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Activation of Dopamine D_4 Receptors Induces Synaptic Translocation of CaMKII in Cultured Prefrontal Cortical Neurons

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Running Title: D_4 regulation of CaMKII localization in prefrontal cortex

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ABBREVIATIONS: PFC, prefrontal cortex; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; PLC, phospholipase C; IP_3Rs, ionsitol-1,4,5-triphosphate receptors; mEPSCs, miniature excitatory postsynaptic currents; PSD, postsynaptic density.

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ABSTRACT

One of the important targets of dopamine D_4 receptors in prefrontal cortex (PFC) is the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). In the present study, we investigated the effect of D_4 receptor activation on subcellular localization of CaMKII. We found that activation of D_4 receptors, but not D_2 receptors, induced a rapid translocation of α-CaMKII from cytosol to postsynaptic sites in cultured PFC neurons. Activated CaMKII (Thr^{286} phospho-CaMKII) was also redistributed to postsynaptic sites following D_4 receptor stimulation. The translocation was blocked by inhibiting the PLC/IP_3R/Ca^{2+} signaling. Point mutation of the calmodulin binding site (Ala^{302}), but not the autophosphorylation site (Thr^{286}), of α-CaMKII prevented the D_4-induced CaMKII translocation. Moreover, D_4 receptors failed to induce CaMKII translocation in the presence of an actin stabilizer, and D_4 activation reduced the binding of CaMKII to F-actin. Concomitant with the synaptic accumulation of α-CaMKII in response to D_4 receptor activation, a D_4-induced increase in the CaMKII phosphorylation of AMPA receptor GluR1 subunits and the amplitude of AMPA receptor-mediated excitatory postsynaptic currents was also observed. Thus, our results show that D_4 receptor activation induces the synaptic translocation of CaMKII through a mechanism involving Ca^{2+}/calmodulin and F-actin, which facilitates the regulation of synaptic targets of CaMKII, such as AMPA receptors.

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INTRODUCTION

Dopaminergic inputs to prefrontal cortex (PFC) are believed to play important roles in many physiological functions such as working memory formation (Brozoski et al., 1979; Goldman-Rakic, 1995; Marie and Defer, 2003). Dysfunction of the dopaminergic system in PFC is considered to be a significant contributor to the pathophysiology of a variety of disorders, including schizophrenia (Grace, 1991; Goldman-Rakic, 1994; Andreasen et al., 1997; Lewis and Lieberman, 2000; Carlsson et al., 2001). The dopamine D_4 receptor, a member of the D_2-family receptors, is highly expressed in PFC (Mrzljak et al., 1996; Wedzony et al., 2000). Several lines of evidence have suggested the involvement of D_4 receptors in normal PFC functioning and neuropsychiatric disorders (Rubinstein et al., 1997; Dulawa et al., 1999; Oak et al., 2000). The expression of D_4 receptors is elevated in PFC of schizophrenia patients (Seeman et al., 1993). Some antipsychotic drugs have high affinities to D_4 receptors (Van Tol et al., 1991; Kapur and Remington, 2001). D_4 receptor antagonists are effective in ameliorating cognitive deficits caused by the psychotomimetic drug phencyclidine (Jentsch et al., 1997; 1999). However, the cellular mechanisms underlying these D_4 actions are yet to be elucidated.

Our recent studies have shown that one of the important targets of dopamine D_4 receptors in PFC is CaMKII (Wang et al., 2003; Gu and Yan, 2004). In PFC neurons with low activity, D_4 receptor stimulation increases CaMKII activity through a PLC/IP_3R-dependent pathway, but not the classical $G_{i/o}$ and PKA signaling pathway, while D_2 receptors fail to increase CaMKII activity (Gu and Yan, 2004). Thus, activation of CaMKII can be one of the features to distinguish D_4 receptor signaling from D_2 receptor signaling.

CaMKII is a multifunctional kinase highly enriched in neurons. It is activated by elevated intracellular Ca^{2+}, which triggers calmodulin binding to CaMKII at Ala^{302}. CaMKII is autophosphorylated at Thr^{286} when the enzyme is activated, leading to the appearance of a sustained, Ca^{2+}-independent activity (Miller and Kennedy, 1986). Many signals can elevate intracellular Ca^{2+} through different pathways, including extracellular Ca^{2+} influx through voltage-gated Ca^{2+} channels and NMDA receptor channels, or Ca^{2+} release from intracellular stores through activation of G_q-coupled receptors. Upon

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activation, CaMKII phosphorylates dozens of substrates throughout the whole cell (Braun and Schulman, 1995). Like other kinases with broad substrate selectivity, CaMKII achieves the efficacy and specificity of signal transduction via compartmentalized localization (Kennedy, 2000; Hudmon and Schulman, 2002). CaMKII located in postsynaptic sites has been found to play a crucial role in synaptic plasticity that is integral for learning and memory, through the regulation of several postsynaptic proteins, such as postsynaptic AMPA receptors and NMDA receptors (Silva et al., 1992; Malenka and Nicoll, 1999; Soderling et al., 2001; Frankland et al., 2001).

Since targeting CaMKII to certain subcellular compartments is crucial for the efficacy and specificity of signals in response to various stimuli, we examined whether D_4 receptor activation in PFC neurons could change the subcellular localization of CaMKII. We found that D_4 receptor stimulation induced a synaptic translocation of CaMKII, and this event was paralleled by a significant increase in the CaMKII phosphorylation of AMPA receptors and the postsynaptic AMPA response following D_4 receptor activation.

MATERIALS AND METHODS

Primary neuronal culture. Rat PFC cultures were prepared as previously described (Wang et al., 2003). Briefly, PFC was dissected from 18 d rat embryos and cells were dissociated using trypsin and trituration through a Pasteur pipette. The neurons were plated on coverslips coated with poly-L-lysine in Dulbecco's Modified Eagle medium (DMEM) with 10% fetal calf serum at a density of 100,000 cells/cm^2. When neurons attached to the coverslip within 24 hr, the medium was changed to Neurobasal with B27 supplement. Neurons were maintained in the same kind of media for two to three weeks. Cultured neurons were treated with various agents for the durations as indicated in texts prior to fixation and immunostaining.

Immunocytochemistry. After treatment, cultured PFC neurons were fixed in 4% paraformaldehyde in PBS for 20 min and were permeabilized with 0.2% Triton X-100 for 5 min. Following 1 hour incubation with 5 % bovine serum albumin (BSA) to block nonspecific staining, the cells were incubated with the

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polyclonal α-CaMKII antibody (Upstate Biotechnology, 1:100) and monoclonal PSD-95 antibody (Affinity BioReagents, 1:100) at 4°C overnight. In some experiments, the cells were incubated with the polyclonal Thr^{286}-p-α-CaMKII antibody (Santa Cruz, 1:100) and monoclonal PSD-95 antibody at 4°C overnight. Alternatively, cells were incubated with the polyclonal β-CaMKII antibody (Zymed Laboratories Inc., 1:100). After washing, the cells were incubated with a fluorescein-conjugated and a rhodamine-conjugated secondary antibody (Sigma, 1:200) for 60 min at room temperature. After three washes in PBS, the coverslips were mounted on slides with VECTASHIELD mounting media (Vector Laboratories, Inc., Burlingame, CA). Fluorescent images were obtained using a 100x objective with a cooled CCD camera mounted on a Nikon microscope, and analyzed with the Image J software. All specimens were imaged under identical conditions and analyzed using identical parameters. To define CaMKII, p-CaMKII and PSD-95 clusters, a single threshold was chosen manually and corresponded to two times the average intensity of fluorescence in the dendritic shaft. Any CaMKII or p-CaMKII cluster that overlapped with a PSD-95 cluster was defined as synaptic. Three to four independent experiments for each of the treatments were performed. On each coverslip, 4-6 neurons were chosen and quantified. For each neuron, three to four neurites (50 μm each) were measured. Quantitative analyses were conducted blindly (without knowledge of experimental treatment).

Receptor ligands PD168077 maleate (PD), L-745870 trihydrochloride (Tocris, Ballwin, MO), quinpirole, dopamine, SCH23390, sulpiride, α-methyl-5-hydroxytryptamine (α-Me-5HT), carbachol (Sigma, St. Louis, MO), as well as second messenger reagents cpt-cAMP, PKI_{14-22}, U73122, genistein, Bis-indolyl-maleimide I (Bisl), KN-62, KN-93, 2-aminoethoxydiphenylborane (2APB) (Calbiochem, San Diego, CA), and actin/microtubule agents phalloidin-oleate, taxol (Sigma), were made up as concentrated stocks and stored at -20ºC. The final DMSO concentration in all applied solutions was no more than 0.1%. Stocks were thawed and diluted immediately prior to use.

Western blot and co-immunoprecipitation. After treatment with different agents, equal amounts of protein from PFC culture homogenates (in 1% SDS lysis buffer) were separated on 7.5% acrylamide gels

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and transferred to nitrocellulose membranes. The blots were blocked with 5% nonfat dry milk for 1 hour at room temperature. Then the blots were incubated with the anti-p^{S831}-GluR1 (Upstate, 1:500) or anti-GluR1 (Upstate, 1:1000) for 1 hour at room temperature. After being rinsed, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit antibodies (Amersham, 1:1,000) for 1 hour at room temperature. Following 3 washes, the blots were exposed to the enhanced chemiluminescence substrate. Quantification was obtained from densitometric measurements of immunoreactive bands on films using NIH Image software.

For co-immunoprecipitation experiments, PFC slices (400 μm) from 3-week-old rats were used. Slices were prepared as previously described (Gu et al., 2003; Gu and Yan, 2004). After treatment with indicated agents, each slice was collected and homogenized in 1 ml lysis buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 50 mM $NaPO_4$, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 mg/ml leupeptin). Lysates were centrifuged at 4°C at 16,000 × g for 30 min. Supernatant fractions were incubated with an anti-actin antibody (Santa Cruz Biotechnology) for 1 hr at 4°C, followed by incubation with 50 μl of protein A/G plus agarose (Santa Cruz Biotechnology) for 1 hr at 4°C. Immunoprecipitates were washed for three times with lysis buffer containing 0.2 M NaCl, then boiled in 2x SDS loading buffer for 5 min, and separated on 7.5% SDS-polyacrylamide gels.

