Molecular Neurobiology (2019) 56:7321–7337
https://doi.org/10.1007/s12035-019-1593-3

Neurogranin Expression Is Regulated by Synaptic Activity and Promotes Synaptogenesis in Cultured Hippocampal Neurons

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Received: 4 December 2018 / Accepted: 2 April 2019 / Published online: 24 April 2019

Abstract

Neurogranin (Ng) is a calmodulin (CaM)-binding protein that is phosphorylated by protein kinase C (PKC) and is highly enriched in the dendrites and spines of telencephalic neurons. It is proposed to be involved in regulating CaM availability in the post-synaptic environment to modulate the efficiency of excitatory synaptic transmission. There is a close relationship between Ng and cognitive performance; its expression peaks in the forebrain coinciding with maximum synaptogenic activity, and it is reduced in several conditions of impaired cognition. We studied the expression of Ng in cultured hippocampal neurons and found that both protein and mRNA levels were about 10% of that found in the adult hippocampus. Long-term blockade of NMDA receptors substantially decreased Ng expression. On the other hand, treatments that enhanced synaptic activity such as long-term bicuculline treatment or co-culture with glial cells or cholesterol increased Ng expression. Chemical long-term potentiation (cLTP) induced an initial drop of Ng, with a minimum after 15 min followed by a slow recovery during the next 2–4 h. This effect was most evident in the synaptosome-enriched fraction, thus suggesting local synthesis in dendrites. Lentiviral expression of Ng led to increased density of both excitatory and inhibitory synapses in the second and third weeks of culture. These results indicate that Ng expression is regulated by synaptic activity and that Ng promotes the synaptogenesis process. Given its relationship with cognitive function, we propose targeting of Ng expression as a promising strategy to prevent or alleviate the cognitive deficits associated with aging and neuropathological conditions.

Keywords Neurogranin · Synaptic plasticity · Glutamate receptors · Synaptogenesis · Hippocampal neurons

Introduction

Our cognitive abilities rely on the generation of new memories, which are made possible by the plastic changes displayed by synapses. Synaptic plasticity is highest during development when a large number of synaptic contacts are created, many of which are subsequently eliminated. In adulthood, several forebrain areas retain moderate to high levels of synaptic plasticity, and their synaptic networks provide the substrate and mechanisms that sustain cognition. Neurogranin (Ng) [1] is a small protein present mainly in the central nervous system with mostly postnatal expression and is restricted to telencephalic areas such as the cerebral cortex, hippocampus, striatum, and amygdala [2, 3]. It is expressed in differentiated neurons, localizes to the somatodendritic compartment, and concentrates in dendritic spines [4]. In the rat brain, Ng expression increases dramatically after birth, peaks during the third postnatal week, and slowly declines thereafter to reach the still high levels of adults. Ng features an IQ motif that mediates its interaction with calmodulin (CaM) [5] and phosphatidic acid (PA) [6]. This motif is phosphorylated by protein kinase C (PKC) at serine 36, rendering phospho-Ng unable to bind either CaM or PA. The current functional model proposes that Ng regulates CaM availability in the post-synaptic environment and drives Ca^{2+}/CaM-dependent signaling toward alternative pathways [7–10].

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12035-019-1593-3) contains supplementary material, which is available to authorized users.

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There is ample evidence demonstrating a correlation between brain Ng and cognitive performance [8]. Ng-deficient knockout mice [7, 11] grow normally and show no apparent anatomical or physiological anomalies. However, they show severe deficits in visual-spatial learning tasks, a marked tendency toward stress and anxiety [12], low basal levels of phospho-CaMKIIα, and substantial alterations in the induction of long-term potentiation (LTP) and long-term depression (LTD) [7]. Such correlation has also been found in zebra finches, birds that learn their characteristic song at the juvenile stage and do not change it throughout life. Ng expression in the song areas of the zebra finch brain increases rapidly during the learning period and decreases drastically when learning has ended [13]. Another condition in which brain Ng and cognitive performance are closely related is hypothyroidism, an endocrine disorder where the thyroid gland produces insufficient levels of thyroid hormones (THs). Hypothyroidism is associated with alterations in dendrite spine morphology and synaptic transmission in the hippocampus [14], and Ng reductions of up to 60% in some brain regions [15, 16]. Sleep deprivation, which impairs the formation and consolidation of new memories and compromises learning [17, 18], induces severe Ng reductions in the rat cerebral cortex and hippocampus [19]. Aging is characterized by cognitive decline and the deterioration of synaptic function in several forebrain regions [20]. Studies undertaken in aging mice and rats have shown that brain Ng levels decline with age, in parallel with severe impairment of LTP and memory [21, 22]. More recently, it has been shown that Ng levels are elevated in the cerebrospinal fluid (CSF) of people suffering from Alzheimer's disease (AD) [23, 24] and mild cognitive impairment (MCI) [25], a condition that precedes the complete development of AD symptoms. Several studies have confirmed a good correlation between the severity of cognitive impairment and a loss of synaptic Ng [26, 27]. In fact, CSF Ng content is quickly becoming a reliable biomarker of early synaptic degeneration and cognitive deterioration, which makes it an excellent prognostic marker for AD and other cognitive disorders [28].

In summary, Ng deficits do not induce important anatomical or physiological alterations, but specifically lead to cognitive performance impairments. These features, namely its expression restricted to the forebrain and its functional specificity, make Ng a neat target of strategies to prevent, alleviate, or even recover from conditions of impaired cognition, particularly during their earliest phases. Moreover, targeting Ng expression should lack the undesired effects that are common in other strategies. For this reason, we need to study in greater depth the molecular mechanisms that modulate expression of Ng.

In the present work, we used cultured hippocampal neurons to study the expression of Ng. We found low levels of Ng expression that could be enhanced in the presence of glial cells or cholesterol. In the long term, Ng expression needed the activity of NMDA receptors whereas, in the short term, chemical LTP (cLTP) led to both Ng proteolysis and new synthesis, most likely from the local translation of dendritic Ng mRNA. Restoring Ng levels to those of the adult hippocampal tissue by lentiviral transduction greatly increased the proportion of cultured neurons expressing Ng and led to almost twice the density of excitatory and inhibitory synapses in the cultures. In summary, we showed that Ng expression is regulated by synaptic activity and promotes synaptogenesis in cultured hippocampal neurons.

Materials and Methods

Animals and Ethics Compliance

Wistar rats were bred at the in-house Animal Core Facility of the 'Centro de Biología Molecular "Severo Ochoa" (Madrid, Spain). All procedures were carried out in accordance with the Spanish Royal Decree 1201/2005 for the protection of animals used in scientific research, and the European Union Directive 2010/63/EU regarding the protection of animals used for scientific purposes. The procedures were approved by local Ethical Committees.

Reagents

Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), 0.25% trypsin, Neurobasal, and B27 media were from Invitrogen, and protease inhibitor cocktail was from Biotools (B14001). Total protein was measured using a Bradford Protein Assay kit (BioRad). Pre-stained protein marker VI (10–245 kDa) was from PanReac-Applichem, and Immobilon-P membranes and ECL western blotting reagent were from Millipore. Oligonucleotides were purchased from IDT (Integrated DNA Technologies) and Sigma. 1-Beta-arabino-furanosylcytosine (AraC) was purchased from Calbiochem (#251010). Cholesterol and methyl-beta-cyclodextrin (MCD) were from Sigma. D-2-Amino-5-phosphonovalerate (AP5), memantine hydrochloride, N-methyl-D-aspartic acid (NMDA), (+)-MK-801 maleate, tetrodotoxin (TTX), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo [f]quinoxaline-7-sulfonamide disodium salt (NBQX), ifenprodil, and (−)-bicuculline methiodide (BIC) were from Tocris. Strychnine was from Sigma (S0532), and paraformaldehyde (PFA) was from Merck. The antibodies used in this study are shown in Supplementary Table 1.

Lentivirus Constructs

For heterologous expression of Ng in cultured hippocampal neurons, we used lentiviral particles prepared from the vector pLOX-Syn-Ng. Starting from the previously described vector pLOX-Syn-DsRed-Syn-GFP [29], the pLOX-Syn-Ng

lentiviral vector (Syn-Ng) was constructed, eliminating the DsRed-Syn-GFP fragment, and inserting a cDNA corresponding to the rat Ng sequence, between the BamHI and EcoRI sites. For packing the lentiviral particles, HEK 293T cells in 10-cm dishes were transfected with 15 μg of pLOX-Syn-Ng, 15 μg of pCMVδR8.74, and 2.25 μg of pMD2.G using the calcium phosphate method. Five hours later, the medium was replaced with Neurobasal + B27 media, and at 24, 48, and 72 h post-transfection, the culture medium was collected, centrifuged, filtered (0.45 μm), titrated, and kept in aliquots at − 80 °C.

