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

K. J. Jackson and M. I. Damaj

Department of Pharmacology and Toxicology, Medical Campus, Virginia Commonwealth University, Richmond, Virginia

Received January 27, 2009; accepted March 30, 2009

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 be-

haviors. 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 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 nic-

otine. 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. Phar-

This work was supported by the National Institutes of Health National Institute on Drug Abuse [Grant DA12610].

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.109.151530.

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; CaMKII, calcium/calmodulin-dependent protein kinase II; CPA, conditioned place aversion; Bay K8644, S-(+)-1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-[trifluoromethyl]phenyl)-3-pyridine carboxylic acid methyl ester; KN93, N-(2-[N-[4-chlorocinnamyl]-N-methylaminomethyl]phenyl)-N-2-hydroxyethyl-4-methoxybenzenesulfonamide; KN92, ({2-[N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine, phosphate); +/-, heterozygote; +/+, wild type; -/-, knockout.

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 $\alpha 4\beta 2^*$ 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, ($\pm$)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 B6.129P3 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, ($\pm$)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 ml/kg body weight. Verapamil, nimodipine, and ($\pm$)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. Unilateral 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 μ l 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); Durect 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 ($\pm$)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 $\times$ 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 ($\pm$)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 μ g/animal i.c.v.) 10 min before testing. For mecamylamine-precipitated studies, mecamylamine (2 mg/kg s.c.) was injected 10 min before testing on the morning of day 15. For KN93-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, jumps, 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 compared on 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 μ g/animal), or KN92 (0.01 μ g/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. 2, B and C). There was no significant difference in time spent on the open arms of the plus maze in nicotine-withdrawn mice treated with nimodipine compared with vehicle, suggesting that nimodipine had no effect on anxiety-related behavior (Fig. 2A). Verapamil, an L-type calcium channel blocker structurally different from nimodipine, had effects similar to nimodipine in nicotine-withdrawn mice. Verapamil (1 mg/kg i.p.) significantly reduced somatic signs ($F_{6,49} = 21.261$, $p < 0.0001$) and significantly increased hot-plate latency in nicotine-withdrawn mice ($F_{6,49} = 3.432$, $p < 0.0001$); however, verapamil had no effect on anxiety-related behavior as measured by the plus maze (Fig. 2). The calcium channel blocker doses used were not behaviorally active in any measure by themselves because there were no significant effects in saline-treated mice (Fig. 2).

In a separate experiment, mice were treated with the L-type calcium channel activator, ($\pm$)Bay K8644, after nicotine withdrawal to complement results observed with L-type calcium channel blockers. Because ($\pm$)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 ($\pm$)Bay K8644, but not 0.25 mg/kg, exhibited sig-

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 $\pm$ S.E.M. for eight mice per group. *, $p < 0.05$ versus vehicle and 1 and 2 mg/kg nimodipine groups.

Fig. 2. L-type calcium channels are involved in physical but not affective withdrawal. Nimodipine (1 mg/kg i.p.) and verapamil (1 mg/kg i.p.) attenuated somatic withdrawal signs (B) and attenuated the hyperalgesia response as measured by a significant increase in hot-plate latency (C); however, they had no effect on anxiety-related behavior as measured by the amount of time spent on the open arms of the plus maze (A). Each point represents the mean $\pm$ S.E.M. of eight mice per group. *, $p < 0.05$ versus saline controls for all tests and versus nicotine (nic)-nimodipine (nim) and verapamil (ver) 1 mg/kg groups for the hot-plate and somatic sign assessments. \$, $p < 0.05$ versus saline controls, nic-veh, and nic-nim, 0.25 mg/kg groups.

nificantly 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 (Fig. 5B). Mice treated with 0.0025 μ g showed a significant reduction in the total number of somatic signs compared with nicotine-withdrawn mice treated with vehicle, whereas somatic signs were attenuated in nicotine-withdrawn mice treated with 0.005 and 0.01 μ g of KN93 ($F_{6,77} =$, $p < 0.0001$) (Fig. 5B). In the hyperalgesia assessment, nicotine-withdrawn, vehicle-treated mice displayed a significant reduction in hot-plate latency compared with saline-infused animals ($F_{6,77} = 3.469$, $p < 0.05$); 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 in KN93 treated nicotine-withdrawn mice compared with vehicle-treated nicotine-withdrawn mice. The highest dose of KN93 (0.01 μ g) 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-

Fig. 3. ($\pm$)Bay K8644 dose-dependently enhances physical withdrawal signs. Nicotine-withdrawn mice treated with ($\pm$)Bay K8644 (BayK, 0.5 mg/kg i.p.) exhibited a significant increase in somatic signs (A) and a significant decrease in hot-plate latency compared with nicotine-withdrawn vehicle (veh)-treated mice (B). Each point represents the mean $\pm$ S.E.M. of eight mice per group. *, $p < 0.05$ versus saline controls. +, $p < 0.05$ versus nicotine-veh mice. MP, minipump.

