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Neurogranin phosphorylation fine-tunes long-term potentiation

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Abstract

Learning-related potentiation of synaptic strength at CA1 hippocampal excitatory synapses is dependent on neuronal activity and the activation of glutamate receptors. However, molecular mechanisms that regulate and fine-tune the expression of long-term potentiation (LTP) are not well understood. Recently it has been indicated that neurogranin, a neuron-specific, postsynaptic protein that is phosphorylated by protein kinase C (PKC), potentiates synaptic transmission in an LTP-like manner. Here, we report that a neurogranin mutant that is unable to be phosphorylated cannot potentiate synaptic transmission in rat CA1 hippocampal neurons and results in a sub-maximal expression of LTP. Our results provide the first evidence that the phosphorylation of neurogranin can regulate LTP expression.

Keywords

calmodulin; rat hippocampus; NMDARs; PKC; synaptic plasticity

INTRODUCTION

Long-term potentiation (LTP) is one of the best-characterized forms of synaptic plasticity (Lisman, 1989; Alkon & Nelson, 1990; Bliss & Collingridge, 1993; Malenka & Nicoll, 1999; Hayashi *et al.*, 2000; Malinow *et al.*, 2000). At CA1 hippocampal excitatory synapses, two different classes of glutamate receptors are crucial for synaptic plasticity: ionotropic NMDA receptors (NMDARs) and metabotropic glutamate receptors (mGluRs). NMDAR activation triggers an influx of Ca^{2+} into the dendritic spine, resulting in a series of Ca^{2+}-dependent events, e.g. Ca^{2+}/CaM-dependent protein kinase II (CaMKII) activation, and ultimately leading to the expression of LTP indicated by insertion of AMPA receptors (AMPARs) into the synapses. The activation of mGluRs, on the other hand, results in the initiation of the phospholipase C/diacylglycerol/protein kinase C (PLC/DAG/PKC) second messenger pathway. Although both NMDAR and mGluR signaling are crucial for LTP, the mechanism by which they crosstalk remains unresolved.

Neurogranin (Ng), a neuron-specific, postsynaptic protein, may provide insight into such crosstalk. Ng is a known PKC substrate that is essential for LTP and potentiates synaptic strength in an NMDAR-dependent manner (Zhong *et al.*, 2009). These effects are attributed to Ng's ability to bind and target CaM in the dendritic spines. Ng is the most abundant CaM-binding protein postsynaptically under basal conditions (Represa *et al.*, 1990; Gerendasy *et al.*, 1994a; Gerendasy *et al.*, 1994b; Watson *et al.*, 1994; Zhabotinsky *et al.*, 2006). Since the amount of CaM in specified cell compartments is a limiting factor for the target activation

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(Zhabotinsky *et al.*, 2006), Ng, through its regulated binding to CaM, can modulate LTP expression.

There are two mechanisms that can regulate Ng-CaM binding and hence the local availability of free unbound CaM. First, CaM can dissociate, reversibly, from Ng when local Ca^{2+} is increased, e.g. via NMDAR activation (Huang *et al.*, 1993; Gerendasy *et al.*, 1995). On the other hand, activation of mGluRs results in PKC-mediated phosphorylation of Ng at its serine 36 (S36) residue within the IQ-motif, rendering CaM incapable of rebinding to phosphorylated Ng (Ramakers *et al.*, 1995). These two mechanisms can be simplified as follows:

$$\mathrm{CaM[bound]{-}Ng} \overset{\mathrm{Ca^{2+}}}{\rightleftharpoons} \mathrm{CaM[unbound]{+}Ng} \xrightarrow{\text{PKC phosphorylation}} \mathrm{CaM[unbound]{+}Ng{-}P}$$

It has been shown that Ng-mediated potentiation is dependent on NMDAR activity as well as Ng's ability to release CaM upon demand (Zhong *et al.*, 2009). However, the role of Ng phosphorylation--the crosstalk point between the two major classes of glutamate receptors in LTP--in synaptic function has not been previously explored.

In this study, we investigated the role of Ng phosphorylation in synaptic function and plasticity in rat CA1 hippocampal neurons. We expressed Ng mutants with different phosphorylation properties and examined their effects on synaptic transmission and LTP expression. Our results show that phosphorylation of Ng plays an important role in determining the magnitude of LTP expression. We propose a novel mechanism through which Ng phosphorylation fine-tunes synaptic plasticity.

MATERIALS AND METHODS

Animals and hippocampal slice preparation

Young Sprague-Dawley rats (postnatal day 5 or 6) were purchased from Charles River Laboratories (Portage, MI, USA) and maintained on a daily 12 h light: 12 h dark cycle. All biosafety procedures and animal care protocols were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee (IACUC). Hippocampal slices were prepared as described previously (Gahwiler *et al.*, 1997).

