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Differential CaMKII regulation by voltage-gated calcium channels in the striatum

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Abstract

Calcium signaling regulates synaptic plasticity and many other functions in striatal medium spiny neurons to modulate basal ganglia function. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a major calcium-dependent signaling protein that couples calcium entry to diverse cellular changes. CaMKII activation results in autophosphorylation at Thr286 and sustained calcium-independent CaMKII activity after calcium signals dissipate. However, little is known about the mechanisms regulating striatal CaMKII. To address this, mouse brain slices were treated with pharmacological modulators of calcium channels and punches of dorsal striatum were immunoblotted for CaMKII Thr286 autophosphorylation as an index of CaMKII activation. KCl depolarization increased levels of CaMKII autophosphorylation ~2-fold; this increase was blocked by an LTCC antagonist and was mimicked by treatment with pharmacological LTCC activators. The chelation of extracellular calcium robustly decreased basal CaMKII autophosphorylation within 5 min and increased levels of total CaMKII in cytosolic fractions, in addition to decreasing the phosphorylation of CaMKII sites in the GluN2B subunit of NMDA receptors and the GluA1 subunit of AMPA receptors. We also found that the maintenance of basal levels of CaMKII autophosphorylation requires low-voltage gated T-type calcium channels, but not LTCCs or R-type calcium channels. Our findings indicate that CaMKII activity is dynamically regulated by multiple calcium channels in the striatum thus coupling calcium entry to key downstream substrates.

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Keywords

synaptic plasticity; L-type calcium channels; T-type calcium channels; medium spiny neuron; subcellular fractionation

Introduction

Refined motor control and cognitive functions such as decision-making and habit learning depend on proper function of the striatum, the major input to the basal ganglia system. Over 90% of striatal neurons are medium spiny neurons (MSNs) which integrate and process converging glutamatergic and dopaminergic inputs via modifications of synaptic efficacy to regulate striatal output. Moreover, alterations of the activity or morphology of striatal MSNs are associated with dysregulation of striatal function in many psychiatric and neurological disorders (Glenn & Yang 2012, Ehrlich 2012, Looi & Walterfang 2013, Roberts *et al.* 1996, Stephens *et al.* 2005, Zaja-Milatovic *et al.* 2005, Plotkin & Surmeier 2015, Day *et al.* 2006). However, signaling mechanisms underlying the regulation of striatal function and plasticity are poorly understood.

The multifunctional serine-threonine kinase, calcium/calmodulin-dependent protein kinase-II (CaMKII), can respond to diverse calcium signals to initiate many responses. Calcium/calmodulin binding to CaMKII activates autophosphorylation at Thr286, inducing prolonged, calcium-independent activation, which allows CaMKII to execute various forms of neuroplasticity following transient increases of postsynaptic calcium. In other brain regions, Thr286 autophosphorylation promotes interactions with and/or phosphorylation of many downstream targets, including NMDA receptor (NMDAR) subunits (Bayer *et al.* 2006, Strack & Colbran 1998, Barria & Malinow 2005) and AMPA receptor (AMPAR) subunits (Barria *et al.* 1997a, Barria *et al.* 1997b, Hayashi *et al.* 2000). CaMKII autophosphorylation at Thr286, and CaMKII binding to GluN2B subunits of the NMDAR are essential for normal hippocampal long-term potentiation and various forms of learning and memory (reviewed in Sanhueza & Lisman 2013, Hell 2014). However, CaMKII also plays an important role in some forms of synaptic depression (Mockett *et al.* 2011, Mayford *et al.* 1995, Shonesy *et al.* 2013, Coultrap *et al.* 2014). Moreover, CaMKII activation is important for the initiation of excitation-transcription coupling (Ma *et al.* 2014) and for the modulation of neuronal excitability (Nelson *et al.* 2005, Klug *et al.* 2012b, Sametsky *et al.* 2009), morphology (Okamoto *et al.* 2009, Jourdain *et al.* 2003, Fink *et al.* 2003, Wu & Cline 1998), and toxicity (Ashpole *et al.* 2012, Vest *et al.* 2010, Hajimohammadreza *et al.* 1995).

CaMKII is critical for normal striatal function. Under basal conditions, Thr286 autophosphorylation of CaMKII is significantly higher in the striatum relative to other brain regions (Baucum *et al.* 2013), though mechanisms behind this are not understood. Striatal CaMKII Thr286 autophosphorylation is further increased following the lesion of dopamine inputs in rodent models of Parkinson Disease, and this increase can be reversed by repeated injections of levodopa, the primary dopamine replacement therapy used to treat patients with Parkinson Disease (Brown *et al.* 2005, Picconi *et al.* 2004). Moreover, CaMKII inhibition reverses the disruption of corticostriatal synaptic plasticity and the deficit in spontaneous

motor behavior caused by dopamine depletion (Picconi et al. 2004). While the links between CaMKII activity and striatal dysfunction are not completely understood, CaMKII is very abundant in striatal MSNs (Fukunaga *et al.* 1988, Erondu & Kennedy 1985, Ouimet *et al.* 1984). Moreover, striatal CaMKII interacts strongly with other proteins (Baucum et al. 2013), and has been found to regulate excitatory transmission and intrinsic excitability (Klug *et al.* 2012a) and control endocannabinoid-dependent plasticity (Shonesy et al. 2013).

Calcium signaling in striatal MSNs is complex. Relative contributions of calcium influx into dendritic spines via multiple ligand- and voltage-gated calcium channels depends on the resting membrane potential, which fluctuates between a downstate membrane potential near -80mV and a depolarized 'upstate' membrane potential near -50mV (Carter & Sabatini 2004). For example, T-type voltage-gated calcium channels (TTCCs) play a substantial role in calcium influx in the downstate, but not in the upstate. In contrast, L-type voltage-gated calcium channels (LTCCs) play little role in the downstate, but are responsible for much of calcium influx from the upstate. R-type voltage-gated calcium channels appear to make significant contributions in both the downstate and upstate (Carter & Sabatini 2004). Notably, calcium influx via specific channels can be linked to distinct downstream responses in striatal MSNs. For example, the CaV1.3 subtype of LTCC is specifically linked to the induction of endocannabinoid-dependent synaptic plasticity (Adermark & Lovinger 2007), as well as to the loss of dendritic spines following dopamine depletion in a parkinsonian mouse model (Day et al. 2006). However, the specific signaling mechanisms that are engaged downstream of calcium entry via these different channels are poorly understood.

Here we utilize *in vitro* slice pharmacology and biochemistry to investigate the link between calcium entry and CaMKII activation in the dorsal striatum. We report that Thr286 autophosphorylation of CaMKII and its synaptic localization under basal conditions is maintained by continual influx of extracellular calcium. Whereas TTCCs significantly contribute to the maintenance of this basal CaMKII autophosphorylation, the activation of LTCCs further increases CaMKII autophosphorylation. Overall, our data begin to define the roles of different VGCCs in regulating striatal CaMKII.