Hippocampal Neuron Cultures

Cultures of rat hippocampal neurons were prepared as previously described [30]. Coverslips were cleaned in 65% nitric acid overnight, extensively washed with distilled water and heat sterilized at 180 °C in an oven. Culture dishes and coverslips were coated with poly-L-lysine (at 0.075 and 0.15 mg/ml, respectively) in sodium borate buffer (25 mM, pH 8.5) for at least 24 h. On the day of culture setup, day in vitro 0 (DIV0), coverslips and dishes were rinsed twice with sterile milliQ water and maintained in DMEM supplemented with 10% FBS, 1 mM pyruvate and 10 mM D-glucose. After euthanizing an 18-day-pregnant rat with CO_2, embryos were collected and maintained at 4 °C in Hanks medium without Ca^{2+} and Mg^{2+} (incomplete Hanks) containing 138 mM NaCl, 5.33 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 5.56 mM D-glucose, and 10 mM HEPES (pH 7.4). Then, the brains were extracted in a horizontal flow hood and, with the help of a stereomicroscope, the hippocampi dissected and transferred to fresh incomplete Hanks. After extensive washing with incomplete Hanks, the hippocampi were incubated for 15 min in a 0.25% trypsin solution at 37 °C, then carefully washed twice with incomplete Hanks and once with complete Hanks (containing 1.25 mM $CaCl_2$ and 0.8 mM $MgSO_4$). The hippocampal cells were then mechanically dissociated with the aid of a fire-polished Pasteur pipette in the presence of 0.04 mg/ml DNAse-I. The suspension was centrifuged (200×*g*, 5 min) and the cells in the pellet resuspended, counted, and plated in culture dishes at an appropriate density (P35 35,000 cells/cm^2, coverslips 10,000 cells/cm^2). After 3–4 h in the CO_2 incubator to allow cell adhesion to the substrate, the medium was replaced by neuronal growth medium (Neurobasal with 2% B27 and 1% GlutaMAX), and cultures were maintained for up to 21–25 days in vitro. When appropriate, on DIV1, some dishes were transduced with previously titrated lentiviral particles (Syn-Ng). At DIV3, when appropriate, AraC (cytosine arabinoside, 1 μM) was added to the culture medium to prevent glial cell growth. After replacing the water lost by evaporation, 50% of the culture medium was replaced by fresh medium first at DIV7 and then every 3–4 days. The glia-conditioned medium used in this study was collected from astrocyte monolayers [31] cultured in neuronal growth medium for 36 h.

Long-Term Drug Treatments and Induction of Chemical LTP and LTD

Induction of cLTP or chemical LTD (cLTD) in hippocampal neurons was performed in the third week of culture and carried out at 37 °C, using procedures based on previously described protocols [32, 33]. At DIV18, the growth medium was collected and replaced by artificial cerebrospinal fluid (ACSF) without Mg^{2+} (132 mM NaCl, 4 mM KCl, 1.6 mM $CaCl_2$, 20 mM D-glucose, 20 mM HEPES; pH 7.3). Fifteen minutes later, 200 μM glycine, 20 μM BIC, and 1 μM strychnine were added and incubated for 3 min. Finally, cultures were returned to their original medium to start recovery. cLTD was induced using the method described in [34], which involves a 3-min incubation with 30 μM NMDA and subsequent recovery by returning the cultures to their original medium.

Collection of Cellular Extracts and Western Blot

Rats of different ages were killed by CO_2, and working at 4 °C, their brains were quickly removed and cleaned from meninges and blood clots. Dissected hippocampi were weighed, homogenized (1:5 *w*/*v*) in a solution containing 50 mM NaCl, 0.5% Triton X-100 (TX-100), 5 mM DTT, 3 mM EDTA, protease inhibitor cocktail, and 50 mM Tris-HCl (pH 6.8) and centrifuged at 17,500×*g*, for 20 min at 4 °C. Cultures of hippocampal neurons were harvested in lysis buffer (0.1% SDS; 5 mM DTT, 1 mM EDTA, protease inhibitor cocktail, 20 mM Tris-HCl; pH 6.8), homogenized by 10 passages through a 23 gauge needle, centrifuged at 17,500×*g*, for 15 min and supernatants collected. Total protein was measured and aliquots were mixed with electrophoresis sample buffer, heated for 3 min at 90 °C, separated by SDS-PAGE, and processed by western blot [35]. Immunoreactive bands were visualized using HRP-conjugated secondary antibodies (Jackson Immunoresearch, 1:25,000) and ECL detection was performed with an Amersham Imager 600 (GE Healthcare Life Sciences).

Subcellular Fractionation

After a quick rinse with PBS, cell cultures were harvested in chilled medium containing 0.32 M sucrose, 1 mM EDTA, protease inhibitor cocktail, and 10 mM MOPS (pH 7.4). The extracts were homogenized by 10 passages through a 23 gauge needle and centrifuged at 1300×*g* for 5 min at 4 °C to obtain pellet 1 (P1) and supernatant 1 (S1). P1 was resuspended and centrifuged again in the same conditions to obtain the nuclear pellet, whereas S1 was centrifuged at 15,000×*g* for 15 min at 4 °C to obtain the synaptosomal pellet (P2) and

cytosolic supernatant (S2). S2 was mixed with eight volumes of cold acetone, kept overnight at − 20 °C, and centrifuged at 5000×*g* for 15 min at 4 °C. After removing the acetone, the pellet was dried and stored at − 20 °C.

Post-synaptic densities (PSDs) fractions were purified essentially as described [36] from hippocampal neuron cultures that were kept in control medium or treated with tetrodotoxin (0.5 μM) or bicuculline (25 μM) for 48 h.

RNA Extraction and qRT-PCR

Total RNA samples were prepared from freshly dissected tissue or extracted from neuronal cultures using Tripure (Roche) isolation reagent. For quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), a 1-μg sample of total RNA was retrotranscribed and analyzed by qPCR in a LightCycler 480 (Roche) using the oligonucleotides 5′-AACACCGGCAATGGACTG-3′ and 5′-GGGATGTCTAGAATATCGTCGTCT-3′, which were designed using ProbeFinder (Applied Biosystems). As an endogenous control, we used GAPDH.

Immunofluorescence

For cultured cells, the IF procedure started with a quick rinse in PBS, cell fixation with 2% PFA/PBS for 10 min and an additional 5-min incubation in 0.1 M glycine/PBS to inactivate free aldehydes. Blocking of non-specific binding and permeabilization was achieved by incubating in blocking buffer (5% heat-inactivated horse serum, 0.2% (*w*/*v*) TX-100 in PBS) for 30 min. Primary and secondary antibody incubations were done in blocking buffer overnight at 4 °C and for 60 min at room temperature, respectively, followed by three 5-min washing steps in PBS. To visualize cell nuclei, an additional incubation with To-Pro3 (1:500, 30 min) or DAPI (1:5000, 5 min) was performed, followed by extensive washing. Finally, coverslips were rinsed in distilled water and mounted in Mowiol; negative controls were prepared by omitting the primary antibody.

Cultured hippocampal neurons were stained to quantify Ng-expressing cells, using rabbit anti-Ng (1:2000) and mouse anti-NeuN (1:1000) as primary antibodies, and donkey anti-rabbit-Alexa555 (1:2000) and goat anti-mouse-Alexa488 (1:1000) as secondary antibodies. Excitatory synapses were quantified using mouse anti-PSD95 (1:250), guinea pig anti-vGluT1 (1:30,000), and rabbit anti-MAP 2 (1:1000) with anti-mouse-Alexa488 (1:1000), anti-guinea pig-Alexa555 (1:2000), and anti-rabbit-Alexa647 (1:500) antibodies. For inhibitory synapses, we used sequential incubation with mouse anti-GAD6 (1:20,000) and rabbit anti-MAP 2 (1:1000), goat anti-mouse-Alexa488 (1:1000) and anti-rabbit-Alexa647 (1:500), and, finally, mouse anti-gephyrin-Oyster555 (1:1000). Additionally, rabbit anti-GluN1 (1:100) and guinea pig anti-MAP 2 (1:10,000) were used to identify excitatory neurons and mouse anti-GFAP (1:1000) to label astrocytes.

Adult Wistar rats were anesthetized with a mixture of xylazine and ketamine and then perfused through the heart with 4% PFA fixative prepared in 0.12 M NaH_2PO_4 (pH 7.3) buffer (PB). All efforts were made to minimize animal suffering. After perfusion, brains were removed and immediately immersed in ice-cold fixative. After 24 h in fixative, the brains were rinsed in PB and then either cut into 50-μm-thick sections with a vibratome (Leica VT1200S) or cryoprotected for 48 h in 20% sucrose, frozen and cut at 50 μm in a cryostat. The sections were washed in PB and incubated overnight at room temperature in rabbit anti-Ng antibody (1:1000) in blocking buffer (0.1% TX-100, 0.1% bovine serum albumin in PB) and rinsed with PB (five changes). After incubation with biotinylated anti-rabbit immunoglobulin G (Jackson, 1:500) for 2 h, sections were rinsed with PB (five changes), incubated with streptavidin-Alexa555 (Thermofisher, 1:500) for 2 h, and finally rinsed with PB (five changes). The sections were mounted on gelatinized slides, allowed to dry sufficiently, and then covered with Mowiol and coverslips; negative controls were prepared by omitting the primary antibody.

Imaging

Visual inspection and image acquisition were done using a motorized Zeiss Axiovert 200M microscope with a Photometrics Coolsnap FX monochrome CCD camera, XY-motorized stage, and MetaMorph 7.10.1 software (molecular devices). The "scan slide" application of MetaMorph was used for the automated acquisition of images. To count Ng-expressing neurons, a tile scan of 15 × 15 frames was captured using a 20× PlanApo oil 0.85 NA objective for each coverslip, in each of three channels: DAPI-470, NeuN-488, and Ng-555. To count synapses, we proceeded in the same way using a 40× NeoFluar oil 1.30 NA objective and imaged four channels: DAPI-470, PSD95-488 or GAD6-488, vGluT1-555 or gephyrin-555, and MAP 2-647. Captured images were automatically stitched and analyzed in MetaMorph, using two different procedures. To quantify cells expressing NeuN and Ng, we used the MetaMorph application "cell scoring." To identify and quantify synapses, we developed a MetaMorph journal that used a spatial matching criterion between presynaptic and post-synaptic markers, combined with a criterion of proximity to the neuronal dendritic tree defined by the MAP 2 marker, and an exclusion criterion defined by the area size expected for a synaptic contact. For these, we employed the MetaMorph applications "granularity" and "neurite outgrowth," respectively. A flowchart example of the procedure used to quantify synaptogenesis in hippocampal neurons in culture is shown in Supp. Fig. 11.

