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PHOSPHORYLATION OF MICROTUBULE-ASSOCIATED PROTEIN STOP BY CALMODULIN KINASE II*

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Running title: Phosphorylation of STOP by CaMKII

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STOP proteins are microtubule-associated, calmodulin-regulated proteins responsible for the high degree of stabilization displayed by neuronal microtubules. STOP suppression in mice induces synaptic defects affecting both short- and long-term synaptic plasticity, in hippocampal neurons. Interestingly, STOP has been identified as a component of synaptic structures in neurons, despite absence of microtubules in nerve terminals, indicating the existence of mechanisms able to induce a translocation of STOP from microtubules to synaptic compartments. Here we have tested STOP phosphorylation as a candidate mechanism for STOP relocalization. We show that, both *in vitro* and *in vivo*, STOP is phosphorylated by the multifunctional enzyme calcium/calmodulin-dependent protein kinase II (CaMKII), which is a key enzyme for synaptic plasticity. This phosphorylation occurs on at least two independent sites. Phosphorylated forms of STOP do not bind microtubules *in vitro* and do not co-localize with microtubules in cultured differentiating neurons. Instead, phosphorylated STOP co-localizes with actin assemblies along neurites or at

branching points. Correlatively, we find that STOP binds to actin *in vitro*. Finally, in differentiated neurons, phosphorylated STOP co-localizes with clusters of synaptic proteins, whereas unphosphorylated STOP does not. Thus, STOP phosphorylation by CaMKII may promote STOP translocation from microtubules to synaptic compartments where it may interact with actin, which could be important for STOP function in synaptic plasticity.

Microtubules are vital components of the cell cytoskeleton, being involved in cell mitosis, intracellular organization and cell morphogenesis. Neurons contain abundant subpopulations of stable microtubules that resist depolymerising conditions such as exposure to cold temperature and to depolymerizing drugs (1-3). In neurons, microtubule stabilization is mainly due to association with a family of proteins known as STOPs (Stable Tubule Only Polypeptides). STOP proteins were initially characterized as microtubule cold-stabilizing factors whose activity was inhibited by interaction with Ca²⁺-calmodulin (4), and subsequent work has shown that STOPs contain bi-functional

modules comprised of overlapping calmodulin-binding and microtubule-stabilizing sequences (5). Neurons contain two major variants of STOP, E-STOP (89 kD) and N-STOP (116 kD). E-STOP is present in mice brain from embryonic stage E16 to adulthood, whereas N-STOP appears at birth and is subsequently expressed in the adult brain (2,6). STOP function has been investigated in the whole animal by studying STOP null mice (7). In these mice, microtubule cold stability is suppressed, with no dramatic consequences for mouse organogenesis, viability or brain anatomy. However, STOP $-/-$ mice display multiple synaptic deficits that affect both long- and short-term synaptic plasticity in the hippocampus. These synaptic defects are associated with depleted vesicular pools in glutamatergic nerve terminals and with severe behavioral disorders (8), which, interestingly, are specifically alleviated with long-term neuroleptic treatment. It has been very surprising that a protein associated with microtubules along whole neurites turns out to be important for glutamatergic synaptic function, despite apparent microtubule absence in nerve terminals. However, from proteomic analysis of synaptosomal fractions, there is evidence that STOP localizes to synapses (8,9) and this raises questions concerning the mechanisms that could promote STOP dissociation from microtubules and re-localization in synaptic structures. Previous evidence has indicated that STOP can be inactivated by calmodulin-dependent phosphorylation (10), correlated with the presence of putative CaMKII phosphorylation sites in the STOP sequence (6). Interestingly, long-term potentiation (LTP), which is known to involve CaMKII, is severely impaired in STOP null mice (7). Here, we have tested both the occurrence and the consequences of STOP phosphorylation. We show that CaMKII phosphorylates STOP on at least two independent sites (S198, S491), both *in vitro* and *in vivo*. Consequently, phosphorylated STOPS do not bind to microtubules *in vitro*. Additionally, the phosphorylated forms of STOP co-localize with actin-rich structures in cultured neurons and bind to polymerized actin *in vitro*. Finally, phosphorylated STOP protein associates with synaptic markers following synaptogenesis, in cultured neurons. Thus, phosphorylation by CaMKII may delocalize STOPS from microtubules and allow STOP

association with synaptic actin, which may be important for synaptic plasticity.

EXPERIMENTAL PROCEDURES

Purification of STOP and CaMKII from mouse brain (CE fraction) - Purification of STOP protein from adult mice brain was performed as described by Pirolet *et al.* (11). All buffers used during STOP purification contained 2 mM DTT and protease inhibitors (1 mM PMSF, 10^4 units/ml aprotinin; 20 μ M leupeptin). Briefly, 40 brains from OF1 mice were homogenized in MEM buffer (100 mM Mes, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.75) and centrifuged at 100,000 g for 40 min at 4° C. The supernatant was loaded on a Sepharose-S Fast Flow column and bound proteins were eluted in MEM containing 0.4 M NaCl. The eluate was adjusted to 2 mM calcium and passed through a calmodulin-agarose column equilibrated in MEM buffer containing 2 mM $CaCl_2$ / 0.4 M NaCl. Bound proteins were eluted in MEM buffer containing 0.4 M NaCl and 1 mM EGTA. The purified fraction (calcium eluate, CE) was stored in elution buffer containing 10% glycerol, at -80° C.

Phosphorylation reaction - CE fraction containing STOPS was buffer-exchanged to 50 mM Pipes, 10 mM $MgCl_2$, pH 7.0. The phosphorylation reaction was performed in the presence of 100 μ M ATP (added of 2 μ Ci γ - ^{32}P -ATP when indicated), 0.7 mM $CaCl_2$, 0.3 μ M calmodulin, 0.4 mM EGTA, 0.5 μ M mycrocystine, 1 μ M okadaic acid and 5 mM 2-mercapto-ethanol, during 30 min at 30° C. For quantitative experiments, gels were dried before exposure on a screen for analysis with a phosphorimager (Molecular Imager[®] FX, Biorad). Relative amounts of incorporated radioactivity were quantified using Quantity One[®] software (Biorad). For control experiments, the buffer-exchanged CE fraction was incubated with alkaline phosphatase for 30 min at 37° C, resulting in de-phosphorylation of STOPS. For experiments using pre-activated CaMKII, CE fraction was incubated with 30 μ M calmodulin and 0.7 mM calcium for 10 minutes at 30° C. Phosphorylation reaction was then performed in the presence or in the absence of 2.5 mM EGTA at 30° C for 30 minutes.