Methods

Animal care

Adult, male mice at postnatal day 35-49 on a C57Bl6/J background (Jackson Laboratories) were used for all experiments and were housed on a 12-h light-dark cycle with food and water *ad libitum*. All experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee and in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH.

Acute slice preparation and treatment

Mice were anesthetized using isofluorane and then decapitated. Brains were removed, cut into left and right hemispheres, and then 300 μm coronal slices were made at 1-4°C in oxygenated (95% v/v O_2, 5% v/v CO_2) dissecting solution (208 mM sucrose, 2.5 mM KCl, 1 mM $CaCl_2$, 4 mM $MgCl_2$, 4 mM $MgSO_4$, 1.6 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM

glucose, and 3 mM Na-pyruvate) using a Vibratome 3000 (The Vibratome Company). Typically, a total of 5-7 striatal hemi-slices (left/right hemisphere combined) were obtained from each mouse brain (1.1 to 0.14 mm from Bregma). Slices were allowed to recover on a nylon mesh for 1 h at 30°C in oxygenated ACSF (113 mM NaCl, 2.5-5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, 20 mM glucose, and 3 mM Na-pyruvate) followed by addition of picrotoxin (50 μM) for 30 min. Slices were then transferred to oxygenated 30°C ACSF solutions supplemented with vehicle or drug for 1-30 min, as defined in the figure legends. The following drugs were used and dissolved in water or DMSO based on manufacturer's instructions: BAPTA (Sigma), (S)-BayK8644 (Tocris), FPL64176 (Tocris), isradipine (National Institute of Mental Health Chemical Synthesis and Drug Supply Program), KCl (Sigma), mibefradil (Tocris), Nickel Chloride (Sigma), nimodipine (MP Biomedicals), SNX-482 (Peptides International), and TTX (Tocris).

Homogenization of striatal tissue punches

Punches (2 mm diameter) of dorsal striatum, containing both lateral and medial regions, were collected from slices on ice after incubation. Total lysates were prepared by immediately homogenizing striatal tissue punches in lysis buffer (2% SDS, 2 mM EGTA, 0.2 mM PMSF, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μM pepstatin, and 1 μM microcystin). Protein concentrations in total striatal lysates were determined by BCA assay (Thermo Scientific), using a bovine serum albumin standard.

Subcellular fractions of freshly dissected striatal tissue punches were prepared as previously described (Gustin et al. 2010). Briefly, two striatal punches were pooled and homogenized in 100 μl Isotonic Buffer (IB: 150 mM KCl, 50 mM Tris-HCl pH 7.5, 1 mM DTT, 0.2 mM PMSF, 1 mM Benzamidine, 1 μM Pepstatin, 10 mg/l Leupeptin, 1 μM microcystin) and incubated at 4°C with rocking for 30 min prior to centrifugation at 100,000 × g for 1 h. The supernatant (S1 fraction, “cytosolic”) was saved, and the pellet was resuspended in 100 μl IB containing 1% (v/v) Triton X-100 and then incubated at 4°C with rocking for 30 min. This lysate was then centrifuged at 18,403 × g, and the resulting supernatant (S2 fraction, “Extrasynaptic membrane”) was saved. The pellet was resuspended in 100 μl isotonic buffer containing 1% Triton X-100 and 1% deoxycholate and sonicated (S3 fraction, “Synaptic”).

Immunoblotting

Striatal lysate protein (10 μg) was mixed with 4× SDS sample buffer, heated at 70°C for 10 min, and then separated by electrophoresis in a 10% SDS polyacrylamide gel. For striatal subcellular fractions, equal volumes were mixed with 4× sample buffer prior to immunoblotting. Gels were transferred in a 0.01 M CAPS/10% methanol buffer to nitrocellulose membranes, which were stained with Ponceau S to detect all proteins and then scanned using a desktop scanner. After blocking for 0.5 hr in 5% milk in Tris-Buffered Solution with 0.01% Tween-20 (TBST), membranes were probed overnight with antibodies to total CaMKIIa (1:1000-1:4000; Thermo, MA1-0147), pThr286 CaMKIIα (1:1000-1:4000; Santa Cruz Biotechnologies, SC-12886-R), pSer1303-GluN2B (1:500; Millipore, 07-398), Total GluN2B (1:1000; BD Transduction, 610417), pSer831-GluA1 (1:500; PhosphoSolutions, P1160-831), GluA1 (1:1000; Santa Cruz Biotechnologies, SC-55509), GAPDH (1:5000; Millipore, MAB374), or PSD95 (1:1000; Neuromab; 75-028).

Membranes were then washed, incubated with the appropriate infra-red fluorescent secondary antibodies (1:10,000; 1 h) and imaged using an Odyssey system (LiCor Biosciences).

In vitro autophosphorylation assay

Purified CaMKIIα (1 μM) was incubated on ice for 2.5 min with 50 mM HEPES, 2 mM $Mg(Ac)_2$, 1.5 mM $CaCl_2$, 2 mM DTT, 2 μM CaM, and various concentrations of ATP (0.2-5 μM) to control the level of Thr286 autophosphorylation. Reactions were stopped by adding 4X SDS sample buffer, and approximately 200 ng of CaMKIIα was then loaded on a SDS-PAGE gel.

Quantification of blots and statistical analysis

For analysis of total lysates, Image J (http://imagej.nih.gov/ij/) was used to quantify total Ponceau staining in each gel lane on the nitrocellulose membrane as well as immunoblot signal intensities. Immunoblot signals for total protein levels were normalized to the Ponceau signal in the corresponding lane to correct for variations in gel loading. Phospho-site specific immunoblot signals were normalized to the immunoblot signal for the corresponding total protein in each lane. For analysis of striatal subcellular fractions, individual values were normalized to the average of the values from the same subcellular fraction of control slices analyzed in parallel.

The raw normalized ratios within each experimental condition on each experimental day (5-8 slices) were first subjected to a Grubbs outlier test, resulting in the exclusion of less than 2% of samples analyzed across all conditions. The mean signal from all vehicle control slices was then used for normalization of the individual values for all of the individual slices analyzed on that day (i.e., as a % of the mean control value on each day). The results are plotted as the mean ± SEM of the % control for the indicated total number of slices per group, collected from 4-11 mice over 1-3 days. In analysis of levels of PSD95, GAPDH, and CaMKIIα in subcellular fractions (Fig. 4F), raw normalized ratios of protein/ponceau from BAPTA treated slices were compared to protein/ponceau raw normalized ratios from control slices from the same fraction of the same animal. For autophosphorylation in subcellular fractions, raw normalized ratios of P-T286/total CaMKIIα from BAPTA treated slices were compared to P-T286/total CaMKIIα raw normalized rations from control slices from the same fraction of the same animal. Data were then analyzed using an unpaired Student's *t* -test, paired *t* -test, or one way ANOVA followed by a Tukey's post-hoc test as appropriate.