Statistical Analysis

Statistical comparisons were performed with paired or unpaired Student's *t* tests or two-way ANOVA as appropriate. The data are expressed as the mean ± SEM and *p* values less than 0.05 were considered significant.

Results

Ng Is Expressed in a Small Fraction of Hippocampal Neurons

The developmental profiles of synapsin, CaMKIIα, β, and other synapse-related proteins (Fig. 1a and Supp. Fig. 1) were analyzed in cultured hippocampal neurons. In general, there was a good correlation between the temporal expression profiles observed in cultured neurons and those of developing hippocampal tissue (Fig. 1b). Most synaptic proteins appeared during the second week in culture and reached maturity levels during the third week [37, 38]. Ng expression in hippocampal neurons was very low in the first week, increased strongly in the second week, and reached peak levels in the third week of culture. Only CaM profiles were found to differ in both preparations, very likely reflecting its ubiquitous expression in neurons, glia, and many other cell types in the tissue. Over the course of the study, we realized that unlike other neuronal proteins such as β3-tubulin, synapsin, or GAP-43, Ng expression levels in hippocampal neurons were very low when compared with its levels in the adult hippocampus (Fig. 1c). Using semi-quantitative western blot analysis, we estimate that the relative abundance of Ng in hippocampal neurons represented about 10% of the Ng present in intact adult hippocampus (Fig. 1d). Furthermore, Ng mRNA levels in the cultures displayed the same profile found for Ng protein, accounting for less than 10% of the mRNA levels measured in the adult hippocampus (Fig. 1d). These results suggest that the low levels of Ng observed in the cultures are due to a reduction in either the transcription or stability of Ng mRNA.

It is known that in hippocampal neuron cultures, pyramidal neurons account for the vast majority of the total neuronal population [30] and GABAergic neurons represent about 6% of the total [39]. Indeed, we analyzed the percentage of excitatory and inhibitory neurons in the cultures using antibodies to NMDA receptor 1 (GluN1) and glutamate decarboxylase (GAD), respectively and found that more than 80% of the neurons were excitatory (Supp. Fig. 2). Moreover, using the excitatory presynaptic marker vGluT1, we observed a dense network of excitatory terminals in DIV16 hippocampal neurons, suggesting a great density of excitatory synapses. (Supp. Fig. 3). Thus, it was not clear whether the overall low expression of Ng was due to a general low expression in the majority

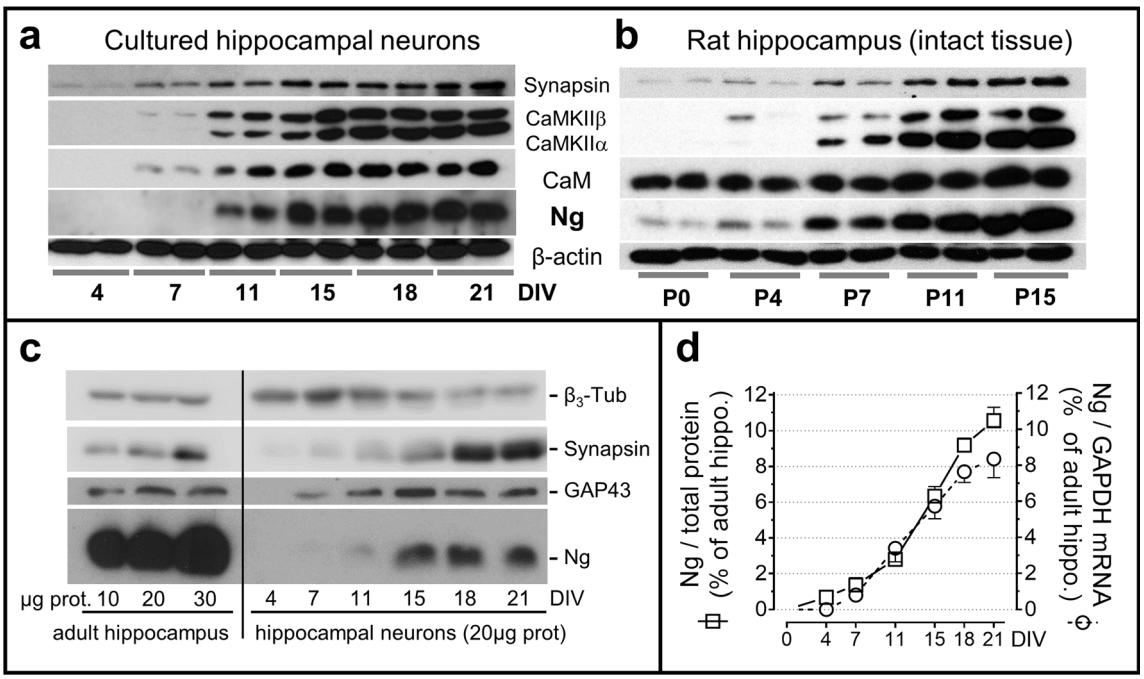


Fig. 1 Level of Ng expression in the rat hippocampus and cultured hippocampal neurons. Cultured hippocampal neurons or hippocampal tissues were sampled at several developmental times and processed for western blot or qRT-PCR. The developmental profile for Ng and other proteins is shown in **a** cultured neurons after different days in vitro (DIV) and **b** intact tissue from rats at different postnatal days (P0-P15). **c** The different expression levels of Ng in adult hippocampal tissue and cultured neurons are highlighted. **d** Quantitative representation of Ng expression in cultured neurons through 3 weeks in culture. The presented values are a percentage of the expression in adult hippocampal tissue, for both protein (square symbols, $n = 10$) and mRNA levels (circles, $n = 3$); plots indicate mean ± SEM

of cultured cells, or rather that Ng was expressed at tissue levels but only in a few of the cultured neurons. Ng expression was analyzed by IF, revealing that none of the Ng-positive cells showed GAD expression and vice versa (Fig. 2a), indicating that Ng is not expressed in GABAergic neurons but are solely expressed in excitatory neurons, in good agreement with previous evidence showing that Ng is expressed by principal cells but not interneurons [40]. Additionally, we observed that only a small proportion of the total number of cells in the culture actually expressed Ng (Fig. 2b and Supp. Fig. 4).

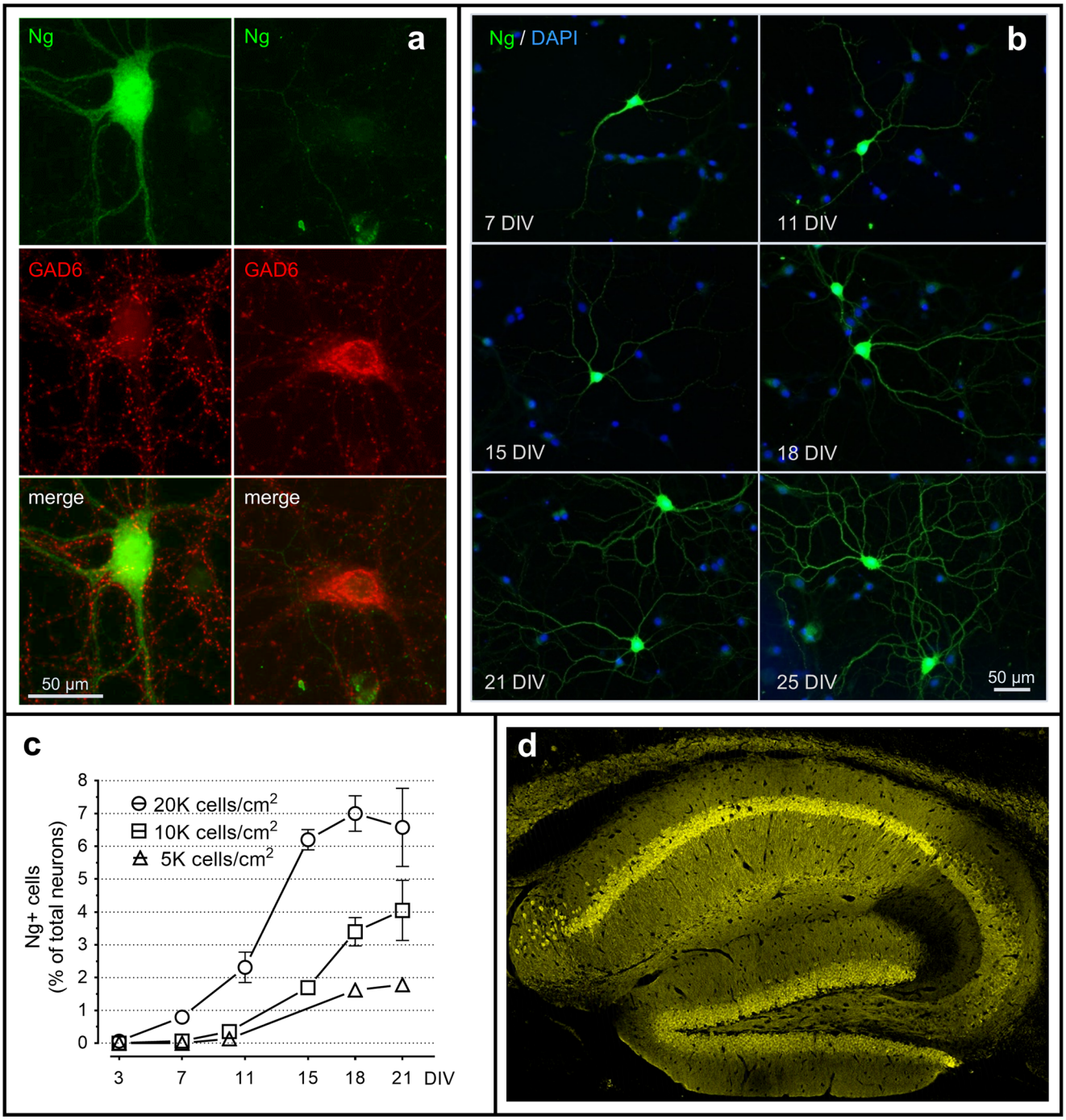


Fig. 2 Ng expression is restricted to a small fraction of excitatory neurons. **a** Immunofluorescence showing neurons expressing Ng (left panel) or glutamate decarboxylase (GAD, right panel), labeled with anti-Ng and anti-GAD-6 antibodies, confirming a lack of co-localization. **b** Maturation of Ng-expressing neurons in culture over time. Note the low number of Ng-positive cells relative to the total number of cells (DAPI stained) in each frame. **c** Quantitation of the percentage of Ng-expressing neurons in cultures grown at 5000, 10,000, or 20,000 cells/cm^2, plotting the mean ± SEM ($n = 3$). The low percentage of Ng-expressing neurons in cell culture is in stark contrast with **d** its widespread expression in the pyramidal and granular layers of the adult hippocampus