Phospho-STOP antibodies and primary antibodies - Rabbit polyclonal antibodies were

raised against phospho-synthetic peptides corresponding to the potential phosphorylated sites in N-STOP protein, as shown in figure 1A. The phospho peptides P1 to P4 used for the immunisation were the following: RPRpSEYQPSDAPFC (aa 136-148); RPQpSQERGPMQLC (aa 195-206); RAWpTDIKPVKPIKC (aa 435-447); RRIRpSLYSEPFKEC (aa 487-499), according to aa numbering of mouse N-STOP (GenBank Accession number CAA75930). These peptides were used to generate sera P1, P2, P3 and P4 respectively (Eurogentec). The C-terminal cystein residue of each peptide was introduced for the purification steps. Crude sera were affinity-purified against the phosphorylated peptide on Sulfolink coupling gel (Pierce). Antibodies were eluted with 100 mM glycine, pH 2.5, neutralised with Tris 1 M, pH 8.0 and then incubated for 1 h with the corresponding non-phosphorylated peptide coupled on Sulfolink gel in order to absorb antibodies reacting with non-phosphorylated peptides. Polyclonal antibodies reacting only with phosphorylated peptides were collected in the flow-through and stored at -80° C in 50% glycerol. Affinity-purified P2 and P4 polyclonal antibodies did not show detectable cross reactivity in enzyme-linked immunoabsorbent assay (ELISA) tests (not shown). The same phospho-peptides P1-P4 were used to generate monoclonal antibodies following standard procedure. Hybridoma culture supernatants were screened for anti STOP phospho-peptides using ELISA assay. The positive hybridomas were selected and screened on western blot for immunoreactivity on phosphorylated and non-phosphorylated STOP proteins. Positives hybridomas were sub-cloned by limiting dilution.

The following primary antibodies were used in this study : mAb against α -tubulin (alpha3a clone (12), ascitic fluid, dilution 1/1,000 for IF); mAb 175 against C-terminal part of N-STOP (6) (dilution 20 μ g/ml for IF and 1 μ g/ml for WB); affinity-purified phospho-STOP P2 and P4 antibodies (dilution 20 μ g/ml for IF and 1 μ g/ml for WB); P1 mAb against phospho-STOP (hybridoma culture supernatant, dilution 1/10 for WB); mAb against CaMKII (Ref C89220, Transduction laboratories), Homer (all isoforms, Chemicon), synapsin1 (BD Biosciences), CaMKI and CaMKIV (Santa Cruz Biotech.) were used following manufacturer's advice.

Sedimentation of STOPs with microtubules or actin - All proteins were pre-clarified at 150,000 g for 15 minutes in a TL-100 ultracentrifuge (Beckman) at 4° C prior start experiments. STOP proteins of the CE fraction were either phosphorylated by CaMKII or dephosphorylated with alkaline phosphatase treatment as described above. Microtubule-binding assay was performed as in Masson and Kreis (13) using taxol-stabilized microtubules (4 μ M) as substrates. Actin-binding assay was performed with CE fraction containing 0.5 μ M phospho or de-phospho STOP (as estimated on Coomassie blue gels, using known amounts of pure STOP as standards) complemented or not with various amount of G-actin (1 μ M, 6 μ M and 15 μ M), in 100 μ l of a 1x F-polymerization buffer (10X stock: 50 mM Tris-HCl, 5 mM DTT, 5 mM ATP, 1 M KCl, 50 mM MgCl₂, pH 7.5) in the presence of 1 mM EGTA. After 90 min of incubation at 22° C, samples were centrifuged at 150,000 g for 15 minutes at 4° C. Supernatants were removed and kept; the pellets were washed twice with polymerization buffer and dissolved in 100 μ l of H₂O. Laemmli buffer was added to the pellet solutions and to the supernatants. Equal amounts of each sample were separated by 7.5% SDS PAGE and either stained with Commassie blue or transferred on nitrocellulose and blotted with mAb 175 or CaMKII antibodies.

Cell culture - Hippocampal cell cultures were prepared according to Dotti *et al.* (14). Briefly, mouse hippocampi (E18.5) were removed and digested in 0.25% trypsin in HBSS (Invitrogen) at 37° C for 15 min. After manual dissociation, cells were plated at a concentration of 5,000–15,000 cells/cm² on poly-L-lysine (Sigma) coated coverslips in DMEM-10% FBS (Invitrogen). One hour after plating, the medium was changed to DMEM containing B27 and N2 supplement (Invitrogen). Inhibitor of CaMKII, KN62 (5 μ M) was applied to neuronal culture for 8 h prior to cell fixation when indicated. In some experiments, neuronal cultures were subjected to cytochalasin treatment (50 μ M, for 10 min). In some experiments, neurons in culture were activated by exposure to glutamate treatment (glutamate 100 μ M, glycine 10 μ M) for 15 min prior to fixation.

Immunofluorescence - Neurons grown on coverslips for 12 to 30 days were fixed 20 min with warmed solution of 4%

paraformaldehyde, 0.12 M sucrose, in PBS, pH 7.4 and then permeabilized for 3 min using 0.2% Triton X100, in PBS, pH 7.4. Cells were processed for immunofluorescence by incubation with primary antibodies for 1 h in PBS, 0.2 % Tween 20 and then with secondary antibodies (Cy3- or Alexa488-coupled from Molecular probes and Jackson Immuno-research, respectively) for 40 min. To visualize F-actin, rhodamine-phalloidin (Molecular Probes) was included with the secondary antibodies. The cells were analyzed with an inverted microscope. Images were digitalized using a Princeton RTE-CCD-1317-K/1 camera (Princeton Instruments) and IPLab Spectrum software (Signal Analytics).

RESULTS

CaMKII Phosphorylation sites in N-STOP - The consensus aa sequence for CaMKII phosphorylation is K/R X X S/T, where either S or T residues are phosphorylated (15). A search for such sequences in murine N-STOP protein sequence (Genbank Accession number CAA75930) revealed four candidate sequences (P1 to P4, Figure 1A) containing serine or threonine residues S139, S198, T438 and S491, respectively. N-STOP contains two classes of microtubule-stabilizing modules, Mc and Mn and multiple calmodulin-binding sites (5). The locations of P1-P4 sites relative to these STOP functional domains (5) is shown in Figure 1A. All four P1-P4 sequences are located within or are overlapping a calmodulin-binding site on N-STOP. P1 and P3 are located into or are overlapping with microtubule-stabilizing modules Mn1 and Mn3, respectively.

STOP phosphorylation by CaMKII in vitro - We used semi-purified fractions to assay STOP protein phosphorylation *in vitro*. These protein fractions (CE fractions) were purified from mouse brain extracts by ion exchange chromatography followed by calmodulin affinity chromatography. The procedure resulted in the isolation of several calmodulin-binding proteins as shown on SDS gels in figure 1B. CE fractions contained one major band at 116 KD and one other prominent band at 52 KD band. According to immunoblots of CE fraction, with anti N-STOP antibody (Figure 1C) and with anti CaMKII antibody (Figure 1D) the 116 KD band corresponded to N-STOP, and the 52 KD and 58 KD bands to

subunits of CaMKII. Thus, CE fractions contained both N-STOP and CaMKII. CE fractions were also immuno-blotted with anti CaMKI and CaMKIV antibodies with negative results (not shown).