CaMKII α subunit cloning and site mutation. CaMKII α subunit cDNA (Genebank accession number NM012920) was amplified by RT-PCR from rat brain total RNA. The primers used for PCR are: 5'-gcgaattctgccaggatggctaccatcacc and 5'-gcggatccctggcctggtccttcaatgg. The PCR product was then cloned to PCR2.1-TOPO vector (Invitrogen) and further subcloned to pEGFP-C1 vector (BD Biosciences Clontech). Point mutants were made with a site mutation kit (Stratagene, La Jolla, CA) using the primers: 5'-catgcacagacaggaggccgtggactgcc (for Thr^{286} to Ala mutation) and 5'-ggaaactgaagggacgcatcctcaccac tatgc (for Ala^{302} to Arg mutation). Transfection of GFP-fused CaMKII constructs to primary PFC cultures

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was performed using Lipofectamine 2000 (Invitrogen) according to the manual. Neurons were used two days after transfection.

Synaptic current recording in cultures. The recording of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) in PFC cultures was similar to what was previously described (Cai et al., 2002). The internal solution consisted of (in mM): 130 Cs-methanesulfonate, 10 CsCl, 4 NaCl, 10 HEPES, 1 $MgCl_2$, 5 EGTA, 2.2 QX-314, 12 phosphocreatine, 5 MgATP, 0.5 Na_2GTP, 0.1 leupeptin, pH = 7.2-7.3, 265-270 mosm/L. The external solution consisted of (in mM): 127 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 12 glucose, 10 HEPES, 0.001 TTX, 0.005 bicuculline, pH = 7.3-7.4, 300-305 mosm/L. D-APV (20 µM) was added to block the NMDAR-mediated component of mEPSCs. The membrane potential was held at -70 mV. The AMPAR-mEPSCs in the absence or presence of agonists were compared. Before recording, cultured neurons were treated with TTX (1 µM) for 24 hours to lower the basal activity. Synaptic currents were analyzed with Mini Analysis Program (Synaptosoft, Leonia, NJ). Statistical comparisons of the amplitude and frequency of synaptic currents (mean ± SEM) were made using the Kolmogorov _Smirnov (K-S) test.

RESULTS

Induction of CaMKII postsynaptic translocation by D_4 receptors in cultured PFC neurons

We assessed the effect of D_4 receptor activation on the subcellular localization of CaMKII in cultured PFC neurons using immunocytochemical approaches. As shown in **Figure 1A**, in the untreated cells (control), CaMKII was mainly distributed uniformly throughout dendritic shafts, similar to what was found in cultured hippocampal neurons (Fong et al., 2002). In the cells treated with PD168077, a selective D_4 receptor agonist (Glase et al., 1997; Wang et al., 2003), CaMKII exhibited an enrichment in punctate structures along dendritic processes. Counting CaMKII puncta along dendrites (**Figure 1B**) showed that the translocation occurred at 2 min (18.3 ± 3.9 clusters/100µm vs. 7.2 ± 1.5 clusters/100µm in untreated neurons; $p < 0.05$, ANOVA), peaked at 5 min (43.5 ± 7.5 clusters/100µm, $p < 0.001$, ANOVA), lasted for about 30 min (17.1 ± 3.8 clusters/100μm, $p < 0.05$, ANOVA), and recovered at about 60 min (8.9 ± 2.2 clusters/100μm).

To further investigate the localization of CaMKII puncta after PD168077 treatment, we co-stained CaMKII with the postsynaptic scaffolding protein PSD-95 (a marker for postsynaptic synapses). In contrast to the translocation of CaMKII, the distribution of PSD-95 was not altered by PD168077 treatment (**Figure 2A**). As illustrated on the merged pictures, the punctate distribution of CaMKII after PD168077 treatment showed a marked colocalization with PSD-95, suggesting that CaMKII was translocated to postsynaptic sites in response to D_4 receptor activation. Quantification (**Figure 2B**) revealed a significant increase in synaptic CaMKII clusters along dendrites after activating D_4 receptors (control: 5.3 ± 1.5 clusters/100μm; PD168077: 35.5 ± 6.4 clusters/100μm; $p < 0.001$, ANOVA; $n = 24$ neurons per group from six cultures), while the density of synaptic PSD-95 clusters along dendrites was unchanged by D_4 receptor activation (control: 52.9 ± 8.5 clusters/100μm; PD168077: 54.8 ± 8.7 clusters/100μm; $n = 10$ neurons per group from three cultures).

We then examined whether D_4 receptor activation triggered the synaptic targeting of activated CaMKII (Thr^{286}-phosphorylated) in cultured PFC neurons. As shown in **Figure 2C** and **2D**, in the untreated cell, p-CaMKII was distributed almost evenly on dendritic arbors. In the PD168077-treated cell, p-CaMKII exhibited a significantly increased clustering at synaptic sites, as indicated by the enhanced co-localization with PSD-95 (synaptic p-CaMKII clusters/100μm dendrite: 6.2 ± 1.7 in controls, 37.3 ± 6.7 in PD168077-treated neurons; $p < 0.001$, ANOVA; $n = 22$ neurons per group from four cultures). These results suggest that activated CaMKII, like CaMKII, was redistributed to postsynaptic sites by D_4 receptor activation.

Mechanisms for the D_4-induced synaptic translocation of CaMKII in PFC cultures

If the PD168077-induced CaMKII translocation to postsynaptic sites was indeed mediated by D_4 receptors, selective D_4 receptor antagonists should prevent the action of PD168077. Consistent with this,

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application of L745870 (20 µM), a selective D_4 receptor antagonist (Kulagowski et al., 1996; Patel et al., 1997), strongly inhibited the synaptic translocation of CaMKII induced by PD168077 (**Figure 3,** synaptic CaMKII clusters/100µm dendrite: 5.2 ± 1.7 in untreated neurons; 35.7 ± 6.2 in PD168077-treated neurons; 6.1 ± 1.6 in L745870+PD168077-treated neurons; n = 18 neurons per group from four cultures). In addition, we found that quinpirole, a dopamine D_2 receptor agonist, failed to induce CaMKII translocation (4.9 ± 1.9 synaptic CaMKII clusters/100µm dendrite, n = 20 neurons from four cultures), indicating that the effect on CaMKII translocation is specific for D_4 receptor activation.

We further tested whether dopamine can mimic the effect of D_4 agonist. PFC cultures were treated with SCH23390 (10 µM) and sulpiride (10 µM) for 15 min to block D_1/D_5 and D_2/D_3 receptors, followed by a 5-min treatment with dopamine (50 µM). SCH23390 and sulpiride treatment alone did not induce CaMKII clustering (5.6 ± 3.8 clusters/100µm dendrite, n = 18 neurons from three cultures). However, in the presence of these D_1/D_5 and D_2/D_3 antagonists, dopamine induced significant CaMKII synaptic translocation (26.8 ± 4.5 synaptic CaMKII clusters/100µm dendrite, n = 20 neurons from three cultures, $p < 0.001$, ANOVA), similar to the effect of PD168077.

It was previously shown that D_4 receptor activation increased CaMKII activity through a mechanism involving the stimulation of PLC/IP_3R pathway, but not the classical D_2 receptor-coupled $G_{i/o}$ and PKA pathway in PFC slices (Gu and Yan, 2004), we then examined the participation of these signaling molecules in D_4-induced CaMKII translocation. As shown in **Figure 4**, neither the PKA activator cpt-cAMP (100 µM) nor the PKA inhibitor PKI_{14-22} (100 nM) prevented the PD168077-induced CaMKII translocation (synaptic CaMKII clusters/100µm dendrite: 32.7 ± 5.7 in cAMP+PD168077-treated neurons; 33.2 ± 5.8 in PKI+PD168077-treated neurons; n = 18 neurons per group from three cultures), indicating the lack of involvement of the PKA pathway in this event. On the other hand, in the presence of the PLC inhibitor U73122 (1 µM), after PD168077 treatment, CaMKII still exhibited the uniform distribution along dendritic processes (5.8 ± 1.3 clusters/100µm in U73122+PD168077-treated

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neurons, n = 16 neurons from three cultures), indicating that the D_4-induced CaMKII synaptic translocation is abolished by the inhibition of the PLC pathway.

Because PLC stimulation leads to the release of intracellular Ca^{2+} via IP_3 receptors and activation of PKC, we further examined the role of these molecules in D_4-induced CaMKII synaptic translocation. As shown in Figure 4, blocking IP_3 receptors with 2APB (15 μM) or inhibiting the elevation of intracellular Ca^{2+} with BAPTA/AM (50 μM) prevented CaMKII from clustering at synaptic sites in response to PD168077 treatment (synaptic CaMKII clusters/100μm dendrite: 6.5 ± 1.4 in 2APB+PD168077-treated neurons; 6.8 ± 1.2 in BAPTA+PD168077-treated neurons; n = 14 neurons per group from three cultures). In contrast, inhibiting PKC activation with the specific inhibitor Bis-indolyl-maleimide (Bisl, 4 μM) or inhibiting tyrosine kinases with genistein (100 μM) failed to alter the PD168077-induced CaMKII synaptic targeting (synaptic CaMKII clusters/100μm dendrite: 31.7 ± 5.3 in Bisl+PD168077-treated neurons; 33.6 ± 6.2 in genistein+PD168077-treated neurons; n = 18 neurons per group from four cultures). Taken together, these results suggest that the D_4 receptor-mediated CaMKII translocation depends on intracellular Ca^{2+} elevation induced by the PLC/IP_3R pathway.

To understand how Ca^{2+} is potentially involved in the D_4 induction of CaMKII translocation, we examined the influence of CaMKII mutants on the translocation. We expressed GFP-fused wild-type α-CaMKII, the autophosphorylation-deficient mutant (T286A) or the calmodulin binding-deficient mutant (A302R) form of α-CaMKII in PFC cultures. As shown in **Figure 5**, similar to the endogenous CaMKII, GFP-fused α-CaMKII diffused throughout dendritic shafts in untreated neurons (synaptic CaMKII clusters/100μm dendrite: 6.4 ± 1.8, 6.8 ± 1.7, and 5.2 ± 1.4 for wild-type, autophosphorylation-deficient, and calmodulin binding-deficient CaMKII, respectively, n = 20 neurons per group from four cultures). PD168077 treatment (5 min) significantly increased CaMKII-GFP puncta along dendrites in neurons transfected with wild-type CaMKII (38.8 ± 6.7 clusters/100μm, $p < 0.001$, ANOVA; n = 20 neurons from four cultures) or autophosphorylation-deficient CaMKII (39.4 ± 6.6 clusters/100μm, $p < 0.001$, ANOVA; n = 20 neurons from four cultures), but not in neurons transfected with the calmodulin binding-deficient

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CaMKII (8.3 ± 1.9 clusters/100μm; n = 20 neurons from four cultures). It suggests that calmodulin binding, but not autophosphorylation, of CaMKII is required to trigger its synaptic translocation in response to D_4 receptor activation.

