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Spatial gene's (Tbata) implication in neurite outgrowth and dendrite patterning in hippocampal neurons

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ABSTRACT

The unique architecture of neurons requires the establishment and maintenance of polarity, which relies in part on microtubule-based kinesin motor transport to deliver essential cargo into axons and dendrites. In developing neurons, kinesin trafficking is essential for delivering organelles and molecules that are crucial for elongation and guidance of the growing axonal and dendritic termini. In mature neurons, kinesin cargo delivery is essential for neuron dynamic physiological functions which are critical in brain development. In this work, we followed Spatial (Tbata) gene expression during primary hippocampal neuron development and showed that it is highly expressed during dendrite formation. Spatial protein exhibits a somatodendritic distribution and we show that the kinesin motor Kif17, among other dendrite specific kinesins, is crucial for Spatial localization to dendrites of hippocampal neurons. Furthermore, Spatial down regulation in primary hippocampal cells revealed a role for Spatial in maintaining neurons' polarity by ensuring proper neurite outgrowth. This polarity is specified by intrinsic and extracellular signals that allow neurons to determine axon and dendrite fate during development. Neurotrophic factors, such as the Nerve Growth Factor (NGF), are candidate extracellular polarity-regulating cues which are proposed to accelerate neuronal polarization by enhancing dendrite growth. Here, we show that NGF treatment increases Spatial expression in hippocampal neurons. Altogether, these data suggest that Spatial, in response to NGF and through its transport by Kif17, is crucial for neuronal polarization and can be a key regulator of neurite outgrowth.

Introduction

Brain development is a complex series of dynamic and adaptive processes that operate throughout the course of development to promote the emergence and differentiation of neural structures and functions (Stiles and Jernigan, 2010). Neuron differentiation occurs through a tight regulation of specific genes, that if disrupted, can fundamentally alter neural outcome (Brumwell and Curran, 2006). Spatial gene (Stromal Protein Associated with Thymii and Lymph node) or Tbata (thymus, brain and testes associated), has been shown to be highly expressed in restricted areas of the brain (Irla et al., 2007).

Spatial was isolated by DNA chip differential screening between immunodeficient mice models, in order to understand the impact of the thymic microenvironment on the T-cell development (Carrier et al., 1999). Spatial generates five alternatively spliced variants: Spatial-α, -β and -γ (short) and Spatial-ε and -δ (long); with a tissue specific distribution (Irla et al., 2004). Spatial is expressed in highly polarized cell types such as epithelial cells in the thymus, germ cells in the testis and neurons in the brain (Irla et al., 2004).

In the thymus, Spatial constitutes a marker of progenitor thymic epithelial cells and acts like a promiscuous gene involved in thymocyte education and in the prevention of autoimmune diseases (Saade et al., 2010). In the testis, Spatial seems to be restricted to spermiogenesis, characterized by drastic morphological events, allowing spermatid differentiation into spermatozoa (Saade et al., 2007). Spatial distribution strongly correlates with nuclear shaping and flagella formation and elongation; thus, suggesting a function for Spatial in spermatid differentiation. In the central nervous system, Spatial expression overlaps with the beginning of neuronal differentiation in both cerebellum and hippocampus. In the cerebellum, only Spatial-β and -ε are presented. They are detected in the external precursor granular layer and persist when these cells migrate and differentiate to form the internal granular layer. In the hippocampus, Spatial-β and -ε are strictly expressed and their expression is spatially and temporally regulated: they are first expressed in CA3, then in CA1 field and later in the dentate gyrus. In cultured hippocampal neurons, Spatial displays a somatodendritic pattern and interacts with the kinesin Kif17, which is implicated in dendrite trafficking (Irla et al., 2007).

Abbreviations: Spatial, Stromal Protein Associated with Thymii and Lymph node; NGF, Nerve Growth factor; d.i.v, days in vitro.

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In this study, we show that Spatial is detected throughout hippocampal neuron development in vitro and it is highly expressed during dendrite specification. Spatial proteins are localized to the cytosol of early developing neurons and are further detected in MAP2 positive dendrites of mature neurons. Several key molecules that are involved in dendrite specification start to accumulate into the distal parts of neurites due to their direct or indirect association with kinesins. Here, we show that Spatial transport to dendrites is directly associated with the kinesin Kif17. Moreover, Spatial's down regulation in primary hippocampal cells resulted in a decrease in neurite outgrowth, which can be modulated by extracellular signals. Here, we show that NGF (Nerve Growth Factor) treatment, which is known to accelerate neuronal polarization by enhancing dendrite growth, upregulates Spatial's transcripts expression. Our data suggest that Spatial is implicated in neurite outgrowth and dendrite patterning via the NGF signaling pathway.