Incubation of CE fraction proteins with γ -³²P-ATP in the absence of Ca²⁺-calmodulin followed by SDS PAGE analysis and autoradiography showed little phosphate incorporation in the bands corresponding to N-STOP or to CaMKII (Figure 1E, left column). Phosphate incorporation was dramatically enhanced in the presence of Ca²⁺-calmodulin with an apparent shift of phosphorylated bands, which migrated at positions corresponding to higher apparent molecular weights (Figure 1E, right column). Band shift of both N-STOP and CaMKII following phosphorylation was confirmed on immunoblots (Figure 1F). The whole N-STOP band was shifted, showing that all STOP molecules had been phosphorylated during the phosphorylation reaction.

In a time course analysis of STOP phosphorylation (Figures 1 G and H), STOP phosphorylation reached a plateau within 10 minutes. After one minute of incubation, a small shift of N-STOP was detectable (Figure 1H), showing that STOP molecules had already incorporated at least one molecule of phosphate at this time point. According to quantitative autoradiography, the ratio of phosphate incorporation in N-STOP at the plateau versus phosphate incorporation at 1 minute was circa 3.7. Assuming that the first shift corresponds to the incorporation of a single phosphate group in STOP molecules, this ratio may reflect the phosphorylation of the four CaMKII phosphorylation consensus sites at the plateau.

We used purified N-STOP and pure CaMKII to test whether N-STOP was truly a substrate for CaMKII. We observed both N-STOP phosphorylation and shift following STOP phosphorylation with CaMKII (Supplementary figure S1). However, pure STOP is hard to prepare in biochemical amounts and cannot be stored, hence all subsequent experiments were run using CE fractions.

High concentrations of Ca²⁺/calmodulin inhibit STOP phosphorylation by CaMKII - The overlap of all P1-P4 putative STOP phosphorylation sites with calmodulin-binding sites on N-STOP (Figure 1A) raised the possibility of an inhibition of STOP

phosphorylation through steric hindrance, in the presence of an excess of Ca^{2+} -calmodulin complex. We examined STOP phosphorylation in CE extracts in the presence of varying amounts of calmodulin ranging from 0.003 to 30 μM . Full activation of CaMKII occurred for calmodulin concentrations above 0.03 μM as indicated by the band shift of CaMKII specific (Figure 2A). STOP phosphorylation was also observed above 0.03 μM calmodulin as indicated by the shift of N-STOP molecular weight on western blots (Figure 2A). However, at calmodulin concentrations above 0.3 μM , there was a small but reproducible decrease in N-STOP shift, indicating inhibition of N-STOP phosphorylation by calmodulin in the micromolar range.

When CaMKII is pre-activated through autophosphorylation in the presence of Ca^{2+} -calmodulin, the enzyme remains activated following Ca^{2+} removal, as long as dephosphorylation does not occur. We made use of this persistent CaMKII activation for further test the inhibition of N-STOP phosphorylation by Ca^{2+} -calmodulin complexes. Pre-activated CaMKII was added to CE fractions, in the presence of 30 μM Ca^{2+} -calmodulin, and in the presence or in the absence of EGTA (Figure 2B) in order to inactivate or not the Ca^{2+} -calmodulin complexes. The N-STOP band shift was markedly reduced in the absence of EGTA (active Ca^{2+} -calmodulin complex) compared to the band shift observed in the presence of EGTA (no active Ca^{2+} -calmodulin complex). Altogether these results show that CaMKII-mediated STOP phosphorylation is inhibited by Ca^{2+} -calmodulin binding to N-STOP, compatible with an overlap of P1-P4 with calmodulin-binding sites, on the N-STOP sequence.

Phosphorylated N-STOP does not bind to microtubules in vitro - We next investigated whether the phosphorylation of STOP by CaMKII could affect STOP binding to microtubules, as phosphorylation often regulates the binding on microtubules of associated proteins (16,17). CE fractions, with or without prior phosphorylation, were mixed with taxol-stabilized microtubules. Microtubules were then pelleted and the STOP content of both supernatant and pellet was analyzed on immunoblots. In the absence of phosphorylation, STOP co-sedimented with

microtubules, being present in microtubule pellets and absent in supernatants (Figure 3, lanes 1-2). In contrast, phosphorylated STOP was absent in microtubule pellets, being only present in the supernatants (Figure 3, lanes 5-6). Thus, phosphorylated STOP does not bind to microtubules *in vitro* indicating a strong regulation by CaMKII of the STOP binding to microtubule *in vivo*.

Phosphorylated STOP antibodies - We designed phospho-peptides corresponding to the four potential CaMKII phosphorylation sequences on STOP (P1 to P4, figure 1A) and used them to produce polyclonal or monoclonal antibodies specific to phosphorylated forms of STOP. On western blots of CE fractions, P2 and P4 polyclonal antibodies reacted with N-STOP (Figure 4). The signal was strongly enhanced when a phosphorylation step was introduced prior to immunoblotting and was erased when CE fractions were exposed to alkaline phosphatase (Figure 4). Additionally we obtained a monoclonal antibody P1, which reacted only very weakly with N-STOP in CE fractions whereas a strong signal was observed when a phosphorylation step was introduced prior to immunoblotting (Figure 4). These results indicate that at least three out of the four P1-P4 peptides are phosphorylated by CaMKII *in vitro*, at S139, S198 and S491 positions. Additionally, the immuno-reactivity of P2 and P4 antibodies on native CE fractions indicates the presence of phosphorylated STOP protein in adult brain extract. This strongly suggests that STOP is phosphorylated at least on S198 and S491 residues *in vivo*.

Phosphorylated STOP co-localizes with actin-rich structures in differentiating neurons - We used one of the polyclonal phospho-STOP antibody, P2 antibody and N-STOP monoclonal antibody to localize phosphorylated STOP and total N-STOP in neuronal cells.

Specificity of P2 antibody for STOP labeling in neuronal culture was assessed by staining wild type and STOP deficient neurons (Supplementary figure S2). Hippocampal neurons from E18.5 embryos were cultured for 12 days *in vitro*, a stage of differentiation at which N-STOP is expressed. At this stage of differentiation neurons are forming a complex network comprising neurite extensions and branching points (18) where individual microtubules are distinct. Total N-STOP

labeling showed co-localization of STOP and tubulin staining in neurites (Figure 5A, upper left panels) whereas phosphorylated STOP staining using P2 antibody showed a diffuse staining, not co-localizing with tubulin staining and concentrated in spike-like structures appended to neurites (Figure 5A, bottom left panels). At branching points, N-STOP staining again co-localized with microtubules (Figure 5A, upper right panels). In contrast, P2 labeling was concentrated in a ring-shaped zone, underneath the cell membrane, not co-localized with microtubules (Figure 5A, bottom right panels). Since neuronal actin concentrates in spikes along neurites and in sub-membrane domains at branching points (18), we investigated the possibility of a co-localisation of phosphorylated STOP with actin. P2 antibody staining co-localized with actin staining in spikes (Figure 5B, bottom left panels). At branching points, P2 stained the internal border of the actin rich extensions (Figure 5B, bottom right panels). Interestingly, cytochalasin treatment, which results in a disorganization of the actin network, also induced a loss of P2 staining indicating an association of phosphorylated STOP with F-actin assemblies (Figure 5C). When neurons were activated by a 15 min exposure to glutamate prior to fixation, the P2 staining of spikes increased two fold, compatible with CaMKII activation and enhanced STOP phosphorylation (Figure 5D). CaMKII phosphorylation sites can be substrate for the cyclic AMP regulated protein kinases PKA, which is also present in synapses and is involved in synaptic plasticity (19). PKA is insensitive to the general CaMkinases inhibitor KN62. In neuronal cells, exposure to KN62 suppresses the P2 staining, indicating inhibition of STOP phosphorylation (Figure 5E). This result argues against a significant contribution of PKA in STOP phosphorylation, *in vivo*.