To determine whether calmodulin binding is sufficient to induce CaMKII redistribution, we examined the effect of several other agents on CaMKII translocation, including glutamate (50 μM), muscarinic receptor agonist carbachol (10 μM), 5-HT_2 receptor agonist α-Me-5HT (20 μM), and the calcium ionophore A23187 (2 μM), all of which can elevate intracellular Ca^{2+} and thus promote the calmodulin binding and activation of CaMKII. Indeed, similar to PD168077, all these agents induced CaMKII activation, as indicated by the increased level of Thr^{286}-phosphorylated CaMKII (**Figure 6A** and **6B)**. However, only glutamate induced a significant increase in synaptic CaMKII clustering along dendrites (**Figure 6C** and **6D**, 38.1 ± 5.5 clusters/100μm, $p < 0.001$, ANOVA; n = 18 neurons from three cultures), consistent with the previous result (Shen and Meyer, 1999). None of the other three agents showed any effect on CaMKII clustering (Figure 6C and 6D, CaMKII clusters/100μm dendrite: 6.5 ± 1.5, 6.3 ± 1.3 and 6.0 ± 1.4 for carbachol, α-Me-5HT and A23187, respectively), suggesting that calmodulin binding is not sufficient to induce CaMKII redistribution.

What is the other factor, in addition to Ca^{2+}/calmodulin binding, that is necessary for CaMKII translocation? Previous studies have shown that CaMKII could associate with actin filaments (Ohta et al., 1986) and microtubules (Vallano et al., 1986). We hypothesize that the D_4-induced elevation of intracellular Ca^{2+} and calmodulin binding of CaMKII may affect the association of CaMKII with the cytoskeletal network, therefore facilitating the translocation of CaMKII. To test this, we exposed neuronal cultures to various agents that disturb F-actin or microtubule before activating D_4 receptors with PD168077 treatment. As shown in **Figure 7A** and **7B**, the F-actin stabilizer phalloidin-oleate (1 μM) prevented PD168077-induced synaptic CaMKII translocation along dendrites (9.4 ± 2.1 clusters/100μm, n = 20 neurons from four cultures), while the microtubule stabilizer taxol (10 μM) was without effect (33.0 ± 4.9 CaMKII clusters/100μm dendrite, $p < 0.001$, ANOVA; n = 20 neurons from four cultures).

Neither of these stabilizers induced CaMKII clustering by themselves. These results prompted us to hypothesize that the F-actin stabilizer may prevent the reorganization of actin cytoskeleton and the dissociation of CaMKII from actin, thus blocking the D_4-induced CaMKII translocation.

To test this, we examined the effects of various agents on the binding between CaMKII and F-actin. As shown in **Figure 7C** and **7D,** both PD168077 and glutamate caused a significant reduction of CaMKII that bound to F-actin (PD168077: 35 ± 8% of control; Glu: 29 ± 6% of control, n = 6; p < 0.001, ANOVA), while carbachol, α-Me-5HT and A23187 had no effect (carbachol: 97 ± 15% of control; α-Me-5HT: 98 ± 13% of control; A23187: 95 ± 19% of control; n = 6). In the presence of phalloidin, both PD168077 and glutamate lost the capability to reduce the level of actin-binding CaMKII. These results suggest that CaMKII redistribution may require its dissociation from actin cytoskeleton, and the different effects of those agents on the binding of CaMKII to F-actin may underlie their distinct effects on CaMKII translocation.

Since β-CaMKII binds to F-actin directly, and α-CaMKII is docked to the actin cytoskeleton by forming heterooligomers with β-CaMKII (Shen et al., 1998), we further examined the possible translocation of β-CaMKII in response to D_4 receptor activation. As shown in **Figure 8A** and **8B**, treatment of PFC culture with PD168077 (20 μM, 5 min) induced a significant increase of β-CaMKII clusters along dendrites (control: 6.8 ± 4.5 clusters/100μm, n = 20 neurons from three cultures; PD168077: 41.3 ± 7.3 clusters/100μm, n = 24 neurons from three cultures; p < 0.001, ANOVA), similar to the effect of glutamate (50 μM, 5 min) treatment (45.8 ± 7.8 clusters/100μm, n = 20 neurons from three cultures). In contrast, carbachol (10 μM, 5 min) treatment had no effect on the distribution of β-CaMKII (7.5 ± 5.5 clusters/100μm, n = 20 neurons from three cultures).

Increased CaMKII phopsphorylation of GluR1 and miniature EPSCs after D_4 receptor activation

The synaptically translocated CaMKII after D_4 receptor activation can phosphorylate many synaptic substrates. One potential target is the AMPA receptor GluR1 subunit (Soderling and Derkach,

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2000). We then investigated whether D_4 receptor activation can modulate the CaMKII phosphorylation of GluR1. Previous studies have identified that both CaMKII and PKC phosphorylate GluR1 at the same site Ser^{831} (Mammen et al., 1997). As shown in **Figure 9A** and **9B**, application of PD168077 (5 min) induced a significant increase in GluR1 phosphorylation at Ser^{831} (3.4 ± 0.3 fold, n = 8, $p < 0.001$, ANOVA). The effect was largely blocked by the D_4 receptor antagonist L-745870 (1.3 ± 0.2 fold, n = 6) or the CaMKII inhibitor KN-62 (1.4 ± 0.2 fold, n = 6), but not by the PKC inhibitor Bis-indolyl-maleimide (3.1 ± 0.3 fold, n = 6, $p < 0.001$, ANOVA). It suggests that D_4 receptor activation increases the CaMKII-mediated phosphorylation of GluR1.

To determine whether the increased phosphorylation of GluR1 is caused by synaptically translocated CaMKII, we then examined the effect on GluR1 phosphorylation by other agents that may activate but not translocate CaMKII. As shown in **Figure 9C** and **9D,** the calcium ionophore A23187 (2 μM, 5 min) was unable to increase GluR1 phosphorylation (1.2 ± 0.2 fold, n = 6). Glutamate (50 μM, 5 min) and carbachol (10 μM, 5 min) induced a strong increase in GluR1 phosphorylation (Glu: 3.7 ± 0.5 fold, carb: 3.9 ± 0.6 fold, n = 6, $p < 0.001$, ANOVA), and α-Me-5HT (20 μM, 5 min) had a moderate effect (2.1 ± 0.4 fold, n = 6, $p < 0.05$, ANOVA). We further found that the glutamate-induced GluR1 phosphorylation was largely blocked by the CaMKII inhibitor KN-62 (1.8 ± 0.3 fold, n = 6, $p < 0.05$, ANOVA), and partially blocked by the PKC inhibitor Bis-indolyl-maleimide (Bisl, 2.2 ± 0.4 fold, n = 6, $p < 0.05$, ANOVA). However, the effect of carbachol or α-Me-5HT on GluR1 phosphorylation was insensitive to CaMKII inhibition, but was almost completely blocked by inhibiting PKC, indicating that the GluR1 phosphorylation induced by carbachol or α-Me-5HT was likely through activated PKC rather than CaMKII. Taken together, our data show that the agents that can induce CaMKII translocation, such as PD168077 and glutamate, increase CaMKII phosphorylation of GluR1; whereas the agents that can not induce CaMKII translocation, such as A23187, carbachol and α-Me-5HT, fail to increase CaMKII-dependent phosphorylation of GluR1.

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To understand the functional consequence of the D_4-induced synaptic translocation of CaMKII, we examined the effect of PD168077 on AMPA receptor-mediated synaptic transmission. Miniature excitatory postsynaptic currents (mEPSCs) are believed to result from the random release of single glutamate packets (quanta), and a significant effect on their amplitude is usually considered good evidence for a modification of postsynaptic AMPA receptor properties. Thus, neuronal cultures were exposed to TTX (1 μM) and mEPSCs were recorded in PFC pyramidal neurons. As shown in **Figure 10A** and **10B**, bath application of PD168077 significantly enhanced the mEPSC amplitude ($p < 0.01$, K-S test), as indicated by a rightward shift of the distribution. In the presence of the CaMKII inhibitor KN-93 (10 μM, 10 min pretreatment), PD168077 failed to enhance the mEPSC amplitude (**Figure 10C** and **10D**). In a sample of neurons we tested, PD168077 increased the mean amplitude of mEPSCs by 18.7 ± 2.4% (n = 7, $p < 0.01$, K-S test), which was abolished by KN-93 (2.7 ± 0.9%, n = 4). The mEPSC frequency was either slightly increased or not changed by PD168077 (data not shown). These results suggest that AMPA receptors are up-regulated by synaptically translocated CaMKII in response to D_4 receptor activation.

DISCUSSION

Given the broad substrate selectivity of CaMKII, the control of specificity becomes a crucial issue in signal transduction. Subcellular targeting has emerged as an important mechanism by which signaling enzymes achieve precise substrate recognition and enhanced efficacy of signal transduction (Pawson and Scott, 1997; Hudmon and Schulman, 2002). Different pools of CaMKII compartmentalized at membrane, postsynaptic density (PSD), nucleus and cytosol are responsible for regulating distinct substrates. A previous study has revealed the CaMKII translocation after NMDA receptor activation (Shen and Meyer, 1999). Little is known about CaMKII translocation in response to other stimuli that can elevate intracellular Ca^{2+} from internal stores. Our previous studies have found that D_4 receptor stimulation changes the enzymatic activity of CaMKII (Wang et al., 2003; Gu and Yan, 2004). In this study, we show that stimulation of D_4 receptors also changes the subcellular localization of CaMKII. Following a short

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treatment with the D_4 receptor agonist, both endogenous CaMKII and transfected CaMKII-GFP exhibit rapid redistribution to synapses. Activated CaMKII (Thr^{286}-phosphorylated) is also recruited to synapses after D_4 receptor stimulation. The synaptic accumulation of CaMKII provides a mechanism for limiting activation of all cellular CaMKII and phosphorylation of all of its substrates when D_4 receptors are activated.