DNA constructs and expression

GFP-tagged Ng mutants (Ng-SA and Ng-SD) were cloned from GFP-Ng plasmid as described (Zhong *et al.*, 2009) using the gene-tailor site-directed mutagenesis system (Invitrogen, Carlsbad, CA, USA). Mutations were made in the serine 36 residue to an alanine for Ng-SA and to an aspartate in Ng-SD. Constructs were re-cloned into pSinRep5 (Invitrogen) for Sindbis virus preparation. Recombinant plasmids have been verified by sequencing. After 2–7 days in culture, the recombinant gene was delivered into the slices. For the experiments shown in Fig. 3, we used the biolistic delivery method (Lo *et al.*, 1994), which allowed us to deliver two plasmids bearing mammalian promoters. For expression of single proteins, we used the Sindbis virus expression system, which is a replication-deficient, low-toxicity and neuron-specific system (Malinow, 1999).

Calmodulin Pull-down

The pull-down assay was performed as described previously (Zhong *et al.*, 2009). Briefly, hippocampal extracts were prepared in homogenization buffer (150 mM NaCl, 20 mM Tris pH 7.5, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml chemostatin, 1 μg/ml antipain, 1 μg/ml pepstatin and 1 % Triton X-100) containing either 2 mM EDTA or 2 mM Ca^{2+}. These

extracts were then incubated with CaM-sepharose beads (GE Healthcare, Uppsala, Sweden) for 3 hours at 4°C followed by three washes in homogenization buffer. Elution buffer contained either 10 mM $CaCl_2$ (to elute Ca^{2+}-sensitive CaM binding proteins, e.g. Ng) or 10 mM EDTA (to elute Ca^{2+}-dependent CaM binding proteins, e.g. CaMKII). Antibodies used for western blot analysis were anti-Ng (rabbit) (Millipore, Billerica, MA, USA, AB5620) and anti-α CaMKII (rabbit) (Millipore, MAB8699).

Electrophysiology

For paired recordings (Fig. 1B), simultaneous double whole-cell recordings were obtained for nearby pairs of infected (fluorescent) and uninfected (non-fluorescent neurons) under visual guidance using differential interference contrast (DIC) illumination as previously described (Zhong *et al.*, 2009). Synaptic responses were evoked with two bipolar electrodes (2-contact, FHC, Bowdoin, ME, USA) placed on the Schaffer collateral fibers between 300 and 500 mm from the recorded cells. The responses obtained from the two stimulating electrodes were averaged for each cell and counted as an 'n' of 1. The recording chamber was perfused with 119 mM NaCl, 2.5 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose, 0.1 mM picrotoxin and 2 μM 2-chloroadenosine, at pH 7.4, and gassed with 5% CO_2, 95% O_2. Patch recording pipettes (3–6 MΩ) were filled with 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 4 mM Na_2ATP, 0.4 mM Na_3GTP, 10 mM sodium phosphocreatine and 0.6 mM EGTA, at pH 7.25. Miniature EPSCs (Figs. 1C and 4) were recorded in the presence of 1 μM tetrodotoxin (TTX) (Tocris, Ellisville, MO, USA) and no chloroadenosine. For rectification experiments (Fig. 3), 0.1 mM spermine (Fisher Scientific, Pittsburg, PA, USA) was added in the intracellular solution, and 0.1 mM DL-2-amino-5-phosphonopentanoate (AP5) (Tocris) was present in the bath solution. LTP was induced by pairing 3 Hz presynaptic stimulation (300 pulses) with 0 mV postsynaptic depolarization. Voltage-clamp whole-cell recordings were acquired with a Multiclamp 700A amplifier (Axon Instruments, Sunnyvale, CA, USA).

Statistical analysis

Comparison of electrophysiological responses between pairs of infected and uninfected neurons (Fig. 1B) was carried out using the paired non-parametric Wilcoxon test. Mean values of mEPSCs (Figs. 1C and 4) and rectification index of AMPAR synaptic responses (Fig. 3) were compared using non-directional Student's *t*-test. Comparison of normalized average steady-state AMPAR-mediated responses between control uninfected neurons and those expressing Ng-SA or Ng-SD (Fig. 2B) was achieved using One-Way ANOVA followed by the Tukey-Kramer *post hoc* test. Values were considered significantly different if $P < 0.05$ and marked with asterisk. Error bars represent standard error of the mean in all figures.

RESULTS

Non-phosphorylatable neurogranin mutant cannot potentiate synaptic transmission

Overexpression of wild-type Ng enhances synaptic strength by increasing synaptic AMPARs through its regulated interaction with CaM (Zhong *et al.*, 2009). In the current study, we assessed the role of Ng phosphorylation in synaptic transmission and LTP. We used a GFP-tagged Ng mutant that cannot be phosphorylated at serine 36 through its mutation to alanine (Ng-SA). First, we tested whether this mutant binds to CaM in a Ca^{2+}-dependent manner, like the endogenous Ng. To do so, we expressed Ng-SA in CA1 hippocampal neurons and performed a CaM-sepharose pull-down assay in the presence of 2 mM EDTA or 2 mM Ca^{2+}. In agreement with previously published data (Gerendasy *et al.*,

1994a), Ng-SA binds to CaM only in the absence of Ca^{2+}, similar to endogenous Ng (Fig. 1A).

