L-Type Calcium Channels and Calcium/Calmodulin-Dependent Kinase II Differentially Mediate Behaviors Associated with Nicotine Withdrawal in Mice

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ABSTRACT

Smoking is a widespread health problem. Because the nicotine withdrawal syndrome is a major contributor to continued smoking and relapse, it is important to understand the molecular and behavioral mechanisms of nicotine withdrawal to generate more effective smoking cessation therapies. Studies suggest a role for calcium-dependent mechanisms, such as L-type calcium channels and calcium/calmodulin-dependent protein kinase II (CaMKII), in the effects of nicotine dependence; however, the role of these mechanisms in nicotine-mediated behaviors is unclear. Thus, the goal of this study was to elucidate the role of L-type calcium channels and CaMKII in nicotine withdrawal behaviors. Using both pharmacological and genetic methods, our results show that L-type calcium channels are involved in physical, but not affective, nicotine withdrawal behaviors. Although our data do provide evidence of a role for CaMKII in nicotine withdrawal behaviors, our pharmacological and genetic assessments yielded different results concerning the specific role of the kinase. Pharmacological data suggest that CaMKII is involved in somatic signs and affective nicotine withdrawal, and activity level is decreased after nicotine withdrawal, whereas the genetic assessments yielded results suggesting that CaMKII is involved only in the anxiety-related response, yet the kinase activity may be increased after nicotine withdrawal; thus, future studies are necessary to clarify the precise behavioral specifics of the relevance of CaMKII in nicotine withdrawal behaviors. Overall, our data show that L-type calcium channels and CaMKII are relevant in nicotine withdrawal and differentially mediate nicotine withdrawal behaviors.

Nicotine withdrawal represents a motivational component of nicotine dependence that promotes continued tobacco use and relapse after smoking cessation. Although there are smoking cessation therapies available, the success rate of these therapies after 1 year remains only approximately 20 to 25% (Gonzales et al., 2006). Severity of the nicotine withdrawal syndrome is a better predictor of unsuccessful smoking and relapse attempts than smoke intake or dependence (West et al., 1989). Because smoking is such a widespread health problem, it is important to understand the molecular and behavioral mechanisms of nicotine withdrawal to generate more effective smoking cessation therapies.

Behaviors associated with nicotine withdrawal are mediated through nicotinic acetylcholine receptors (nAChRs), which are calcium-permeable, and the initial targets for nicotine. Recent studies from our lab indicate that nAChR subtypes have differential roles in physical and affective nicotine withdrawal behaviors (Jackson et al., 2008). Activation of these receptors leads to increases in intracellular calcium via various routes. Upon nicotine binding, there is a direct influx of calcium through nAChRs, which leads to an indirect calcium influx through voltage-gated calcium channels and intracellular calcium stores (Rathouz and Berg, 1994; Dajas-Bailador et al., 2002). The subsequent rise in intracellular calcium leads to activation of various downstream second messengers, including calcium/calmodulin-dependent protein kinase II (CaMKII), the most abundant calcium-dependent kinase in the neuron (Deisseroth et al., 1998), and a protein involved in several essential processes, including neurotransmitter release (Schulman and Hanson, 1993) and induction of long-term potentiation (Lisman et al., 2002).