Results

Ca^{2+}/calmodulin-dependent Thr286 autophosphorylation of CaMKIIα results in generation of an autonomously active form of the kinase that is critical for normal neuronal function. Therefore, we assessed CaMKIIα activation by comparing the levels of Thr286 autophosphorylation by immunoblotting extracts of striatal slices incubated under a variety of conditions using phospho-Thr286 site-specific and total CaMKII antibodies and measuring the ratio of signals detected. In initial pilot studies, slices were immediately flash frozen on dry ice prior to collecting punches of dorsal striatum, followed by immediate

homogenization. However, when processing tissue in this manner we could not reliably detect increased levels of CaMKII autophosphorylation after incubating slices in a variety of pharmacological conditions to increase intracellular calcium (data not shown). In order to investigate whether this may reflect a ceiling effect dues to high basal levels of autophosphorylation, we compared samples collected in this manner from control (untreated) slices with samples of purified CaMKIIα that were autophosphorylated with different limiting concentrations of ATP *in vitro* (Fig 1A). Surprisingly, levels of Thr286 autophosphorylation in basal striatal samples were similar to the maximal levels attained *in vitro*, suggesting that CaMKII is near maximally phosphorylated in extracts prepared in this manner. Therefore, we optimized the collection of dorsal striatal punches after chilling slices to ∼0°C on wet ice, followed by immediate homogenization. Notably, in tissue punches collected on ice the levels of CaMKIIα autophosphorylation at Thr286 were ∼15% of the levels observed in samples collected after freezing (Fig. 1B). We interpret these observations to indicate that freezing and thawing during the isolation of striatal tissue punches can induce rapid increases in intracellular calcium concentrations, which are sensed by calmodulin to near-maximally activate CaMKIIα. Consequently, all the remaining data reported in this paper were collected from analyses of striatal tissue punches collected near 0°C on ice.

CaMKII autophosphorylation is increased by LTCC activation

We initially used a pharmacological approach to promote calcium influx. Depolarization of striatal slices using 40 mM KCl for 1 min resulted in a significant ∼2-fold increase in CaMKIIα Thr286 autophosphorylation in whole tissue lysates (Fig. 2A). Since CaMKII and LTCCs are both enriched in dendritic spines, (Fukunaga et al. 1988, Hell *et al.* 1993, Ouimet et al. 1984), we pretreated slices with the LTCC blocker nimodipine (10 μM, >25 min) and found that this prevented the KCl-induced increase of CaMKIIα Thr286 autophosphorylation. Moreover, treatment of slices with structurally distinct direct pharmacological LTCC activators (0.5 μM FPL64176 or 5 μM BayK8644) for 2 min significantly increased striatal CaMKIIα autophosphorylation in whole tissue lysates by ∼25% and ∼17%, respectively as compared to vehicle treated slices (Fig. 2B, 2C). However, CaMKII activation by FPL64176 or BayK8644 was transient, with no significant increases in levels of Thr286 autophosphorylation after 10 min (data not shown). Taken together, these results indicate that calcium influx via LTCCs transiently activates striatal CaMKIIα.

Blocking spontaneous activity promotes CaMKII autophosphorylation

In order to explore the role of intrinsic neuronal activity in determining the set point of striatal CaMKIIα autophosphorylation under basal conditions, slices were incubated for 30 min with 1 μM tetrodotoxin (TTX), a sodium channel blocker. Interestingly, TTX treatment increased induced levels of CaMKIIα Thr286 autophosphorylation by ∼18% in whole tissue lysates compared to vehicle treated controls (Fig 3). Thus, it appears that spontaneous firing of action potentials reduces CaMKIIα autophosphorylation under basal conditions.

Calcium influx is necessary to maintain basal phosphorylation of CaMKII, GluA 1, and GluN2B

Since intrinsic neuronal activity was not responsible for the maintenance of significant CaMKIIα autophosphorylation in dorsal striatum under basal conditions, we investigated whether extracellular calcium was required to maintain basal CaMKIIα autophosphorylation. BAPTA (5 mM final) was added to the slice incubation media (ACSF) to rapidly chelate extracellular calcium and prevent basal calcium influx (Fig 4A). Notably, Thr286 autophosphorylation of CaMKIIα in whole tissue lysates was decreased by ∼70% after 5 min and by ∼95% after 15 min (Fig 4A), indicating that CaMKIIα was rapidly and progressively dephosphorylated following the prevention of calcium influx. Thus, continual calcium influx under basal conditions is important in driving CaMKIIα activation to maintain basal levels of Thr286 autophosphorylation.

We also examined the impact of removing extracellular calcium on the phosphorylation of two physiologically relevant synaptic substrates of CaMKII, Ser831 in the AMPAR GluA1 subunit (Mammen *et al.* 1997, Barria et al. 1997a) and Ser1303 in the NMDAR GluN2B subunit (Omkumar *et al.* 1996). Phosphorylation of both substrates was readily detected under basal conditions. The addition of BAPTA significantly reduced GluA1 phosphorylation at Ser831 (Fig. 4B) to 40% and 35% of control after 5 and 15 min, respectively. Similarly, GluN2B phosphorylation at Ser1303 (Fig 4C) was significantly reduced to 57% and 39% of control, respectively. These data indicate that ongoing calcium influx under basal conditions drives not only CaMKIIα activation, but also the phosphorylation of two key synaptic targets of CaMKII that are important for synaptic plasticity.

Calcium influx is required for normal CaMKII localization

CaMKII activation drives its translocation to dendritic spines and the postsynaptic density in the hippocampus (Shen & Meyer 1999, Strack *et al.* 1997c). To determine if striatal CaMKII localization is also sensitive to its activation state, we prepared subcellular fractions from striatal slices that had been treated for 15 min with 5 mM BAPTA or vehicle. Under basal conditions, CaMKIIα was detected in all three fractions, but was most abundant (normalized to volume loaded) in the S3 fraction, and least abundant in the S1 fraction (see representative blots in Fig. 5A), consistent with prior studies of dissected tissues (Baucum et al. 2013, Gustin *et al.* 2011). Moreover, the relative enrichment (normalized to protein) of CaMKIIα in S1, S2, and S3 fractions from control dorsal striatum was 0.54 ± 0.13, 0.91 ± 0.15, and 1.42 ± 0.18, respectively, demonstrating significant enrichment in the synaptic fraction (n=8-9. P=0.0024 by ANOVA). Thr286 phosphorylation of CaMKIIα also was readily detected in all three fractions from control slices, and the P-T286/total CaMKIIα ratio was highest in synaptic fractions (S1, 1.28 ± 0.17; S2, 0.62 ± 0.09; S3, 1.80 ± 0.23; N=9. ANOVA P=0.0003). Extracellular calcium chelation using BAPTA decreased the levels of Thr286 autophosphorylation by >85% in all three fractions, when compared to the corresponding fraction from untreated control slices (Fig. 5A, B). Interestingly, BAPTA treatment also resulted in a significant translocation of total CaMKIIα to the cytosolic fraction when quantified as the S1/S2 ratio (Con: 0.59 ± 0.10, BAPTA: 0.78 ± 0.07; paired *t* -test, p=0.02) or as the S1/S3 ratio (Con: 0.35 ± 0.10, BAPTA: 0.47 ± 0.08; paired *t*test,