The effect of Ng-SA on synaptic transmission was evaluated by simultaneous double whole-cell recordings from pairs of nearby infected and uninfected neurons under voltage-clamp configuration. As shown in Fig. 1B, Ng-SA neither enhanced AMPAR- (39.78 ± 7.06 pA vs. 44.80 ± 8.57 pA for control, $n = 10$, $P = 0.40$, Wilcoxon test) nor changed NMDAR-mediated responses (38.64 ± 7.80 pA vs. 34.56 ± 6.51 pA for control, $n = 12$, $P = 0.36$, Wilcoxon test). As an independent method to test the role of Ng-SA on synaptic transmission, we measured miniature excitatory postsynaptic currents (mEPSCs). In agreement with the whole-cell double recordings shown in Fig. 1B, Ng-SA overexpression did not enhance the mEPSC amplitude (Fig. 1C, control: 24.21 ± 1.22 pA, Ng-SA: 26.30 ± 2.05 pA, $n = 13$ for each condition, $P = 0.39$, Student's *t*-test) nor did it change the frequency (control: 0.19 ± 0.048 Hz, Ng-SA: 0.16 ± 0.036 Hz, $P = 0.61$, Student's *t*-test). These results indicate that Ng-SA overexpression does not enhance AMPAR-mediated responses. This is in contrast to the wild-type Ng, whose overexpression results in enhanced synaptic transmission (Zhong *et al.*, 2009). This enhancement is likely due to the ability of the wild-type Ng (and not Ng-SA) to be phosphorylated, thus preventing CaM from rebinding to then-phosphorylated wild-type Ng and allowing more time for CaM to activate its targets. These results suggest that Ng phosphorylation is necessary for Ng-mediated synaptic potentiation.

Neurogranin phosphorylation is essential for maximum LTP expression

Ng-mediated potentiation of synaptic transmission is dependent on regulated CaM binding (Zhong *et al.*, 2009). Since Ng-SA binds to CaM only in the absence of Ca^{2+}, similar to wild-type Ng, its inability to potentiate synaptic transmission suggests phosphorylation is required for Ng-mediated potentiation (Zhong *et al.*, 2009). To directly investigate the role of Ng phosphorylation in LTP induction, we tested the effect of Ng-SA expression on LTP. LTP was induced in infected (Ng-SA-expressing) and uninfected CA1 neurons by pairing presynaptic stimulation (3 Hz, 1.5 min) with postsynaptic depolarization (0 mV). As shown in Fig. 2A, uninfected neurons exhibited robust LTP (207.9 ± 22.6%, $n = 11$, $P = 0.00075$, Student's *t*-test). Interestingly, while neurons expressing Ng-SA were able to produce LTP (140.1 ± 15.8%, $n = 8$, $P = 0.039$, Student's *t*-test), they exhibited significantly smaller magnitude of potentiation than control neurons (Fig. 2B, $P = 0.0032$, ANOVA and Tukey-Kramer *post hoc* test). These results suggest that the inability of Ng to be phosphorylated interferes with the ability of neurons to maximally express LTP. It is worth mentioning that overexpression of wild-type Ng mimics and occludes LTP (Zhong *et al.*, 2009). Nonetheless, when Ng-mediated potentiation was blocked by concurrent application of NMDAR blocker, AP5, robust LTP was induced in neurons overexpressing wild-type Ng, indicating that overexpression *per se* does not interfere with LTP induction. It is important to note that the lack of maximal LTP in Ng-SA-expressing neurons is not due to partial occlusion of LTP since Ng-SA overexpression was not able to enhance basal transmission, in contrast to wild-type Ng.

Ng-SA expression does not interfere with AMPA receptor insertion

The inefficient induction of LTP, when Ng-SA is expressed, signifies the relevance of Ng phosphorylation in fine-tuning the level of LTP expression. However, it is also possible that overexpression of Ng-SA may interfere with AMPAR insertion to some degree, thus preventing a full expression of LTP. To examine this possibility, we used the "electrophysiological tagging" to monitor the effects of Ng-SA on CaMKII-mediated synaptic delivery of GluR1. To do so, we used the biolistic method to co-transfect CA1 neurons with GFP-GluR1 and truncated CaMKII (tCaMKII, a constitutively active form of

CaMKII that has been shown to induce GluR1 insertion into the synapse) in the absence or presence of Ng-SA. Delivery of GFP-GluR1 receptors to synapses was then quantified as an increase in the ratio of the evoked postsynaptic current at −60 mV relative to the current at +40 mV (rectification index = I_{-60}/I_{+40}) (Hayashi *et al.*, 2000; Gerges *et al.*, 2005). As expected, neurons co-expressing GluR1 and tCaMKII showed an increase in rectification index relative to non-transfected neurons (Fig. 3, untransfected: 1.39 ± 0.14, $n = 14$, GluR1-tCaMKII transfected: 2.40 ± 0.20, $n = 6$, $P = 0.0017$, Student's *t*-test), indicating that expression of constitutively active CaMKII results in GluR1 synaptic delivery. Importantly, neurons co-expressing GluR1 and tCaMKII in the presence of Ng-SA showed a similar degree of rectification (Fig. 3), indicating that the presence of Ng-SA did not interfere, even partially, with CaMKII-mediated GluR1 synaptic delivery. These results demonstrate that the reduced expression of LTP seen in the presence of Ng-SA expression (Fig. 2) is not due to its interference with AMPAR insertion into the synapse but rather due to its inability to be phosphorylated.