We then tested whether STOP could interact directly with polymeric actin *in vitro*, using standard sedimentation assays. CE STOP fractions, either phosphorylated or treated with alkaline phosphatase, were mixed with actin under polymerizing conditions. Then, polymerized actin was pelleted and STOP content was analyzed on immunoblots, in both supernatants and pellets (Figure 6). The concentrations of F-actin at which

approximately half of STOP co-sedimented with actin were of circa 1 μ M and 6 μ M for un-phosphorylated and phosphorylated STOP, respectively (Figure 6B-C). In the same sedimentation assays, CaMKII co-sedimented with F-actin when non-phosphorylated, whereas phosphorylated CaMKII remained in the supernatants, as expected from previous study (20). Affinity purified STOP fractions, devoid of detectable amounts of CaMKII also bound to F-actin (Figure 6B), indicating that actin binding is a genuine property of STOP. These results indicate that STOP and phosphorylated STOP both interact directly with polymerized actin *in vitro*, although with different Kds.

Phosphorylated forms of STOP co-localize with clusters of synaptic proteins in differentiated neurons - Hippocampal neurons were cultured for 45 days *in vitro*, a stage of differentiation at which neurons are forming a complex network and are connected by synapses. In such cultures, synaptic proteins form clusters. Clusters corresponding to different synaptic proteins are both present in transport vesicles and at synapses. In differentiated neurons, the presynaptic vesicle-associated protein synapsin and the post-synaptic PSD-associated protein homer, showed a typical cluster distribution (Figure 7). As these markers, phosphorylated STOP (STOP-P) was distributed in clusters and phosphorylated STOP staining partially overlapped with synapsin or homer stainings (Figure 7, arrows). This overlap was never observed with N-STOP. Hence, these results are compatible with a presence of phosphorylated STOP in both the pre- and post-synaptic compartments.

DISCUSSION

STOP proteins are important for synaptic plasticity, as demonstrated by the synaptic defects in both long- and short-term plasticity exhibited by STOP deficient mice (8). Given the apparent absence of microtubules in nerve terminals, it has been surprising to find a synaptic function for a protein tightly associated with microtubules in neurites (2). STOP function in synaptic plasticity may arise indirectly from modifications of microtubule dynamics and their resulting downstream effects on synaptic functions (7). However, there are indications for a synaptic localization

of STOPs which are present in synaptosomal fractions (8) and in the PSD proteome (9). Here, we find that CaMKII, which is a key enzyme for synaptic plasticity (21,22), can inhibit STOP binding to microtubules through phosphorylation, thereby offering a possible mechanism for persistent STOP dissociation from microtubules and localization in other compartments, during synaptic activation. N-STOP is phosphorylated by CaMKII *in vitro* on at least three independent sites (S139, S198 and S491) out of four consensus CaMKII phosphorylation sites, in the sequence. Previous studies have shown that N-STOP interaction with microtubules can be inhibited by direct Ca²⁺-calmodulin binding to microtubules (4). These data suggest a model in which N-STOP dissociates from microtubules in two separate ways, either by binding Ca²⁺-calmodulin in response to transient Ca²⁺ influxes induced by synaptic activation, or through phosphorylation by active CaMKII, in the absence of Ca²⁺-calmodulin. The latter mechanism could be particularly relevant during short or long term synaptic potentiations, which both involve active CaMKII, after the Ca²⁺ peak dropped back to basal levels.

In cultured neurons, we find that phosphorylated STOP co-localizes with actin spikes along neurites and at branching points during differentiation. We also find that both phosphorylated and non-phosphorylated forms of STOP bind to polymerized actin *in vitro*, although phosphorylated STOP has apparently less affinity for F-actin. In cells, the overall concentration of F-actin is circa 100µM (23), higher than the F-actin concentrations found to absorb both dephosphorylated and phosphorylated STOP quantitatively, *in vitro* (6 µM and 16 µM, respectively). Additionally, local F-actin concentrations in cellular actin

assemblies are much higher than 100 µM, so high in fact, that once bound to F-actin, actin-binding proteins do not dissociate measurably from actin assemblies. Dissociation requires actin disassembly (23). In this context, our data suggest a model in which phosphorylated STOP, which has no detectable affinity for microtubules, binds to F-actin. Subsequent STOP dephosphorylation could re-enforce the interaction between STOP and F-actin, without causing STOP re-association with microtubules, as long as F-actin does not depolymerize.

It is uncertain whether STOP binding to actin at actin spikes or at branching points is important physiologically since we have not detected any obvious perturbation of neurite morphogenesis in STOP null mice (7). A major signature of STOP inactivation, is a dramatic depletion of synaptic vesicle pools in glutamatergic synapses, which could be central to some of the synaptic disorders observed in STOP null mice (8). Actin is important for the localization and regulation of synaptic vesicle pools and binding to actin may be important for STOP effect on the size of vesicular pool (24-28). Additionally, there is evidence for a burst of actin polymerization during synaptic potentiation, both in the pre- and post-synaptic compartments (24,27,29,30), and we find phosphorylated STOP in the vicinity of pre- and post-synaptic protein clusters, in differentiated neurons. Finally, cross talks between microtubule and actin assemblies have been a subject of great interest in recent times and several proteins with dual microtubule / actin binding properties have been identified as crucial integrators of the cytoskeleton (31-33). Our findings suggest that STOP protein may function as such integrators in nerve terminals.

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FOOTNOTES

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The abbreviations used are: CE, calcium eluate; E-STOP, Early STOP; N-STOP, neuronal adult STOP; PSD, post-synaptic density; STOP, Stable Tubule Only Polypeptide, IF, immunofluorescence, WB, western blot.