CaMKII has multiple interacting partners in the PSD, including actin (Shen et al., 1998) and NMDA receptors (Gardoni et al., 1998; Leonard et al., 1999; Strack et al., 2000). It has been shown that the regulated CaMKII interaction with NR2B subunits provides a mechanism for the glutamate-induced synaptic translocation of CaMKII-GFP (Strack and Colbran, 1998; Bayer et al., 2001). Possible mediators for the D_4-induced CaMKII translocation include synaptic activity, actin reorganization, kinase activation, and an alteration in the affinity of protein interactions. We show that the D_4-induced CaMKII synaptic translocation depends on the stimulation of PLC pathway, elevation of intracellular Ca^{2+}, but not PKC or tyrosine kinases. Similar to NMDA-induced CaMKII translocation (Shen and Meyer, 1999), mutation of the calmodulin binding site, but not the autophosphorylation site, of CaMKII prevented CaMKII synaptic accumulation after D_4 receptor activation, suggesting that Ca^{2+}/calmodulin binding is the driving force for CaMKII translocation under these conditions. However, although calmodulin binding is necessary for the D_4-induced CaMKII synaptic translocation, it is not a sufficient factor. Many other agents, such as the agonists for G_q-coupled muscarinic and serotonerigic receptors (carbachol and α-Me-5HT) and the calcium ionophore A231087, show no effect on CaMKII distribution, despite their capability to elevate intracellular Ca^{2+} and promote calmodulin binding to CaMKII. Additional mechanisms must be involved in the D_4-induced CaMKII translocation.

CaMKII has been found to bind to actin filaments in a calmodulin-sensitive manner (Ohta et al., 1986). The predominant α-CaMKII is docked to the actin cytoskeleton by forming heterooligomers with β-CaMKII, which is bound to F-actin (Shen et al., 1998). It also binds to the actin network through α-actinin, an actin-binding protein (Walikonis et al., 2001; Dhavan et al., 2002). We speculate that the D_4-

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induced Ca^{2+}/calmodulin binding to CaMKII leads to the decreased association of CaMKII with F-actin and increased association of CaMKII with PSD-localized binding partners, therefore facilitating the clustering of CaMKII at PSD. In agreement with this, our data show that the D_4-induced CaMKII translocation is prevented by an F-actin stabilizer, and the binding of CaMKII to F-actin is reduced by D_4 receptor activation. The lack of effect of carbachol, α-Me-5HT and A231087 on CaMKII association with F-actin provides a possible reason to explain why not all agents that can promote calmodulin binding of CaMKII will induce CaMKII redistribution. Taken together, these results suggest that the dissociation of CaMKII from F-actin is required for the D_4-induced synaptic translocation of CaMKII.

By inducing synaptic targeting of CaMKII, D_4 receptors could more effectively modulate synaptic efficacy through the specific regulation of CaMKII substrates that are enriched in PSD, such as AMPA receptors (Barria et al., 1997; Hayashi et al., 2000). Indeed, we observed a CaMKII-mediated increase of GluR1 phosphorylation after D_4 receptor activation. Interestingly, other agents that do not induce CaMKII synaptic translocation fail to increase CaMKII-dependent GluR1 phosphorylation, suggesting that synaptically translocated CaMKII is a more effective regulator of AMPA receptors. The D_4-induced potentiation of mEPSC amplitudes is consistent with the finding that CaMKII phosphorylation of GluR1 enhances the AMPA channel conductance (McGlade-McCulloh et al., 1993; Derkach et al., 1999). Therefore, D_4 receptors could achieve efficient and specific regulation of synaptic strength through modulating postsynaptic AMPA receptors by PSD-accumulated CaMKII.

This study mechanistically links together D_4 receptors and CaMKII, both of which have been implicated in cognitive and emotional processes (Oak et al., 2000; Chen et al., 1994; Hudmon and Schulman, 2002) associated with PFC. In addition to the dynamic regulation of CaMKII activity (Gu and Yan, 2004), D_4 receptors cause the synaptic translocation of CaMKII through a mechanism depending on the PLC pathway and intracellular Ca^{2+} release. This dynamic trafficking of CaMKII enables D_4 receptors to facilitate the preferential regulation of CaMKII substrates localized at postsynaptic sites, including AMPA and NMDA receptors. By focusing the range of CaMKII substrates, D_4 receptors should be able to achieve an increased “signal-to-noise” ratio in signal transduction. Since “loss of central filtering” caused

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by unbalanced excitatory and inhibitory activity has been postulated as a mechanism for the sensorimotor gating deficiencies in schizophrenia (McGhie and Chapman, 1961; Braff and Geyer 1990), the accurate regulation of CaMKII signaling provides a cellular mechanism for the functional roles of D_4 receptors in neuropsychiatric disorders.

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FOOTNOTES

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FIGURE LEGEND

Fig. 1. PD168077 induced α-CaMKII clustering along dendrites of cultured PFC neurons in a time-dependent manner. **A.** Immunocytochemical images of α-CaMKII in cultured PFC pyramidal neurons treated with or without PD168077 (20 μM) for different durations. Diffused distribution of CaMKII staining was shown in untreated neurons. CaMKII clusters were formed 2 min after PD168077 application, peaked at 5 min, and returned to basal level at 60 min. Enlarged versions of the boxed regions of dendrites are shown beneath each of the images. **B.** Quantitative analysis of α-CaMKII clusters along dendrites under different treatment. *: $p < 0.001$ *vs.* control, **: $p < 0.05$ *vs.* control, ANOVA.

Fig. 2. PD168077-induced CaMKII clusters were largely co-localized with PSD-95 in cultured PFC neurons, and autophosphorylated CaMKII (p^{T286}-CaMKII) was also translocated to synapses by PD168077 treatment. **A.** Images of cultured PFC pyramidal neurons co-immunostained with antibodies against α-CaMKII (green) and PSD-95 (red). Before staining, cultured neurons were either untreated (top) or treated with PD168077 (bottom, 20 μM, 5 min). The merged pictures showed great co-localization of CaMKII clusters and PSD-95 after PD168077 treatment. **B.** Quantitative analysis of CaMKII clusters, PSD-95 clusters and co-localized CaMKII and PSD-95 clusters along dendrites with or without PD168077 treatment. *: $p < 0.001$ *vs.* control, ANOVA. **C.** Images of cultured PFC pyramidal neurons co-immunostained with antibodies against p^{T286}-CaMKII (green) and PSD-95 (red). Similar to total CaMKII, p^{T286}-CaMKII was clustered after application of PD168077 (5 min). The clusters of p^{T286}-CaMKII were also co-localized with PSD-95. **D.** Quantitative analysis of p-CaMKII clusters and co-localized p-CaMKII and PSD-95 clusters along dendrites with or without PD168077 treatment. *: $p < 0.001$ *vs.* control, ANOVA.

Fig. 3. D_4 receptor, but not D_2 receptor, activation induced CaMKII synaptic translocation. **A.** Immunocytochemical images of α-CaMKII in cultured PFC pyramidal neurons treated with PD168077

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(20 μM), PD168077 plus the D_4 receptor antagonist L-745870 (20 μM), quinpirole (20 μM), or dopamine (50 μM) in the presence of the D_1/D_5 antagonist SCH23390 (10 μM) and D_2/D_3 antagonist sulpiride (10 μM). **B.** Quantitative analysis of CaMKII synaptic clusters along dendrites under different treatment. *: $p < 0.001$ *vs.* control, ANOVA.

Fig. 4. The PD168077-induced CaMKII synaptic translocation was depended on the PLC/IP_3R/Ca^{2+} pathway, but not PKA or PKC. **A.** Immunocytochemical images of α-CaMKII in cultured PFC pyramidal neurons pre-incubated (20 min) with various agents followed by the PD168077 treatment (20 μM, 5 min). Agents include: the PKA activator cpt-cAMP (100 μM), the PKA inhibitor PKI_{14-22} (100 nM), the PLC inhibitor U73122 (1 μM), the IP_3 receptor inhibitor 2APB (15 μM), the membrane permeable Ca^{2+} chelator BAPTA/AM (50 μM), the PKC inhibitor Bis-indolyl-maleimide (Bisl, 4 μM), or the tyrosine kinase inhibitor genistein (100 μM). **B.** Quantitative analysis of CaMKII synaptic clusters along dendrites under different treatment. *: $p < 0.001$ *vs.* control, ANOVA.

Fig. 5. Mutation of the calmodulin binding site (Ala^{302}), but not the autophosphorylation site (Thr^{286}), of CaMKII prevented the PD168077-induced CaMKII synaptic translocation. **A.** Immunocytochemical images of GFP-CaMKII in cultured PFC pyramidal neurons transfected with wild-type or site mutants of CaMKII. All three kinds of GFP-CaMKII were distributed evenly throughout the dendrites in untreated neurons, similar to the distribution pattern of endogenous CaMKII. Application of PD168077 induced a dramatic synaptic translocation of the wild-type GFP-CaMKII and the autophosphorylation-deficient GFP-CaMKII ($Thr^{286}\rightarrow$Ala), but not the calmodulin binding-deficient GFP-CaMKII ($Ala^{302}\rightarrow$Arg). **B.** Quantitative analysis of GFP-CaMKII synaptic clusters along dendrites in neurons transfected with different CaMKII constructs before (control) and after PD168077 treatment (PD). *: $p < 0.001$ *vs.* control, ANOVA.

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Fig. 6. Various agents that could activate CaMKII had different effects on CaMKII translocation. **A.** Immunoblots of phospho-CaMKII and CaMKII in PFC slices incubated with PD168077 (PD, 20 μM), glutamate (50 μM), muscarinic receptor agonist carbachol (10 μM), 5-HT_2 receptor agonist α-Me-5HT (20 μM), or the calcium ionophore A23187 (2 μM) for 10 min. Extracts of slices were immunoblotted with an anti-phospho-α-CaMKII antibody. Following stripping out signals, membranes were reblotted with an antibody recognizing the total α-CaMKII. **B.** Quantification of p-CaMKII induced by different treatment. *: $p < 0.001$ *vs.* control, ANOVA. **C.** Immunocytochemical images of α-CaMKII in cultured PFC pyramidal neurons treated for 5 min with various agents, including glutamate (50 μM), carbachol (10 μM), α-Me-5HT (20 μM), and A23187 (2 μM). Among them, only glutamate induced CaMKII translocation. **D.** Quantitative analysis of CaMKII synaptic clusters along dendrites under different treatment. *: $p < 0.001$ *vs.* control, ANOVA.