p=0.05) (Fig. 5A, C). Alternatively, when total levels of CaMKIIα in S1 fractions (normalized to Ponceau) were quantified as a BAPTA/control ratio, there is a significant 68 ± 12% increase in CaMKIIα in the cytosolic S1 fraction (p = 0.0017, by Student's *t*-test). However, there was no significant effect of BAPTA treatment on the levels of total CaMKIIα in the S2 or S3 fractions, or in the S2/S3 ratio (Fig. 5C). To verify the effective fractionation of control and BAPTA treated tissue, samples were also blotted for PSD95 (Fig. 5A, D) and GAPDH (Fig. 5A, E). Treatment with BAPTA did not alter the levels of PSD95 in the S2 (Triton-soluble) membrane fraction or the (higher) levels in the S3 (Triton-insoluble) synaptic fraction. Moreover, levels of GAPDH in the S1 (cytosolic) fraction and S2 membrane fraction were unaffected by BAPTA. Taken together, these data indicate that chelation of extracellular calcium and dephosphorylation of Thr286 resulted in a significant translocation of total CaMKIIα to the cytosolic S1 fraction.

T-type voltage gated calcium channels (TTCCs) contribute to basal CaMKII autophosphorylation

Since calcium influx is essential for maintenance of basal CaMKIIα autophosphorylation, we tested for the involvement of specific calcium channels under basal condition. Striatal MSNs express both CaV1.2 and CaV1.3 LTCCs, and a stronger membrane depolarization is required to activate CaV1.2 in comparison to CaV1.3 channels. Therefore, we tested the effects of two different dihydropyridines, nimodipine and isradipine, which are reported to differentially block CaV1.2 and CaV1.3 LTCCs (Bonci *et al.* 1998, Fitton & Benfield 1990, Sinnegger-Brauns *et al.* 2009, Koschak *et al.* 2001). However, basal CaMKIIα Thr286 autophosphorylation was unaffected by a 30 min incubation with either 10 μM nimodipine (Fig. 6A) or 5 μM isradipine (Fig. 6B) as compared with vehicle treated controls. RTCCs make a significant contribution to calcium influx into MSN dendritic spines at both down- and up-state membrane potentials (Carter & Sabatini 2004), but Thr286 phosphorylation of CaMKIIα was unchanged by treatment with the RTCC blocker SNX-482 (400 nM; 30 min) (Fig. 6C). Finally, we examined the role of TTCCs, which appear to be active at downstate membrane potentials in the striatum (Carter & Sabatini 2004). TTCCs can be selectively inhibited using mibefradil or $NiCl_2$ compared to other VGCCs (Martin *et al.* 2000, Todorovic & Lingle 1998, Fox *et al.* 1987). Interestingly, incubation of striatal slices for 30 min with mibefradil (5 μM) or $NiCl_2$ (100 μM) significantly decreased Thr286 autophosphorylation of CaMKIIα by 37% (Fig. 6D) and 35% (Fig. 6E), respectively. In combination, these data indicate that basal CaMKIIα activation/autophosphorylation in the striatum is at least partially supported by calcium influx through TTCCs.

Discussion

Calcium influx via numerous channels dynamically regulates multiple MSN functions by recruiting diverse signaling proteins. While extensive work has identified several channels underlying striatal plasticity, our understanding of the proteins that couple calcium influx to synaptic function remains incomplete. We began to explore this by focusing on the calcium-dependent activation of CaMKII, which modulates synaptic function in multiple brain regions, including dorsal striatum (Klug et al. 2012a, Shonesy et al. 2013). We found that basal influx of extracellular calcium promotes Thr286 autophosphorylation and synaptic

localization of CaMKIIα as well as the phosphorylation of physiologically relevant targets, NMDAR GluN2B and AMPAR GluA1 subunits, at CaMKII sites. We also identified a novel role for striatal TTCCs in maintaining basal striatal CaMKIIα activity, with calcium influx via LTCCs inducing a further activation of CaMKIIα following depolarization. These findings demonstrate a dynamic, calcium-dependent CaMKIIα regulation that presumably connects striatal calcium channel activity with key downstream signaling pathways.

Our studies utilize the calcium-dependent Thr286 autophosphorylation site as a proxy for CaMKIIα activation. The calcium sensitivity of CaMKIIα introduced unusual technical difficulties in that we found that freezing brain slices prior to isolating dorsal striatal tissue induced a near maximal autophosphorylation at Thr286. Previous work also found that flash freezing of rat hippocampal slices causes similar artificially elevated CaMKIIα activity as measured by increases in Thr286 autophosphorylation and CaMKII substrate peptide phosphorylation (Lengyel *et al.* 2001). Furthermore, this freezing-induced elevation of CaMKII activity masked experimental treatment effects and was not reversed by exclusion of phosphatase inhibitors, changes in buffer components, or by freezing on dry ice versus liquid nitrogen (Lengyel et al. 2001). Our data emphasize the importance of avoiding freeze/thaw of striatal samples prior to homogenization to avoid artificial activation of CaMKII in these types of experiments.

The sensitivity and rapid response of CaMKII autophosphorylation to increases of calcium are well known, enabling CaMKII to function as an effective integrator of repetitive calcium spikes that occur during action potential firing. Our studies examined if intrinsic spontaneous activity in striatal slices modulates CaMKII activity. Previous work using neurons cultured from other brain regions has shown that spontaneous neuronal activity induces glutamate release to promote CaMKII activation (Murphy *et al.* 1994, Padmanabhan *et al.* 2008, Eshete & Fields 2001). Thus, it was somewhat surprising that TTX treatment slightly increased CaMKIIα autophosphorylation in the striatum. The mechanism of this activation is likely complex, perhaps involving components at the molecular and system levels. At the molecular level, TTX treatments have been shown to increase the phosphorylation of striatal DARPP-32 at Thr34 (Nishi *et al.* 1997). Enhanced phosphorylation of DARPP-32 would be expected to inhibit protein phosphatase 1, which is known to dephosphorylate CaMKII at Thr286 (Shields *et al.* 1985, Strack *et al.* 1997a). However, the response to TTX treatment might also reflect the complex neuromodulatory environment of the striatum. As a central mediator of the basal ganglia circuit, striatal MSNs receive excitatory inputs from cortical and thalamic pathways. However, dopaminergic, cholinergic, and GABA-ergic transmission also exert potent inhibitory control of the glutamatergic inputs to MSNs (Pakhotin & Bracci 2007, Kawaguchi *et al.* 1995, Gerfen & Surmeier 2011, Do *et al.* 2013). Furthermore, these excitatory and inhibitory inputs converge on both major subtypes of striatal MSNs, which either express the Dopamine 1 Receptors (D1R-MSNs) or Dopamine 2 Receptors (D2R-MSNs). Striatal D1R-MSNs and D2R-MSNs have quite distinct properties, particularly with respect to excitability (Kreitzer & Malenka 2007b, Gertler *et al.* 2008, Planert *et al.* 2013), with D1R signaling promoting LTP (Centonze *et al.* 2003, Kerr & Wickens 2001) and D2R signaling promoting LTD (Kreitzer & Malenka 2007a, Calabresi *et al.* 1997). Dopamine enhances calcium influx via