These Ng-positive cells were barely seen during the first week of culture but were easily distinguished later in the second and third weeks in culture due to their high expression, quite similar to that observed in the pyramidal layer of the hippocampus (Fig. 2d). In many cases, the localization of Ng to presumptive dendritic spines could be easily seen (Supp. Fig. 5). To get a quantitative estimate of the percentage of Ng-expressing neurons, hippocampal cells were grown at three different densities and then processed for IF with antibodies against Ng and NeuN (a neuronal marker) and stained with DAPI. Using an automated image capture and analysis procedure (see "Methods" and Supp. Fig. 6), we first confirmed that more than 75% of the cells present in the cultures at each density were actually neurons. All Ng-expressing cells were also positive for NeuN, indicating that Ng is expressed exclusively in neurons. Further, the proportion of Ng-expressing neurons did not exceed 8% of the total number of neurons at any maturation stage or neuronal density tested (Fig. 2c), which fits well with the profile obtained for Ng protein and mRNA levels (Fig. 1d), both in shape and relative magnitude. These results indicate that the low overall Ng expression observed in hippocampal cultures is due to expression in only a small fraction of the neurons, albeit at relatively high levels similar to that of hippocampal tissue. Such heterogeneity in Ng expression among cultured neurons was not expected, since none of the other synaptic proteins analyzed, such as GAP-43 or synapsin, and exhibited such different expression levels between neuronal cultures and intact tissue.

Ng Expression Depends on Cell Density

One big difference between primary cultures and intact tissue is their cell density. We suspected that cell density might actually affect Ng expression from our data in Fig. 2c, which showed that the fraction of Ng-expressing neurons decreases with cell density. Following this thread, we tested the effect of cell density on Ng expression (Fig. 3a), showing that higher densities indeed led to increased Ng levels during the second and third weeks in vitro. However, Ng levels tended to equalize by the end of the third week. The onset of Ng expression was observed 3 days earlier in higher density cultures, whereas lower cell density cultures featured a similar 3-day delay. It should be noted that cell density affects the maturation rate of the cultures, as could be appreciated by visual inspection of the neuronal morphology and the timing of expression of other synaptic proteins (data not shown). Additionally, it is known that density affects network connectivity and spontaneous activity in cultured hippocampal neurons [41], which in turn could affect Ng expression. We also tried cell densities above 70,000 cells/cm^2 and below 17,000 cells/cm^2, finding lower levels of Ng expression in both cases. Densities of 100,000 cells/cm^2 and above resulted in a dramatic decay of neuronal survival in the second week of culture, whereas densities below 5000 cells/cm^2, resulted in great numbers of immature neurons and cell death (data not shown).

Ng Expression Is Modulated by Astrocytes

Another important difference between cultures and intact tissue is the presence of glial cells. It is well known that astrocytes promote neuronal growth, survival, and synaptogenesis [42, 43]. Addition of the mitotic inhibitor AraC to the cultures inhibits cell proliferation. Without AraC, the cells that proliferate most are astrocytes, whose growth can be recognized by visual inspection in the second and third weeks of culture. We measured Ng levels in mixed cultures, with no added AraC, and found a significant increase when compared with AraC-treated cultures (Fig. 3b), indicating a positive effect of astrocytes. To investigate whether cell contact was required, AraC-treated cultures were grown in glia-conditioned medium from DIV7 and Ng content was analyzed at DIV18 (Fig. 3c). A clear increase in expression was found, pointing to an involvement of soluble factors secreted by astrocytes to the medium. It is known that one of these factors is astrocyte-derived cholesterol, which promotes presynaptic function, transmitter release [44], and contributes to dendrite development and synaptogenesis [45]. Thus, we explored the effect of cholesterol on AraC-treated cultures, finding that it promotes a clear increase in Ng expression (Fig. 3d). Interestingly, synaptogenesis and Ng expression share nearly identical developmental profiles in both intact tissue [2] and primary cultures [37]. Since cholesterol enhances both, we hypothesized that Ng expression, synaptogenesis, and thereby synaptic activity are related. To test this, we cultured hippocampal neurons in BrainPhys™, a chemically defined medium that improves neurophysiological activity and increases the proportion of neurons that are synaptically active [46]. Hippocampal neurons grown in BrainPhys from DIV7 showed an enhanced expression of Ng and other synaptic markers (Fig. 3e), thus supporting a relationship between synaptic function and Ng expression.

A number of alternative scenarios and signaling mechanisms that may be involved in the regulation of Ng expression were evaluated. Ng is a brain target of THs and 9-cis-retinoic acid action [47, 48]. However, short- and long-term treatments (from 6 h to 7 days) with the TH T3 (50 nM), 9-cis-retinoic acid (1 μM), or the combination of both did not affect Ng expression in the third week of culture (data not shown). We were not surprised by these findings since Ng expression is not regulated by TH in the pyramidal layers of the hippocampus [16, 49], which is the major source of neurons in our cultures. Several growth factors including neurotrophins and fibroblast growth factor have been linked to the formation of functional synapses between cultured hippocampal neurons [50, 51]. We tried short- and long-term treatments using brain-derived neurotrophic factor (BDNF, 50 ng/ml), the

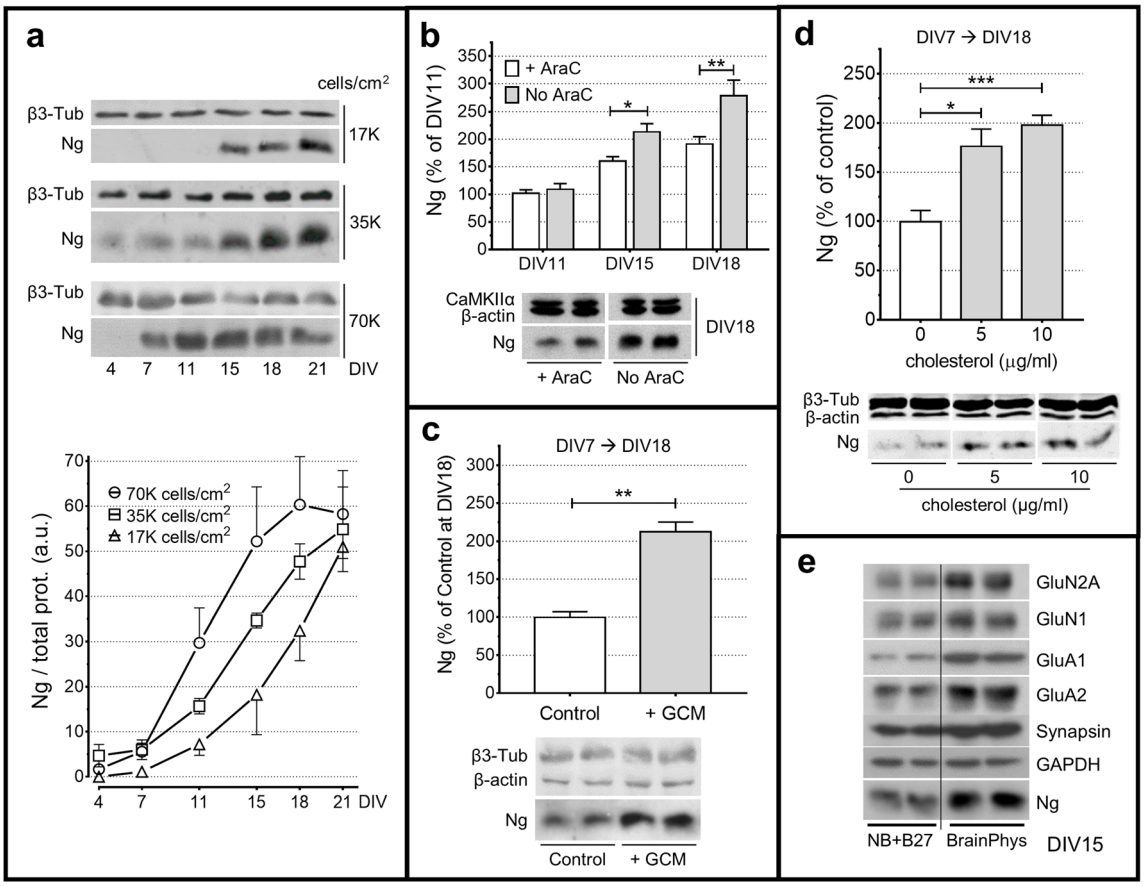


Fig. 3 Ng expression increases under conditions that promote synaptic activity. **a** Hippocampal neurons grown at 17,000, 35,000, and 70,000 cells/cm^2 were harvested after different days in vitro (DIV) and analyzed by western blot (top panel). The ratio of Ng to total protein obtained for each culture is plotted over time (mean ± SEM, $n = 3$). **b** Hippocampal neurons were treated with 1 μM 1-beta-arabino-furanosylcytosine (+ AraC) or not (No AraC) at DIV3 and harvested at DIV11, 15 or 18. Ng content is expressed as a percentage of the mean value obtained at DIV11 in cultures treated with AraC (mean ± SEM, $n = 4$). An example blot image illustrates the comparison of AraC- and No AraC-treated cultures at DIV18. **c** Hippocampal neurons were cultured from DIV7 with glia-conditioned medium (GCM). Lysates from treated and non-treated cultures were obtained at DIV18, and Ng content expressed as a percentage of the values obtained in non-treated cultures (mean ± SEM, $n = 7$). **d** Hippocampal neurons were treated with specified concentrations of cholesterol at DIV7 and maintained until DIV18, when they were harvested and analyzed for Ng content. Values are expressed as a percentage of the non-treated controls (mean ± SEM, $n = 4$). **e** Hippocampal neurons were grown from DIV7 in BrainPhys medium or Neurobasal + B27 medium and harvested at DIV15. The relative content of several synaptic proteins was analyzed by western blot: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

