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Neuronal calcium/calmodulin-dependent protein kinase-II mediates nicotine reward in the conditioned place preference test in mice

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Abstract

Several recent studies indicate involvement of calcium-dependent mechanisms, in particular the abundant calcium activated kinase, calcium/calmodulin dependent kinases II (CaMKII), in behaviors associated with nicotine dependence in mice. Behavioral and biochemical studies show that CaMKII is involved in acute and chronic nicotine behaviors and nicotine withdrawal; however, evidence of a role for CaMKII in nicotine reward is lacking. Thus, the goal of the current study was to examine the role of CaMKII in nicotine reward. Using pharmacological and genetic tools, we tested nicotine conditioned place preference (CPP) in C57Bl/6 mice after administration of CaMKII antagonists and in α -CaMKII wild-type (+/+) and heterozygote (+/-) mice. CaMKII antagonists blocked expression of nicotine CPP, and the preference score was significantly reduced in α -CaMKII +/- mice compared to +/+ counterparts. Further, we assessed CaMKII activity in the ventral tegmental area (VTA), nucleus accumbens (NAc), prefrontal cortex, and hippocampus after nicotine CPP, and found significant increases in CaMKII activity occurring in the mouse VTA and NAc that were blocked by CaMKII antagonists. The findings from this study show that CaMKII mediates nicotine reward, and suggest that increases in CaMKII activity in the VTA and NAc are relevant to nicotine reward behaviors.

Keywords

CaMKII; calcium signaling; nicotine reward; conditioned place preference; nucleus accumbens; ventral tegmental area; mouse

Introduction

Nicotinic acetylcholine receptors (nAChRs) are pentameric, calcium-permeable, cholinergic receptors which form ligand gated ion channels, and are the primary targets through which

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nicotine exerts its effects. Nicotine binding to its receptors leads to an influx and subsequent rise in intracellular calcium. This, in turn, activates several downstream pathways and second-messengers, including calcium/calmodulin-dependent protein kinase II (CaMKII), a highly abundant serine threonine specific kinase that is essential for long term potentiation and neurotransmitter release (Schulman and Hanson 1993;Lisman *et al.* 2002), and calcium/calmodulin-dependent protein kinase IV (CaMKIV), a less abundant kinase involved in the regulation of activity-triggered gene expression (Deisseroth *et al.* 1998). Several lines of recent evidence have emerged, suggesting an important role for these kinases in mediating nicotine-associated behaviors.

L-type calcium channels and CaMKII are involved in nicotine-induced antinociception in mice (Damaj 2000, 2005, 2007). In addition, L-type calcium channels, CaMKII, and CaMKIV mechanisms are involved in physical and affective nicotine withdrawal behaviors (Biala and Weglinska 2005;Jackson and Damaj 2009;Jackson *et al.* 2012). Molecular studies show that $\beta 2$ -containing nAChRs mediate acute nicotine-induced increases in CaMKII activity in the ventral tegmental area (VTA), nucleus accumbens (NAc), and amygdala (Jackson *et al.* 2009). Further, phosphorylated CaMKII levels are increased in the NAc following exposure to chronic nicotine in mice (Jackson and Damaj 2013). In contrast, significant reductions in phosphorylated CaMKII levels in the NAc are observed after cessation of nicotine treatment in nicotine-dependent mice (Jackson and Damaj 2013). Human genetic studies also indicate that variants in the CaMKIV gene are associated with a protective effect in nicotine dependence (Jackson *et al.* 2012). While there is an emerging consensus that calcium-dependent mechanisms facilitate aspects of nicotine addiction-related behaviors, the role of these mechanisms in nicotine reward is limited. Recent studies show that nicotine reward, as measured by the conditioned place preference (CPP) test, is attenuated in CaMKIV knockout (-/-) mice, and CaMKIV level is increased in the NAc after nicotine CPP in mice (Jackson *et al.* 2012); however, the role of the more abundant kinase, CaMKII, in nicotine reward, is unclear.