Fig. 4. L-type calcium channels are not involved in development of mecamlamine-precipitated CPA. Mecamlamine (mec) precipitates aversion in nicotine-infused mice. Pretreatment with nimodipine (nim) had no effect on the development of mecamlamine-precipitated aversion. Each point represents the mean $\pm$ S.E.M. of 12 mice per group. *, $p < 0.05$ versus saline group. sal, saline; nic, nicotine; MP, minipump.

sponse after KN93 treatment ($F_{6,77} = 6.139$, $p < 0.001$) (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 μ 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 μ 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, mecamlamine, or KN93. Pretreatment with mecamlamine (2 mg/kg s.c.) or KN93 (0.01 μ g/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 mecamlamine also precipitated a significant increase in so-

Fig. 5. KN93 attenuates somatic signs but enhances anxiety-related behavior. Nicotine-withdrawn mice were treated with various doses of KN93. Results show that KN93 dose-dependently reduces time spent on the open arms of the plus maze, suggesting an enhanced anxiety-related response (A) and a dose-dependent attenuation of somatic signs after KN93 treatment (B), but no effect on the hyperalgesia response after any KN93 dose tested (C). The highest dose of KN93 (0.01 μ g/animal) did not produce behavioral effects in saline-treated mice, indicating that the dose is not behaviorally active in the measures used for this evaluation. The inactive analog KN92 also did not produce behavioral effects in any withdrawal assessment. Each point represents the mean $\pm$ S.E.M. of 10 mice per group. *, $p < 0.05$ versus saline groups. +, $p < 0.05$ versus the nicotine-vehicle group. MP, minipump.

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 $\pm$ S.E.M. for eight mice per group.

Treatment Group	Average Number of Arm Crosses
Saline MP- vehicle	2.4 $\pm$ 0.3
Saline MP- KN93, 0.01 μ g	2.5 $\pm$ 0.6
Nicotine MP- vehicle	2 $\pm$ 0.5
Nicotine MP- KN93, 0.0025 μ g	2.2 $\pm$ 0.6
Nicotine MP- KN93, 0.005 μ g	2 $\pm$ 0.6
Nicotine MP- KN93, 0.01 μ g	2 $\pm$ 0.3
Nicotine MP- KN92, 0.01 μ g	2.2 $\pm$ 0.4

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 signif-

icantly higher plus maze baseline activity than their +/+ counterparts, and mecamylamine did not precipitate an anxiety-related response in nicotine-infused +/- mice ($F_{1,44} = 61.604$, $p < 0.0001$ for genotype effects; no significant treatment 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 ($F_{4,55} = 6.376$, $p < 0.001$) (Fig. 8). KN93 (0.01 μ g/animal i.c.v.), but not KN92 (0.01 μ g/animal i.c.v.), showed a strong

Fig. 6. KN93 precipitates anxiety-related behavior but not physical withdrawal signs. Withdrawal was precipitated with KN93 or mecamylamine in chronic nicotine-infused mice. **A**, mice show significant decrease in the amount of time spent on the open arms of the plus maze, indicating an anxiety-related response after KN93 and mecamylamine treatment. KN93 did not precipitate somatic signs (**B**) or hyperalgesia response in chronic nicotine-infused mice as seen after mecamylamine injection (**C**). Each point represents the mean $\pm$ S.E.M. of eight mice per group. *, $p < 0.05$ versus saline group. +, $p < 0.05$ versus mecamylamine group. MP, minipump.

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, α -CaMKII(+/-) mice were evaluated using the CPA model. α -CaMKII(+/-) mice were bred with female +/+ B6129P3 hybrid mice to produce F2 α -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 α -CaMKII(+/+) and α -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

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 $\pm$ S.E.M. of 12 mice per group. *, $p < 0.05$ versus saline groups. +, $p < 0.05$ versus +/+ mice.