Currently, the role of calcium-dependent second messenger systems in nicotine withdrawal-mediated behaviors is poorly understood; however, some studies suggest a role for L-type calcium channels and CaMKII in nicotine dependence. Pharam...
macological blockade of L-type calcium channels attenuates mecamylamine-precipitated somatic signs of nicotine withdrawal in mice (Biala and Weglinska, 2005). Studies from our lab showed that L-type calcium channel blockers and CaMKII inhibitors block development and expression of nicotine-induced antinociception at the spinal level (Damaj, 2005). Furthermore, L-type calcium channels and e4β2* nAChRs are up-regulated in mouse cerebral cortical neurons after 7 days of chronic nicotine exposure, leading to an increased calcium influx (Katsura et al., 2002). In addition, the signaling pathway that results in nicotine-induced extracellular signal-regulated kinase phosphorylation in mouse primary cortical neurons involves L-type calcium channels and CaMKII (Steiner et al., 2007). In PC12 cells, an increase in intracellular calcium after stimulation of nicotinic receptors activates CaMKII (MacNicol and Schulman, 1992), and data from our laboratory show that an acute systemic injection of nicotine is sufficient to elevate CaMKII in the spinal cord (Damaj, 2000). Although the current studies evaluating calcium-dependent mechanisms in nicotine dependence provide evidence of a potential role for calcium signaling, the behavioral relevance of these mechanisms in nicotine withdrawal remains unclear. In addition, most of the behavioral studies only assess the physical aspect of nicotine withdrawal and not the affective component, which is suggested to be of greater motivational significance in contributing to relapse (Koob et al., 1993; Markou et al., 1998). Therefore, the goal of the current study was to elucidate the behavioral relevance of L-type calcium channels and CaMKII in physical and affective nicotine withdrawal. Using our adapted spontaneous nicotine withdrawal and conditioned place aversion (CPA) models (Jackson et al., 2008), mice were treated with one of two structurally different L-type calcium channel blockers, nimodipine or verapamil, the L-type calcium channel activator, (±)Bay K8644, the CaMKII inhibitor KN93, or its inactive analog KN92, and physical and affective withdrawal signs were measured. To complement our studies of CaMKII inhibition, we measured physical and affective nicotine withdrawal signs in nicotine-dependent α-CaMKII heterozygote (+/−) mice in our mecamylamine-precipitated model.

Materials and Methods

Animals. Male C57BL/6J mice, male and female B6.129P2-Camk2atm1Sva/J(+−) mice, and male and female B6129P3 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). α-CaMKII(+/−) mice were generated as described by Silva et al. (1992a) and were backcrossed at least 16 generations. Animals were 8 to 10 weeks of age and were group-housed in a 21°C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care-approved animal care facility with ad libitum access to food and water. Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Drugs. (−)-Nicotine hydrogen tartrate salt, mecamylamine hydrochloride, verapamil hydrochloride, (±)Bay K8644, and KN93 were purchased from Sigma-Aldrich (St. Louis, MO). Nimodipine was purchased from Sigma/RBI (Natick MA). KN92 was purchased from Seikagaku Corporation (Tokyo, Japan). Nicotine and mecamylamine were dissolved in physiological saline (0.9% sodium chloride) and injected subcutaneously at a volume of 10 μl/kg body weight. Verapamil, nimodipine, and (±)-Bay K8644 were dissolved in a vehicle solution made of 5% ethyl alcohol, 5% Emulphor oil, and 90% saline and administered by intraperitoneal injection. KN92 and KN93 were diluted in saline and administered by intracerebroventricular injection. Doses are expressed as the free base of the drug.

Intracerebroventricular Surgery. Mice were anesthetized with sodium pentobarbital (45 mg/kg i.p.) on the evening before testing, and a scalp incision was made to expose the bregma. Uni-lateral injection sites were prepared using a 26-gauge needle with a sleeve of polyurethane tubing to control depth of the needle at a site 2 mm rostral and 2 mm lateral to the bregma at a depth of 2 mm. The scalp was sutured in such a way to enable an injection volume of 5 μl using a 26-gauge needle with a sleeve of polyurethane tubing into the lateral ventricle on the morning of testing. Animals were allowed to recover overnight. For intracerebroventricular injections the next morning (test day), the needle was held in place for 30 s to ensure drug delivery.

