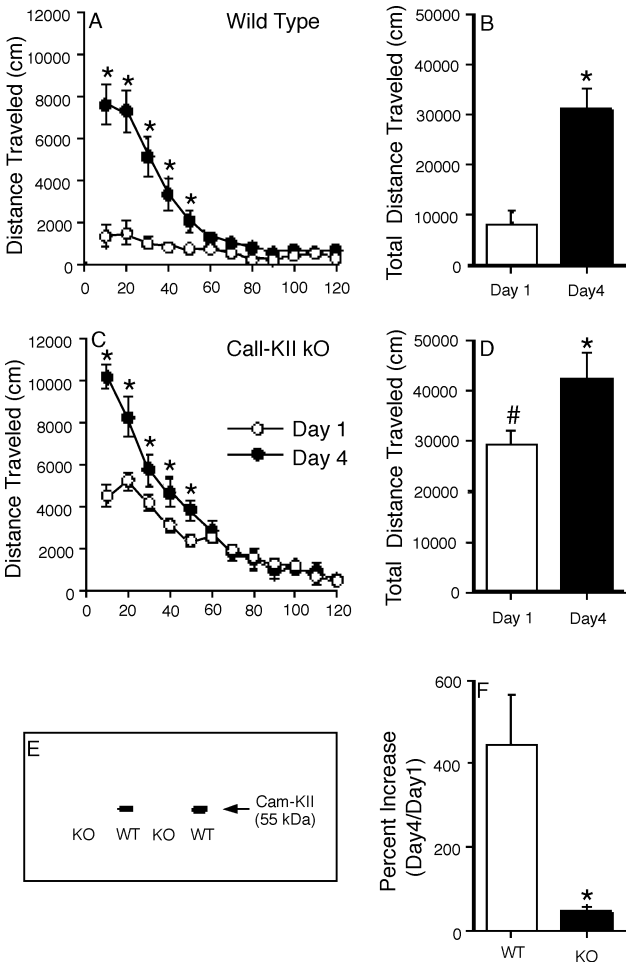


FIG. 5. Behavioural sensitization to cocaine is attenuated in α-CaM-KII knockout mice. On days 1–4 both wild-type and α-CaM-KII knockout mice received systemic injections of cocaine (20 mg/kg, i.p.) and their behaviour was monitored for the following 2 h. The data summarized in A and C are the time courses of the distance travelled (mean ± SEM) following cocaine administration in the wild-type mice (A) and α-CaM-KII knockout mice (B) on days 1 and 4. The asterisks indicate significant differences from day 1 at that time point (Fisher's LSD, $P < 0.05$). The data shown in B (wild-type mice) and D (α-CaM-KII knockout mice) represent the total distance travelled (mean ± SEM) over the 120 min following the cocaine injection on days 1 and 4. The asterisks indicate a significant difference from day 1 within the same group, whereas the pound symbol represents a significant difference from the data collected on day 1 from wild-type mice (from B). (E) Representative phenotyping of α-CaM-KII and wild-type mice. Lanes show either the absence (knockout) or presence (wild-type) of a band corresponding to CaM-KII. Each lane contained 40 μg protein. (F) Percentage increase in total distance travelled on day 4 relative to day 1 for wild-type (WT) and knockout (KO) mice. The asterisk represents a significant difference from wild-type (*t*-test, $P < 0.05$). There were five knockout mice and eight wild-type mice for this experiment.

knockout mice are shown in panels C and D. The results of the analysis of the data shown in panel C revealed significant main effects of treatment day ($F_{1,8} = 5.566$, $P < 0.046$), time ($F_{11,88} = 97.014$, $P < 0.0001$) and a significant interaction between these variables ($F_{11,88} = 13.744$, $P < 0.0001$). Subsequent pairwise analyses (Fisher's LSD) revealed a significant difference between the distance travelled following cocaine on days 1 and 4 during the first 50 min after cocaine administration. Analysis of the total distance travelled in the α-CaM-KII knockout mice on days 1 and 4 of cocaine treatment revealed a significant difference ($t_4 = 3.405$, $P < 0.0272$). In addition, an unpaired *t*-test ($t_{11} = 5.575$, $P < 0.0002$) showed that the total distance travelled on day 1 was significantly greater among α-CaM-KII knockout mice relative to wild-type mice (see day 1 in panels B and D). There were five knockout mice and eight wild-type mice in these experiments.