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-precipitated withdrawal. Despite the complimentary results, in our study, we show that the doses used in the Biala study (2005) significantly depressed locomotor activity in mice, which could affect data interpretation in a withdrawal study. Furthermore, the Biala study used the mecamylamine-precipitated model to evaluate expression of nicotine withdrawal. It is unknown whether the combination of mecamylamine and L-type calcium channel blockers produces a drug interaction that influences the results; thus, we utilized a spontaneous nicotine withdrawal model to avoid potential drug interactions and L-type calcium channel

blocker doses that do not significantly affect locomotor activity. L-type calcium channel blockers have also been shown to inhibit $\alpha 3^*$ and $\alpha 7^*$ 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, ($\pm$)Bay K8644. Nicotine-withdrawn mice treated with ($\pm$)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

Fig. 8. Pharmacological inhibition of CaMKII enhances the aversion associated with nicotine withdrawal. Nicotine-infused mice treated with the CaMKII inhibitor KN93 on test day express a strong trend that did not reach statistical significance toward enhanced CPA compared with nicotine-infused mice treated with vehicle (veh) or KN92 ($p = 0.06$). Each point represents $\pm$ S.E.M. of 12 mice per group. *, $p < 0.05$ versus the corresponding saline group.

TABLE 2

Average activity counts in the CPA model for the CaMKII assessment. Mice were treated with vehicle or KN93 (0.01 µg/animal i.c.v.) for the pharmacological assessment on test day. Activity counts were taken for each compartment in the CPA chamber. Numbers represent the total activity counts in drug-paired compartment on test day for each group and are presented as the average activity count on test day (postconditioning day) $\pm$ S.E.M. for 12 mice.

Treatment Groups	Average Activity Counts/Drug-Paired Compartment
Saline MP-vehicle	438 $\pm$ 78.1
Saline MP-KN93	369.9 $\pm$ 34.2
Nicotine MP-vehicle	437 $\pm$ 97.8
Nicotine MP-KN93	455.8 $\pm$ 185.1
Nicotine MP-KN92	492.9 $\pm$ 92.9
Saline α -CaMKII(+/-)	624.1 $\pm$ 45
Nicotine α -CaMKII(+/-)	671 $\pm$ 50.5
Nicotine α -CaMKII(+/-)	692.9 $\pm$ 36.4

MP, minipump.

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 aversive response after KN93 treatment from reaching significance. Using a lower dose of nicotine (24 instead of 36 mg/kg/day) in the CPA model for future studies may prevent this floor effect and provide 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

Fig. 9. Evaluation of the role of CaMKII using a genetic approach. A, background strain of our CaMKII(+/-) mice, B6129P3 hybrid mice, was assessed in the development of aversion in the CPA model. Nicotine-infused male and female B6129P3 hybrid mice develop aversion after mecamylamine conditioning in the CPA model. B, mecamylamine precipitates aversion in α -CaMKII(+/-) and α -CaMKII(+/-) mice. There was no significant difference in the level of aversion between +/+ and +/- mice. Each point represents the mean $\pm$ S.E.M. of 12 mice per group. *, $p < 0.05$ versus saline groups.

roles 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 $\alpha 3^*$ and $\alpha 7^*$ 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 $\alpha 3$ -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(-/-) 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 CaMKIV activity at an IC_{50} of 3 μ M, a concentration only 4-fold higher than the IC_{50} value for CaMKII (0.8 μ M) (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 μ g/animal) would correspond to a tissue concentration of $\sim$ 0.7 μ M, which is below the IC_{50} value sufficient to block CaMKIV 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 -CaMKII(-/-) 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