In the current study, we sought to complement previous behavioral findings from our lab indicating a role for CaMKII in acute and chronic nicotine (Damaj 2000, 2005, 2007;Jackson *et al.* 2009;Jackson and Damaj 2013) and nicotine withdrawal (Jackson and Damaj 2009) by examining the involvement of brain CaMKII activity and function in nicotine reward using the CPP test. To this end, biochemical and behavioral approaches were used, as well as genetically modified mice. We first investigated the involvement of CaMKII in nicotine reward in the CPP test following intracerebroventricular (i.c.v.) administration of CaMKII membrane-permeable selective inhibitors, KN-62 and KN-93, and the inactive analog KN-92. α -CaMKII -/- mice have deficits in spatial learning, decreased anxiety-related responses, and increased susceptibility to seizures (Silva *et al.* 1992;Butler *et al.* 1995). Although memory retention is impaired in α -CaMKII heterozygote (+/-) mice at longer retention delays (10-50 days), learning and recent memory are normal in these mice at 1-3 days after training (Frankland *et al.* 2001). Thus, to complement our pharmacological approach and avoid the potential factors in α -CaMKII -/- mice that may confound our results, we tested nicotine preference in α -CaMKII +/- mice and wild-type (+/+) counterparts. Food reward was also conducted as a control to assess the potential for

learning deficits in α -CaMKII +/- mice in the CPP test. Finally, we measured changes in CaMKII activity in several brain regions in nicotine treated mice after the CPP test.

Materials and Methods

Subjects

Adult male C57BL/6J mice, male α -CaMKII +/- mice (Camk2atm1Sva targeted mutation), and male +/+ littermates were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in groups of six in a 21°C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care-approved animal care facility with food and water freely available. The rooms were on a 12 h light/dark cycle (lights on at 07:00 h). Mice were 8-10 weeks of age and weighed approximately 25-30 g at the start of the experiment. All experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

I.c.v. surgery

Mice undergoing i.c.v. surgeries for administration of CaMKII antagonists were anesthetized with an i.p. injection of 45 mg/kg sodium pentobarbital. An incision was made at the scalp of the mouse midway between the eyes and ears to expose cranial sutures. The skin that was cut resulted in a flap that was put back into place at the end of the surgery to keep the site free of debris. Using the stereotaxic apparatus, the injection site was made through the dura mater with the following coordinates: -0.6 mm AP; +1.3 mm ML, with respect to bregma, and -2.1 mm DV from the surface of the skull. After the surgery, animals were returned to clean home cages and were allowed to recover for 24 h.

Nicotine CPP

Nicotine CPP (n= 6-8 per group) was conducted on independent groups of C57Bl/6 mice and α -CaMKII +/- and +/+ mice using an unbiased design, as previously described by Kota et al (2007). In brief, mice were handled for three days prior to initiation of CPP testing. The CPP apparatus consisted of a three-chambered box with a white compartment, a black compartment, and a center grey compartment. The black and white compartments also had different floor textures to help the mice further differentiate between the two environments. On day 1, mice were placed in the grey center compartment for a 5 min habituation period, followed by a 15 min test period to determine baseline responses. A pre-preference score was recorded and used to counterbalance and randomly pair each mouse with either the black or white compartment so that an even number of mice received drug in either environment. Over the next 3 days, mice were conditioned twice a day; once in the morning (07-09.00 h) and once in the afternoon (14-16.00 h), for 20 min with the saline group receiving saline on both sides of the boxes and drug groups receiving nicotine (0.5 mg/kg, s.c.) on one side of the box and saline on the opposite side. On the evening of Day 4, after removal from the CPP chambers, C57Bl/6 mice underwent i.c.v. surgery for administration of the CaMKII inhibitors.

On CPP test day (Day 5), the CaMKII inhibitor KN-62 (0.01 and 0.05 μ g), a structurally different CaMKII inhibitor KN-93 (0.05 μ g), and its inactive analog KN-92 (0.05 μ g) were

injected i.c.v. directly into the skull of C57Bl/6 mice using a Hamilton syringe. Sterile saline soaked cotton swabs were used to lightly nudge the flap of skin to expose the injection site while gently restraining the mouse by holding the nape of the neck. 5 μ l of the CaMKII inhibitors, inactive analog, or vehicle were given, with the syringe being held in place for 30 s to allow time for drug diffusion into the lateral ventricle. Following the injection period, animals were returned to home cages and allowed 5 min to recover from the light restraint required for injection before being placed into the CPP chamber. α -CaMKII +/- and +/- mice did not undergo surgery at any time during the experiment or receive any drug treatment on test day. The test day procedure was conducted the same as the day 1 procedure. Data were expressed as time spent on the drug-paired side post-conditioning minus time spent on the drug-paired side pre-conditioning. A positive number indicated a preference for the drug-paired side, whereas a negative number indicated an aversion to the drug-paired side. A number at or near zero indicated no preference for either side.