high-affinity TrkB agonist 7,8-dihydroxyflavone (DHF, 0.5 μM) [52], nerve growth factor (NGF, 50 ng/ml), neurotrophin-3 (NT3, 50 ng/ml), basic fibroblast growth factor (bFGF, 50 ng/ml), and epidermal growth factor (EGF, 50 ng/ml). None of these treatments consistently altered the amount of Ng present in the cultures extract (data not shown).

Ng Expression Is Regulated by Synaptic Activity

Next, we focused on how short- and long-term changes in synaptic activity affected Ng expression. Cultured hippocampal neurons develop spontaneous electrical activity that gradually increases from the end of the first week throughout the second and third weeks in vitro [41]. The spiking activity can be inhibited by TTX, a sodium channel blocker that blocks action potentials, or by glutamate receptor antagonists, and can be upregulated by BIC, a γ-aminobutyric acid type A (GABA-A) receptor antagonist that blocks the major inhibitory input. These reagents, when maintained in the culture for prolonged periods, can trigger homeostatic plasticity mechanisms, the best known being synaptic scaling [53]. We tested the effects of TTX (1 μM), AP5 (50 μM, a competitive NMDA receptor antagonist), and NBQX (10 μM, a competitive AMPA receptor antagonist), either alone or in several combinations. Initially, the treatment period was chosen to start at DIV7 and end at DIV18, since this would include both the onset and maturation of Ng expression (Fig. 4a). Both TTX and AP5 independently reduced Ng content to less than 50% of the controls, and their combination had a seemingly additive effect. NBQX, which blocks a large fraction of the excitatory post-synaptic currents and leads to a reduction in spiking activity [54], did not reduce Ng expression. We then

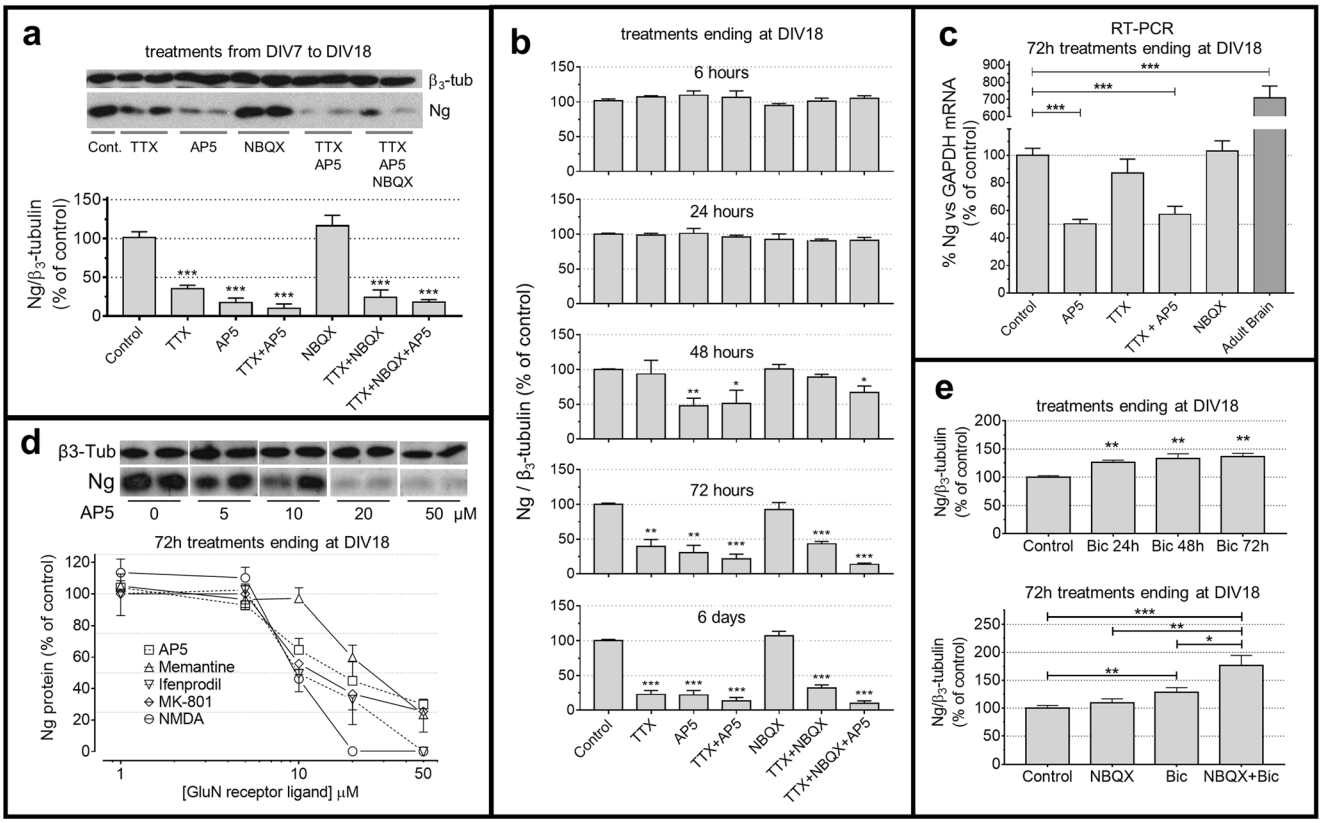


Fig. 4 Long-term manipulation of endogenous synaptic activity modulates Ng expression. **a** Hippocampal neurons were treated after 7 days in vitro (DIV7) with 1 μM TTX, 50 μM AP5, or 10 μM NBQX, either alone or in several combinations, and then harvested at DIV18 to analyze Ng content by western blot. Upper panel shows a western blot of a typical experiment. The lower panel shows the quantitative results obtained. The ratio of Ng:β3-tubulin was normalized to the ratio obtained for the untreated controls (mean ± SEM, $n = 4$). **b** Hippocampal neurons were treated as in (**a**) for the indicated periods, starting at different times and all of them collected at DIV18 for western blot analysis. Data are ratios of Ng:β3-tubulin normalized to the ratio obtained for the untreated controls (mean ± SEM, $n = 3$). **c** Hippocampal neurons treated as in (**a**) starting from DIV15 were collected at DIV18 for RT-PCR analysis. Data shown are Ng:GAPDH mRNA ratios, normalized to the untreated controls (mean ± SEM, $n = 5$). **d** Hippocampal neurons were treated with NMDA, or several NMDA receptor antagonists at 1, 5, 10, 20, or 50 μM starting at DIV15, and collected at DIV18 for western blot analysis. Upper panel shows a western blot of a typical experiment using AP5. The lower panel shows the quantitative results obtained. Ng content is expressed as a percentage of the levels in untreated controls (mean ± SEM, $n = 4$). **e** Hippocampal neurons were treated with (upper panel) 25 μM bicuculline (BIC) for 24, 48, or 72 h (mean ± SEM, $n = 4$) or with (lower panel) 25 μM BIC, 10 μM NBQX, or both for 72 h (mean ± SEM, $n = 10$), and then harvested at DIV18 for western blot analysis. Ng content is expressed as Ng:β3-tubulin ratio normalized to untreated controls: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

determined the effects of shorter treatments, first realizing that alterations of Ng content were only seen in the third week of culture. Treatments with TTX, AP5, or NBQX that end in the second week (DIV11) did not affect Ng levels, regardless of their duration (data not shown). In the third week of culture, we found that the treatments that significantly decreased Ng levels (TTX and AP5) required at least 48 h of drug exposure (Fig. 4b and Supp. Fig. 7). Then, we measured Ng mRNA levels following 72-h treatments in DIV18 cultures (Fig. 4c), showing that treatment with AP5 led to a 50% reduction of Ng mRNA levels, whereas NBQX and TTX showed no significant alteration. We then analyzed the protein content of postsynaptic densities (PSDs) purified from cultures treated with either TTX or BIC. We found that GluN1 and GluN2B content decreased after 48-h treatment with BIC, whereas PKCε levels did not change (Supp. Fig. 8), in good agreement with previous studies [55]. These results support the notion that NMDA- and not AMPA- receptor activity is needed for Ng expression. Besides, an NMDA receptor blockade most likely determines reduced transcription or decreased stability of Ng mRNA. To assess the involvement of NMDA receptors in Ng expression, we performed dose-response assays using MK-801 and memantine (activity-dependent channel blockers) and ifenprodil, which acts at the polyamine-binding site and is more selective for the GluN2B subunit. All of them exhibited similar potency in reducing Ng after a 72-h treatment (Fig. 4d). Next, we thought that the decreased Ng expression observed after prolonged treatment could be a side effect of reduced or compromised metabolism. To rule this out, we exposed cultures to AP5, TTX, or NBQX for up to 72 h and checked whether Ng expression recovered when the cultures were returned to normal growth medium and analyzed 3 days

later. Cultures exposed to AP5 but not TTX regained control Ng levels at DIV21 (Supp. Fig. 9). However, TTX-treated neurons showed signs of morphological deterioration (dendritic retraction, reduced arborization, varicosities) after recovery at DIV21, suggesting that 72-h TTX treatments were stressful and compromised long-term neuronal survival (data not shown). No changes in Ng content were observed during NBQX treatments or subsequent recovery (Supp. Fig. 9).