Chronic Nicotine Administration Protocol. Mice were anesthetized with sodium pentobarbital (45 mg/kg i.p.) and implanted with Alzet osmotic minipumps [model 2002 (14 days) or model 2004 (28 days); Duract Corporation, Cupertino, CA] filled with (−)-nicotine or saline solution as described by Jackson et al. (2008). The concentration of nicotine was adjusted according to animal weight and minipump flow rate. For all withdrawal studies, mice received 36 mg/kg/day for 14 days; however, a lower dose (24 mg/kg/day) was used in the (±)-Bay K8644 assessment because an enhancement in the extent of withdrawal was expected. For all CPA studies, animals were implanted with 28-day minipumps containing 36 mg/kg/day nicotine.

Locomotor Activity. Mice were injected with vehicle intraperitoneally and immediately placed into individual photocell activity cages (28 × 16.5 cm; Omnitech, Columbus, OH) for a 30-min habituation period. After habituation, mice were injected with vehicle or the assigned dose of nimodipine (1–10 mg/kg i.p.) and immediately returned to the locomotor cages. Interruptions of the photocell beams (two banks of eight cells each) were recorded for the next 30 min. Data are expressed as the number of photocell interruptions during the total 30-min time frame after drug administration.

Nicotine Withdrawal Assessment. Withdrawal studies were conducted as described previously by Jackson et al. (2008). In brief, for calcium channel and pharmacological CaMKII studies, minipumps were removed on day 14, and testing was initiated on day 15, approximately 18 to 24 h after minipump removal. Mice were injected with vehicle, nimodipine (0.25 or 1 mg/kg i.p.), verapamil (1 mg/kg i.p.), or (±)-Bay K8644 (0.25 and 0.5 mg/kg i.p.) 15 min before initiation of testing or with KN93 or KN92 (0.0025–0.01 μg/mm i.c.v.) 10 min before testing. For mecamylamine-precipitated studies, mecamylamine (2 mg/kg s.c.) was injected 10 min before testing. For KN92-precipitated studies, KN93 was administered i.c.v. 10 min before initiation of testing. The mice were first evaluated for 5 min in the plus maze test for anxiety-related behavior, followed by a 20-min observation of somatic signs measured as paw and body tremors, head shakes, backing, ramps, curls, and ptosis. Hyperalgesia was evaluated immediately after the somatic sign observation period. The specific testing sequence was chosen based on our prior studies showing that this order of testing reduced within-group variability and produced the most consistent results.

Nicotine CPA. The CPA protocol was conducted over the course of 4 days in a biased fashion as described in Jackson et al. (2008). In brief, mice were implanted with 28-day minipumps 14 days before initiation of CPA testing to induce dependence. Infusion continued throughout the duration of testing. On day 1 of the CPA procedure, after a 5-min habituation period in the center compartment, mice were allowed to roam freely between compartments for 15 min to determine baseline responses. The pre-preference score was used to pair each mouse with mecamylamine (3.5. mg/kg s.c.) to its initially preferred compartment. On days 2 and 3 of CPA training, all mice received injections of saline in the morning and mecamylamine in the afternoon. Mice were confined to their assigned chambers for
30-min conditioning sessions. After the mecamylamine conditioning session on day 3, intracerebroventricular injection sites were prepared. On day 4, mice moved freely between compartments as on day 1. Activity counts and time spent on each side were recorded via photosensors using the MED Associates (St. Albans, VT) interface and software. A reduction in time spent in the initially preferred compartment compared with the postconditioning day compared with the preconditioning day was interpreted as CPA.

**Nimodipine CPA Assessment.** On days 2 and 3 of CPA training, all mice received injections of saline in the morning. In the afternoon, mice received an injection of nimodipine (1 mg/kg i.p.) or vehicle 15 min before mecamylamine injection. Mice were placed in the assigned chamber immediately after mecamylamine injection for 30 min.

**KN93 CPA Assessment.** On day 4, mice received intracerebroventricular injections of vehicle, KN93 (0.01 μg/animal), or KN92 (0.01 μg/animal) 10 min before being placed in the test chambers. Mice moved freely between compartments as on day 1, and time spent in each compartment was recorded for each mouse. A reduction in time spent in their initially preferred compartment was interpreted as CPA.