Results

Spatial is induced during neuronal development

In order to follow Spatial's distribution and expression during neuronal differentiation, we worked on mice embryonic pyramidal hippocampal neurons, a frequently adapted model used for neuronal polarity studies. These cells express in vitro almost all their key phenotypic features and their morphological changes occur during five developmental stages (DS1-5) (Dotti et al., 1988). Immunofluorescence co-staining with anti-Spatial and MAP-2 (dendrite marker) antibodies show that at 3 h in vitro (i.v; DS1), when hippocampal neurons extend a lamellipodia all around the cell body Spatial proteins are cytosolic. At 6 h i.v (DS2), when minor neurites begin to extend, Spatial is also localized in these neurites (Fig. 1A). During axon patterning (2 days in vitro (d.i.v); DS3), dendrite formation (4 d.i.v; DS4) and neuron maturation (10 d.i.v; DS5), Spatial stained all MAP-2 positive processes (Fig. 1A). In contrast, Spatial was not detected, neither during DS4 nor DS5, in axons which are positive for the axonal marker SMI31 (Supplementary Fig. 1).

Thus, during neuron development Spatial is localized to cell bodies, neurites and specifically dendrites of hippocampal neurons. These results confirm the somatodendritic distribution previously reported in vivo in adult CA3 and CA1 pyramidal hippocampal cells and in vitro at 15 d.i.v cultured hippocampal neurons (Irla et al., 2007). To specifically know which isoform is expressed during neuronal development and polarization, we conducted quantitative RT-PCR with primers specific for Spatial-β short and -ε long isoforms. Analyses show that both Spatial variants are expressed during neuronal development. They are transcriptionally upregulated during lamellipodia (DS1) and dendrite formation (DS4), and slightly down regulated during axon patterning (DS3) (Fig. 1B).

Altogether, these data show that Spatial distribution is polarized during neuronal development. Spatial is highly expressed during dendrite patterning, suggesting that this gene could participate in neurite outgrowth from primary hippocampal cells.

Kif17 is necessary for Spatial transport

Differentiated neurons contain several compartments of distinct molecular composition and function. Kinesin proteins are mainly implicated in sorting and delivering specific proteins for axon and dendrite specification (Hirokawa and Takemura, 2004; Hirokawa et al., 2009). Spatial, is one of the proteins presenting a polarized distribution, it is localized in the cell body and in the outgrowing and matured processes. Previous work has shown that Spatial colocalizes and interacts with the neuron-specific molecular motor kinesin Kif17 via the Spatial-ε and -β isoforms in dendrites (Irla et al., 2007). Several studies have shown that certain kinesins transport specific cargos to dendrites (Guillaud et al., 2003; Kayadjanian et al., 2007). In order to understand the contribution of Kif17 to Spatial dendritic targeting, we sought to assess whether Spatial transport is specific to Kif17. To do so, we transfected 11 d.i.v dissociated hippocampal neurons with dominant negative (DN) variants of Kif17 and four other kinesins Kif5A, Kif5B, Kif21B, and KifC2 that have been found to be implicated in dendrite transport