We hypothesized that if antagonism of NMDA receptors decreased Ng expression, their activation should potentiate it. We first tested NMDA, and although a very slight increase in Ng was observed at concentrations below 10 μM (Fig. 4d), higher concentrations negatively affected neuronal morphology and survival, as expected. So we tried increasing the endogenous excitatory activity by means of BIC treatment, which increases the firing rates of cultured neurons [56]. We found that neurons could not withstand 50 μM BIC treatments for long periods (48–72 h), and it did not affect Ng expression at concentrations of 15 μM or lower (data not shown). Therefore, we tried BIC at 25 μM, which proved to be compatible with long-term neuronal survival inducing a substantial increase in excitatory activity [56]. In these conditions, BIC treatments induced a small but significant increase in Ng levels (+ 30%) as early as 24 h (Fig. 4e). Interestingly, when BIC was combined with NBQX, a greater increase of Ng expression was observed. These results support the notion that an increase in excitatory activity positively modulates Ng expression in the long term. At the same time, they reveal the thin line that exists between plasticity and excitotoxicity in cultured hippocampal neurons. The increased expression observed by the combination of BIC and NBQX may be explained by a more efficient NMDA receptor activation, possibly facilitated by the anti-excitotoxic action of NBQX. In summary, these results show that NMDA receptor inhibition leads to decreased Ng expression and that increases of excitatory activity increase it, suggesting that Ng expression is regulated by synaptic activity mediated by activation of NMDA receptors.

Induction of cLTP Alters Ng Expression

Next, we analyzed the effect of short-term changes of synaptic activity on Ng expression, using established protocols to induce cLTP [32, 33] and cLTD [34] in our cultures. Within 15 min of cLTP induction, Ng levels were drastically reduced to almost 50% of controls, followed by a gradual recovery in the following 2–4 h, whereas induction of cLTD did not significantly affect Ng content measured in the following 4 h (Fig. 5a). The changes of Ng content observed after cLTP indicate an underlying initial proteolysis with subsequent stimulation of new synthesis to regain control levels. It is known that excitatory activity triggers proteolytic events in the synaptic environment that are critical for synaptic remodeling, learning, and memory [57]. To test this, we measured Ng after cLTP in the presence of protease inhibitors. Calpeptin, a cell-permeable calpain inhibitor, totally prevented the Ng decline observed 15 min after cLTP (Fig. 5b). In addition, a significant increase above the pre-cLTP control level was observed 4 h later, suggesting that cLTP triggers calpain activation, which in turn cleaves Ng. This notion is also supported by recent data showing that Ng fragments present in CSF are mostly generated by calpain-1 cleavage [58]. MG132, a selective proteasome inhibitor, was also tested but was not equally effective in preventing the cLTP-mediated loss of Ng (Supp. Fig. 5a). Next, we challenged our hypothesis using two well-known protein synthesis inhibitors: cycloheximide (CHX) and anisomycin. Both inhibitors completely prevented the Ng recovery after cLTP (Fig. 5b, and Supp. Fig. 10a), indicating that new Ng synthesis must take place to regain control levels after cLTP induction. Thus, these results showed that cLTP induction in hippocampal neurons triggers fast calpain-mediated proteolysis of Ng, followed by a slow recovery that requires translation of Ng mRNA. As expected, both AP5 and NBQX independently inhibited the changes induced by cLTP on Ng proteolysis and new synthesis, whereas (S)-α-methyl-4-carboxyphenylglycine (MCPG), a metabotropic GluR antagonist, did not (Fig. 5b).

Our next objective was to determine the subcellular localization of the proteolysis and new synthesis elicited by the induction of cLTP. Ng mRNA is transported to dendrites [59, 60] where its translation is likely regulated by synaptic activity [61, 62]. We measured Ng after cLTP in nuclear, synaptosomal, and cytosolic fractions. As shown in Fig. 5c, Ng recovered in the synaptosomal fraction exhibited the most characteristic profile, showing a decrease 15 min after cLTP followed by a recovery that was already evident 60 min after cLTP. The cytosolic profile followed a similar but much more attenuated time course, whereas Ng recovered in the nuclear fraction exhibited a distinct profile, increasing shortly after cLTP and then decaying slowly. These latter results fit well with our previous observation reporting a nuclear translocation of Ng in response to excitatory synaptic activity in acute hippocampal slices [63].

Ng Overexpression Enhances Synaptogenesis

Recent studies have proposed the involvement of Ng in the mechanisms underlying synaptogenesis and synaptic remodeling [62, 64]. The dramatic increase of Ng that occurs during the second postnatal week in rats and mice in several forebrain areas coincides with a developmental period characterized by rapid dendritic growth and very active synaptogenesis [2, 65]. Thus, we wanted to know if changes of Ng expression in cultured hippocampal neurons could influence the process of synaptogenesis. For that, we developed a lentiviral construct driving expression of Ng under control of the human synapsin

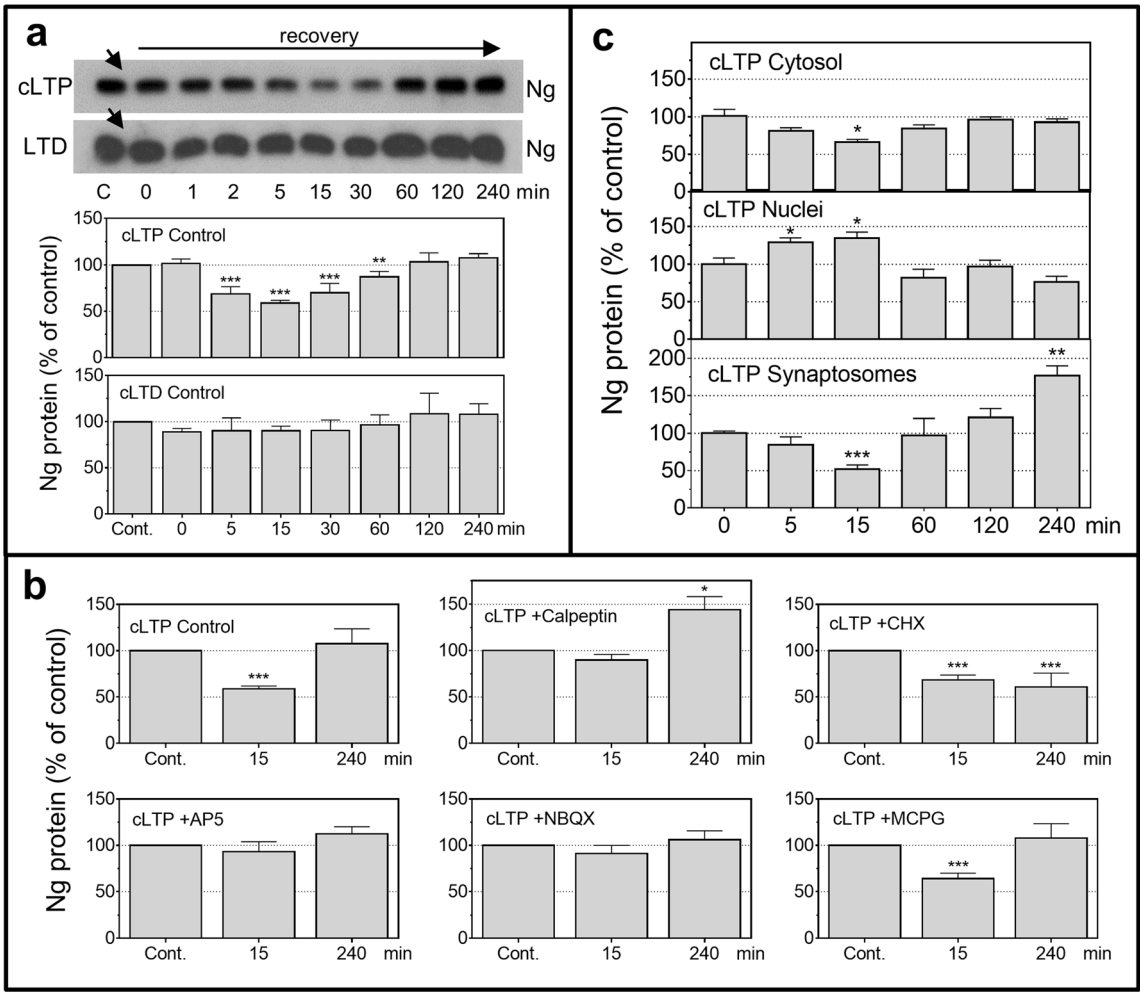


Fig. 5 LTP triggers Ng degradation and a new synthesis. **a** Cultured hippocampal neurons after 17 or 18 days in vitro (DIV) were processed for induction of chemical long-term potentiation (cLTP) or chemical long-term depression (cLTD). Lysates were obtained at indicated times of the recovery period and analyzed by western blot, with Ng content expressed as the percentage of levels in untreated control cultures (mean ± SEM, $n = 4$). **b** Hippocampal neurons were submitted to cLTP and sampled at 15 and 240 min; inhibitors (cycloheximide (CHX), 25 μg/ml; calpeptin, 10 μM; AP5, 100 μM; NBQX, 20 μM; and MCPG, 125 μM) were added 15 min before cLTP induction and maintained thereafter. Ng content is expressed as a percentage of the level measured in control cultures with no inhibitors added (mean ± SEM, $n = 4$). **c** Hippocampal neurons at DIV18 were submitted to cLTP induction and the lysates fractionated to obtain nuclear, cytosolic, and synaptosomal fractions for western blot analysis of Ng content. Results are percentages of the control values obtained before cLTP induction (mean ± SEM, $n = 3$): $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