LTCCs in D1R-MSNs (Hernandez-Lopez *et al.* 1997), but inhibits LTCCs in D2R-MSNs (Hernandez-Lopez *et al.* 2000). Additionally, D1R activation can inhibit sodium channels, whereas D2R activation can activate sodium channels (Surmeier *et al.* 1992, Surmeier *et al.* 1993). Future studies would need to differentiate the contributions of these D1R- and D2R-MSN subtypes to better elucidate the regulation of CaMKII activity in the striatum.

Next, we examined the role of calcium entry in regulating striatal CaMKII. We found that membrane depolarization increases CaMKIIα autophosphorylation, indicating that striatal CaMKII can be further activated from basal conditions. We also identified an important role for calcium influx in sustaining basal levels of CaMKII autophosphorylation by adding BAPTA to the ACSF. The rapid chelation of extracellular calcium by BAPTA presumably decreases the amount of calcium in specific intracellular pools that continually drive CaMKII autophosphorylation in the face of significant basal phosphatase activity(ies) that can dephosphorylate Thr286 (Strack *et al.* 1997b, Fukunaga *et al.* 1993, Ishida *et al.* 1998). These findings are consistent with prior studies in cultured neurons showing that removal of extracellular calcium profoundly decreases CaMKII autophosphorylation (Scholz & Palfrey 1998, Cohen & Fields 2006). We also found that extracellular calcium chelation decreases the phosphorylation of GluN2B and GluA1, key synaptic substrates of CaMKII (Hunt & Castillo 2012, Santos *et al.* 2009), suggesting that basal CaMKII activity is driving the phosphorylation of physiological CaMKII substrates. However, our data cannot exclude possible contributions from broader effects of BAPTA addition on other signaling pathways or on the kinetics of calmodulin dissociation. Since BAPTA reduced Thr286 autophosphorylation of CaMKIIα in whole lysates by ∼90% within 15 min, it was not surprising that pools of CaMKIIα holoenzymes in the cytosolic (S1), membrane (S2), and synaptic (S3) fractions were similarly dephosphorylated. However, BAPTA treatments increased total levels of CaMKIIα in the cytosolic fraction as compared with control treatments, which we interpret to reflect a translocation of CaMKII out of the synaptic fraction because it is well established that calcium/calmodulin-dependent activation and Thr286 autophosphorylation drives CaMKII translocation to synapses (Strack et al. 1997c, Shen *et al.* 2000, Shen & Meyer 1999). While total levels of CaMKIIα in membrane (S2) or synaptic (S3) fractions were not significantly reduced by BAPTA treatment, this may reflect the higher levels of CaMKIIα in these fractions. Thus, dissociation of a statistically insignificant fraction of CaMKIIα from the synaptic or membrane fraction could significantly increase levels of CaMKIIα in the cytosolic S1 fraction. Furthermore, we recently identified over 100 proteins associated with synaptic CaMKII holoenzymes (Baucum *et al.* 2015), some of which could limit CaMKII translocation to other fractions following decreases in Thr286 autophosphorylation. However, we cannot exclude other contributions to changes in cytosolic CaMKII levels such as degradation or synthesis. Taken together these findings suggest that the ongoing influx of extracellular calcium under steady-state basal conditions promotes basal CaMKIIα autophosphorylation and localization to intracellular compartments in striatal MSNs where it could act on substrates necessary for synaptic plasticity.

We also examined the roles of specific VGCCs thought to mediate calcium entry in CaMKII regulation. Prior calcium imaging studies have shown that LTCCs, RTCCs, and TTCCs can

make significant contributions to total calcium influx into striatal MSN spines and dendrites, depending on the resting membrane potential (Carter & Sabatini 2004). Although we have no direct control of the resting membrane potential in our *in vitro* slice experiments, our data indicate that LTCCs and TTCCs, but not RTCCs, make distinct contributions to CaMKIIα activation under different conditions.

First, we found that pretreatment of slices with nimodipine blocked depolarization-induced increases in CaMKIIα autophosphorylation, suggesting that LTCCs are key regulators of CaMKII during depolarization. Furthermore, CaMKII autophosphorylation was also transiently increased by the selective activation of LTCCs using FPL64176 and BayK8644. Notably, FPL64176 and BayK8644 only modestly activated CaMKII in comparison with the response to global depolarization using KCl, likely due to their mechanism of action. These compounds enhance calcium influx by prolonging LTCC opening (Kunze & Rampe 1992, Bechem & Hoffmann 1993, Rampe *et al.* 1993), rather than directly opening the channel, so it seems likely that FPL64176 and BayK8644 will induce more modest levels of calcium influx in comparison to 40 mM KCl. Although calcium influx via activated LTCCs can recruit CaMKII autophosphorylation, LTCCs did not play a significant role in driving CaMKIIα autophosphorylation under basal conditions because neither nimodipine, a generic LTCC blocker, nor isradipine a somewhat CaV1.3-selective LTCC blocker, had a significant effect. This is not entirely surprising becasue LTCCs would be expected to be largely closed under basal conditions. Thus, the role of LTCCs in CaMKIIα regulation is dependent on the upstate (depolarized) or downstate (basal) membrane potentials, which could have implications for understanding the essential roles of LTCCs in spine loss following dopamine depletion (Day et al. 2006), endocannabinoid dependent synaptic depression (Adermark & Lovinger 2007), and depolarization-induced spine loss (Tian *et al.* 2010). Indeed, CaMKII has an important role in regulating the synthesis of the major striatal endocannabinoid, 2-arachidonylglycerol (Shonesy et al. 2013) and spine morphology (Xie *et al.* 2007, Zheng *et al.* 2010). Furthermore, CaMKII could also function as a feedback regulator of striatal LTCCs, as CaMKII binds to and phosphorylates multiple subunits of the CaV1.2 and CaV1.3 LTCCs to facilitate calcium entry (Hudmon *et al.* 2005, Koval *et al.* 2010, Erxleben *et al.* 2006, Lee *et al.* 2006, Wang *et al.* 2009, Gao *et al.* 2006, Jenkins *et al.* 2010). This positions CaMKII to regulate LTCCs through phosphorylation or binding, in addition to acting on downstream synaptic targets that mediate striatal LTCC functions.