**Statistical Analysis.** For all data, statistical analyses were performed using StatView (SAS Institute, Cary, NC). Data were analyzed with one-way analysis of variance with treatment as the between-subject factor or two-way analysis of variance with treatment and genotype or sex as between-subject factors. Significant results were further analyzed using the Neuman-Keuls post hoc test. A p value of less than 0.05 was considered significant.

**Results**

**Role of L-Type Calcium Channels in Physical and Affective Nicotine Withdrawal.** Before beginning our studies using L-type calcium channel blockers, we wanted to determine the appropriate doses to use for our studies that would not have significant effects on locomotor activity. Nimodipine dose-dependently decreased locomotor activity in mice ($F_{4,35} = 7.794, p < 0.0001$) (Fig. 1). Mice treated with 3 or 10 mg/kg nimodipine displayed a significant decrease in activity compared with vehicle-treated mice, yet there was no significant difference in activity between vehicle-treated mice or mice treated with 1 or 2 mg/kg nimodipine. Based on these data, nimodipine doses no higher than 2 mg/kg were used for withdrawal studies.

As expected, nicotine-withdrawn mice pretreated with vehicle showed a significant decrease in the amount of time spent on the open arms of the plus maze, indicating an anxiety-related response ($F_{6,49} = 5.395, p < 0.001$). Significant somatic signs ($F_{6,49} = 21.261, p < 0.0001$) and a significant decrease in hot-plate latency ($F_{6,49} = 3.432, p < 0.05$) compared with saline controls were also observed (Fig. 2). Nimodipine dose-dependently reduced physical signs in nicotine-withdrawn mice. Mice treated with 1 but not 0.25 mg/kg nimodipine showed a significant reduction in total somatic signs ($F_{6,49} = 21.261, p < 0.0001$) and a significant increase in hot-plate latency at the 1 mg/kg dose ($F_{6,49} = 3.432, p < 0.05$) compared with vehicle-treated mice, indicating a loss of the hyperalgesia response (Fig. 2A). Nimodipine had no effect on anxiety-related behavior (Fig. 2A).

In a separate experiment, mice were treated with the L-type calcium channel blocker, Bay K8644, after nicotine withdrawal to complement results observed with L-type calcium channel blockers. Because Bay K8644 decreased the number of arm crosses on the plus maze (data not shown), animals were not evaluated in this test. Compared with vehicle-treated nicotine-withdrawn mice, mice treated with 0.5 mg/kg (±)Bay K8644, but not 0.25 mg/kg, exhibited sig-

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**Fig. 1.** Nimodipine dose-dependently reduces locomotor activity in mice. Doses of 3 and 10 mg/kg nimodipine significantly reduced locomotor activity during the 30-min test period; therefore, doses no higher than 2 mg/kg nimodipine were used for our studies. Data are expressed as the number of photocell interruptions ± S.E.M. for eight mice per group. *, $p < 0.05$ versus vehicle and 1 and 2 mg/kg nimodipine groups.
significantly more somatic signs \( F_{4,35} = 38.853, p < 0.0001 \) (Fig. 3A) and a significant decrease in hot-plate latency \( F_{4,35} = 6.088, p < 0.0001 \) (Fig. 3B), indicating an enhanced response in both behavioral tests.

**Evaluation of L-Type Calcium Channels in Nicotine Withdrawal Using the CPA Model.** Results suggest that L-type calcium channels are involved in physical but not affective nicotine withdrawal. To further assess this effect, the aversion associated with nicotine withdrawal was measured using the CPA model. Mice were pretreated with nimodipine (1 mg/kg i.p.), 15 min before mecamylamine injection during conditioning. Mecamylamine (3.5 mg/kg s.c.) precipitated significant aversion in chronic nicotine-infused mice compared with saline-infused mice \( F_{3,44} = 2.964, p < 0.05 \) (Fig. 4). Pretreatment with nimodipine had no effect on development of nicotine dependence because mice pretreated with nimodipine expressed significant aversion compared with saline counterparts at a dose that did not produce significant behavioral effects in saline-infused mice (Fig. 4).