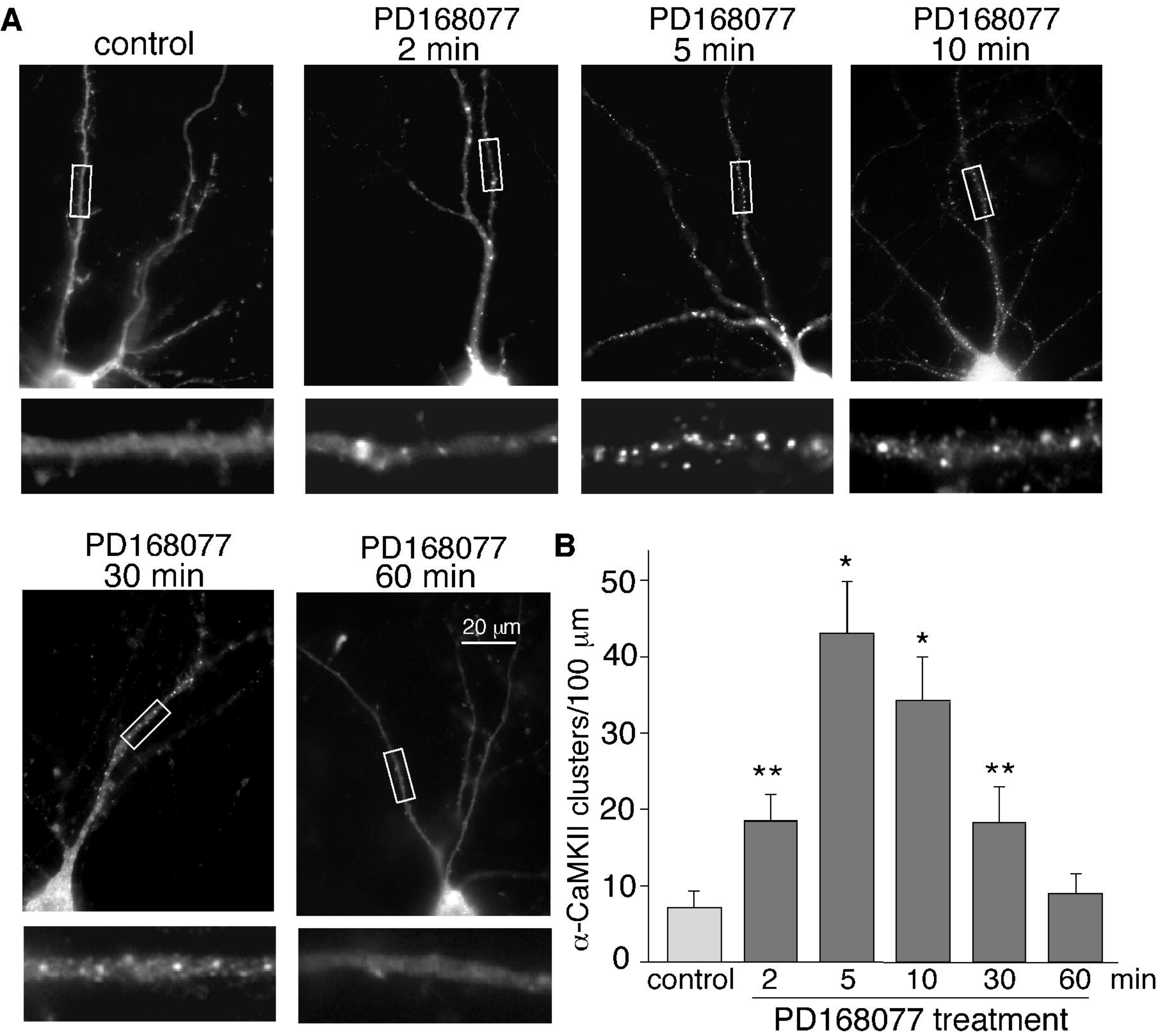
Fig. 7. The CaMKII synaptic translocation involved F-actin. **A.** Immunocytochemical images of α-CaMKII in cultured PFC pyramidal neurons pre-incubated (1 hr) with the F-actin stabilizer phalloidin-oleate (1 μM) or the microtubule stabilizer taxol (10 μM), followed by the PD168077 treatment (20 μM, 5 min). Phalloidin, but not taxol, prevented the PD168077-induced CaMKII synaptic translocation. **B.** Quantitative analysis of CaMKII synaptic clusters along dendrites under different treatment. *: $p < 0.001$ *vs.* control, ANOVA. **C.** Effect of various agents on the interaction of CaMKII with F-actin. Cell lysates from PFC slices were incubated with various agents in the absence or presence of phalloidin-oleate (1 μM), followed by immunoprecipitation with anti-actin antibody and Western blot analysis for CaMKII and actin. PD168077 and glutamate, but not cabachol (10 μM), α-Me-5HT (20 μM) or A23187 (2 μM), disrupted the binding of CaMKII to F-actin. Phalloidin blocked this effect of PD168077 and glutamate. **D.** Bar graphs showing the levels of CaMKII bound to F-actin under different treatment. *: $p < 0.001$ *vs.* control, ANOVA.

Fig. 8. PD168077 induced β-CaMKII clustering along dendrites of cultured PFC neurons. **A.** Immunocytochemical images of β-CaMKII in cultured PFC pyramidal neurons treated with or without PD168077 (20 μM, 5 min), glutamate (50 μM, 5 min) or carbachol (10 μM, 5 min). Enlarged versions of the boxed regions of dendrites are shown beneath each of the images. **B.** Quantitative analysis of β-CaMKII clusters along dendrites under different treatment. *: $p < 0.001$ *vs.* control, ANOVA.

Fig. 9. PD168077 increased CaMKII phosphorylation of GluR1 in PFC culture. **A.** Immunoblots of Ser^{831} phospho-GluR1 (top) and total GluR1 (bottom) in PFC neurons untreated (ctl) or treated with PD168077 (PD, 20 μM) or quinpirole (quin, 20 μM) in the absence or presence of various agents. Agents include: the D_4 receptor antagonist L-745870 (20 μM), the CaMKII inhibitor KN-62 (10 μM), and the PKC inhibitor Bis-indolyl-maleimide (Bisl, 4 μM). **C.** Immunoblots of Ser^{831} phospho-GluR1 in PFC neurons treated with various agents in the absence or presence of KN-62 or Bisl. Agents used: A23187 (2 μM), glutamate (Glu, 50 μM), carbachol (10 μM), and α-Me-5HT (20 μM). **B, D.** Bar graphs showing the fold increase of GluR1 phosphorylation at Ser^{831} under different treatment. *: $p < 0.001$ *vs.* ctl, **: $p < 0.05$ *vs.* ctl, #: $p < 0.05$ *vs.* Glu alone, ANOVA.

Fig. 10. PD168077 increased the mEPSC amplitude in PFC cultures. **A, C.** Representative mEPSC traces obtained from cultured PFC pyramidal neurons treated with PD168077 (PD, 40 μM) in the absence or presence of KN-93 (10 μM). Scale bars: 25 pA, 1 sec. **B, D.** Cumulative plots of the distribution of mIPSC amplitude before and during PD168077 treatment in the absence or presence of KN-93.

Fig. 1


A
control
PD168077
2 min
PD168077
5 min
PD168077
10 min
PD168077
30 min
PD168077
60 min
20 μm
B
α-CaMKII clusters/100 μm
0
10
20
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40
50
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*
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control
2
5
10
30
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min
PD168077 treatment