Phosphomimic neurogranin does not change synaptic transmission but blocks LTP

The reduced magnitude of LTP due to Ng-SA expression supports a model in which Ng can regulate the local unbound CaM through two distinct steps. First, the increase in Ca^{2+} will lead to CaM dissociation from Ng. The released CaM can locally activate targets (e.g. CaMKII). If Ng is phosphorylated, CaM will have more time to activate its targets resulting in maximal LTP. However, if Ng is not phosphorylated (as in the case of Ng-SA), CaM will re-bind to Ng and become sequestered, limiting the activation of its targets and resulting in reduced LTP expression. Alternatively, it is possible that phosphorylation of Ng may have a direct role in potentiating synaptic strength, in a CaM-independent manner. To directly test this possibility, we assessed the effects of a phosphomimic mutant of Ng (Ng-SD) on synaptic transmission. As shown in Fig. 4, Ng-SD expression did not increase the mEPSC amplitude (control: 27.46 ± 1.43 pA, Ng-SD: 30.12 ± 2.22 pA, $n = 10$ for each condition, $P = 0.33$, Student's *t*-test) nor did it change the frequency (control: 0.20 ± 0.094 Hz, Ng-SD: 0.31 ± 0.15 Hz, $P = 0.55$, Student's *t*-test), strongly suggesting that Ng phosphorylation *per se* does not potentiate synaptic transmission. This is consistent with the previous finding that Ng-SD does not enhance AMPAR- or NMDAR-mediated responses (Zhong *et al.*, 2009). It is important to note that Ng-SD does not bind to CaM in the presence or absence of Ca^{2+} (Zhong *et al.*, 2009). These results show that the phosphomimic Ng does not have a direct effect on synaptic transmission.

We have previously shown that Ng-mediated effects on synaptic plasticity are dependent on its regulated binding to CaM. For example, mutants that lack the CaM binding ability (e.g. Ng mutant that lacks the IQ motif) or mutants that constitutively bind to CaM in the presence or absence of Ca^{2+} are unable to potentiate synaptic transmission, in contrast to Ng wild-type (Zhong *et al.*, 2009). This property of Ng predicts that expression of a phosphomimic Ng will block LTP induction due to the inability of this mutant to bind CaM. To test this, we induced LTP in control CA1 neurons and neurons expressing Ng-SD as described above. As predicted, Ng-SD completely abolished LTP induction (Fig. 2). Taken together, the ability of Ng to enhance synaptic plasticity is due to its ability to target CaM and its phosphorylation provides a novel mechanism to regulate LTP.

DISCUSSION

Our study has identified a novel regulatory mechanism by which neurons can fine-tune learning-correlated plasticity through the regulation of Ng phosphorylation. Previous studies have shown that Ng plays a critical role in LTP induction (Huang *et al.*, 2004; Zhong *et al.*, 2009). It has also been increasingly recognized that cognitive abnormalities associated with many disorders are correlated with abnormal levels or distribution of Ng (Iniguez *et al.*,

1993; Piosik *et al.*, 1995; Chang *et al.*, 1997; Mons *et al.*, 2001). For example, a recent genome-wide scan of thousands of schizophrenia and control cases identified Ng as one of four major variants associated with schizophrenia (Stefansson *et al.*, 2009). It has thus been suggested that the cognitive deficits associated with schizophrenia are due, at least partly, to reduced levels of Ng. It is not surprising then to observe cognitive deficits in Ng knockout mice as well as a positive correlation between memory function and Ng levels (Pak *et al.*, 2000; Huang *et al.*, 2004). Thus, the regulation of Ng levels can be critical in determining synaptic plasticity and memory function. In the current study, we unravel a novel alternative molecular mechanism by which Ng can regulate the expression of LTP, a synaptic model of learning and memory.

Here, we show that the inability of Ng to be phosphorylated, as demonstrated by a non-phosphorylatable Ng mutant (Ng-SA), results in reduced LTP expression compared to control neurons. This reduced LTP is unlikely due to a disruption of AMPAR trafficking, as expression of Ng-SA does not prevent CaMKII-mediated GluR1 insertion into to the synapse. The inability of Ng-SA to allow for the full expression of LTP (Fig. 2) is most likely due to the faster rebinding of CaM to Ng-SA after the initial dissociation, rather than the lack of the direct effect of the phosphorylated Ng. While previous studies show that there is enhanced phosphorylation of Ng during LTP (Chen, 1994;Ramakers *et al.*, 2000), the functional relevance of Ng phosphorylation remained to be determined.