FIGURE LEGENDS

Fig. 1. Phosphorylation of N-STOP by CaMKII. *A*, Schematic representation of mouse N-STOP protein showing the location and sequences of the four peptides P1 to P4, containing potential CaMKII phosphorylation sites. Phosphorylatable serine or threonine residues of P1 to P4, (S139, S198, T438 and S491, respectively) are in red. Note that all the phosphorylation sites are overlapping with calmodulin-binding sites (underlined residues). Mc and Mn modules are microtubule-binding sites. *B*, Purified proteins from brain extract after ion exchange and calmodulin affinity chromatography (CE fraction) on Commassie blue-stained SDS-PAGE gel. *C-D*, Western blot and immuno-detection of mouse N-STOP (116 kD)(C) and of CaMKII protein (52 kD and a doublet at 58 kD)(D) in CE fraction using antibody mAb 175 against N-STOP and anti CaMKII antibody, respectively. *E-F*, *In vitro* phosphorylation of N-STOP by CaMKII. Phosphorylation reaction was performed using CE fraction with γ -³²P-ATP in the presence or in the absence of Ca²⁺. Samples were analyzed by SDS/PAGE and either autoradiography or western blot. (E) Autoradiogram showing ³²P-ATP incorporation, indicating auto-phosphorylation of CaMKII (apparent molecular weights of 55 and 60 kD, stars) and phosphorylation of N-STOP (apparent molecular weight of 125 kD, star). (F) Western blot analysis of CaMKII and STOP with antibodies as in (C) and (D). Note that in the presence of Ca²⁺ and ATP the phosphorylation of both CaMKII and N-STOP produces a shift of their molecular weight. *G-H*, Time course of STOP phosphorylation, analyzed by ³²P autoradiogram (G) and by Western blot (H), using antibodies as in (C).

Fig. 2. Effect of calmodulin on N-STOP phosphorylation by CaMKII. *A*, Phosphorylation reaction in CE fraction was performed with various concentration of calmodulin, ranging from 0 to 30 μ M, in the presence or in the absence of Ca²⁺. Proteins of the phosphorylation reaction were separated by SDS PAGE and analyzed by Western blot using N-STOP antibody mAb 175 and anti CaMKII antibody. *B*, CaMKII was pre-activated in the presence of 30 μ M Ca²⁺/calmodulin and phosphorylation reaction was then performed in the presence or in the absence of EGTA. STOP and CaMKII were analyzed by Western blot using antibodies as in (A).

Fig. 3. Microtubule-binding assay. CE fractions were subjected (CE-P) or not (CE) to phosphorylation and were next incubated in the presence of taxol-stabilized microtubules (lanes 1-2 and 5-6) or without microtubules (lanes 3-4 and 7-8). After centrifugation, equal amount of the pellets (P) and supernatants (S) were separated by SDS/PAGE and analyzed by western blot to detect the presence of N-STOP.

Fig. 4. Phosphorylated N-STOP antibodies. CE fractions (CE) were subjected either to phosphorylation reaction (CE-P) or to alkaline phosphatase treatment (CE-AP). Equal amount of proteins from fractions CE, CE-P and CE-AP were analyzed in the same immunoblots, with total N-STOP antibody mAb 175, phosphorylated N-STOP antibodies P2 (S198), P4 (S491) or P1 (S139). All phosphorylated N-STOP antibodies are highly specific of the phosphorylated form of N-STOP.

Fig. 5. Phosphorylated N-STOP (N-STOP-P) in differentiating neurons. *A-B*, Distribution of STOP proteins in cultured mouse hippocampal neurons after 12 days of differentiation *in vitro*, showing either neuritic network (left panels) or branching points (right panels). (A) Double N-STOP/ tubulin or phosphorylated N-STOP/tubulin staining was performed using STOP antibodies (mAb 175 for N-STOP and P2 antibody for phosphorylated STOP). Merge is the result of tubulin labeling in red and

STOP (STOP or STOP-P) labeling in green. *B*, Double immunostaining of either N-STOP/ actin or phosphorylated STOP/actin were performed. Merge is the result of actin labeling in red and STOP (STOP or STOP-P) labeling in green. *C*, Triple immunostaining of phosphorylated N-STOP, tubulin and actin, using antibodies as in (A) and (B), after cytochalasin treatment. *D*, Effect of neuronal activation: Phosphorylated STOP staining was quantified in both control neurons and neurons exposed to glutamate prior to fixation, using equal exposure time during image acquisition, in both conditions. 130 spikes from 30 independent neurons from three independent experiments were selected in both control and activated neurons. A region of interest was traced around each spike, and the maximum fluorescence intensity value (a.u.) was measured in each region. Values were averaged for each condition and plotted on the figure (mean \pm s.e.m.). *** $p \leq 0.001$ t test. *E*, Double immunostaining of phosphorylated N-STOP and actin after incubation of the neuronal culture with the inhibitor of CaMKinases, KN62 for 8 h. Antibodies and merge were as in (B). Bar: 20 μ m.

Fig. 6. Actin binding assay. Actin binding assays were performed starting with various amount of G-actin (1 μ M, 6 μ M and 15 μ M) and with N-STOP (from CE fraction or after affinity purification). *A*, Actin polymerization. Various amount of G-actin were polymerized in the presence of CE fraction and then centrifuged. Supernatant and pellets were separated by 7.5% SDS PAGE and were stained with Commassie blue, showing no detectable amount of actin in the supernatant. *B*, Analysis of N-STOP and CaMKII binding to F-Actin. *CE fraction*: various amounts of G-actin (1 μ M, 6 μ M and 15 μ M) were polymerized in the presence of CE fraction containing N-STOP and CaMKII proteins, and then centrifuged. Equal amounts of supernatants and pellets were separated by 7.5% SDS PAGE, transferred on nitrocellulose and blotted with mAb 175 or anti CaMKII antibody. *Affinity-purified N-STOP*: G-actin (1 μ M and 15 μ M) was polymerized in the presence of affinity purified N-STOP and then centrifuged. Equal amounts of supernatant and pellet were analyzed as above. *C*, Analysis of N-STOP and CaMKII binding to F-Actin after phosphorylation. Various amounts of G-actin (1 μ M, 6 μ M and 15 μ M) were polymerized in the presence of phosphorylated CE fraction and then centrifuged. Equal amounts of supernatants and pellets were analyzed as in (B).

Fig. 7. Phosphorylated STOP in differentiated neurons. Distribution of STOP proteins in cultured mouse hippocampal neurons after 45 days of differentiation *in vitro*. *A*, Double immunostaining of N-STOP/synapsin or phosphorylated STOP/synapsin (mAb 175 for N-STOP and P2 antibody for phosphorylated STOP). Merge is the result of synapsin labeling in red and STOP (N-STOP or STOP-P) labeling in green. *B*, Double immunostaining of phosphorylated STOP/synapsin and of phosphorylated STOP/homer. Merge is the result of either synapsin or homer labeling in red, and STOP-P labeling in green.