Fig. 2

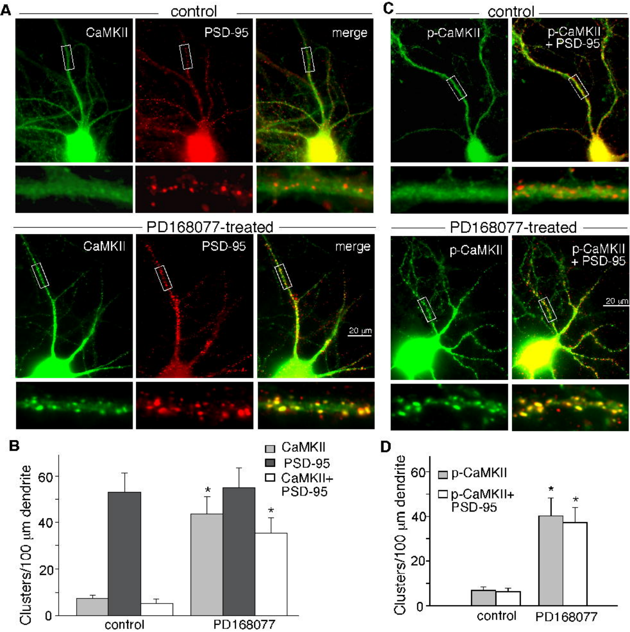
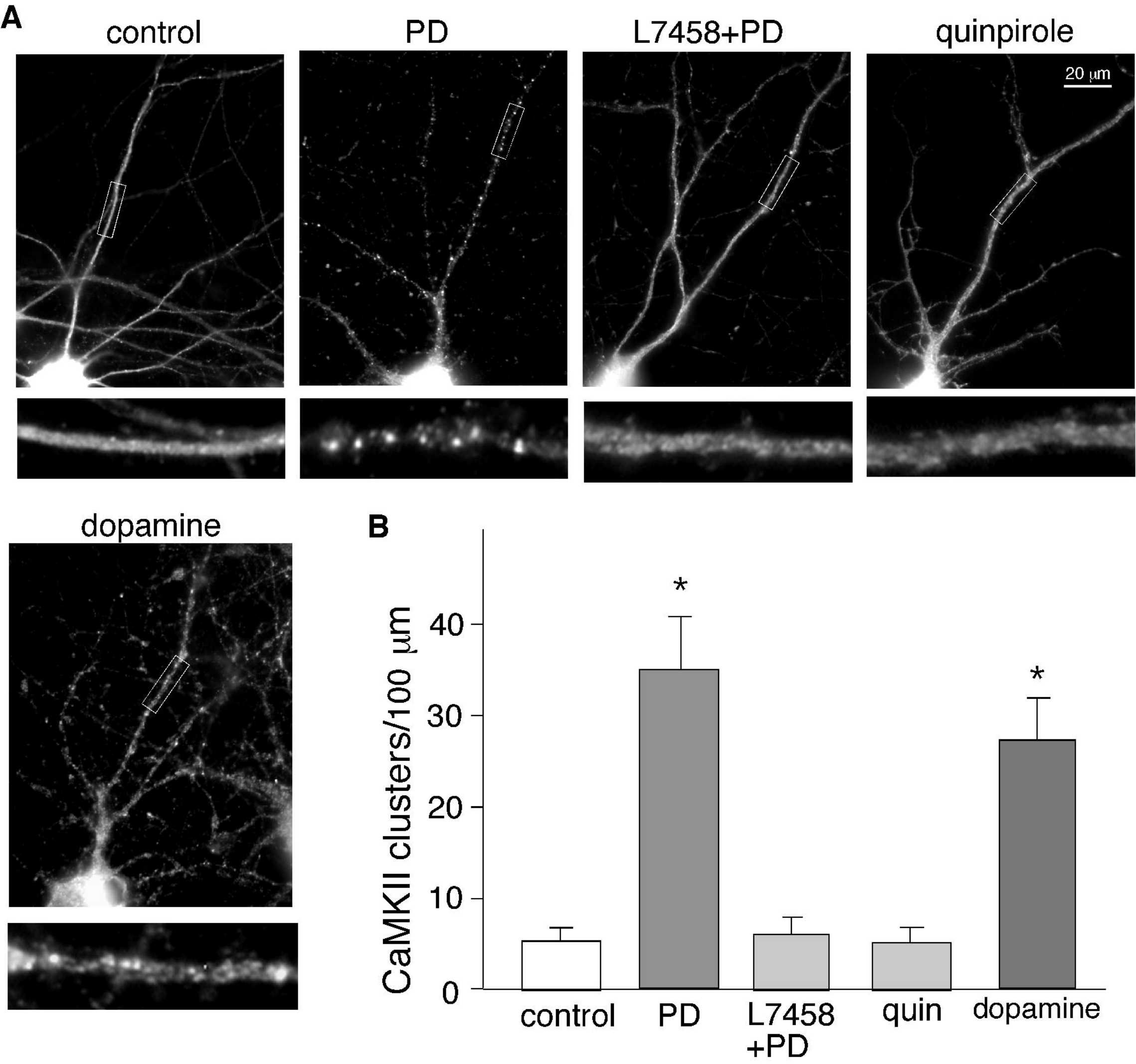

A
control
CaMKII
PSD-95
merge
PD168077-treated
CaMKII
PSD-95
merge
20 µm
C
control
p-CaMKII
p-CaMKII
+ PSD-95
PD168077-treated
p-CaMKII
p-CaMKII
+ PSD-95
20 µm
B
CaMKII
PSD-95
CaMKII+
PSD-95
Clusters/100 µm dendrite
0
20
40
60
*
*
control
PD168077
D
Clusters/100 µm dendrite
p-CaMKII
p-CaMKII+
PSD-95
0
20
40
60
*
*
control
PD168077

Fig. 3



A
control
PD
L7458+PD
quinpirole
20 μm
dopamine
B
CaMKII clusters/100 μm
0
10
20
30
40
*
*
control
PD
L7458
+PD
quin
dopamine

Fig. 4

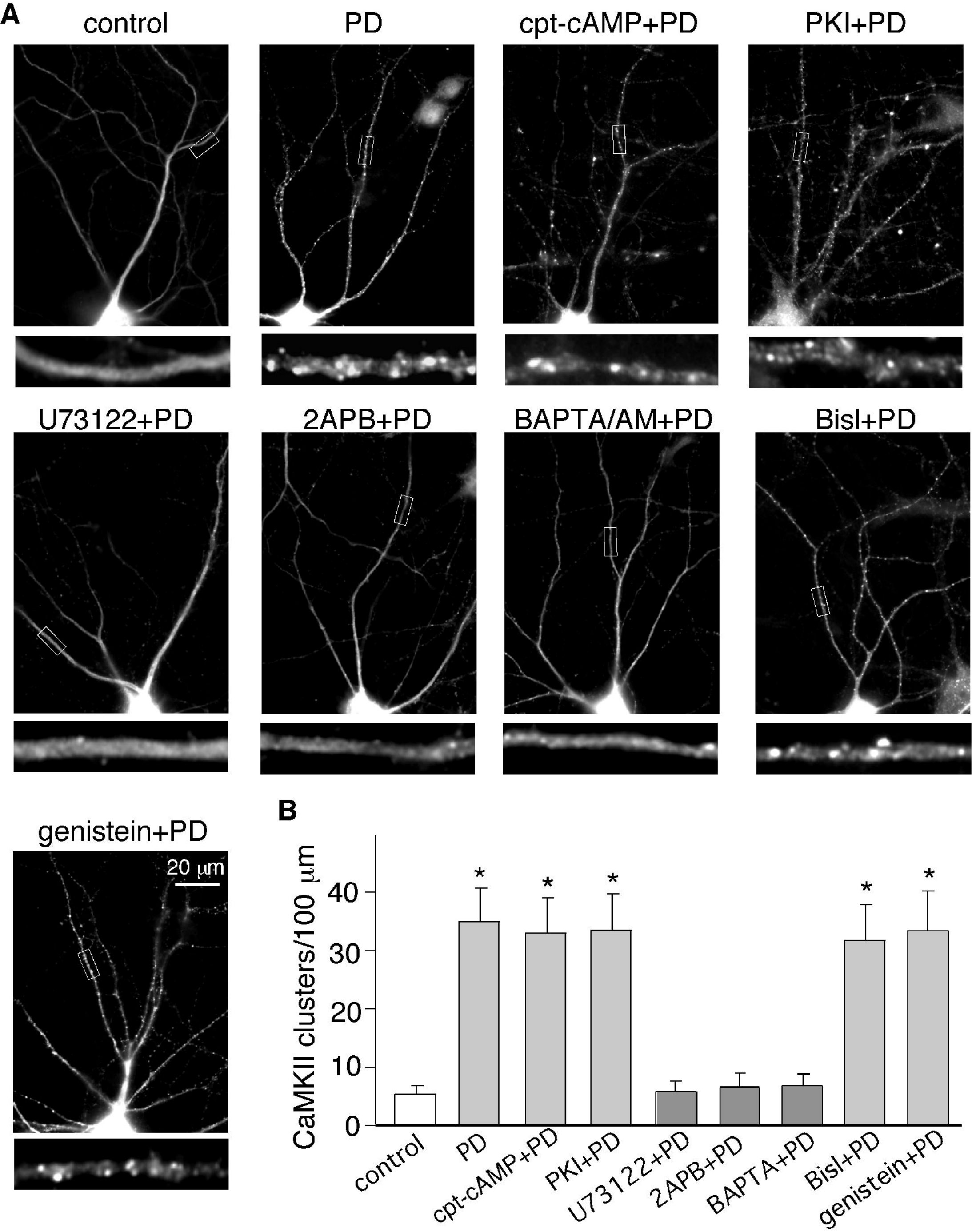

A
control
PD
cpt-cAMP+PD
PKI+PD
U73122+PD
2APB+PD
BAPTA/AM+PD
Bisl+PD
genistein+PD
20 μm
B
CaMKII clusters/100 μm
0
10
20
30
40
*
control
PD
cpt-cAMP+PD
PKI+PD
U73122+PD
2APB+PD
BAPTA+PD
Bisl+PD
genistein+PD

Fig. 5

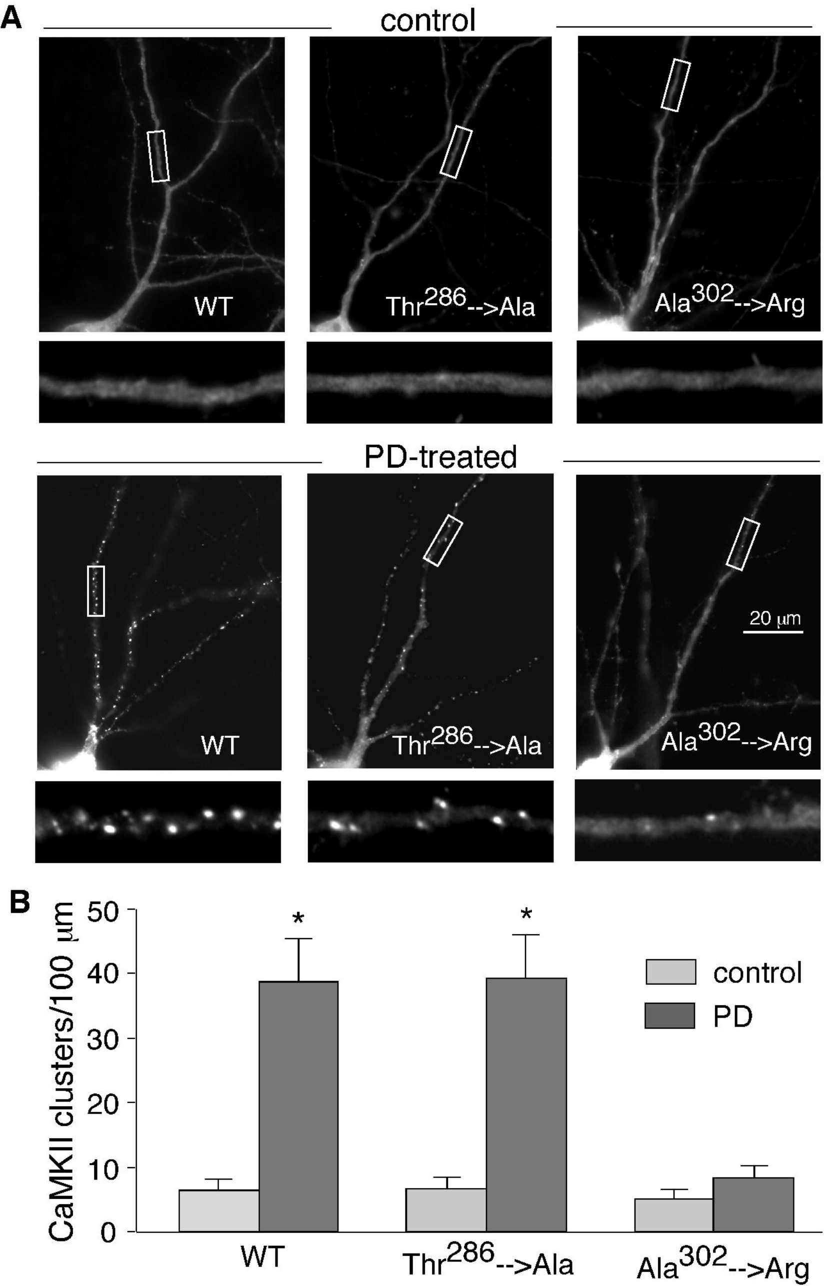

A
control
WT
Thr286-->Ala
Ala302-->Arg
PD-treated
WT
Thr286-->Ala
Ala302-->Arg
20 μm
B
CaMKII clusters/100 μm
0
10
20
30
40
50
*
*
control
PD
WT
Thr286-->Ala
Ala302-->Arg