Two main views exist concerning the function of Ng phosphorylation. One view suggests Ng phosphorylation plays a regulatory role in synaptic function through the modulation of free CaM levels (Li *et al.*, 1999; Wu *et al.*, 2002). This view is based on phosphorylated Ng's reduced affinity for CaM, which would render CaM available for many CaM-dependent enzymes involved in regulating synaptic plasticity and function. The other view, however, is that phosphorylated Ng can directly influence cellular functions independently of CaM regulation. In *Xenopus* oocytes, for example, it has been shown that phosphorylation of Ng can enhance mobilization of intracellular Ca^{2+} (Cohen *et al.*, 1993). The lack of direct effects of Ng-SD on basal transmission supports the former view that Ng phosphorylation plays a regulatory role in synaptic function. Thus, Ng phosphorylation may act as a checkpoint that can give CaM more time to be in the unbound state. Given the importance of CaM signaling and the diversity of its targets, it is likely that multiple levels of regulation exist. Several lines of evidence support this notion. First, the abundant CaM-binding postsynaptic protein, Ng, concentrates and targets CaM within dendritic spines (Zhabotinsky *et al.*, 2006; Zhong *et al.*, 2009). Second, an increase in the local Ca^{2+} frees CaM from Ng (Huang *et al.*, 1993; Gerendasy *et al.*, 1995). Finally, since the spike of local Ca^{2+} increase is rather transient, phosphorylation of Ng allows CaM to be dissociated for a longer period of time. This functionality can give the system a lot of flexibility in determining the level of activation of subsequent targets. The degree of Ng phosphorylation may ultimately influence the level of unbound CaM after the initial dissociation. Therefore, this phosphorylation can allow an ample range of modification within the synapse, which can undergo various degrees of plasticity rather than an all-or-none outcome. This is supported by previous findings indicating that intermediate expression of LTP can be induced by interfering with particular pathways (Zhu *et al.*, 2005).

Our data support a model in which Ng fine-tunes synaptic plasticity through its phosphorylation (see Fig. 5 for illustration). Under normal conditions, local transients of Ca^{2+} spikes free CaM from Ng, which can then activate relevant targets, such as CaMKII, until it re-associates with Ng. The PKC-mediated phosphorylation of Ng prevents CaM from rebinding to Ng and thus allowing it to activate its targets for a prolonged time frame, leading to maximal expression of LTP. However, if Ng is incapable of being phosphorylated (e.g. Ng-SA), CaM re-associates with Ng relatively quickly, attenuating CaM signaling and

resulting in sub-maximal LTP. Finally, the inability of neurons expressing Ng-SD to induce LTP emphasizes that Ng's major molecular role in synaptic function can be attributed to its CaM-binding ability. The current model also supports the idea that Ng can act as a coincidence-detector (Hayashi, 2009) integrating NMDAR and mGluR signaling, through CaM localization and PKC phosphorylation, thereby fine-tuning learning-correlated plasticity.

An important question surfaces from this study is the relevance of Ng phosphorylation in cognitive function. Our model predicts that a lack of Ng phosphorylation, while may not result in total cognitive impairment, would lead to some degree of cognitive deficits. A Ng-SA knock-in mouse is currently unavailable. However, knockout mice for PKC gamma (PKCγ), a neuron-specific PKC isozyme that specifically phosphorylates Ng (Ramakers *et al.*, 1999), exhibit mild to moderate deficits in spatial and contextual learning (Abeliovich *et al.*, 1993a). Moreover, in agreement with the LTP results of neurons expressing Ng-SA, PKCγ knockout mice showed a reduced ability to induce LTP in hippocampal slices (Abeliovich *et al.*, 1993b). It has been hypothesized that memory deficits, associated with some cognitive disorders, are attributed to decreased Ng levels (Iniguez *et al.*, 1993; Piosik *et al.*, 1995; Chang *et al.*, 1997; Mons *et al.*, 2001). In here, we speculate that cognitive deficits can result from not only decreased Ng levels but also from a disturbance in the signaling necessary for Ng phosphorylation. Further studies are needed to test this hypothesis.

In conclusion, our results highlight the importance of Ng phosphorylation in LTP induction and provide a novel mechanism by which neurons can regulate plasticity.

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ABBREVIATIONS

AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
CA1	*Cornu Ammonis* subfield 1
CaM	calmodulin
CaMKII	Ca^{2+}/CaM-dependent protein kinase II
DAG	diacylglycerol
GFP	green fluorescence protein
LTP	long-term potentiation
mEPSC	miniature excitatory postsynaptic current
mGluR	metabotropic glutamateric receptor
Ng	neurogranin
NMDAR	N-methyl-D-aspartate receptor
PKC	protein kinase C
PKCγ	protein kinase C gamma
PLC	phospholipase C