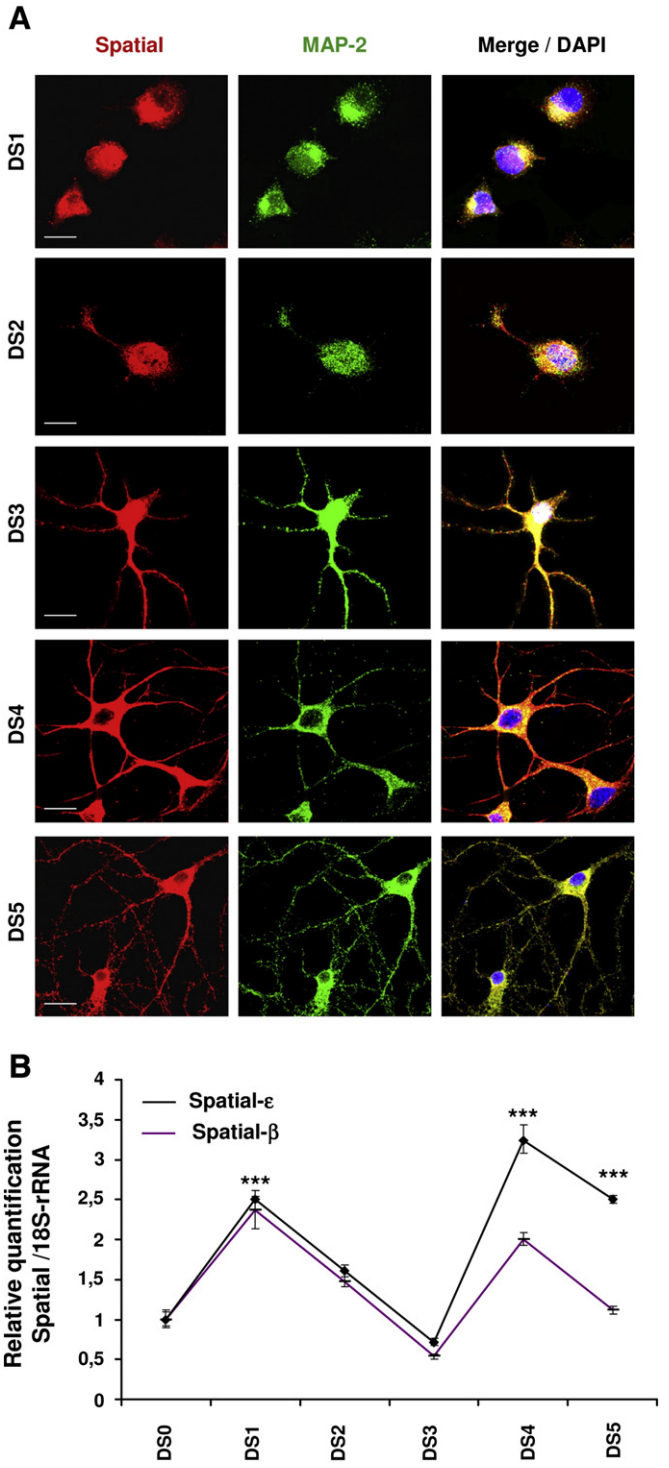

Fig. 1. Spatial expression during hippocampal neurons development. (A) Spatial is present in the cytosol as cells attach to substrate (developmental stage DS1). It persists and extends to neuron processes as cells extend their first neurites (DS2), acquire polarity and mature (DS3-5). (B) Quantitative RT-PCR for Spatial expression shows that both variants are highly expressed during lamellipodia formation and dendrite patterning. Spatial mRNAs/18S-rRNA ratios were standardized to Spatial expression at 0 h *i.v.* (DS0) which was considered as 1. Mean values ± SEM bars are shown on graphs. Experiments were repeated three times. *** $p < 0.001$. Scale bars, 15 μm.

(Hanlon et al., 1997; Setou et al., 2002). Since DNKifs motor domains are replaced with fluorescent proteins, kinesins are able to bind to their cargos but cannot move along the microtubules to assure their dendritic transport (Chu et al., 2005). Analysis of Spatial distribution in MAP2 positive neurons in which DNKifs have been expressed for 48 h revealed that in GFP-DNKif17 expressing cells, Spatial was almost completely confined to the cell body. Nevertheless, in GFP-DNKif5A, YFP-DNKifC2, or GFP-DNKif21B expressing cells, Spatial was present throughout the dendrites (Fig. 2A). As controls, we examined the localization of endogenous Spatial following expression of GFP-pCAGGS. Endogenous Spatial was localized throughout the cell body and dendrites stained by MAP2 marker, indicating that transport was not affected. We