Collectively, these data indicate that the hyperactive behavioural response to cocaine was increased initially in α-CaM-KII knockout relative to wild-type mice. Although the knockout mice displayed behavioural sensitization to cocaine, in that the behavioural response on the fourth day of cocaine administration was significantly greater than the first cocaine injection, the magnitude of this increase was weak relative to that observed in the wild-type mice. As shown in panel F, there was nearly a 450% increase in cocaine-induced behavioural activation following 4 days of cocaine administration among the wild-type mice. In contrast, the increase in behavioural hyperactivity on day 4 in the knockout mice was only 46% greater than the behavioural response observed on day 1. An unpaired *t*-test performed on the data presented in panel F revealed a significant difference between the percentage increase in behavioural activation following cocaine administration on day 4 relative to day 1 in the wild-type relative to CaM-KII knockout mice ($t_{11} = 2.555$, $P < 0.0268$).

The phenotyping of the α-CaM-KII and wild-type mice is shown in Fig. 5E. As expected, α-CaM-KII was not present in the α-CaM-KII knockout mice. In two of the five knockout mice there was a very faint band (see first lane of Fig. 5E) that might reflect labelling of the β isoform of CaM-KII as the antibody used in these experiments recognizes both of the CaM-KII isoforms. However, because the molecular weights of the α and β isoforms of CaM-KII differ, this very faint labelling is most likely to be an artifact.

Repeated cocaine administration increases total CaM-KII protein levels in the VTA

The data presented in Fig. 6 summarize the effects of acute saline, acute cocaine, repeated saline, or repeated cocaine on total protein levels of CaM-KII in the VTA 24 h after a single injection or after the last of seven daily injections. The statistical analysis revealed no significant difference between the groups treated with either acute saline or cocaine ($t_{10} = 0.473$, $P < 0.57$; see Fig. 6A). In the group of rats treated repeatedly with cocaine there was one rat that showed a decrease in CaM-KII in the VTA whereas the other five showed an increase. Only the five animals in which CaM-KII was increased were included in the analysis, which revealed a significant treatment effect ($t_9 = 2.272$, $P < 0.049$). The data presented in Fig. 6B show representative immunoblots generated from these samples.

Discussion

The present results indicate that intra-VTA administration of an AMPA receptor or NMDA receptor antagonist blocked the initiation of behavioural sensitization to cocaine. Administration of an L-type calcium channel antagonist (diltiazem) or a CaM-KII inhibitor (KN-93) directly into the VTA enhanced the acute behavioural hyperactivity induced by cocaine. Cocaine-induced behavioural hyperactivity also was enhanced in homozygous α-CaM-KII knockout mice. These results suggest that reducing CaM-KII activity in the VTA mimics behavioural sensitization. However, behavioural sensitization to cocaine was impaired in rats pretreated with either diltiazem or KN-93. Consistent with the KN-93 findings, behavioural sensitization to cocaine was attenuated in α-CaM-KII knockout mice. In addition, in rats receiving repeated cocaine injections there was an increase in CaM-KII protein levels in the VTA. Taken together, these results indicate that blocking L-type calcium channels or impairing CaM-KII activity in the VTA augments the acute behavioural hyperactivity