Food CPP

For food-induced CPP, palatable food [Kraft Classic Philadelphia Cheesecake (Deerfield, IL)] was used to induce place preference in mice. Briefly, mice underwent pre-conditioning on day 1 and were divided into groups of equal bias, as described above. Mice were immediately introduced to palatable food for the next 4-6 h. The conditioning days followed the same experimental design as described above except that mice received cheesecake or a standard chow pellet control instead of drug or saline, one extra day of conditioning was required in order to obtain significant place preference scores (days 2-5), and each conditioning session lasted for 40 min.

CaMKII activity assay

A separate group of C57Bl/6 mice receiving vehicle, nicotine, and KN-62 (0.05 μ g, i.c.v.) (n= 5 per group) were sacrificed by cervical dislocation immediately after removal from the CPP chambers on test day. Brains were removed and the NAc, VTA, prefrontal cortex (PFC) and Hippocampus (Hipp) were dissected and homogenized in calcium-free Tris buffer containing 1 mM phenylmethylsulfonyl fluoride. Samples were centrifuged to separate the membrane and the cytosol-containing kinase. Cytosol homogenates were normalized for protein concentration. CaMKII activity was measured using a CaMKII-specific assay kit (Millipore, Billerica, MA). Standard phosphorylation reaction solutions contained 70 μ g of protein, 10 mM MgCl₂, 0.1 mM EGTA, 1 μ Ci of [³²P]ATP, 10 mM PIPES pH 7.4, 5 μ M CaCl₂, and 5 μ g of calmodulin. Standard reactions were performed in a shaking water bath at 30°C. CaMKII activity was determined using the following calculation: [(count-specific binding) $\times$ (correcting factor)]/[(specific radioactivity) $\times$ time (10 min)].

Drugs

(-)-Nicotine hydrogen tartrate salt was purchased from Sigma Chemical Company (Milwaukee, WI). 2-(N-(4-Methoxybenzenesulfonyl))amino-N-(4-chlorocinnamyl)-N-methylbenzylamine, phosphate (KN-92), 2-(N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl))amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-93) and 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl--tyrosyl]-4-phenylpiperazine (KN-62), were purchased from Calbiochem (San Diego, CA). KN-62, KN-93 and KN-92 were prepared in

dimethylsulfoxide (2.5% DMSO). Nicotine was dissolved in physiological saline (0.9% sodium chloride) and injected subcutaneously (s.c.) at a volume of 10 ml/kg body weight. The pH of the nicotine solution was checked and neutralized when necessary. All doses are expressed as the free base of the drug.

Statistical analysis

For all data, statistical analyses were performed using StatView®. Data from CPP studies using CaMKII inhibitors or food, and the CaMKII activity assays were analyzed using one-way analysis of variance (ANOVA) with treatment as the between subject factor. CPP studies using α -CaMKII +/- and +/+ mice were analyzed using two-way ANOVA with treatment and genotype as between subject factors. Significant results and interactions for the two-way ANOVA were further assessed using a Newman-Keuls post hoc test. P-values less than 0.05 were considered significant.

Results

CaMKII inhibitors block expression of nicotine CPP in the mouse

Male C57Bl/6 mice were conditioned with saline or nicotine (0.5 mg/kg, s.c.) and treated with the structurally different CaMKII inhibitors, KN-62 (0.01 μ g or 0.05 μ g, i.c.v.) or KN-93 (0.05 μ g, i.c.v.), or the inactive KN-93 analog, KN-92 (0.05 μ g, i.c.v.) on test day. Results are shown in Figure 1. Nicotine produced a robust CPP in C57Bl/6 mice ($F_{(9,63)} = 10.99$, $p < 0.001$). Expression of nicotine CPP was significantly blocked by the highest doses of KN-62 (0.05 μ g, i.c.v.) and by KN-93 (0.05 μ g, i.c.v.). KN-92 did not significantly alter expression of nicotine CPP. The doses of KN-62 and KN-93 used in this assessment did not produce a significant behavioral response in saline treated mice.