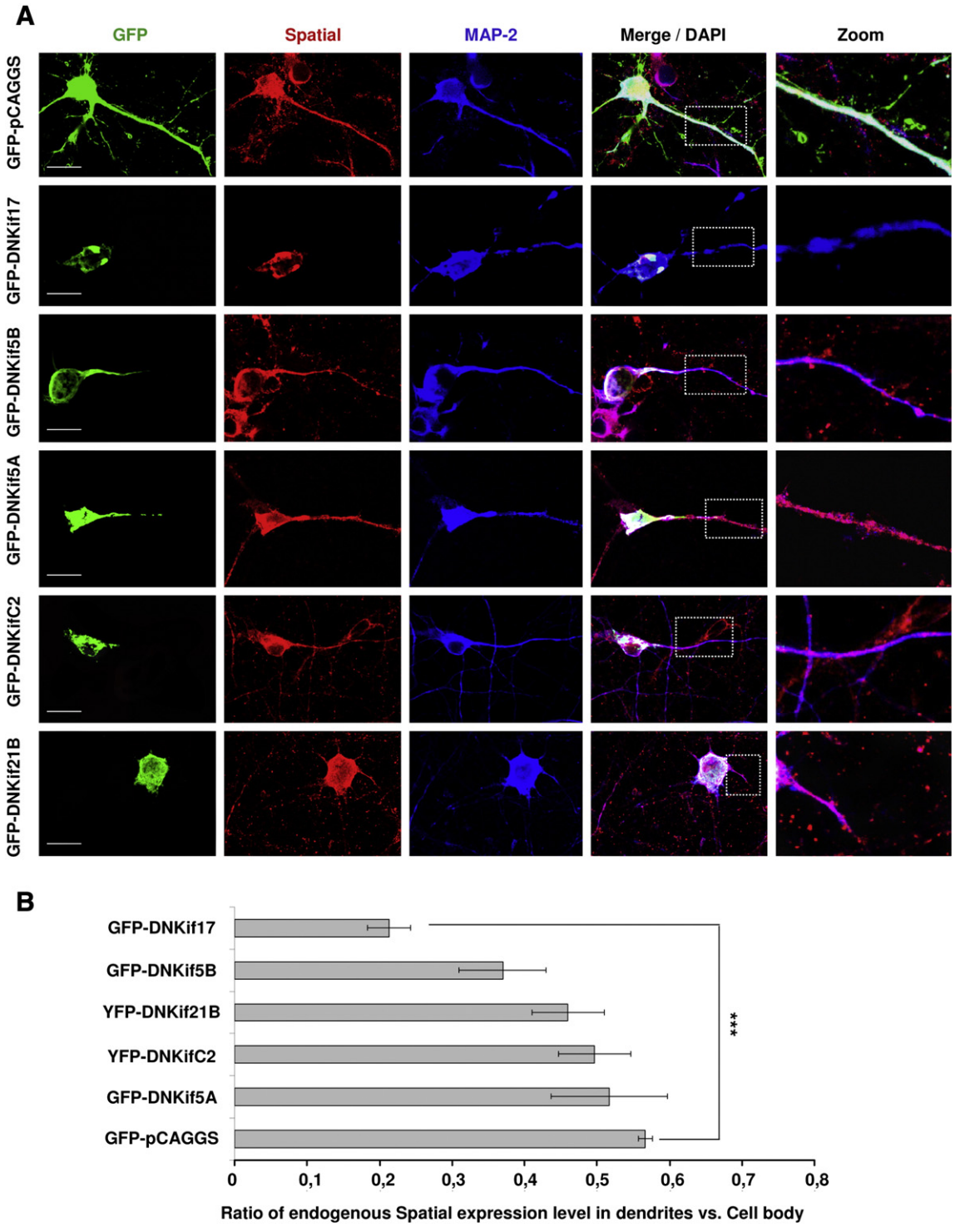

Fig. 2. Effects of various DNKifs on Spatial subcellular localization. (A) DNKifs constructs were expressed in 11 d.i.v hippocampal neurons for 24 h, allowing dimerization with endogenous kinesins. In GFP-pCAGGS transfected neurons, Spatial is present throughout the entire cell; as high magnification also shows. GFP-DNKif17 blocked Spatial localization to dendrites. GFP-DNKif5B, GFP-DNKif5A, YFP-DNKifC2 and GFP-DNKif21B transfected cells show a high density of Spatial in the cell body and in dendrites. In several conditions, Spatial was absent from some dendritic compartments. (B) Expression of GFP-DNKif17 caused Spatial to concentrate in the cell body, with a dendrite/cell body ratio lower than cells expressing other constructs. Mean values ± SEM bars are shown on graphs. Results for each experimental condition are from three independent preparations and 20 neurons were evaluated each time. *** $p < 0.001$. Scale bars: 25 μm.

also observed that DNKif21B transfected neurons presented shorter dendrites and weaker MAP-2 levels compared to other DNKif expressing cells, suggesting that Kif21B trafficking has an important impact on dendrite formation. We further quantified the effect of particular DNKifs on Spatial localization by calculating the ratio of the endogenous Spatial distribution in the dendrites versus that in the cell body. Lower ratios indicate that the protein is localized preferentially to the cell body, consistent with preventing its transport to dendrites. In neurons expressing GFP-DNKif17, the dendrite/cell body ratio of endogenous Spatial was 0.21 ± 0.03, which is significantly lower than that of the ratio of either control (0.56 ± 0.01) or GFP-DNKif5B (0.37 ± 0.06), GFP-DNKif5A (0.51 ± 0.05), YFP-DNKifC2 (0.49 ± 0.05), or GFP-DNKif21B (0.46 ± 0.05) expressing cells (Fig. 2B). From these results, we conclude that Kif17 is essential for Spatial localization to dendrites.

Spatial silencing inhibits neurite outgrowth from primary hippocampal cells

The maintenance and establishment of neuron polarity involve coordinated regulation of the cytoskeleton and protein trafficking. Thus, proteins presenting specific subcellular compartments in neurons allow them to perform specific functions that modulate specification (da Silva and Dotti, 2002). Since Spatial presents a somatodendritic distribution and is specifically transported by Kif17, we were interested to know if it has an impact on neurite outgrowth. For this, we knocked down Spatial expression in freshly isolated primary hippocampal neurons with specific Small Interfering RNAs, siRNA1 and siRNA2 targeting all Spatial isoforms. For this, dissociated hippocampal neurons were electroporated with a GFP expression vector (GFP-pCAGGS), to identify transfected neurons, together with siRNA control or siRNA1 or siRNA2. After 24 h, quantitative analyses of Spatial and GFP levels show that target siRNAs lead to a loss of only Spatial immunoreactivity compared to GFP staining (Fig. 3A and B). Furthermore, cell morphology analysis on GFP-stained neurons showed a 55% reduction in neurite number for siRNA1 or siRNA2 transfected neurons compared to GFP-pCAGGS and siRNA control (Fig. 3C and D). Spatial knocked down expression also resulted in a slight 25% decrease in neurite length (Fig. 3C and E). These findings show that attenuation of Spatial proteins results in decreased neurite number; thus Spatial contributes to neurite outgrowth from primary hippocampal cells.