**Role of CaMKII in Physical and Affective Nicotine Withdrawal.** To investigate the role of CaMKII in nicotine withdrawal, we began with a pharmacological assessment using KN93, a CaMKII inhibitor, and its inactive analog, KN92, which does not inhibit kinase activity. Nicotine-withdrawn mice were treated with vehicle or a range of KN93 doses before initiation of testing. KN93 dose-dependently reduced somatic signs in nicotine-withdrawn mice \( F_{6,77} = 3.469, p < 0.05 \); however, treatment with KN93 had no effect on hot-plate latency at any dose tested (Fig. 5C). It is interesting that there was a significant reduction in the amount of time spent on the open arms of the plus maze compared with vehicle-treated nicotine-withdrawn mice. The highest dose of KN93 (0.01 mg/kg) reduced the amount of time spent on the open arms of the plus maze compared with vehicle-treated nicotine-withdrawn mice, indicating an enhanced anxiety-related re-
response after KN93 treatment ($F_{2,77} = 6.139, p < 0.001$) (Fig. 5A). There was no significant difference in the number of arm crosses between groups, suggesting that the effect was not attributed to differences in locomotor activity (Table 1). Nicotine-withdrawn mice treated with the inactive analog, KN92 (0.01 g/animal i.c.v.), did not differ from their vehicle-treated counterparts in any behavioral test, and the highest dose of KN93 (0.01 g) was not behaviorally active in saline-infused animals.

**Precipitated Assessment.** The results suggest that CaMKII has opposite roles in nicotine withdrawal. Although there was an attenuation of somatic signs, we observed an enhancement of the anxiety-related response. To further examine this response, we tested the effect of KN93 in a precipitated nicotine withdrawal model. Minipumps were not removed on day 14, and withdrawal signs were measured the morning of day 15 after administration of saline, mecamylamine, or KN93. Pretreatment with mecamylamine (2 mg/kg s.c.) or KN93 (0.01 µg/animal i.c.v.) precipitated a significant decrease in the open arms of the plus maze in chronic nicotine-infused mice, indicating an anxiety-related response ($F_{2,21} = 19.276, p < 0.0001$) (Fig. 6A). Although mecamylamine also precipitated a significant increase in so-
TABLE 1

Average number of arm crosses in the KN93 plus maze assessment

Nicotine-withdrawn mice were treated with vehicle, KN93, or the inactive analog KN92 and the total number of crosses between open and closed arms of the plus-maze test was counted. Numbers are presented as the total average number of arm crosses ± S.E.M. of eight mice per group.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Average Number of Arm Crosses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline MP- vehicle</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>Saline MP- KN93, 0.01 µg</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>Nicotine MP- vehicle</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>Nicotine MP- KN93, 0.0025 µg</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>Nicotine MP- KN93, 0.005 µg</td>
<td>2 ± 0.6</td>
</tr>
<tr>
<td>Nicotine MP- KN93, 0.01 µg</td>
<td>2 ± 0.3</td>
</tr>
<tr>
<td>Nicotine MP- KN92, 0.01 µg</td>
<td>2.2 ± 0.4</td>
</tr>
</tbody>
</table>

MP, minipump.

matic signs ($F_{2, 21} = 12.138, p < 0.0001$) and a significant decrease in hot-plate latency in nicotine-infused mice ($F_{2, 21} = 14.071, p < 0.05$), KN93 failed to precipitate physical nicotine withdrawal signs (Fig. 6, B and C).