- Biala G and Weglinska B (2005) Blockade of the expression of mecamylamine-precipitated nicotine withdrawal by calcium channel antagonists. *Pharmacol Res* **51**:483–488.
- Butler LS, Silva AJ, Abeliovich A, Watanabe Y, Tonegawa S, and McNamara JO (1995) Limbic epilepsy in transgenic mice carrying a Ca^{2+} /calmodulin-dependent kinase II α -subunit mutation. *Proc Natl Acad Sci U S A* **92**:6852–6855.
- Dajas-Bailador FA, Mogg AJ, and Wonnacott S (2002) Intracellular calcium signals evolved by stimulation of nicotinic acetylcholine receptors in SH-SY5Y cells: contribution of voltage-operated calcium channels and calcium stores. *J Neurochem* **81**:606–614.
- Damaj MI (2000) The involvement of spinal calcium/calmodulin-protein kinase II in nicotine-induced antinociception in mice. *Eur J Pharmacol* **404**:103–110.
- Damaj MI (2005) Calcium-acting drugs modulate expression and development of nicotine-induced antinociception in mice. *J Pharmacol Exp Ther* **315**:959–964.
- Deisseroth K, Heist EK, and Tsien RW (1998) Translocation of calmodulin to the nucleus supports CREB phosphorylation in hippocampal neurons. *Nature* **392**:198–202.
- Frankland PW, O'Brien C, Ohno M, Kirkwood A, and Silva AJ (2001) α -CaMKII-dependent plasticity in the cortex is required for permanent memory. *Nature* **411**:309–313.
- Gao L, Blair LA, and Marshall J (2006) CaMKII-dependent effects of KN93 and its inactive analog KN92: reversible inhibition of L-type calcium channels. *Biochem Biophys Res Commun* **345**:1606–1610.
- Gonzales D, Rennard SI, Nides M, Oncken C, Azoulay S, Billing CB, Watsky EJ, Gong J, Williams KE, and Reeves KR (2006) Varenicline, and $\alpha 4 \beta 2$ nicotinic acetylcholine receptor partial agonist vs. sustained-release bupropion and placebo for smoking cessation. *JAMA* **296**:47–55.
- Hook SS and Means AR (2001) Ca^{2+} /CaM-dependent kinases: from activation to function. *Annu Rev Pharmacol Toxicol* **41**:471–505. Review.
- Jackson KJ, Martin BR, Changeux JP, and Damaj MI (2008) Differential role of nicotinic acetylcholine receptor subunits in physical and affective nicotine withdrawal signs. *J Pharmacol Exp Ther* **325**:302–312.
- Katsura M, Mohri Y, Shuto K, Hai-Du Y, Amano T, Tsujimura A, Sasa M, and Ohkuma S (2002) Up-regulation of L-type voltage-dependent calcium channels after long term exposure to nicotine in cerebral cortical neurons. *J Biol Chem* **277**:7979–7988.
- Koob GF, Heinrichs SC, Pich EM, Menzaghi F, Baldwin H, Miczek K, and Britton KT (1993) The role of corticotropin-releasing factor in behavioral responses to stress. *Ciba Found Symp* **172**:277–289; discussion 290–295.
- Lisman J, Schulman H, and Cline H (2002) The molecular basis of CaMKII function in synaptic and behavioral memory. *Nature* **3**:175–190.
- Liu Q and Berg DK (1999) Actin filaments and the opposing actions on CaM kinase II and calcineurin in regulating $\alpha 7$ -containing nicotinic receptors on chick ciliary ganglion neurons. *J Neurosci* **19**:10280–10288.
- MacNicol M and Schulman H (1992) Multiple Calcium signaling pathways converge on CaM kinase in PC12 cells. *FEBS Lett* **304**:237–240.
- Markou A, Kosten TR, and Koob GF (1998) Neurobiological similarities in depression and drug dependence: A self-medication hypothesis. *Neuropsychopharmacology* **13**:135–174.
- Matta SG, McCoy JG, Foster CA, and Sharp BM (1995) Nicotinic agonists administered into the fourth ventricle stimulate norepinephrine secretion on the hypothalamic paraventricular nucleus: an in vivo microdialysis study. *Neuroendocrinology* **61**:383–392.
- Rathouz MM and Berg DK (1994) Synaptic-type acetylcholine receptors raise intracellular calcium levels in neurons by two mechanisms. *J Neurosci* **14**:6935–6945.
- Schulman H and Hanson PI (1993) Multifunctional Calcium/calmodulin-dependent protein kinase. *Neurochem Res* **18**:65–77.
- Silva AJ, Paylor R, Wehner JM, and Tonegawa S (1992a) Impaired spatial learning in α -calcium-calmodulin kinase II mutant mice. *Science* **257**:206–211.
- Silva AJ, Stevens CF, Tonegawa S, and Wang Y (1992b) Deficient hippocampal long-term potentiation in α -calcium calmodulin kinase II mutant mice. *Science* **257**:201–206.
- Steiner RC, Heath CJ, and Picciotto MR (2007) Nicotine-induced phosphorylation of ERK in mouse primary cortical neurons: evidence for involvement of glutamatergic signaling and CaMKII. *J Neurochem* **103**:666–678.
- West RJ, Hajek P, and Belcher M (1989) Severity of withdrawal symptoms as a predictor of outcome of an attempt to quit smoking. *Psychol Med* **19**:981–985.
- Wheeler DG, Barrett CF, and Tsien RW (2006) L-type calcium channel ligands block nicotine-induced signaling to CREB by inhibiting nicotinic receptors. *Neuropharmacology* **51**:27–36.

Address correspondence to: Kia J. Jackson, Department of Pharmacology and Toxicology, Virginia Commonwealth University, Box 980613, Richmond, VA 23298-0613. E-mail: jacksonkj@vcu.edu