Fig. 6

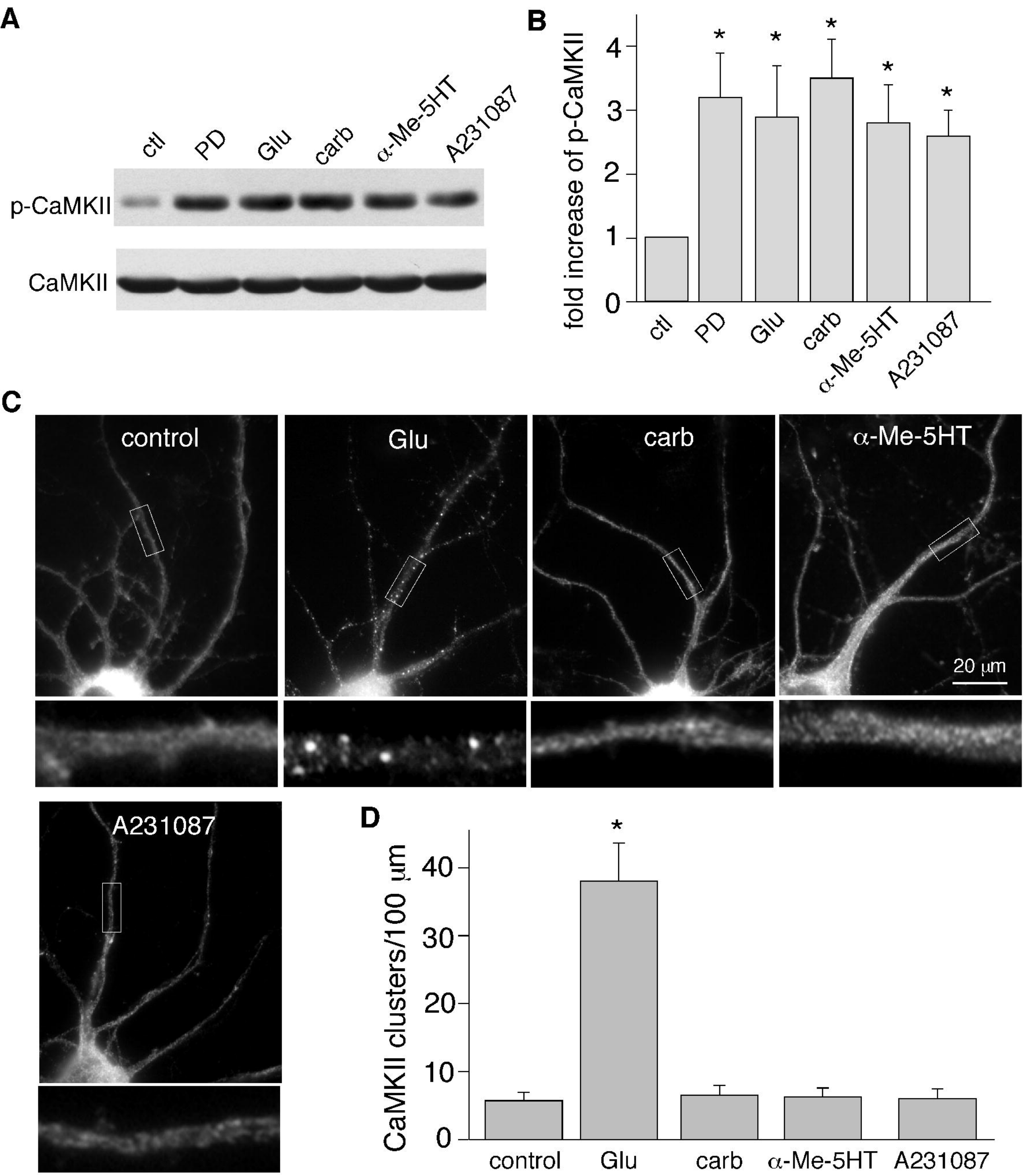
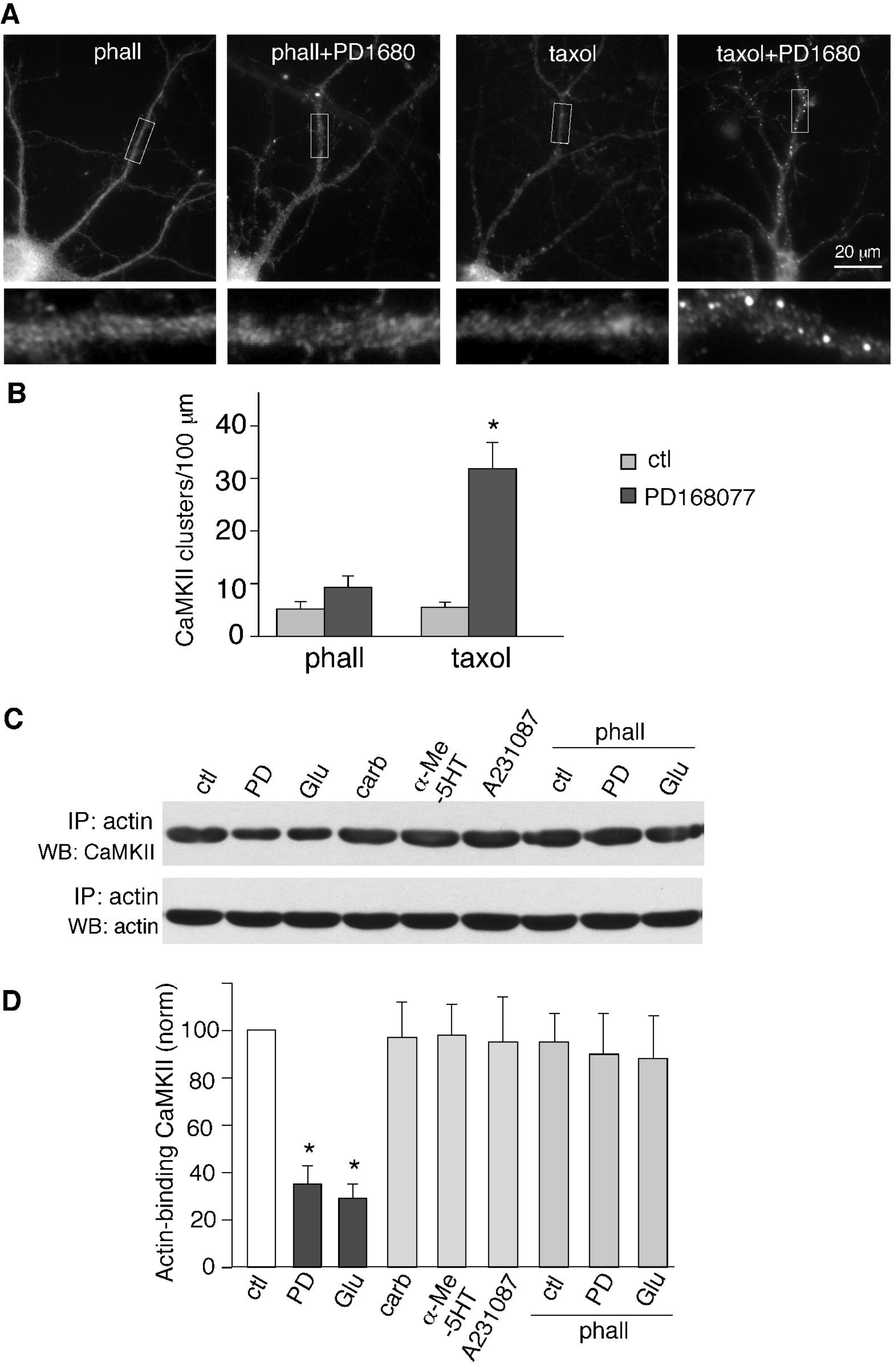

A
ctl
PD
Glu
carb
α-Me-5HT
A231087
p-CaMKII
CaMKII
B
fold increase of p-CaMKII
0
1
2
3
4
*
ctl
PD
Glu
carb
α-Me-5HT
A231087
C
control
Glu
carb
α-Me-5HT
20 μm
A231087
D
CaMKII clusters/100 μm
0
10
20
30
40
*
control
Glu
carb
α-Me-5HT
A231087

Fig. 7



A
phall
phall+PD1680
taxol
taxol+PD1680
20 μm
B
CaMKII clusters/100 μm
40
30
20
10
0
*
ctl
PD168077
phall
taxol
C
ctl
PD
Glu
carb
α-Me -5HT
A231087
phall
ctl
PD
Glu
IP: actin
WB: CaMKII
IP: actin
WB: actin
D
Actin-binding CaMKII (norm)
100
80
60
40
20
0
*
*
ctl
PD
Glu
carb
α-Me -5HT
A231087
ctl
PD
Glu
phall