Given that Spatial is highly expressed during dendrite development and its silencing has an impact on neurite outgrowth, we asked whether Spatial overexpression was able to ablate siRNAs observed phenotypes. For this, each Spatial isoform was cloned in pDEST-26 expression vector. It is important to note that siRNA2, which is designed in the common 5′ UTR region for all Spatial isoforms, could have no specific effects and attenuates expression of other proteins leading to decreased neurite number. To address this issue, we constructed all Spatial isoform expression vectors lacking 5′ and 3′UTR regions. If siRNA2 specifically knocks down Spatial protein expression, then Spatial constructs should serve to rescue the phenotype. Each Spatial-pDEST-26 construct was transfected into SK-Br-3 cell line and protein expression was validated by western-blotting (Supplementary Fig. 2). Then, primary hippocampal neurons were electroporated with the GFP expression vector (GFP-pCAGGS), siRNA2 and either Spatial-β or -ε constructs. As controls, neurons were also electroporated with siRNA2 and either Spatial-α or -γ constructs; since these isoforms are not expressed in the hippocampus. After 24 h, cells were fixed and immunostained for GFP (Fig. 3F). We found that Spatial-β and -ε expression returned neurite number to control values, indicating that the amount of Spatial-β and -ε that were produced from expression plasmids replaced endogenous Spatial that had been knocked down (Fig. 3G). It is also important to note that Spatial-α and -γ expression did not have any impact on neurite number suggesting that Spatial-β and -ε specifically lead to a decrease in neurite number. In addition, a slight effect was observed on neurite lengths (Fig. 3F and G). Altogether, these data show that Spatial-β and -ε may have a role in regulating neurite outgrowth.

Spatial and dendrite stimulating treatments

If Spatial has a physiological role in neurite outgrowth, we expect that its expression will increase when neurons are treated with factors that have been reported to accelerate neuronal polarization and increase dendritogenesis, such as NGF (Vaillant et al., 2002). To address this hypothesis, we treated cultured hippocampal neurons (7 d.i.v) with increasing amounts of NGF for 2 days. We then assayed by immunostaining and quantitative RT-PCR for studying protein distribution and mRNA levels for all Spatial isoforms in the treated neurons. After NGF treatment, all extended MAP-2 dendrites stained also positive with the anti-Spatial antibody (Fig. 4A). To quantify NGF effect on neurite outgrowth, MAP-2 and Spatial labeled neurons were obtained from each treatment, and the number of neurite processes in these neurons was measured. The resulting histograms confirmed that NGF caused a robust and dose-dependent increase in neurite number of MAP-2 and Spatial positive processes (Fig. 4B). Interestingly NGF stimulation increased both Spatial-β and -ε isoform expression (Fig. 4C). β-actin levels were stable showing that NGF treatment does not upregulate all proteins in the neuron (data not shown). Thus, Spatial may act as a signal transducer for extracellular signals that influences dendrite outgrowth via the NGF pathway. To reinforce the importance of Spatial for neurite outgrowth, primary hippocampal cells were co-transfected with Spatial siRNA and GFP-pCAGGS vectors (Fig. 4D). After, NGF (100 ng/ml) was added to the medium and 24 h later, neurite outgrowth was evaluated by confocal microscopy in GFP/MAP-2 positive neurons. Our data show that 70% of cells transfected with GFP-pCAGGS vectors and control siRNA extended neurites; whereas only 12% of cells transfected with Spatial siRNAs did so (Fig. 4E). Thus, Spatial facilitates neurite outgrowth from primary hippocampal cells.