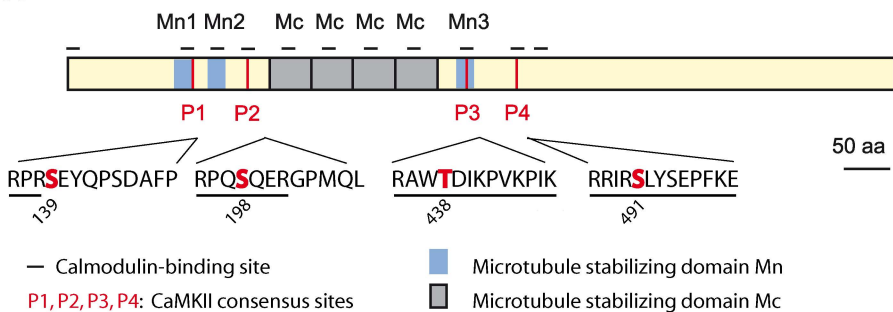
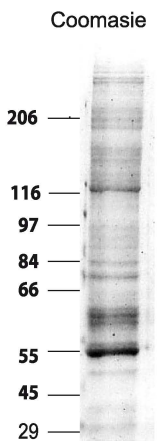
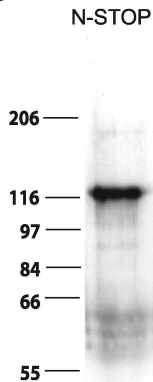
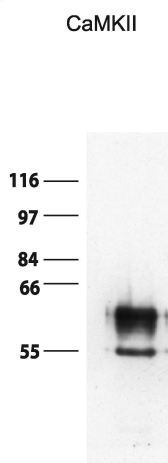
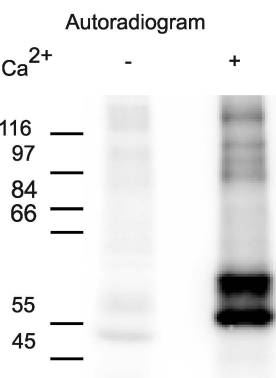
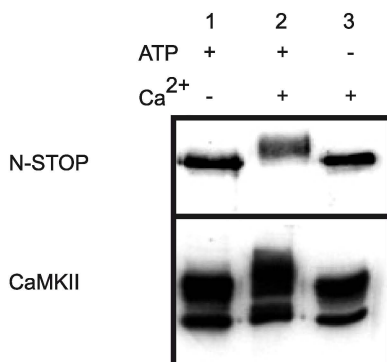
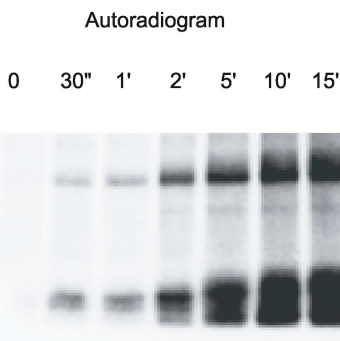
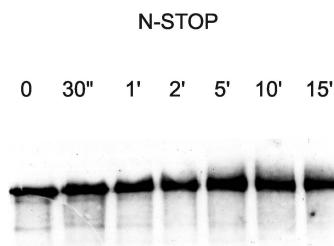
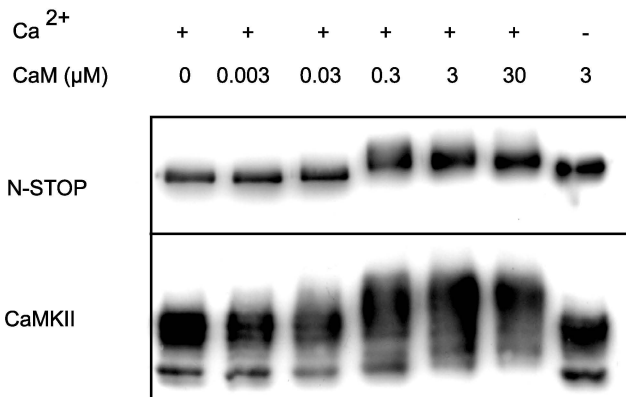
Figure 1**A** N-STOP**B****C****D****E****F****G****H**

Figure 2**A****B**

Kinase pre-activation	-	+	+
EGTA	-	-	+

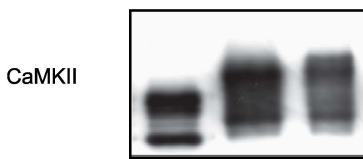
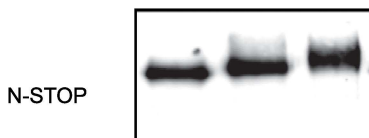