Second, our data indicate that TTCCs, but not RTCCs, make a significant contribution to calcium entry that is important for driving activation and Thr286 autophosphorylation of CaMKIIα under basal conditions. RTCCs and TTCCs are activated at much more hyper-polarized membrane potentials than LTCCs. Our data using SNX-482, which selectively inhibits RTCCs over other VGCCs (Newcomb *et al.* 1998, Bourinet *et al.* 2001), do not support a significant role for RTCCs in basal CaMKIIα activation, although potential off target effects on potassium channels may confound this interpretation (Kimm & Bean 2014). However, basal CaMKIIα autophosphorylation was partially blocked by mibefradil, a selective TTCC blocker. Mibefradil has been reported to also block LTCCs at somewhat higher concentrations (Martin et al. 2000, Jiménez *et al.* 2000), but the lack of effect of dihydropyridines (see above) strongly suggests that LTCCs are not involved under basal

conditions. Moreover, CaMKIIα autophosphorylation was also decreased by incubating slices with $NiCl_2$, which at the dosage used preferentially blocks TTCC currents over other VGCC subtypes (Todorovic & Lingle 1998, Fox et al. 1987). While $NiCl_2$ can also inhibit RTCCs (Zamponi *et al.* 1996), the lack of effect of SNX-482 strongly supports a role for TTCCs in mediating the effect of $NiCl_2$. TTCCs are less well characterized than other VGCCs, though they have been linked to spontaneous neuronal burst firing (Cain & Snutch 2010, Isope & Murphy 2005, Contreras 2006, Broicher *et al.* 2008) and synaptic depression (Bender *et al.* 2006, Birtoli & Ulrich 2004, Oliet *et al.* 1997, Nevian & Sakmann 2006), and were recently shown to increase CaMKII autophosphorylation in hippocampus (Moriguchi *et al.* 2012). The coupling of CaMKIIs to TTCC activation could mediate specific downstream signals or also be important in feedback facilitation of the CaV3.2 TTCC subtype (Yao *et al.* 2006, Welsby *et al.* 2003, Wolfe *et al.* 2002). Future studies further examining the coupling of TTCC and CaMKII activity could provide key insights into how these proteins contribute to striatal plasticity.

One limitation of our biochemical studies is that we are measuring the overall average CaMKIIα activation across all striatal cell types. CaMKII is highly expressed in striatal MSNs (Erondu & Kennedy 1985, Fukunaga et al. 1988, Ouimet et al. 1984), which constitute ≥90% of striatal neurons, likely reflecting the source of most of the CaMKII we assayed here. However, as mentioned previously, there are two major MSN subtypes, the D1R-MSNs or the D2R-MSNs (Gerfen *et al.* 1990, Surmeier *et al.* 1996). These dopamine receptors couple to distinct biochemical signaling pathways and are generally considered to play opposing roles in coordinating basal ganglia functions (Gerfen & Surmeier 2011, Lovinger 2010, Kreitzer 2009, Calabresi *et al.* 2014). Moreover, MSNs expressing D1Rs or D2Rs differ from each other in dendritic morphology (Gertler et al. 2008). Preliminary immunostaining studies have not indicated substantial differences in CaMKII expression in D1R-MSNs and D2R-MSNs (data not shown), but our current data likely reflect the combined effects of these calcium channels in both MSN subtypes. It is possible that these calcium channels selectively modulate CaMKII in one MSN subtype, but not the other. In this regard, it is interesting to note that CaV1.3 LTCCs are selectively implicated in spine loss from D2R-MSNs following dopamine depletion (Day et al. 2006) and that CaMKII can modulate both spine morphology (Xie et al. 2007, Zheng et al. 2010) and the activity of CaV1.3 LTCCs (Jenkins et al. 2010). Future studies should investigate CaMKII regulation using immunohistochemical approaches in mice expressing DrD2-eGFP or Drd1a-tdTomato transgenes to differentiate striatal MSN subtypes, and examine dendritic pools of CaMKII. Such an approach would also provide insights into CaMKII regulation in lateral and medial portions of the dorsal striatum, as well as the ventral striatum (Nucleus Accumbens), which have distinct behavioral roles.

Overall, our results highlight the complexity of CaMKII regulation by multiple calcium channels in the striatum. This could implicate CaMKII in a number of calcium mediated striatal functions, such as regulating membrane potential, integrating dopaminergic signaling, influencing gene expression, and inducing synaptic plasticity. Dysregulation between CaMKII activation and calcium entry via select VGCCs could also be important in understanding neurological diseases (e.g., Parkinson disease, Huntington disease) and

psychiatric disorders involving changes in striatal function. Future studies determining how these upstream calcium entry points couple with downstream calcium-dependent signaling components could be important in the development of new therapeutic targets to neurological and psychiatric disorders.

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Abbreviations

AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
CaMKII	calcium/calmodulin-dependent protein kinase II
LTCC	L-type calcium channel
MSN	medium spiny neuron
RTCC	R-type calcium channel
TTCC	T-type calcium channel
VGCC	Voltage-gated calcium channel

Highlights

- We explored CaMKII regulation by pharmacologically treating striatal slices.
- Depolarization recruits L-type calcium channels to increase CaMKII activation.
- Calcium influx partly by T-type channels sustains CaMKII activity and location.

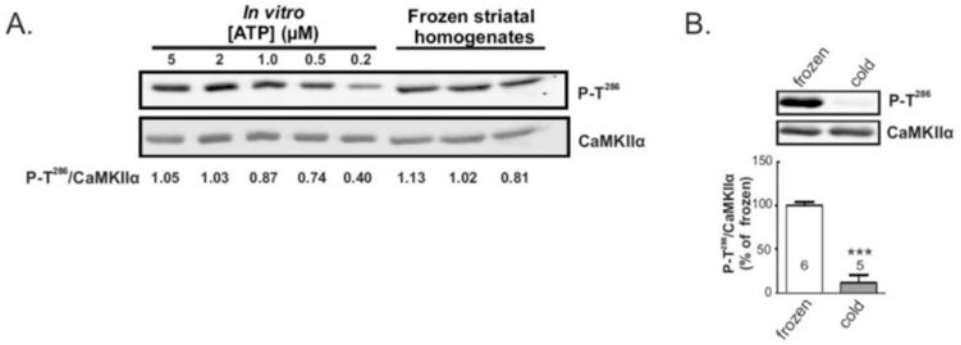

Figure 1.

A) Comparison of Thr286 phosphorylation in purified CaMKIIα autophosphorylated *in vitro* and CaMKIIα in whole lysates of dorsal striatum collected after freezing slices. Numbers below each lane of the blot indicate the P-T286/total signal ratio. Thr286 autophosphorylation of purified CaMKIIα (1 μM) saturates with ATP concentration higher than 2 μM. Notably, CaMKIIα in these striatal samples appears to be Thr286 phosphorylated to similar saturating levels based on the P-T286/total ratio. B) Representative immunoblots of whole lysates of dorsal striatal punches collected either from flash frozen slices on dry ice (frozen) or from slices chilled on regular ice (cold). Quantitation of P-T286 and total CaMKIIα signals from 6 and 5 slices, respectively, revealed that Thr286 phosphorylation in slices dissected on ice is approximately 15% of the levels detected from frozen slices (below). Significance determined by unpaired Student's *t*test (***$p<0.001$).