Discussion

Much progress has been made in identifying the key players and elucidating the signaling mechanisms implicated in dendrite morphology and patterning. Dendrite outgrowth is regulated by the interplay between extrinsic and intrinsic factors. Our laboratory has focused on one of them, Spatial, a gene expressed during mouse brain development in the cerebellum, hippocampus and cortex (Irla et al., 2007). In the hippocampus, Spatial expression is spatially and temporally regulated: it is first expressed in the CA3 field, then in CA1 and later in the dentate gyrus. Spatial presents a vesicle-like cellular distribution in dendrites due to its interaction with the motor protein Kif17 (Irla et al., 2007). Previous studies have shown that Spatial presented a differential expression profile during hippocampal development (Irla et al., 2007). Interestingly, Spatial expression begins at late embryonic stages (E14–E16) where the highest rate of neurogenesis occurs to Ammon's horn pyramidal cells. Then, Spatial is only re-expressed during the intensive period of neurogenesis of the dentate gyrus granule cells (post-natal stages P1-P9) and persists till P20 where this process is almost completed (Irla et al., 2007). In this work, we show that Spatial also presents a differential expression throughout hippocampal cells differentiation: it is highly expressed during lamellipodia (DS1) and dendrite (DS4) formation and down regulated during axon patterning (DS3). Altogether, these data strongly suggest that Spatial could be implicated in neuronal morphogenesis. Interestingly, many genes have been recently identified as potential regulators for either axon or dendrite formation (Barnes and Polleux, 2009; Ye et al., 2011). For example, PI3K (the lipid kinase phosphatidylinositol 3-kinase) is crucial for axon specification while SIRT1 (a NAD + dependent histone deacetylase) is important for dendritic development and maintenance of Codocedo et al. (2012); Yoshimura et al. (2006). Since Spatial is down regulated during axon