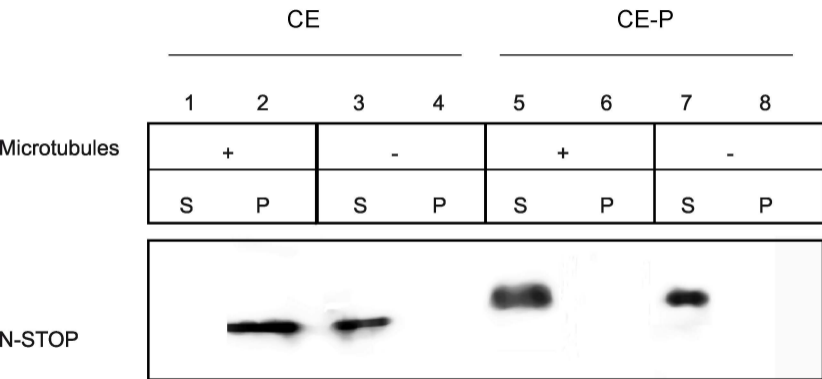
Figure 3

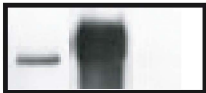
Figure 4

CE CE-P CE-AP

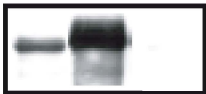
N-STOP



Purified P2 Ab



Purified P4 Ab



P1 mAb

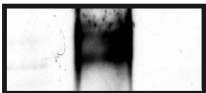


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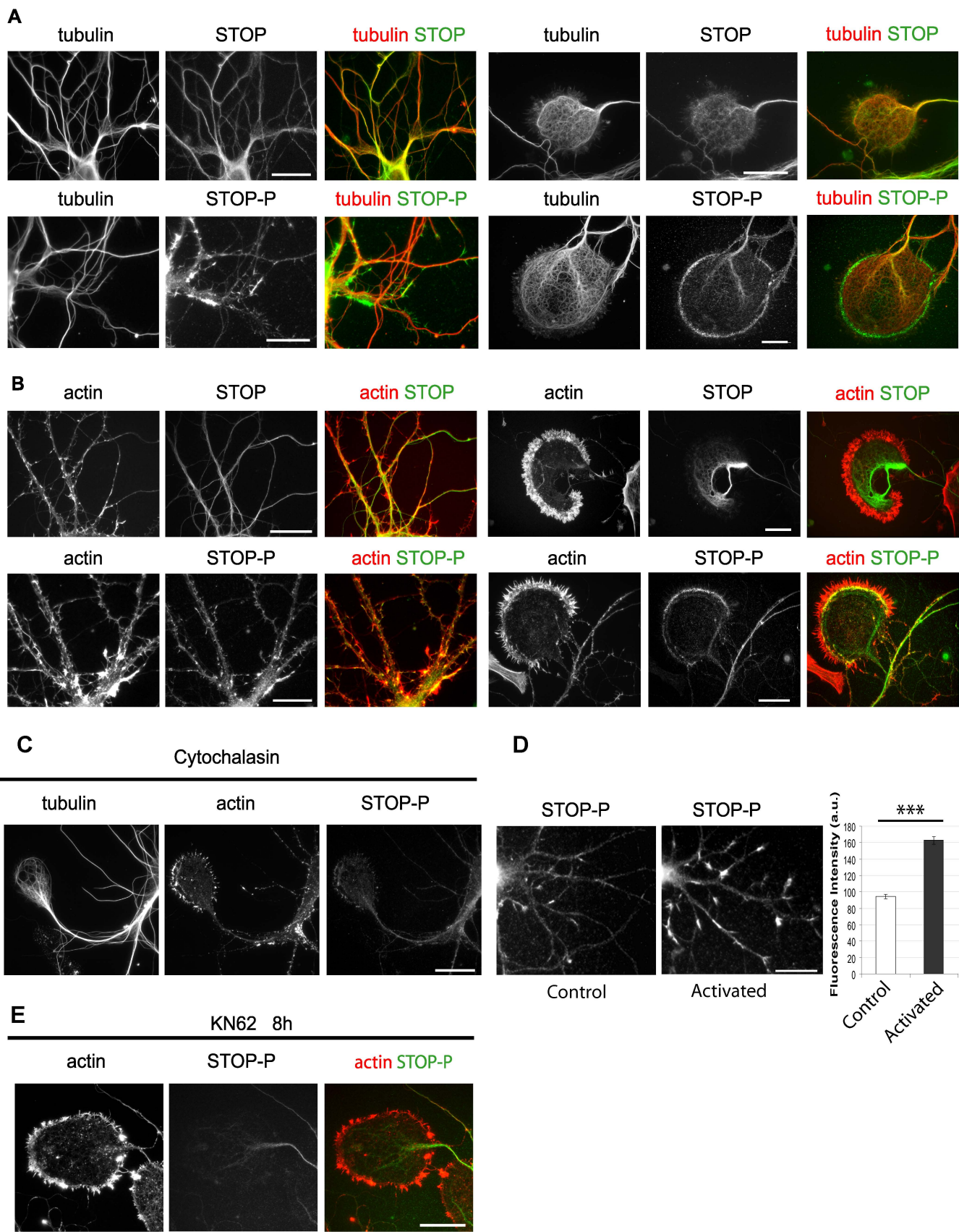


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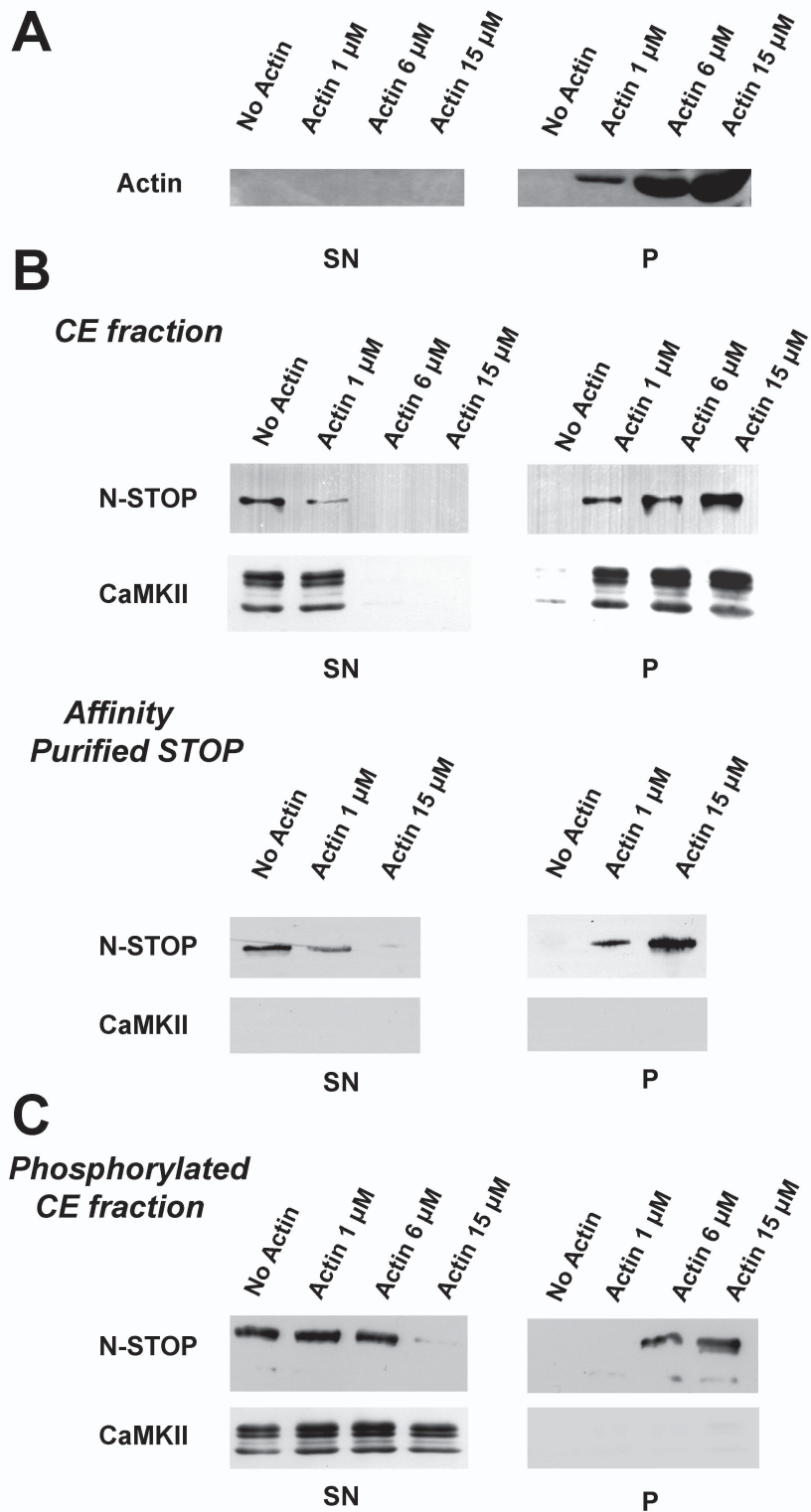
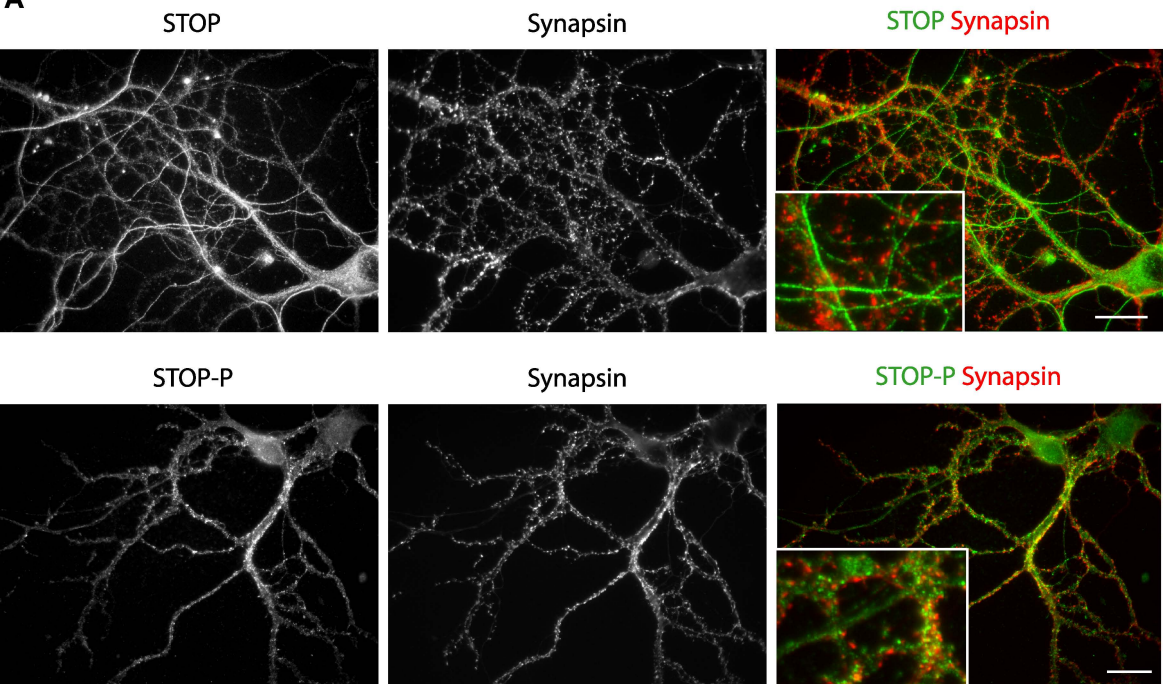