tCaMKII truncated CaMKII

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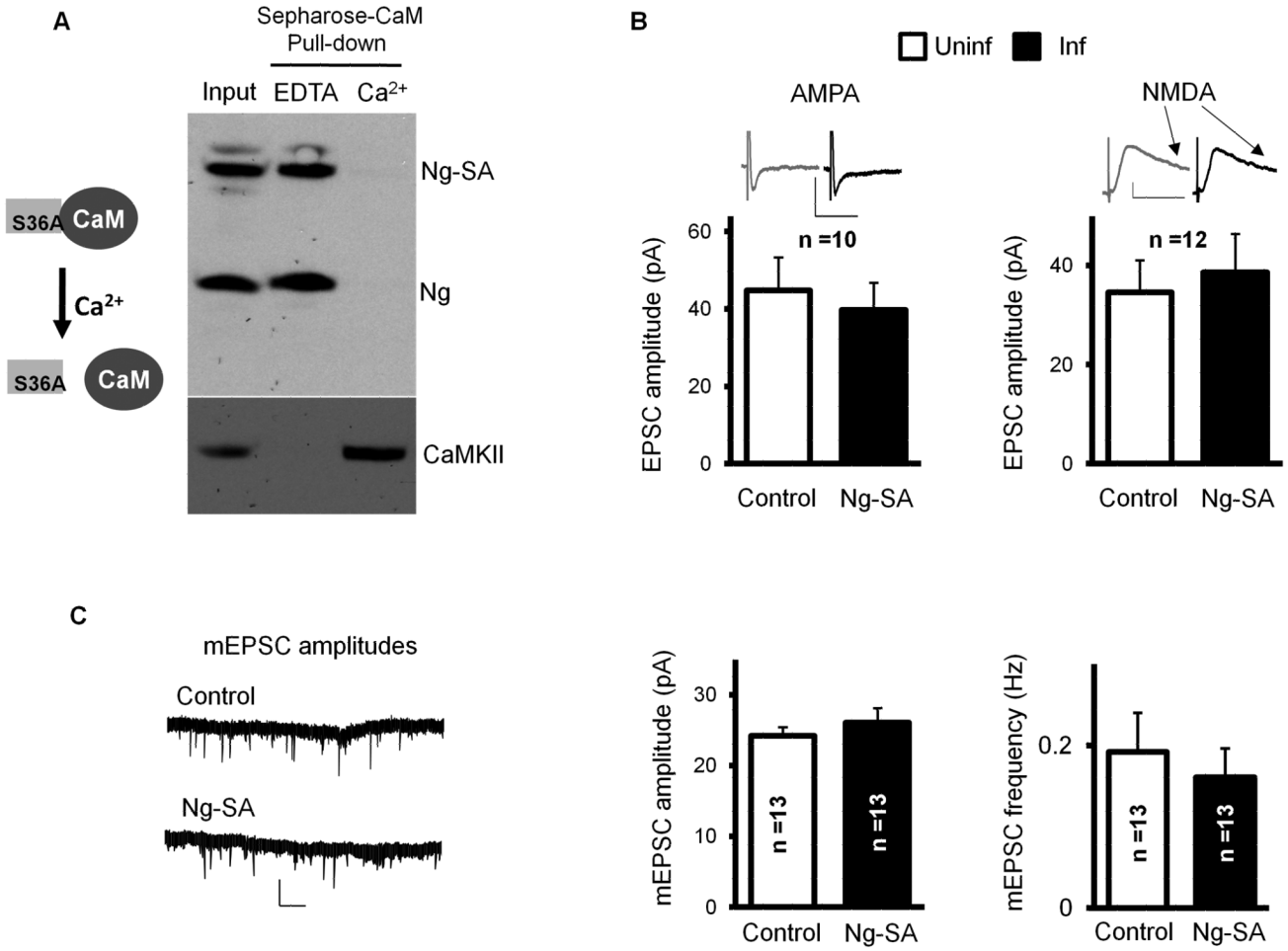

Fig.1. Non-phosphorylatable mutant of neurogranin cannot enhance synaptic transmission

(**A**) Western blot analysis of CaM pull-down assay from hippocampal extracts of slices injected with a non-phosphorylatable mutant of Ng, Ng-SA, in the presence of EDTA or Ca^{2+}. Note that both Ng-SA and endogenous Ng were pulled down only in the absence of Ca^{2+}. As a control, the membrane was stripped and re-probed for CaMKII, which is pulled down with CaM only in the presence of Ca^{2+}. (**B**) Data represent the average evoked EPSCs recorded simultaneously from pairs of nearby uninfected (control) and infected neurons expressing GFP-tagged Ng-SA (Ng-SA). *Insets*, sample traces of evoked AMPAR- and NMDAR-mediated synaptic responses recorded at −60 and +40 mV, respectively. Arrows indicate NMDAR-mediated responses (the amplitude at 60 ms latency, when AMPAR responses have decayed). Scale bars, 40 pA, 40 ms. (**C**) *Left panel*, sample traces of spontaneous miniature excitatory postsynaptic currents (mEPSCs) recorded at −70 mV from uninfected neurons (control, $n = 13$) or Ng-SA-expressing neurons (Ng-SA, $n = 13$). *Scale bars*, 25 pA, 10 s. Data represent average mEPSC amplitudes (*left graph*) and frequencies (*right graph*).