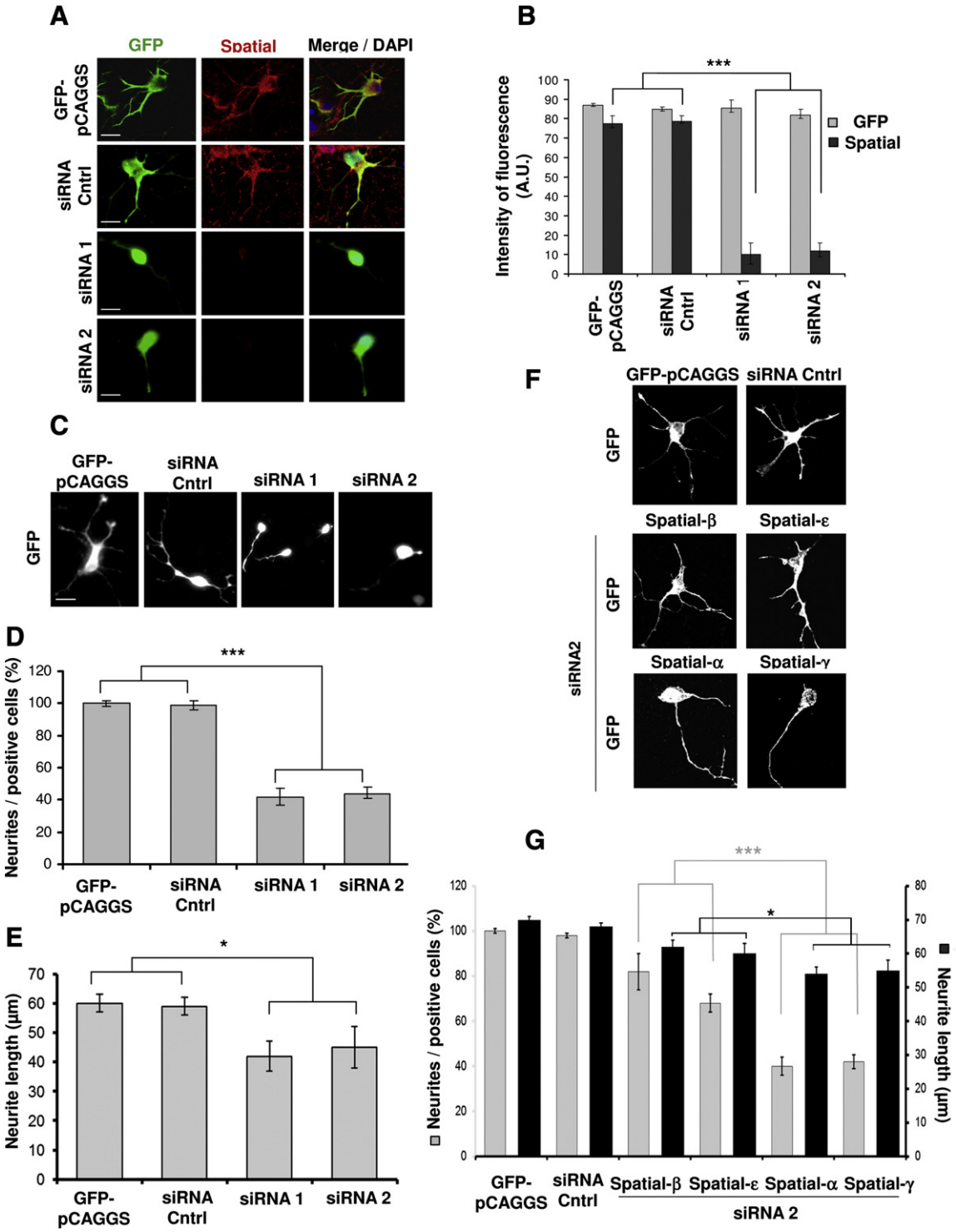

Fig. 3. Spatial regulates neurite outgrowth from primary hippocampal cells. (A) SiRNAs specificity controls of Spatial knockdown. Dissociated hippocampal neurons were electroporated with a GFP expression vector GFP-pCAGGS to identify transfected neurons together with control or Spatial siRNA 1 and 2. Targeted, but not control, siRNAs led to the disappearance of immunoreactivity for Spatial without affecting GFP immunoreactivity. (B) Quantitative analysis of GFP levels in the experiments illustrated in (A). (C) Analysis of neurite outgrowth in: untreated, siRNA control and siRNA1 electroporated neurons. (D–E) Quantification of neurite number and neurite length in GFP positive electroporated neurons, respectively. The percentage of neurite number was obtained by dividing neurite formation from cells transfected with control siRNA, or Spatial siRNA, by that from untransfected cells and multiplying by 100. The average of neuron length (μm) was calculated for each condition. (F) Spatial-β and -ε overexpression rescued siRNA-mediated silencing and enhanced neurite outgrowth. (G) The bar graph shows the number and length of neurites in F panel. Mean values ± SEM bars are shown on graphs. Experiments were repeated at least three times and 100 transfected neurons were assessed for each condition. *** $p < 0.001$. * $p < 0.01$. Scale bars: Scale bars, 25 μm (A,C); 20 μm (F).

formation and over expressed during dendritogenesis, we suggest that this gene is crucial for dendrite specification and maintenance.

We also found that Kif17 is necessary for Spatial localization to dendrites of primary hippocampal neurons. Screening DNKifs against specific dendrite kinesins showed that Kif17 specifically blocks Spatial transport to dendrites. This result is in accordance with the well established role of Kif17 in memory and learning via transporting vesicles containing N-methyl-d-aspartate receptor and K^+ channel subunits to dendrites (Yin et al., 2011). Kif17 also transports Kv1.5 in cardiac myocytes and a cyclic nucleotide gated channel subunit to primary