Evaluation Using α-CaMKII(+/−) Mice. Mecamylamine precipitated an anxiety-related response in nicotine-infused α-CaMKII(+/−) mice ($F_{2, 21} = 14.071, p < 0.05$) (Fig. 7A). It is interesting that saline-infused α-CaMKII(+/−) mice had significantly higher plus maze baseline activity than their +/+ counterparts, and mecamylamine did not precipitate an anxiety-related response in nicotine-infused +/+ mice ($F_{1, 44} = 5.146, p < 0.05$ for treatment effects; no significant genotype effects; no significant interaction) (Fig. 7A). There was also no significant difference between nicotine-infused +/− and +/+ mice in either physical measure because mecamylamine precipitated a significant increase in somatic signs ($F_{1, 44} = 5.146, p < 0.05$ for treatment effects; no significant genotype effects; no significant interaction) and decrease in hot-plate latency ($F_{1, 44} = 10.048, p < 0.05$ for treatment effects; no significant genotype effects; no significant interaction) in both nicotine-infused +/− and +/+ mice (Fig. 7, B and C).

Role of CaMKII in Affective Withdrawal Using the CPA Model. For pharmacological CaMKII assessment of CPA expression, vehicle, KN93, or KN92 were administered to mice on test day, 10 min before entering the chamber. Mecamylamine (3.5 mg/kg s.c.) precipitated aversion in nicotine-infused mice treated with vehicle intracerebroventricularly ($F_{2, 55} = 6.376, p < 0.001$) (Fig. 8). KN93 (0.01 µg/animal i.c.v.), but not KN92 (0.01 µg/animal i.c.v.), showed a strong
trend toward enhanced expression of mecamylamine-precipitated CPA that did not reach statistical significance \( (p = 0.06) \). The dose of KN93 used did not produce significant behavioral effects in saline-infused mice. Activity counts showed no significant between group differences in activity on test day (Table 2), suggesting that results were not attributed to differences in chamber activity. To complement the pharmacological approach, \( \alpha \)-CaMKII\(+/-\) mice were evaluated using the CPA model. \( \alpha \)-CaMKII\(+/-\) mice were bred with female +/- B6129P3 hybrid mice to produce F2 \( \alpha \)-CaMKII\(+/-\) mice on a mixed background for our studies; therefore, before initiating studies, we evaluated development of CPA in the hybrid background strain to determine the background strain contribution to the phenotype of our CaMKII\(+/-\) mice. Results show that both male and female B6129 mice develop a significant mecamylamine-precipitated CPA \((F_{1,44} = 3.223, p < 0.05\) for treatment effects; no significant sex effects; no significant interaction\) (Fig. 9A). Mecamylamine precipitated significant CPA in both \( \alpha \)-CaMKII\(+/+\) and \( \alpha \)-CaMKII\(+/-\) mice \((F_{1,32} = 2.589, p < 0.05\) for treatment effects; no significant genotype effects; no significant interaction\) (Fig. 9B). There was no significant difference in activity count between groups (Table 2).

**Discussion**

The main goal of this study was to investigate the role of L-type calcium channels and CaMKII in nicotine withdrawal. The overall results suggest that calcium-dependent mechanisms are involved in physical and affective nicotine withdrawal. L-type calcium channel blockers attenuated nicotine withdrawal somatic signs and the hyperalgesia response, and \((\pm)\)Bay K8644, an L-type calcium channel activator, enhanced these signs. There was no effect in the plus maze or CPA model, suggesting a role for L-type calcium channels in physical, but not affective, withdrawal. Although our studies do suggest a potential role for CaMKII in nicotine withdrawal behaviors, the results were less clear. The pharmacological CaMKII assessment revealed that KN93 dose-dependently attenuated somatic withdrawal signs but enhanced the affective response, suggesting differential roles for CaMKII in somatic and affective nicotine withdrawal.
withdrawal; however, surprisingly, the transgenic data using α-CaMKII(+/−) mice suggest a role opposite that of the pharmacological assessment.