Fig. 8

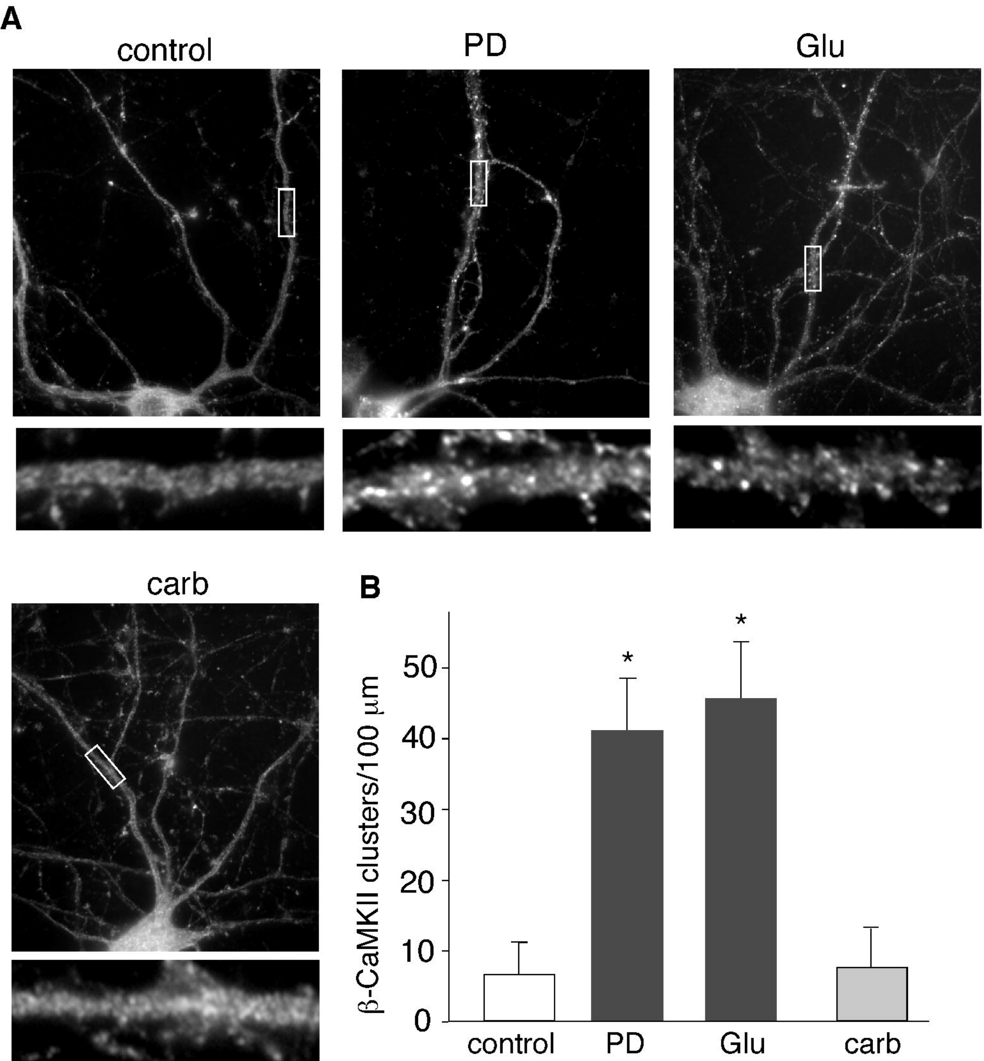
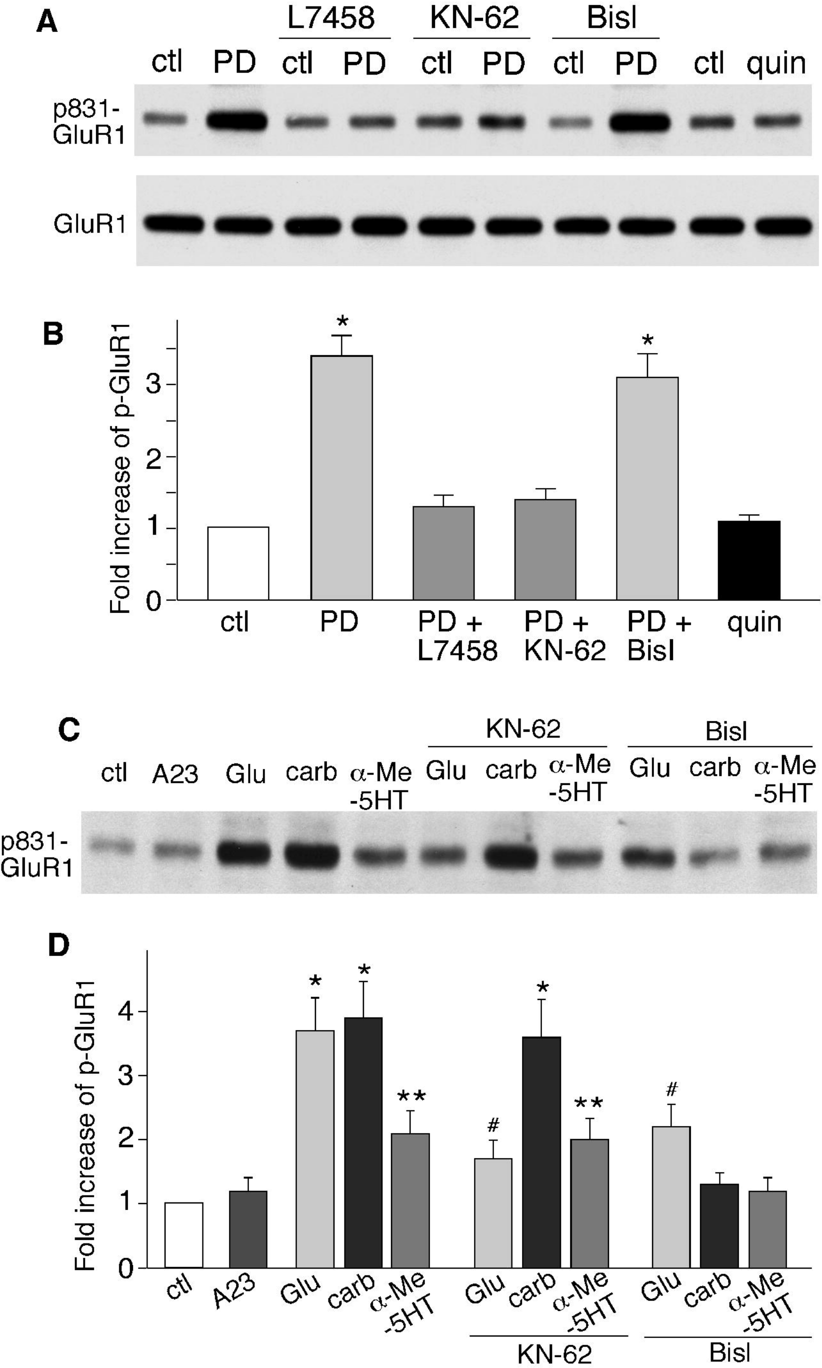
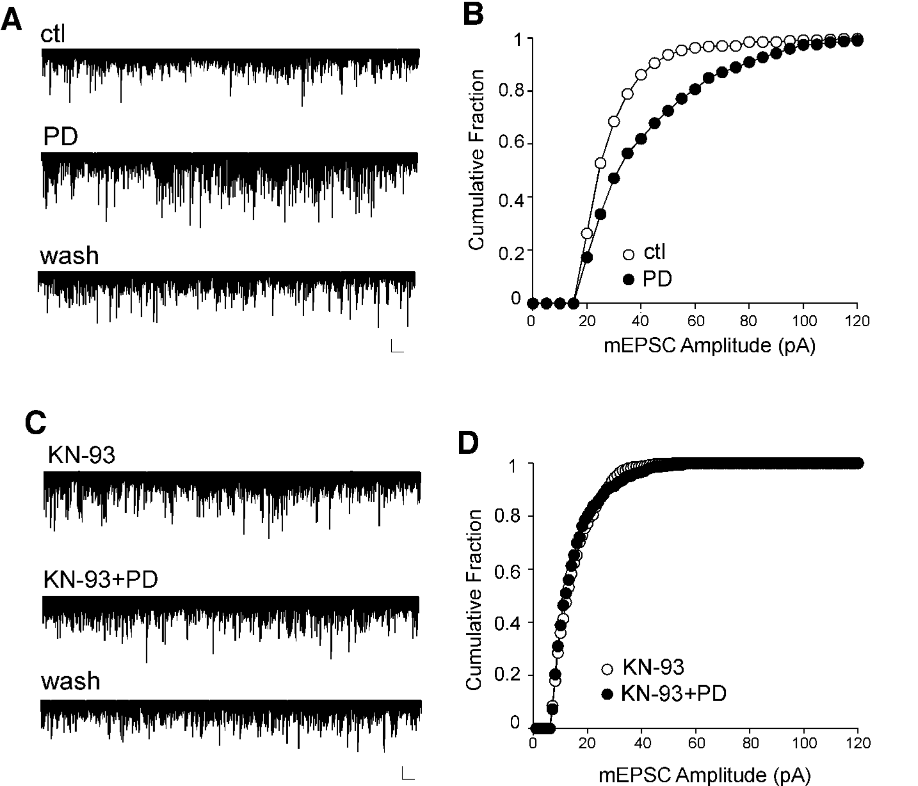

A
control
PD
Glu
carb
B
β-CaMKII clusters/100 μm
0
10
20
30
40
50
*
*
control
PD
Glu
carb

Fig. 9



A
L7458
KN-62
BisI
ctl
PD
ctl
PD
ctl
PD
ctl
PD
ctl
quin
p831-GluR1
GluR1
B
Fold increase of p-GluR1
0
1
2
3
*
*
ctl
PD
PD + L7458
PD + KN-62
PD + BisI
quin
C
KN-62
BisI
ctl
A23
Glu
carb
α-Me -5HT
Glu
carb
α-Me -5HT
Glu
carb
α-Me -5HT
p831-GluR1
D
Fold increase of p-GluR1
0
1
2
3
4
*
*
**
#
*
**
#
ctl
A23
Glu
carb
α-Me -5HT
Glu
carb
α-Me -5HT
Glu
carb
α-Me -5HT
KN-62
BisI

Fig. 10


A
ctl
PD
wash
B
Cumulative Fraction
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
mEPSC Amplitude (pA)
ctl
PD
C
KN-93
KN-93+PD
wash
D
Cumulative Fraction
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
mEPSC Amplitude (pA)
KN-93
KN-93+PD