Figure 7

A



B

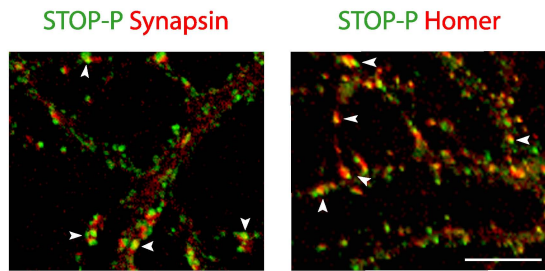


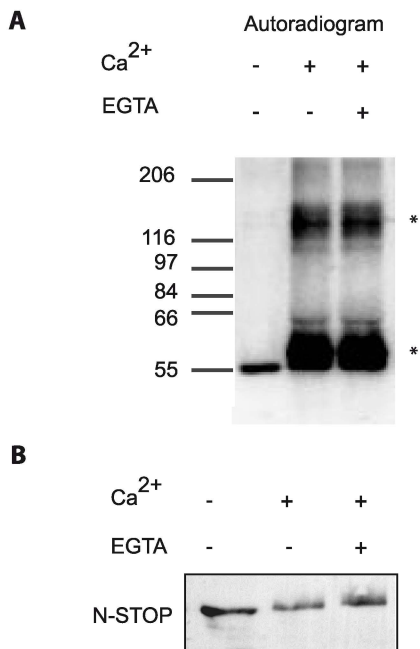
Figure S1

Fig. S1. Phosphorylation of purified N-STOP by exogenous CaMKII. N-STOP protein was purified from CE fraction by affinity purification using STOP mAb 175 coupled to Amino Rgel (Uptima). CE fraction was loaded on the column and the elution was performed using the peptide KDQGPV. This peptide corresponds to the sequence recognized by the monoclonal antibody 175 within the N-STOP sequence, as determined by peptide spot analysis. Phosphorylation reaction was performed using purified N-STOP and recombinant α -CaMKII (60 nM, generous gift from Dr T. Soderling), with γ -³²P-ATP, in the presence or in the absence of Ca²⁺. Phosphorylation reaction was also performed using pre-activated CaMKII (with ATP and Ca²⁺) in the presence of EGTA. *A*, Autoradiogram showing ³²P-ATP incorporation, indicating auto-phosphorylation of α -CaMKII and phosphorylation of N-STOP (stars). *B*, Western blot analysis of N-STOP after phosphorylation reaction showing that in the presence of Ca²⁺ and ATP the phosphorylation of N-STOP produces a shift of the molecular weight. Note that the molecular weight shift of phosphorylated N-STOP is higher in presence of EGTA (no active Ca²⁺/calmodulin complexes).

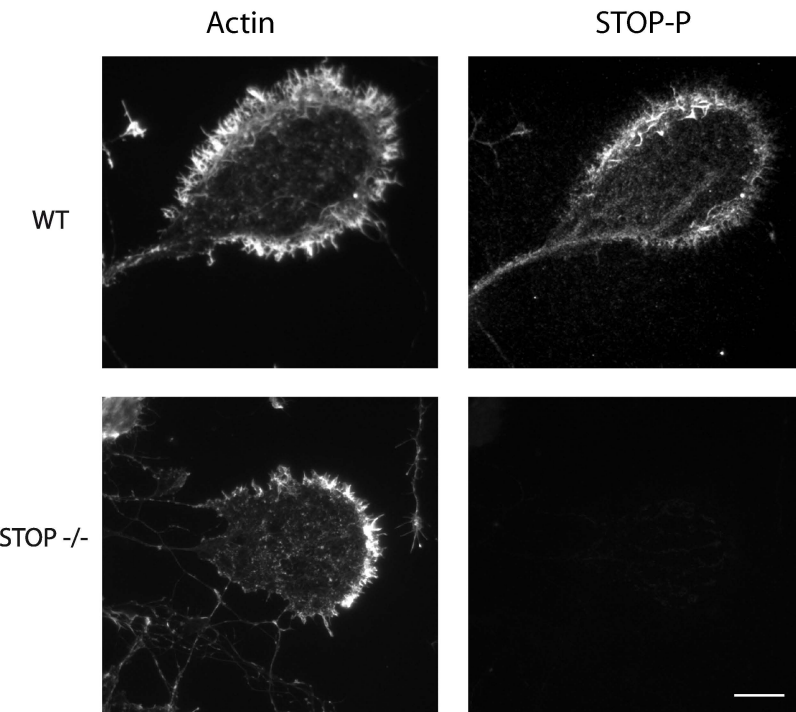


Fig. S2. Specificity of phosphorylated STOP antibody P2. Double immunostaining of phosphorylated STOP/actin in either wild type or STOP^{-/-}-cultured mouse hippocampal neurons, after 12 days of differentiation *in vitro*, showing branching points. Using P2 antibody, phosphorylated STOP is detected in wild type neurons whereas no signal is found in STOP^{-/-} neurons.