The L-type calcium channel blockers, nimodipine and verapamil, attenuated the expression of nicotine withdrawal-induced somatic signs and the hyperalgesia response but had no effect on the anxiety response or CPA, suggesting a role for L-type calcium channels in physical, but not affective, nicotine withdrawal. These results are consistent with a previous study by Biala and Weglinska (2005) in which mice were treated with various L-type calcium channel blockers after mecamylamine (mec) injection compared with saline counterparts. Mecamylamine precipitates a significant hyperalgesia response in α-CaMKII(+/−) and α-CaMKII(+/+) mice. Each point represents the mean ± S.E.M. of 12 mice per group. *p < 0.05 versus saline groups. +, p < 0.05 versus +/+ mice.

Fig. 7. Evaluation of the role of CaMKII in nicotine withdrawal using α-CaMKII(+/−) mice. A, saline (sal)-infused α-CaMKII(+/−) mice spend significantly more time on the open arms compared with +/+ mice, indicating higher baseline levels on the plus maze, yet nicotine-infused α-CaMKII(+/−) mice did not display an anxiety response. B, nicotine-infused α-CaMKII(+/−) mice and α-CaMKII(+/+) mice both exhibit significant somatic signs after mecamylamine (mec) injection compared with saline counterparts. C, mecamylamine precipitates a significant hyperalgesia response in α-CaMKII(+/−) and α-CaMKII(+/+) mice. Each point represents the mean ± S.E.M. of 12 mice per group. *p < 0.05 versus saline groups. +, p < 0.05 versus +/+ mice.

blocker doses that do not significantly affect locomotor activity. L-type calcium channel blockers have also been shown to inhibit α3* and α7* nAChR currents and downstream signaling in vitro at doses typically used to block L-type calcium channels (Wheeler et al., 2006); therefore, mice were treated with the L-type calcium channel activator, (+)Bay K8644. Nicotine-withdrawn mice treated with (+)Bay K8644 showed an enhancement in somatic signs and hyperalgesia. Taken together, these results suggest an important role for L-type calcium channels in physical, but not affective, nicotine withdrawal. The results also implicate the potential involvement of indirect sources in nicotine-induced calcium influx. Upon nicotine binding, there is a direct calcium influx through calcium-permeable nAChRs. The resulting increase in intracellular calcium leads to an indirect calcium influx through voltage-gated calcium channels as a result of membrane depolarization after nAChR activation. Because physical signs are altered by L-type calcium channel pharmacological agents, this would suggest an important role for a mechanism of calcium influx that occurs as a result of nAChR activation.

The intracellular rise in calcium through nAChRs leads to
activation of CaMKII. Previous data report increases in CaMKII function in PC12 cells (MacNicol and Schulman, 1992) and in the spinal cord membrane after acute nicotine treatment (Damaj, 2000). Our pharmacological data showed that the CaMKII inhibitor KN93 dose-dependently attenuated somatic signs but enhanced the expression of the anxiety-related response. There was also a trend toward enhancement of CPA expression after KN93 treatment on test day. It is possible, in the CPA model, that a floor effect prevented the observation of this effect and provided clearer results. Overall, these results imply that CaMKII has differential roles in somatic and affective nicotine withdrawal because the inhibitor decreased somatic signs, suggesting an increase in CaMKII function after withdrawal, but enhanced the anxiety-related response, suggesting a decrease in CaMKII function after withdrawal.

In previous studies, we found that somatic and affective nicotine withdrawal signs are mediated by different subtypes (Jackson et al., 2008); thus, it is possible that the opposing
oles of CaMKII in somatic and affective withdrawal could reflect the involvement of different nAChR subtypes. It may also be possible that the effect of KN93 is attributed to blockade of nAChRs. KN93 has been shown to reversibly block α3 and α7 nAChR responses in vitro (Liu and Berg, 1999); thus, it may be possible that the attenuation of somatic signs by KN93 is attributed to blockade of α3-containing nAChRs. In addition, KN93 blocks L-type calcium channels in vitro (Gao et al., 2006). Although KN93 had no effect on the hyperalgesia response, as was observed with L-type calcium channel blockers, we cannot rule out the possibility that the effects on somatic signs may involve inhibition of this target.