Nicotine CPP is reduced in α -CaMKII +/- mice

Male α -CaMKII +/+ and +/- mice were conditioned with saline or nicotine (0.25 mg/kg or 0.5 mg/kg, s.c.). Results are shown in Figure 2. There were significant main effects of treatment ($F_{(2,36)} = 17.59$, $p < 0.001$) and genotype ($F_{(1,36)} = 4.36$, $p < 0.05$), and a significant treatment $\times$ genotype interaction ($F_{(2,36)} = 3.44$, $p < 0.05$). Nicotine induced a significant CPP in α -CaMKII +/+ mice at 0.25 and 0.5 mg/kg, and α -CaMKII +/- mice at 0.5 mg/kg; however, CPP was significantly reduced at 0.5 mg/kg in α -CaMKII +/- mice compared to +/+ counterparts. Baseline CPP scores did not significantly differ between genotypes or treatments.

Food Conditioned Preference in α -CaMKII +/- and +/+ mice

We assessed palatable food-induced CPP in α -CaMKII +/+ and +/- mice as a control to assess the potential for learning deficits (Figure 3). After four days of conditioning, both α -CaMKII +/+ and +/- mice displayed similar place preference scores for the context associated with palatable food [$F_{(3,21)} = 18.52$; $p < 0.001$].

Nicotine CPP-induced increases in CaMKII activity in the VTA and NAc are blocked by KN-62

To complement our behavioral approach, CaMKII activity was analyzed in the C57Bl/6 mouse VTA, NAc, PFC, and Hipp after nicotine CPP using a CaMKII-specific assay kit. The results are shown in Figure 4. CaMKII activity was significantly increased in the VTA (Figure 4A; $F_{(3,16)} = 9.77$, $p < 0.001$) and NAc (Figure 4B; $F_{(3,16)} = 44.32$, $p < 0.001$) membrane portions after nicotine CPP, but not in the PFC (Figure 4C) or Hipp (Figure 4D). KN-62 significantly decreased the nicotine-induced increases in the VTA and NAc after the CPP test, but did not significantly alter CaMKII activity in the NAc or VTA of saline conditioned mice.

Discussion

Recent evidence indicates an important role for calcium-dependent mechanisms in nicotine-mediated behaviors. Thus, the goal of the current study was to complement previous studies supporting a role for such mechanisms in nicotine responses by determining the role of the most abundant calcium-mediated protein in the brain, CaMKII, in nicotine reward. Pharmacological and genetic studies using CaMKII antagonists and genetically modified α -CaMKII mice show that blockade or reduction of CaMKII reduces nicotine reward as measured in the CPP test. Further, biochemical studies indicate that increases in CaMKII activity in the VTA and NAc are blocked by CaMKII antagonists. Our findings support a role for CaMKII in mediating nicotine reward, and show that increases in CaMKII activity in the VTA and NAc are relevant in nicotine reward mechanisms. A limitation to the study is the use of α -CaMKII +/- mice, which could lead to some difficulties in interpretation since the mice still possess 50% of the enzyme activity. However, use of pharmacological agents, i.e., CaMKII inhibitors, complements the genetic studies and strengthens the validity of these findings.

The decreased nicotine CPP observed after administration of CaMKII antagonists and in α -CaMKII +/- mice coincides with the increase in CaMKII activity in the VTA and NAc in the CPP test. These two brain regions are part of the mesolimbic dopaminergic pathway, and play a central role in nicotine reward behaviors. NACHRs in the VTA mediate nicotine-induced release of the neurotransmitter dopamine in the NAc (Sziraki *et al.* 2002). CaMKII is essential for neurotransmitter release (Schulman and Hanson 1993), thus may mediate changes in dopamine function relevant to nicotine reward. It is noted that while CaMKII inhibitors were injected i.c.v. into the lateral ventricle, likely penetrating the whole brain, nicotine CPP induced increases in CaMKII activity were region specific. The blockade of nicotine CPP induced increases in CaMKII activity in the VTA and NAc further indicates that CaMKII-mediated mechanisms specifically in these two brain regions contribute to alterations in nicotine reward.

CaMKII is necessary for long term potentiation, a cellular mechanism which underlies learning and memory (Lisman *et al.* 2002). Based on this, another explanation for our results is that pharmacological inhibition of CaMKII may disrupt memory recall on test day, as CaMKII has been shown to be involved in this process (Wang *et al.* 2003). However, our complementary genetic studies using α -CaMKII +/- mice show a similar reduction in CPP

on test day as the pharmacological studies. Additionally, the food reward studies reveal a similar expression of food CPP in α -CaMKII $+/+$ and $+/-$ mice, suggesting that disruption in learning and memory processes may not be a major contributing factor in the current study. Importantly, findings of similar place preference profiles to palatable food in α -CaMKII $+/+$ mice and $+/-$ littermates suggest that partial blockade of α -CaMKII does not result in a general decrease in reward, specifically pertaining to the natural incentive for food.