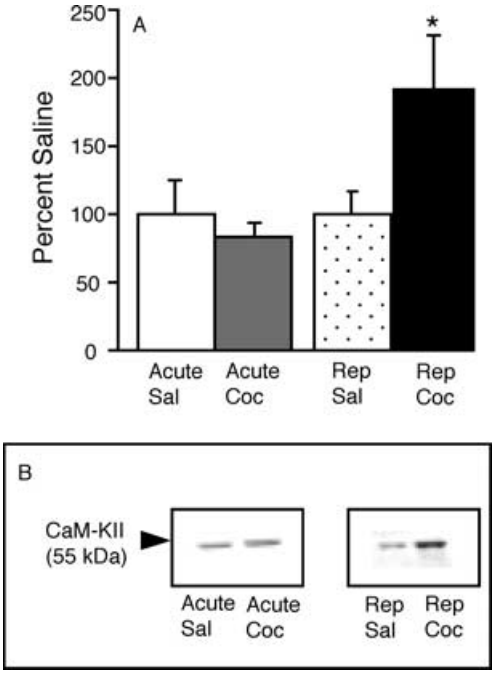


FIG. 6. The effect of cocaine administration on total protein levels of CaM-KII in the VTA. Rats were killed 24 h after receiving either an acute injection of cocaine (or saline) or after the last of seven daily injections of cocaine (or saline). The data in (A) represent the percentage change in CaM-KII immunoreactivity as measured by densitometric band volume (mean ± SEM) relative to saline-treated animals. (B) shows representative immunoblots for each treatment group. Lane 1 corresponds to acute saline, lane 2 corresponds to acute cocaine, lane 3 corresponds to repeated saline, and lane 4 corresponds to repeated cocaine treatment. A total of 100 μg protein was loaded in each lane. The asterisk in Figure 6A represents a significant difference compared to repeated saline (*t*-test, $P < 0.05$). There were 4–7 rats per group.

induced by cocaine. However, the present findings also suggest that calcium influx through AMPA, NMDA and L-type calcium channels located on dopaminergic neurons in the VTA might influence the initiation of behavioural sensitization to cocaine through activation of calcium-mediated second messengers including CaM-KII.

Dopamine, glutamate and the initiation of behavioural sensitization

A number of studies indicate that there is an enhancement of cocaine-induced increases in extracellular dopamine and glutamate in the VTA during the initiation of behavioural sensitization. Repeated cocaine injections enhance the ability of cocaine to increase extracellular dopamine in the VTA (Kalivas & Duffy, 1993; Parsons & Justice, 1993). This change was transient in that it was observed at 1 but not at 14 days after the cessation of repeated cocaine injections (Kalivas & Duffy, 1993; Parsons & Justice, 1993). There also is an increase in cocaine-induced glutamate release in the VTA following repeated injections of cocaine (Kalivas & Duffy, 1998). With amphetamine, acute and repeated injections both produced delayed and prolonged increases in glutamate efflux in the VTA (Xue *et al*., 1996; Wolf & Xue, 1999). Interestingly, the increases in VTA glutamate produced by both cocaine and amphetamine are dopamine D1 receptor-dependent (Kalivas & Duffy, 1998; Wolf & Xue, 1999), which suggests that psychostimulant-induced increases in VTA glutamate efflux could result from stimulation of D1 dopamine receptors located on glutamatergic terminals (Kalivas & Duffy, 1995; White & Kalivas, 1998).

Given the fact that repeated cocaine or amphetamine injections result in enhanced glutamate transmission in the VTA, it is not surprising that AMPA and NMDA receptor antagonists impair the development of behavioural sensitization to cocaine or amphetamine (Karler *et al*., 1989; Stewart & Druhan, 1993; Wolf & Jeziorski, 1993; Karler *et al*., 1994; Ida *et al*., 1995; Li *et al*., 1997, 1999; Jackson *et al*., 1998). Moreover, administration of an AMPA receptor antagonist (present results) or an NMDA receptor antagonist (Kalivas & Alesdatter, 1993; Vezina & Queen, 2000) directly into the VTA blocked the initiation of psychostimulant-induced behavioural sensitization. Consonant with these findings, following repeated amphetamine or cocaine injections there was a transient increase in the excitatory effect of glutamate (White *et al*., 1995) or AMPA (Zhang *et al*., 1997) on dopaminergic neurons in the VTA. Taken together, these findings indicate that enhanced excitatory transmission through ionotropic glutamate receptors located on dopaminergic neurons in the VTA contribute to the initiation of behavioural sensitization.