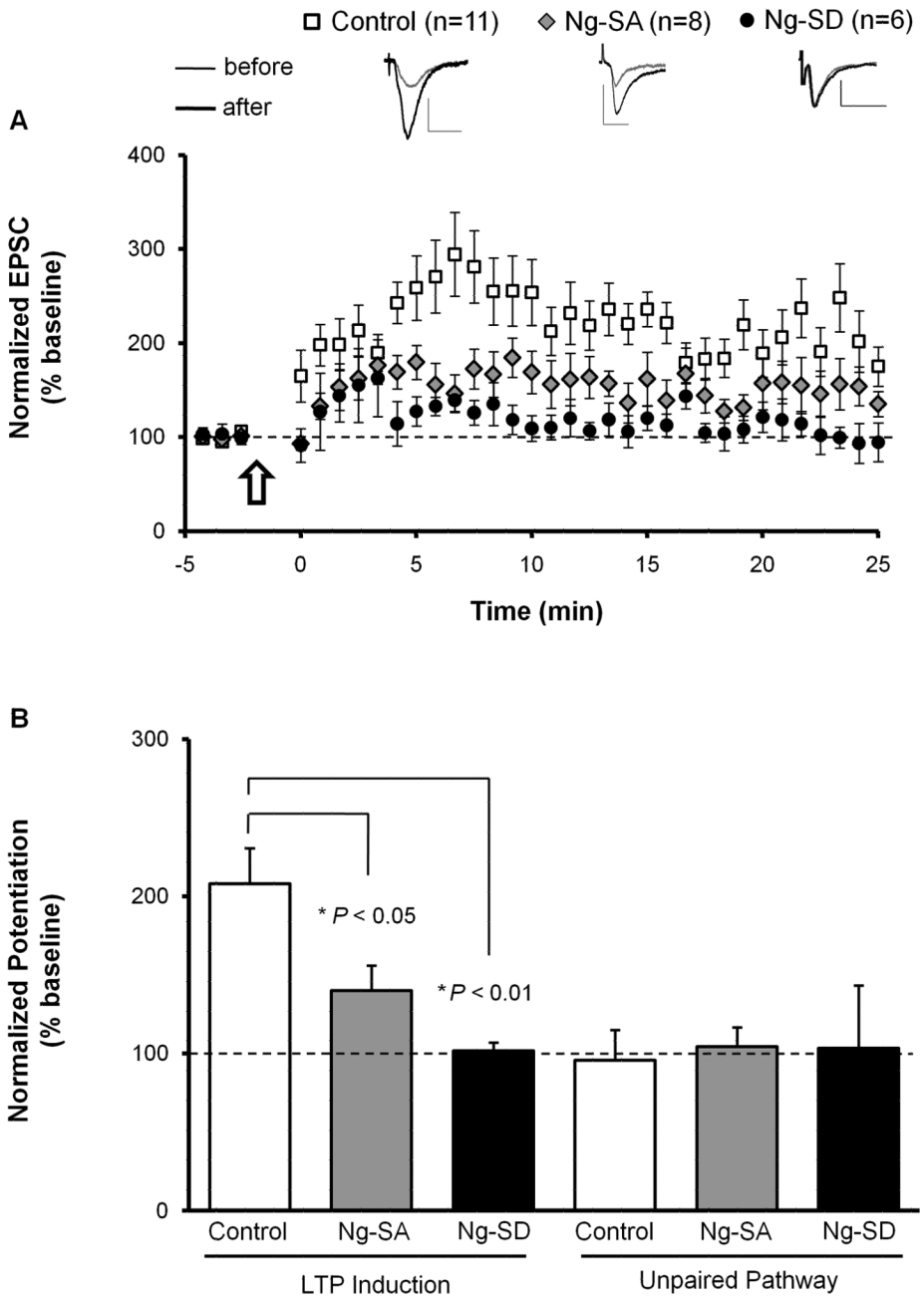

Fig.2. Phosphorylation of neurogranin fine-tunes LTP expression

(**A**) LTP was induced by pairing 3 Hz presynaptic stimulation (300 pulses) with 0 mV postsynaptic depolarization (indicated with an arrow) in CA1 neurons expressing Ng-SA (grey diamonds, $n = 8$), Ng-SD (black circles, $n = 6$) or uninfected neurons (white squares, $n = 11$). *Insets*, sample traces of evoked AMPAR-mediated synaptic responses recorded at −60 mV before pairing (grey line) and 20 min after pairing (black line) from control or infected cells as indicated. *Scale bars*, 20 pA, 20 ms. (**B**) Normalized average steady-state AMPAR-mediated responses in paired (LTP induction) and unpaired (control pathway) pathways for uninfected neurons and neurons expressing Ng-SA or Ng-SD were evaluated by One-Way ANOVA ($P = 0.0032$).

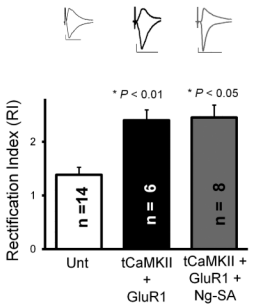

Fig.3. Non-phosphorylatable mutant of neurogranin does not interfere with AMPA receptor insertion

The rectification index (RI) is calculated as the ratio of the amplitude of AMPAR-mediated responses at −60 mV over that at +40 mV. Endogenous receptors conduct currents at −60 and +40 mV, whereas recombinant receptors conduct currents only at negative potentials. Therefore, delivery of the recombinant GluR1 homomeric receptors is accompanied by an increase in the RI (similar results were obtained for the control neurons recorded with each condition and data were pooled). *Inset*, sample traces of evoked AMPAR-mediated synaptic responses recorded at −60 and +40 mV from control (n = 14), tCaMKII + GluR1 (n = 6), or tCaMKII + GluR1 + Ng-SA (n = 8). *Scale bars*, 25 pA and 10 ms.