promoter (Syn) (Fig. 6a top) [29]. In our cultures, the expression profiles of synapsin and Ng are very similar (Fig. 1a), so we expected that lentivirus-expressed Ng would also show a similar profile. Indeed, after transducing hippocampal neurons with lentiviral pLox-Syn-Ng (Syn-Ng) particles, Ng displayed a typical developmental profile but with levels 8–9 times higher than the corresponding controls, while other proteins such as CaM or CaMKIIα were not affected (Fig. 6a). Additionally, cultures transduced with Syn-Ng showed a much higher proportion of neurons expressing mature levels of Ng, with almost 70% Ng-positive neurons (Fig. 6b and c). We then measured the density of excitatory and inhibitory synapses during culture maturation by measuring the density of co-localizing PSD95 and vGluT1 and of GAD6 and gephyrin, respectively, which is close to the somatodendritic compartment. To do that, we followed the procedure illustrated in Supp. Fig. 11 and the "Methods" section. Typical captures for analyzing excitatory synapses are shown in Supp. Fig. 12. Both control and transduced cultures presented a similar synaptogenesis profile: excitatory synaptogenesis showed up at the end of the first week of culture, increased during the second week, and stabilized during the third week, in good agreement with previously reported data [38]. Inhibitory synaptogenesis showed a similar profile, but with a 4-day delay. However, cultures transduced with Syn-Ng showed a significant increase in the overall density of both excitatory and inhibitory synapses (Fig. 6d, f and e, g), with a maximal effect observed in the third week of culture, the time at which Ng

expression and synaptic density also displayed their maximum levels. These results therefore strongly support a close relationship between Ng expression and synaptogenesis in cultured hippocampal neurons.

Discussion

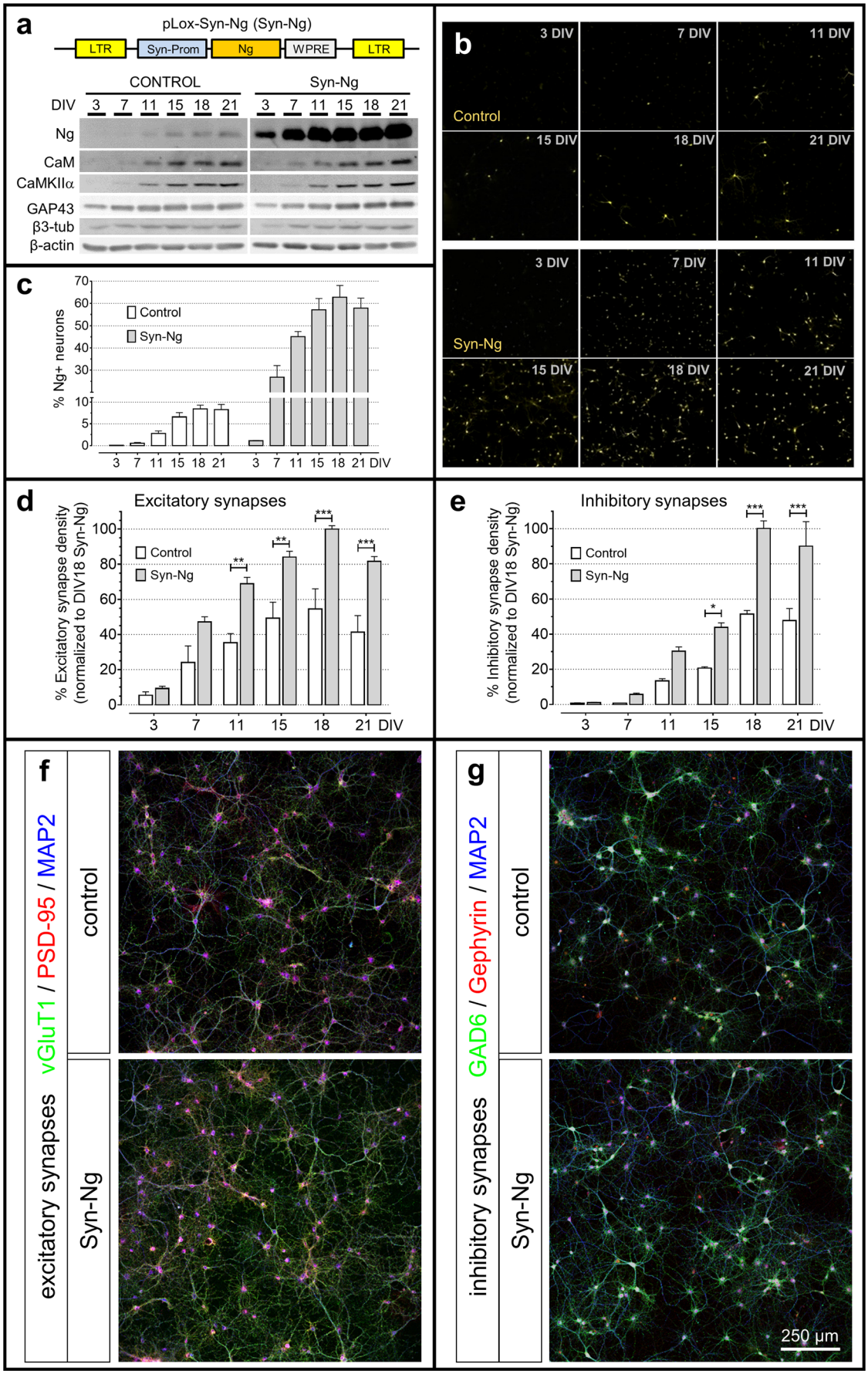
Our intellectual abilities rely on the functional and structural plasticity of the forebrain synapses. Ng is an abundant forebrain synaptic protein whose expression has been mostly studied in intact tissue [2, 3, 16]. These studies have revealed its regional distribution and developmental profiles, which are typically postnatal and coincident with periods of high synaptogenic activity. In adults, different evidences have shown that Ng expression is directly associated with cognitive performance. On the one hand, environmental enrichment enhances Ng expression and hippocampal learning and memory [66]. On the other hand, a number of pathologies featuring reduced cognitive performance are associated with low levels of Ng, such as hypothyroidism [67, 68], sleep deprivation [18, 19], aging [20], or AD [69]. Moreover, Ng-deficient mice show no obvious neuroanatomical abnormalities but demonstrate severe deficits in visual-spatial learning and LTP induction [7, 12]. Despite all this evidence, not much is known about the regulation of Ng expression. THs promote Ng expression [15], but not in all brain regions and cell types; they are ineffective in the upper layers of the cerebral cortex, the pyramidal layer of the hippocampus, and the amygdala [49]. Therefore, there must be additional mechanisms regulating Ng expression in these areas that are so closely associated with memory and learning.

In the present work, we have explored Ng expression in cultured hippocampal neurons and its relationship to synaptogenesis. These cultures are mostly composed of pyramidal neurons that display a well-known differentiation program [30] and establish synaptically active neural networks [70]. We found that the Ng expression profiles in cultures and in intact tissue are very similar. However, only 8–10% of the neurons expressed mature levels of Ng, whereas most neurons in the "stratum pyramidale" of the adult hippocampus strongly express Ng [2]. We believe that the low expression is due to reduced transcription since both Ng mRNA and protein showed similarly low levels (Fig. 1). Several results presented in this study support the notion that synaptic activity is involved in the regulation of Ng expression. First, long-term blockade of evoked release and NMDA receptor activity dramatically reduced Ng expression, both at the protein and mRNA levels. Second, higher cell culture density, which advances the onset of endogenous synaptic activity [41], led to increased Ng levels when analyzed in the period from DIV11 to DIV18. Third, conditions that promote synaptogenesis and synaptic activity such as co-culturing neurons with glial cells, the addition of glia-conditioned medium, cholesterol, or BrainPhys medium also increased Ng expression. Finally, long-term BIC treatment, which increases endogenous excitatory synaptic activity [56, 71], also resulted in a moderate but significant increase of Ng expression.

We must point out that, although we found that Ng expression increases with synaptic activity, we could not bring it up to the levels expected in the adult tissue. Disruption of the spatial organization of tissue by proteolytic and mechanical means to obtain dissociated neurons may be responsible. Synaptic connections in the hippocampus develop with exquisite precision and reliability [72], whereas synaptic connections in cultures are random and prone to develop autapses and recurrent circuits. Correct connectivity and development of the right patterns of synaptic activity could be needed to achieve physiological expression levels of Ng, as happens in the intact hippocampus. If this is the case, the synaptic activity would also play a fundamental role.