L-type calcium channels and the initiation of behavioural sensitization

L-type calcium channels also play an important role in the initiation of behavioural sensitization. Systemic administration of an L-type calcium blocker attenuates psychostimulant-induced behavioural sensitization (Karler *et al*., 1991; Reimer & Martin-Iverson, 1994). Moreover, repeated intra-VTA microinjections of an L-type calcium channel agonist cross-sensitize to a subsequent cocaine challenge injection (Licata *et al*., 2000). The present results indicate that intra-VTA administration of an L-type calcium channel antagonist enhances the acute behavioural hyperactivity induced by cocaine but attenuates the development of behavioural sensitization to cocaine.

Calcium signalling and the initiation of behavioural sensitization

AMPA and NMDA glutamate receptors and L-type calcium channels are calcium permeable (Dunlap *et al*., 1995; Jones, 1998). The mechanisms by which these receptors and channels influence the neuroadaptations that underlie behavioural sensitization to cocaine are unclear, but might involve increased activation of calcium-mediated second messengers. Consistent with this hypothesis, the present results indicate that repeated, daily cocaine injections increase total CaM-KII protein in the VTA. In addition, cocaine self-administration significantly increased α-CaM-KII mRNA levels in dopaminergic neurons in the rat VTA (Backes & Hemby, 2003).

The present results also provide evidence that this increase in CaM-KII levels in the VTA influences the initiation of behavioural sensitization. Thus, administration of a CaM-KII inhibitor into the VTA prior to daily injections of cocaine impaired the development of behavioural sensitization. In addition, behavioural sensitization to cocaine was attenuated in homozygous α-CaM-KII knockout mice. However, similar to the results obtained with the L-type calcium channel antagonist, administration of the CaM-KII inhibitor into the VTA enhanced the acute behavioural hyperactivity produced by cocaine. Likewise, the acute behavioural response to cocaine was increased significantly following the first administration of this psychostimulant in α-CaM-KII knockout mice relative to wild-types. Consonant with this observation, a gain-of-function mutation in the CaM-KII gene resulted in 'severely lethargic locomotion' in *C. elegans* (Robatzek & Thomas, 2000). Although it is unclear what specific mechanism is responsible for increased behavioural activation following reduced CaM-KII activity in the VTA, the increase in behavioural activity is likely to be because of an increase in dopaminergic cell firing secondary to changes in the phosphorylation of some combination of the numerous proteins, enzymes and transcription factors influenced by CaM-KII. The observation that suppression of CaM-KII either pharmacologically or through genetic deletion

enhances cocaine-induced behavioural hyperactivity complicates the interpretation of the effect of these experimental manipulations on behavioural sensitization in that it could be argued that reducing CaM-KII activity in the VTA mimics behavioural sensitization. However, the fact that CaM-KII is known to play a critical role in other forms of neuronal plasticity (Lisman, 1994; Frankland *et al*., 2001; Lisman *et al*., 2002) coupled with the present finding that repeated cocaine injections increase CaM-KII levels in the VTA lead us to conclude that increased CaM-KII activation in the VTA contributes to the initiation of behavioural sensitization.