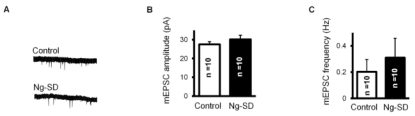

Fig.4. A phosphomimic mutant of neurogranin does not enhance basal transmission

(**A**) Sample traces of spontaneous miniature excitatory postsynaptic currents (mEPSCs) recorded at −70 mV from uninfected neurons (control, *n* = 10) or neurons expressing Ng-SD (Ng-SD) (*n* = 10). *Scale bar*, 50 pA, 20 s. (**B and C**) Data representing average mEPSC amplitudes (B) and frequency (C) of control and Ng-SD-expressing neurons.

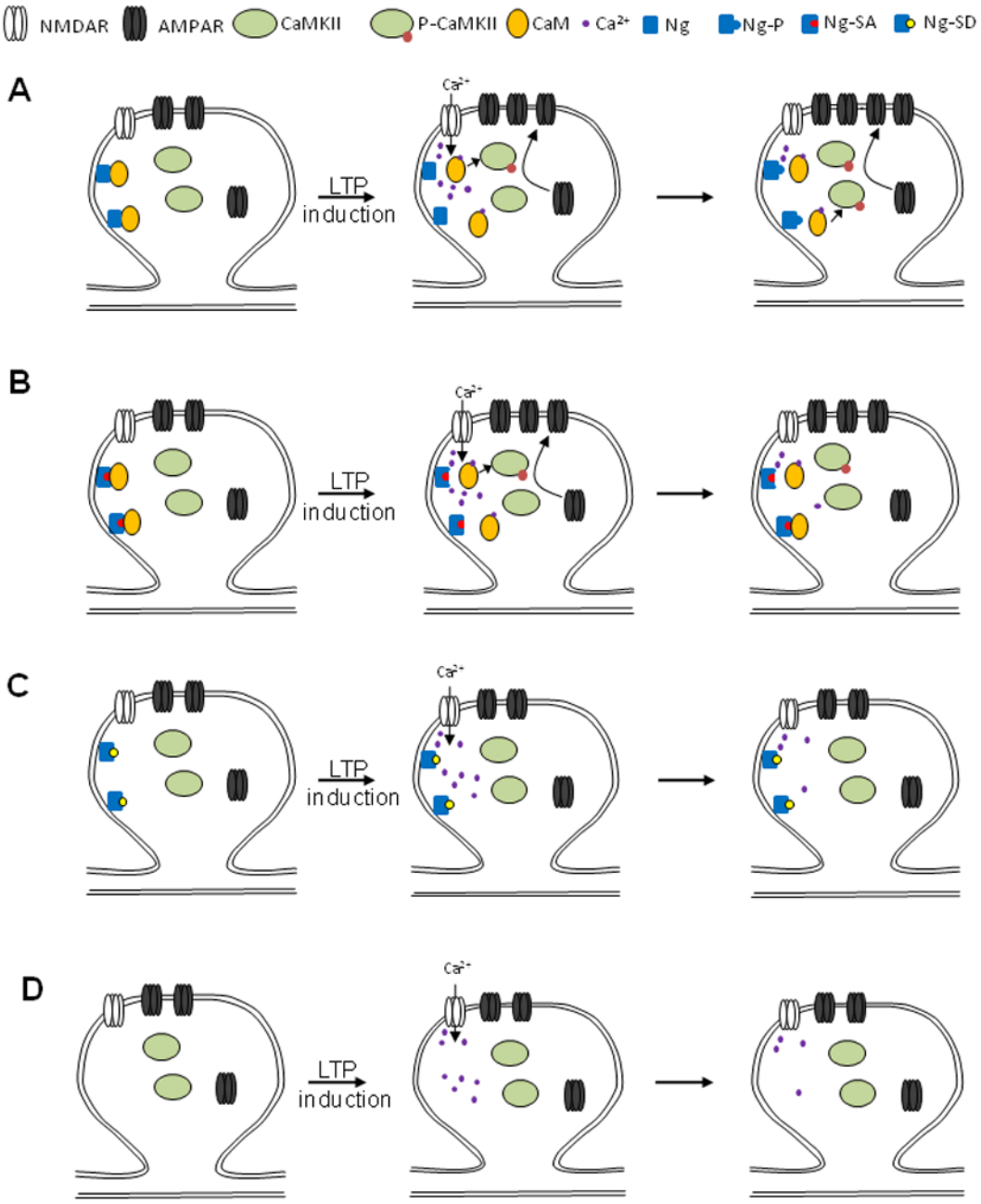

Fig.5. Ng phosphorylation fine-tunes the magnitude of LTP expression

(**A**) Under normal conditions, phosphorylation of Ng gives CaM enough time to be in its unbound state and affect subsequent targets, resulting in maximal LTP expression. (**B**) Inability of Ng to be phosphorylated shortens the time of CaM in its free state as it re-binds with unphosphorylated Ng, resulting in sub-maximal LTP. (**C**) Phosphomimic mutant of Ng results in a loss of spatial targeting of CaM, thus, LTP cannot be induced. This effect is similar to that when Ng is absent (**D**).