An important finding of this study is that Ng expression in the cultures depends on the activity of NMDA receptors. The results that support this claim were obtained by blocking the endogenous activity of these receptors and suggest that highly compartmentalized calcium signaling events unleash Ng expression. Larger and more widespread calcium loads such as those that follow cLTP induction or BIC treatments most likely favor Ng degradation over Ng expression. This is in fact what we observed after cLTP induction, which triggered an initial loss of Ng followed by a substantial recovery (Fig. 5). Both the incipient proteolysis and subsequent new synthesis could be prevented using either calpain or protein synthesis inhibitors, respectively. Additionally, AMPA and NMDA receptor antagonists were able to block both the loss and the recovery of Ng content after cLTP, thus suggesting the need for both depolarization and neurotransmitter release to occur simultaneously. We also observed that long-term BIC induced a moderate increase in Ng expression that was enhanced when

Fig. 6 Ng overexpression enhances synaptogenesis in cultured hippocampal neurons. **a** Hippocampal neurons were infected at 1 day in vitro (DIV1) with Syn-Ng lentiviral particles (top panel) and the expression of several synaptic and housekeeping proteins examined at the indicated times of culture. **b** Typical fields of view of control (upper panel) and Syn-Ng-infected (lower panel) hippocampal neurons, processed for Ng immunofluorescence after different times in culture. **c** Quantification of the percentage of Ng-expressing neurons in control (upper panel) and Syn-Ng-infected (lower panel) cultures at several different time points (mean ± SEM, $n = 3$). Hippocampal neurons growing at 15,000 cells/cm^2 on coverslips were fixed and processed to quantify **d** excitatory and **e** inhibitory synapse density. The total number of synapses measured were divided by the total imaged area on each coverslip, and then normalized to the maximum density value obtained, which was at DIV18 in both cases (mean ± SEM, $n = 4$): $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. The lower panel shows example images of DIV21 hippocampal neurons labeled for excitatory (**f**) (vGluT1, PSD-95, and MAP 2) and inhibitory (**g**) (GAD6, gephyrin, and MAP 2) synapses, in both control and Syn-Ng cultures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AMPA receptor activity was blocked by NBQX. To explain the effect of NBQX, we propose that the depolarization induced by AMPA receptor activation opens voltage-dependent calcium channels, which in turn generate a greater calcium influx and promote the activation of calpains. Thus, in the presence of NBQX, BIC treatment resulted in a more efficient accumulation of Ng. As other authors have shown [73], for synaptic plasticity, the amount of calcium that flows into the neuron, its entry route, and the affected intracellular compartment are all important. Functional compartmentalization of Ng is supported by the results obtained after cLTP induction and subcellular fractionation. The synaptosomal fraction underwent the most striking changes in Ng content after cLTP, suggesting that for the most part, both Ng proteolysis and new synthesis are localized in the synaptic environment. As Ng is distributed throughout the somatodendritic compartment, this behavior also implies that the post-synaptic environment is where Ng function is more relevant.

An important finding in this study is that significant reductions of Ng in the cultures are not observed before 2 days of the continuous blockade of NMDA receptors. Although these results fit with the hypothesis that Ng expression is regulated by synaptic activity, they are difficult to reconcile in a more general context of synaptic scaling, wherein persistent NMDA receptor blockade leads to increased post-synaptic excitability [56, 74, 75]. Indeed, we think that synaptic scaling and Ng expression are independent processes. Scaling can be observed after 6 h of excitatory activity alteration whereas changes of Ng expression need at least 48 h of NMDA receptor blockade. Moreover, AMPA receptor blockade can induce scaling but does not change Ng expression, even in the long term. Since increased levels of Ng have been associated with increased CaM availability and greater synaptic strength [7–9], it could be expected that prolonged NMDA receptor blockade would lead to increased Ng expression. However, this was not what we found. One explanation is that Ng expression responds to the increasing needs of compartmentalization of CaM signaling as synaptogenesis proceeds. Ng has been shown to sequester CaM and reduce the activity of Ca^{2+}/CaM-regulated targets in vitro [76]. Therefore, in a tightly packed synaptic environment, Ng could act in preventing the leakage of Ca^{2+}/CaM signaling to neighboring synapses when activity is high. On the other hand, when synaptic activity is persistently low, less Ng would allow a broader spread of Ca^{2+}/CaM signaling and enhanced excitability. In this hypothetical context, Ng expression would be a late event of the homeostatic response, involved in the synaptic remodeling and consolidation needed to adapt to the new activity regime.

Two previous reports have studied Ng expression in cultures of dissociated neurons. The most recent one shows that novel-context memory formation needs a rapid translation of Ng in the hippocampus [62]. The authors found that BIC treatments (40 μM, 15 min) induced a significant increase of Ng in cultures of cortical neurons. Such increase was blocked by cycloheximide, but not by actinomycin D, indicating that translation and not transcription was required. They confirmed these results using puromycylation and the proximity-ligase assay to visualize newly synthesized Ng. In the present study, we showed a moderate increase of Ng levels in hippocampal neurons after long-term BIC treatment (25 μM, 24 h, and longer). Despite the different time frames, both sets of results are on the same line and suggest that excitatory activity stimulates the expression of Ng. Here, we find good agreement with the functional model that favors a positive effect of Ng on synaptic excitability [77]. Another study used cultures of rat cortical neurons to analyze how the long-term elevation of intracellular cAMP affects Ng expression, for periods ranging from 6 to 96 h starting at DIV14 [78]. They found a progressive downregulation of Ng mRNA and protein levels that attributed to lower transcription rates. Further, 30 min pre-treatments with either bicuculline (20 μM) or tetrodotoxin (1 μM), despite having opposing effects on synaptic function, decreased Ng mRNA levels and occluded the inhibitory effect of a 24-h elevation of intracellular cAMP. Finally, they showed that AP5 (30 min + 24 h, 50 μM) induced a slight decrease of Ng mRNA, but no effect was observed with similar treatments with CNQX (40 μM), nifedipine (10 μM), or BAPTA (1 μM). These results have in common with ours the fact that prolonged (+ 24 h) NMDA receptor blockade reduces Ng expression. In addition to the different experimental conditions used (cultures of cortical vs hippocampal neurons, timing of treatments), it is important to note that this later study mainly analyzes Ng mRNA levels whereas our study focuses more on protein levels. There is evidence showing a different regulation of Ng mRNA and protein levels after sleep deprivation [19] and in CA3 and CA1 regions of the hippocampus [40]. Therefore, consistencies and discrepancies among these results must be made with caution. Regarding fine-tuning experimental setup, we should point out on our behalf that finding the appropriate conditions of neuronal density, timing, and BIC concentration that enabled us to measure net increases of Ng in our cultures was not easy. Actually, it was very comforting to see that the blockade of AMPA receptors by NBQX enhanced the stimulatory effect of BIC.

One of the clearest exponents of long-term plasticity is synaptic remodeling. Neuronal networks featuring higher densities of synaptic contacts have a greater capacity to process information and can elaborate more complex and better-adapted responses. There are strong links between Ng and the processes of synaptogenesis and synaptic remodeling in the literature. Thus, a close parallel was found between Ng expression and high rates of synaptogenesis throughout development [2]. In addition, Ng levels in the brain decline in the asymptomatic onset period of neurodegenerative diseases, which share features of robust synaptic degeneration in the forebrain and increased levels of Ng in CSF [25]. The so-called critical periods of development are probably the times of most highly active synaptic remodeling [79]. A recent study has demonstrated that Ng is key to coordination of

synapse elimination and synapse unsilencing, which are driven by visual experience and lead to normal rearing [64]. As cultures of hippocampal neurons exhibited relatively low Ng expression, we reasoned that this would be a good opportunity to manipulate and raise Ng levels up to those typical of the adult hippocampus so that the role of Ng in synaptogenesis could be analyzed. Cultures transduced with Syn-Ng lentiviruses at DIV1 showed the same maturation profile but with 8–9 times more Ng expression, increasing both the number of Ng-positive neurons (to more than 60% of the culture) and the density of both excitatory and inhibitory synaptic contacts (double of that observed in non-transduced cultures). Therefore, we have shown that greater expression of Ng leads to a greater synaptic enrichment, and this should mean a greater cognitive capacity. In fact, there is already evidence indicating that enhancing Ng expression in the hippocampus restores the cognitive decline of 5XFAD mice, a mouse model of AD [80].

In summary, our results indicate that (1) synaptic activity, and more specific activity of NMDA receptors, is needed for Ng expression in cultured hippocampal neurons; (2) activity-dependent Ng expression is regulated at the transcriptional level in the long-term and most likely at the translational level in the short-term; (3) different patterns of synaptic activity can trigger either Ng proteolysis or new synthesis in different proportions; and (4) a greater expression of Ng leads to increased synaptic density. This experimental evidence adds to that already available in the literature, placing Ng in an advantageous position as a therapeutic target for the development of strategies aimed at improving cognitive function.

Acknowledgments We thank Dr. FG Scholl (IBiS, Sevilla, Spain) and P. Scheifelle (Biozentrum, Basel, Switzerland) for providing the lentivector pLOX-Syn-DsRed-Syn-GFP. We would like to thank the Advanced Light Microscopy Core Facility, from Centro de Biología Molecular Severo Ochoa (CSIC-UAM), for assistance with the imaging studies.

Author Contributions AG-G, RA, AJ-P, PS, DS-F, and EM-B carried out the experiments and analyzed the data. FJD-G conceived the study and wrote the manuscript. All the authors have read and approved the final version of the manuscript submitted.

Funding Information This work was supported by the Spanish Ministry of Science and Innovation and MINECO (grants BFU2010-18297 and SAF2014- 55686-R). We also thank the "Fundación Ramón Areces" for providing institutional support to CBMSO.

Compliance with Ethical Standards

All procedures were carried out in accordance with the Spanish Royal Decree 1201/2005 for the protection of animals used in scientific research, and the European Union Directive 2010/63/EU regarding the protection of animals used for scientific purposes. The procedures were approved by local Ethical Committees.

Conflict of Interest The authors declare that they have no conflict of interest.

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