Once activated, CaM-KII can phosphorylate a number of intracellular targets including, but not limited to, AMPA receptors (Poncer *et al*., 2002), NMDA receptors (Bayer *et al*., 2001), L-type calcium channels (Dzhura *et al*., 2000) and tyrosine hydroxylase (Griffith & Schulman, 1988), the rate-limiting enzyme in dopamine synthesis. Tyrosine hydroxylase immunoreactivity, tyrosine hydroxylase enzymatic activity and tyrosine hydroxylase mRNA are all increased in the VTA of rats treated repeatedly with cocaine (Beitner-Johnson & Nestler, 1991; Sorg *et al*., 1993; Vrana *et al*., 1993), which indicates that dopamine synthesis is increased in this structure during the development of behavioural sensitization. A key nuclear target of CaM-KII is cAMP response element binding protein (CREB; Curtis & Finkbeiner, 1999), a transcription factor that has been linked to several forms of synaptic plasticity including psychostimulant-induced changes in the mesotelencephalic dopamine systems (Konradi *et al*., 1994; Turgeon *et al*., 1997; Self *et al*., 1998; Pliakas *et al*., 2001). Interestingly, calcium influx through L-type calcium channels increases catecholamine synthesis (McCullough & Westfall, 1996) and CREB phosphorylation (Rajadhyaksha *et al*., 1999), presumably through CaM-KII-mediated phosphorylation (Griffith & Schulman, 1988). Although the influence of psychostimulants on CREB has thus far focused primarily on the striatal complex, one study showed that a single injection of amphetamine had no influence on total or phosphorylated CREB in the VTA (Dalley *et al*., The status of CREB in the VTA during a sensitizing regimen of psychostimulant injections has not yet been addressed. Interestingly, CREB is known to regulate tyrosine hydroxylase transcription (Lim *et al*., 2000). Taken together, these results suggest that the calcium influx through ionotropic glutamate receptors and L-type calcium channels during the development of behavioural sensitization might increase tyrosine hydroxylase activity via CaM-KII, either directly or through CREB-mediated transcription.

Conclusion

Taken together, the present data are consistent with the following working hypothesis. The sequential activation of AMPA receptors, NMDA receptors and L-type calcium channels on dopaminergic neurons in the VTA amplifies calcium transmission during the initiation of behavioural sensitizationg Enhanced glutamate transmission in the VTA associated with the initiation of behavioural sensitization would activate AMPA receptors resulting in membrane depolarization via enhanced sodium transmission (Boulter *et al*., depolarization is necessary to remove the tonic block of NMDA receptor channels by magnesium (Sucher *et al*., in an amplification of calcium transmission. The calc through AMPA receptors is limited because only AMPA lacking the GluR2 subunit are calcium permeable (Tanaka *e* rapidly (Jones, induced by AMPA and NMDA receptor st whereby

L-type calcium channels might be activated during the initiation of behavioural sensitizationg In contrast to ionotropic glutamate receptors and other voltage-dependent calcium channels, L-type calcium channels inactivate slowly, producing a more sustained influx of calcium (Jones, dopaminergic neurons during the initiation of behavi tion would increase CaM-KII levels. The present data demonstrate that CaM-KII contributes significantly to the processes that underlie behavioural sensitizationg There are numerous cellular and nuclear targets of CaM-KII that could be relevant to behavioural sensitization, including tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis (Griffith & Schulman, 1988), and the transcription factor CREB (Curtis & Finkbeiner, It is interesting to note that inhibition of CaM-K nucleus accumbens impairs both the long-term expression of cocaine-induced behavioural sensitization and the enhanced dopamine release in this structure observed following repeated cocaine injections (Pierce & Kalivas, Pierce *et al*., results, these data suggest that CaM-KII contr transient cellular and molecular events that take during the initiation of behavioural sensitization to the long-term expression of this phenomenon (Licat It seems possible therefore that CaM-KII could chemical mechanism that translates the numerous tr siological changes associated with initiation alterations in neuronal function that characterize psychostimulant-induced behavioural sensitizationg

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Abbreviations

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; ANOVA, analysis of variance; AP-5, 2-amino-5-phosphonovaleric acid; CaM-KII, calcium/calmodulin-dependent protein kinase II; CNQX, 6,7-dinitroquinoxaline-2,3-dione; CREB, cAMP response element binding protein; NMDA, *N*-methyl-D-aspartic acid; VTA, ventral tegmental areag

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