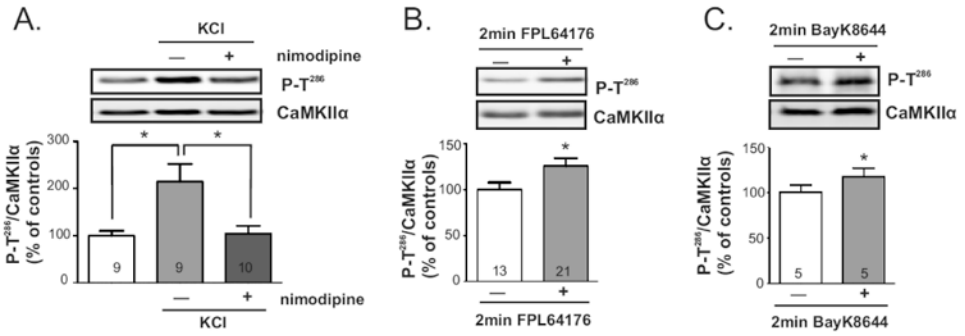

Figure 2.

Striatal CaMKII is regulated by membrane depolarization or by selective activation of LTCCs. Brain slices were incubated with (light/dark gray bars) or without (white bars): A) 40 mM KCl for 1 min (light gray) or nimodipine (10 μM, >25 min) followed by 40 mM KCl for 1 min (dark gray). B) 500 nM FPL64176 for 2 min. C) 5 μM BayK8644 for 2 min. Whole lysates of dorsal striatum were immunoblotted for P-Thr286 and total CaMKIIα. Representative blots are shown above bar graphs reporting P-T286/total CaMKIIα ratios as the mean ± SEM for the indicated number of replicates. Significance determined by ANOVA followed by Tukey post-test (A) or by unpaired Student's -test (B,C) (*$p<0.05$).

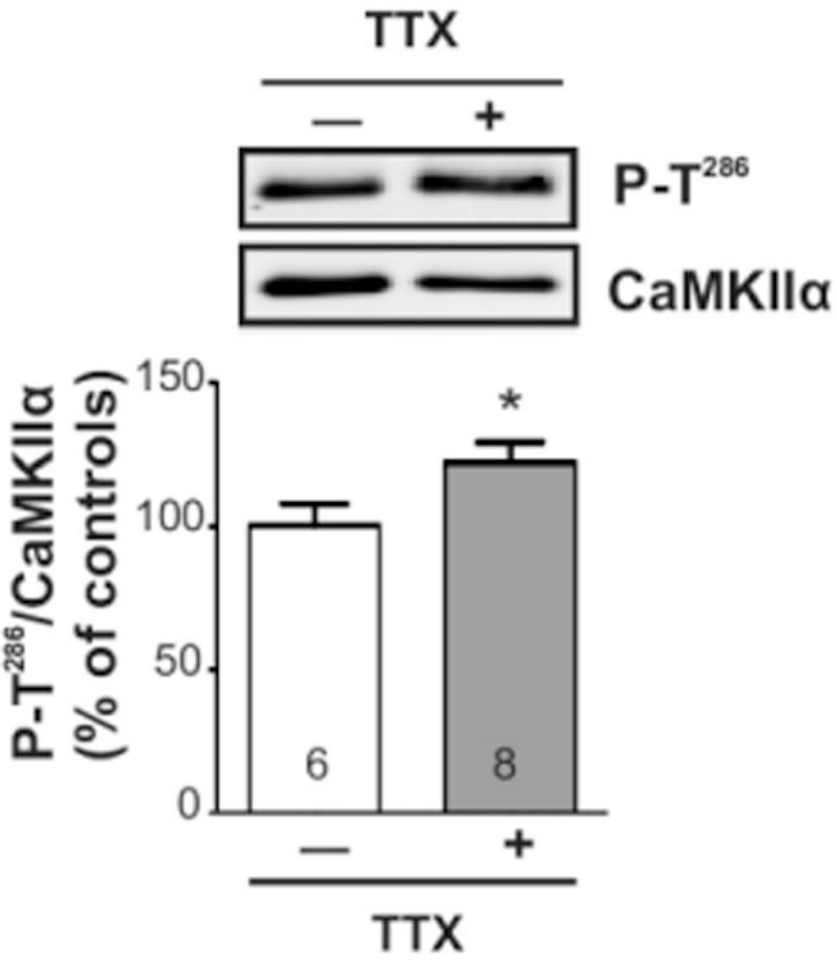

Figure 3.

Blocking intrinsic activity increases striatal CaMKII activity. Mouse brain slices were incubated for 30 min with tetrodotoxin (TTX; 1 μM; gray bars) or vehicle (white bars). Lysates of dorsal striatal punches were immunoblotted for P-Thr286 and total CaMKIIα. Representative blots are shown above the bar graph reporting the P-Thr286/total CaMKIIα ratio as the mean ± SEM for the indicated number of replicates. Significance determined by unpaired Student's *t*-test (*p<0.05).

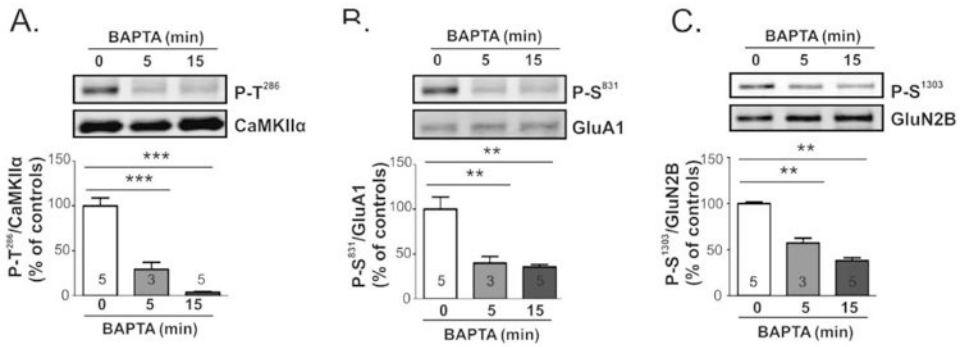

Figure 4.

Extracellular calcium is required to sustain basal CaMKII activity and phosphorylation of CaMKII substrates. Mouse brain slices were incubated with BAPTA (5 mM) or vehicle (white bars) for 0, 5, or 15 min. Lysates of dorsal striatum were immunoblotted for P-Thr286 and total CaMKIIα (A), P-Ser831 and total GluA1 (B), or P-Ser1303 and total GluN2B (C). Representative blots are shown above bar graphs reporting P-Thr286/CaMKIIα, P-Ser831/total GluA1, and P-Ser1303/total GluN2B ratios as the mean ± SEM for the indicated number of replicates. Significance determined by one-way ANOVA (**p<0.001) followed by Tukey's post-hoc analysis (**p<0.001).