Although KN-62 and KN-93 were reported previously to be selective inhibitors of CaMKII (Tokumitsu *et al.* 1990; Sumi *et al.* 1991), they were more recently shown to also block CaMKIV (Enslin *et al.* 1994). As previously shown in our lab, CaMKIV also mediates nicotine reward mechanisms, as nicotine CPP is attenuated in CaMKIV $-/-$ mice, and CaMKIV protein level is increased in the NAc after nicotine CPP (Jackson *et al.* 2012); therefore, it is possible that some of the observed behavioral effects may also be mediated by CaMKIV, as we did not observe total blockade of nicotine CPP in α -CaMKII $+/-$ mice. However, α -CaMKII $+/-$ mice possess 50% of the active CaMKII enzyme, and this level may be sufficient to maintain some development of nicotine CPP. Results may also suggest that α -CaMKII $+/-$ mice are less sensitive to the rewarding effects of nicotine, and may acquire nicotine CPP at higher doses. It is also possible that CaMKII inhibition decreases the effectiveness of nicotine as a stimulus in general. Another explanation, though less likely, is that genetic reduction of α -CaMKII may have potentiated the aversive effects of nicotine, which could explain the decrease in CPP nicotine responses. Future studies using higher doses of nicotine would address these possibilities. Nonetheless, we suggest that our pharmacological and genetic behavioral results support involvement of CaMKII in mechanisms of nicotine reward.

In summary, the pharmacological, genetic, and biochemical findings from this study indicate that CaMKII activity mediates nicotine reward, and emphasize the relevance of CaMKII activity in the VTA and NAc in mediating these behaviors. This work further supports the importance of calcium-dependent mechanisms, in particular CaM Kinases, in behaviors that contribute to nicotine dependence.

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Figure 1. CaMKII inhibitors attenuate expression of nicotine CPP in the mouse

A significant CPP expressed in nicotine conditioned vehicle treated C57Bl/6 mice is blocked by KN-62 and KN-93 in mice treated with the CaMKII inhibitors. The inactive analog KN-92 had no significant effects on expression of nicotine CPP in mice. Each point represents the mean + S.E.M. of 6-8 mice per group. KN-62, KN-93, and KN-92 doses are expressed in μg . Nicotine dose is expressed in mg/kg . * denotes $p < 0.05$ vs. saline controls and nicotine KN-62 and KN-93 treated groups. Veh, vehicle; Sal, saline; Nic; nicotine

Figure 2. Nicotine CPP is reduced in α -CaMKII +/- mice

Nicotine induced a significant CPP in α -CaMKII +/+ at 0.25 and 0.5 mg/kg nicotine, and in α -CaMKII +/- mice at 0.5 mg/kg nicotine; however, CPP was reduced in α -CaMKII +/- mice at 0.5 mg/kg compared to +/+ counterparts. Each point represents the mean + S.E.M. of 6-8 mice per group. * denotes significant main effects of treatment. + denotes significant interaction, $p < 0.05$ vs. α -CaMKII +/- nicotine (0.5 mg/kg) counterparts. Sal, saline; Nic; nicotine

Figure 3. Food CPP is maintained in α -CaMKIV +/- mice

α -CaMKII +/- and α -CaMKII +/+ mice displayed similar place preference scores for the context associated with the appetitive food stimulus. Results are expressed as mean preference scores + S.E.M.s. * denotes $p < 0.05$ vs. standard chow controls.

Figure 4. KN-62 blocks nicotine CPP-induced increases in CaMKII in the mouse VTA and NAc CaMKII activity is increased in the mouse **A. VTA** and **B. NAc** membrane portions after nicotine CPP (0.5 mg/kg), but not in the **C. PFC** or **D. Hipp**. The nicotine CPP-induced increases in CaMKII activity were significantly blocked by KN-62 (0.05 μg) in both brain regions. The x-axis represents the treatment groups. The y-axis represents CaMKII activity measured as picomoles per min per μg of protein. Each point represents the mean + S.E.M. of 5 mice per group. * denotes $p < 0.05$ vs. saline controls and KN-62 treated groups. Veh, vehicle; Sal, saline; Nic; nicotine