To complement our pharmacological approach, we measured withdrawal signs in nicotine-infused α-CaMKII(+/−) mice after mecamylamine injection. α-CaMKII knockout (−/−) mice have deficits in spatial learning, decreased anxiety-related responses, and increased susceptibility to seizures (Silva et al., 1992a,b; Butler et al., 1995); however, it was noted that α-CaMKII(+/−) mice exhibited normal learning and recent memory (Frankland et al., 2001); thus, to avoid the potential factors in α-CaMKII(−/−) mice that may confound our results, we used α-CaMKII(+/−) mice for our studies. Although the phar-
macological results suggest opposing roles for CaMKII in somatic and affective nicotine withdrawal, the assessment using α-CaMKII(+/−) mice produced different results. There was no significant difference in somatic signs between nicotine- and saline-infused α-CaMKII(+/−) and α-CaMKII(+/+) mice after mecamylamine treatment, suggesting that CaMKII is not involved in the development of nicotine withdrawal somatic signs. It is interesting that there was a loss of the anxiety-related response in nicotine-infused α-CaMKII(+/−) mice because there was no decrease in time spent on the open arms after mecamylamine treatment. It is noted that α-CaMKII(+/+) mice had a significantly higher baseline increase in time spent on the open arms of the plus maze compared with controls, which may reflect the phenotypic decrease in anxiety-related response noted in α-CaMKII(−/−) mice. Furthermore, no difference between α-CaMKII(+/−) and α-CaMKII(+/+) mice was observed in the development of significant aversion in the CPA model. These results suggest that CaMKII is not involved in the somatic signs or aversion associated with nicotine withdrawal but is involved in the nicotine withdrawal-induced anxiety-related response; however, the +/− mouse data suggest CaMKII function is increased in nicotine withdrawal-induced anxiety-related responses, which is the opposite of what was observed in the pharmacological KN93 assessment. One possible explanation for the discrepancy between our pharmacological and genetic data is the lack of selectivity of KN93. KN93 blocks CaMKII activity at an IC50 of 3 μM, a concentration only 4-fold higher than the IC50 value for CaMKII (0.8 μM) (Hook and Means, 2001). Therefore, it is possible that the observed effects are attributed to blockade of CaMKIV activity rather than CaMKII. However, based on diffusion studies by Matta et al. (1995), the highest dose of KN93 (0.01 μg/animal) would correspond to a tissue concentration of ~0.7 μM, which is below the IC50 value sufficient to block CaMKII activity. Another possibility is that the comparison of α-CaMKII(+/−) mice to the pharmacological data are unclear. The α-CaMKII(+/−) mice possess 50% of the CaMKII enzyme, and it is unknown whether this enzyme level is sufficient for expression of the alterations in nicotine withdrawal observed after KN93 treatment. It is possible that there is a gene-dosage effect among the +/+, +/−, and −/− mice, and total blockade of CaMKII activity is necessary for expression of attenuated somatic signs and an enhanced anxiety-related response. We also cannot rule out the role of compensatory mechanisms in these mice that may confound interpretation of our results. Lastly, it is possible that the phenotype of these mice may complicate their use in such studies. Future studies may benefit from the use of conditional α-CaMKIII(−/−) to complement the pharmacological approach.

The overall results of this study suggest that different calcium-dependent mechanisms are involved in physical and affective nicotine withdrawal. L-type calcium channels mediate somatic signs and hyperalgesia, suggesting the potential importance of the indirect calcium influx in expression of physical nicotine withdrawal. The role of CaMKII in nicotine withdrawal behaviors appears more complex. Pharmacological data support a role for CaMKII in having opposing roles in nicotine withdrawal because an antagonist attenuates somatic signs, yet enhances affective signs; however, the transgenic assessment did not elucidate a role for CaMKII in nicotine withdrawal. Although the current study, taken together with previous biochemical studies, does implicate a role for CaMKII in nicotine dependence, further work is necessary to examine the behavioral specifics of CaMKII in nicotine withdrawal.

References

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