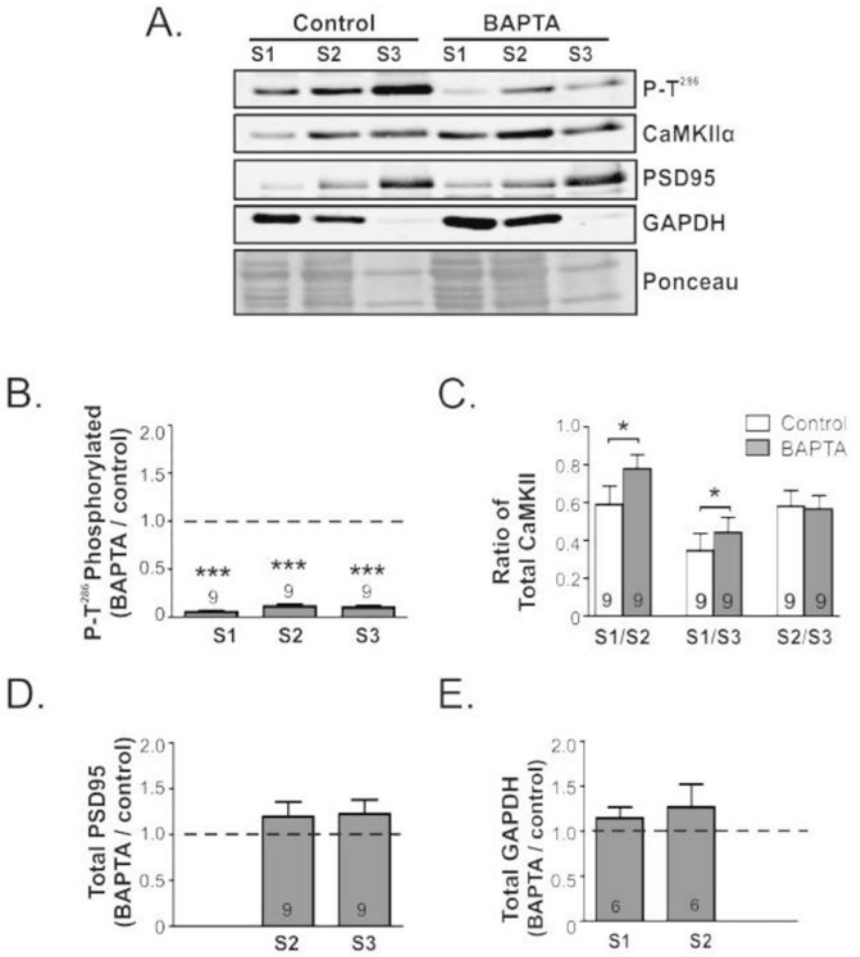

Figure 5.

Depletion of extracellular calcium induces CaMKII translocation to the cytosolic fraction. Mouse brain slices were incubated for 15 min with BAPTA (5 mM) or vehicle. Equal volumes of cytosolic (S1), membrane (S2, Triton-Soluble), and synaptic (S3, Triton-Insoluble) fractions isolated from dorsal striatum were immunoblotted for: P-Thr286, total CaMKIIα, and also GAPDH and PSD-95 as cytosolic and synaptic markers, respectively. Representative images of each blot are shown, as well as a segment of the Ponceau stained membrane prior to immunoblotting. The relative enrichment of the P-Thr286/total CaMKIIα ratio (B) as well as the total CaMKIIα (C) PSD95 (D) GAPDH (E) normalized to the Ponceau stain in each fraction from control and BAPTA treated slices were then quantified. All bar graphs report the mean ± SEM from the indicated number of replicates. The dashed line indicates a value of 1.0 (F); the theoretical value if BAPTA had no effect. Significance determined by one sample -test (*$p<0.05$, ***$p<0.001$).

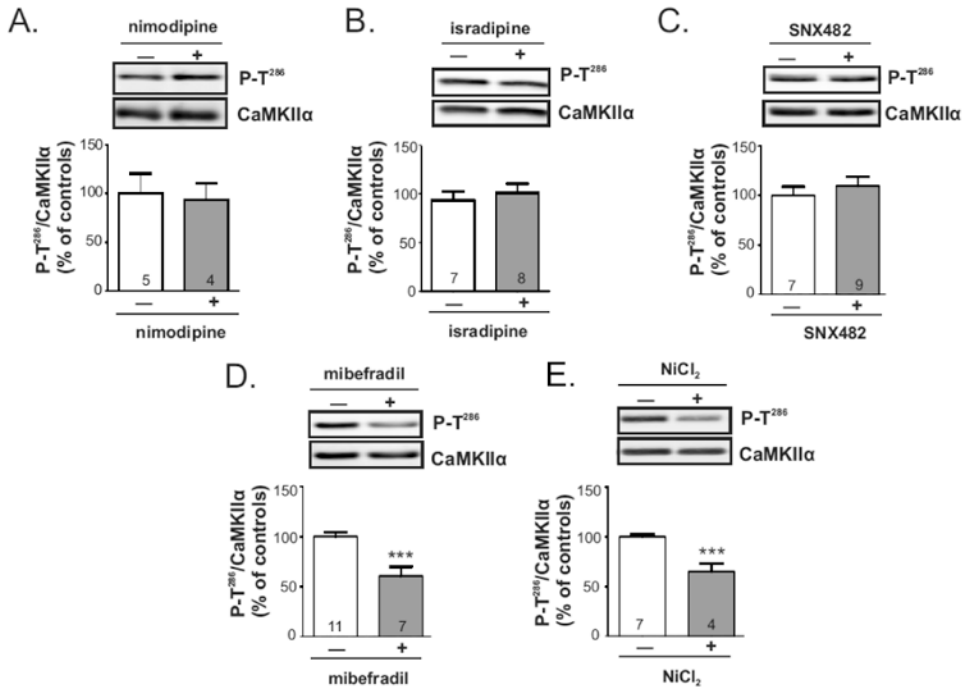

Figure 6.

Maintenance of basal CaMKII autophosphorylation requires TTCC activity. Mouse brain slices were incubated for 30 min with A) nimodipine (10 μM), B) isradipine (5 μM), C) SNX482 (400 nM), D) mibefradil (5 μM), E) $NiCl_2$ (100 μM), or vehicle. Whole lysates of dorsal striatum were immunoblotted for P-Thr286 and total CaMKIIα. Representative blots are shown above bar graphs reporting the P-Thr286/total CaMKIIα ratios as the mean ± SEM for the indicated number of replicates. White bars are vehicle controls and gray bars are drug-treated samples. Significance determined by unpaired Student's *t*test (***p<0.001).