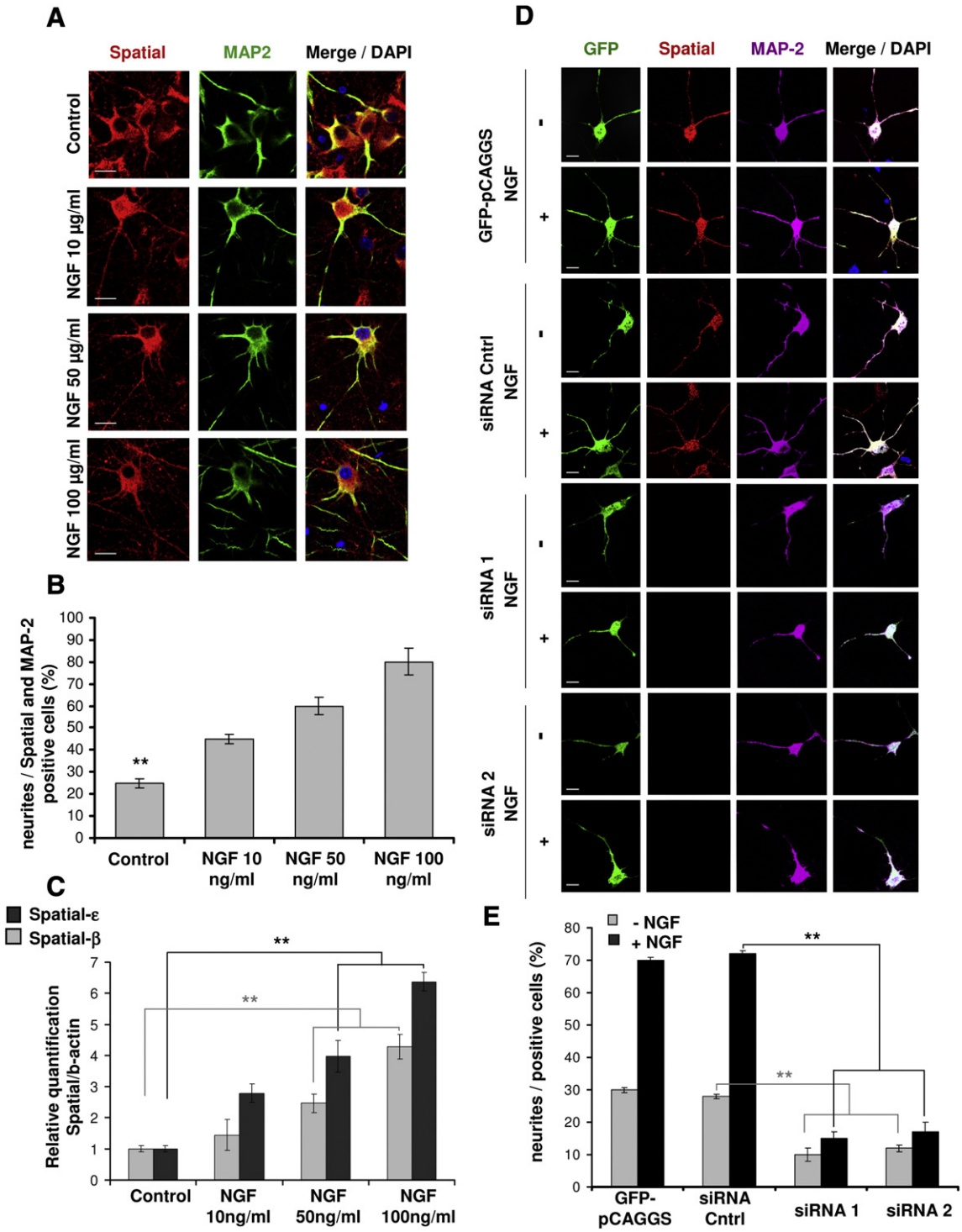

Fig. 4. Spatial expression is stimulated in NGF treated hippocampal neurons. (A) Neurons were treated with NGF (10, 50 and 100 ng/ml). Immunostaining shows that all extended MAP2 dendrites are Spatial positive. (B) NGF treatment increased GFP/MAP-2 positive dendrite number. (C) Quantitative RT-PCR illustrates that at high NGF concentration, Spatial-β and -ε expression increases. (D) Primary hippocampal cells were electroporated with a GFP expression vector GFP-pCAGGS together with control or Spatial siRNAs and then cultured in differentiation medium containing 100 ng/ml NGF. Despite NGF stimulation, Spatial silencing has a drastic effect on neurite outgrowth. (E) A bar graph showing the percentage of neurite number and neurite length in GFP and MAP-2 positive electroporated neurons. Mean values ± SEM bars are shown on graphs. Experiments were repeated three times and at least 60 neurons were assessed for each condition. ** $p < 0.005$. Scale bars, 20 µm.

cilia in epithelia (Cai et al., 2009; Jenkins et al., 2006). In *C. elegans* and *Danio rerio*, Kif17 homologues also play critical roles in intraflagellar particle transport in neuronal sensory cilia and in photoreceptors (Insinna et al., 2008; Snow et al., 2004). In metazoan, Kif17 has been recently shown to play a direct role in epithelial morphogenesis by stabilizing microtubule with the EB1-APC complex (Jaulin and Kreitzer, 2010). We believe that Kif17 may also contribute indirectly to epithelial morphogenesis by orienting intracellular trafficking of cargos playing essential role in maintaining normal cellular morphogenesis (Jaulin and Kreitzer, 2010).

Spatial knockdown in hippocampal cells impairs neurite outgrowth as 55% decrease in neurite number was detected. Interestingly, Spatial-β and -ε overexpression returned neurite number to normal values. Previous studies have shown that Spatial-KIF17 interaction is

mediated through Spatial-ε and -β. It was also suggested that Spatial-ε and -β could bind to KIF17 through their common N-Terminal motif (amino acids 19–259; with 70.8% identity) (Irla et al., 2007). We concluded that through KIF17 interaction, Spatial-ε and -β are both crucial for maintaining normal cellular morphogenesis and hence its activity needs to be dynamically regulated.

Spatial is segregated into somatodendritic compartments of polarized neurons. In cultured hippocampal neurons, we observed that dendritic entry was allowed by KIF17. Furthermore, KIF17 is also found to transport vesicles containing the *N*-methyl-d-aspartate receptor NR2B subunit from the cell body specifically to the dendrites (Setou et al., 2000; Song et al., 2009). These subunits are intimately associated with glutamatergic neurotransmission as well as with learning, memory and synaptic plasticity (Yin et al., 2012). Several studies have shown that transgenic mice overexpressing KIF17 exhibited enhanced learning and memory. In these mice, NR2B expression was up-regulated with an increased phosphorylation of the cAMP-dependent response element-binding protein (CREB) (Dhar and Wong-Riley, 2011; Wong et al., 2002). CREB is known to bind certain DNA sequences called cAMP response elements (CRE); thereby regulating the transcription of the downstream genes (Wong et al., 2002). Since previous bioinformatic predictions have shown the existence of a CRE sequence in the Spatial promoter sequence, we propose that its expression could be upregulated in KIF17 transgenic mice via the nuclear transcription factor CREB (Irla et al., 2004; Saade et al., 2010). To investigate this hypothesis, future studies will be focused on chromatin immunoprecipitation sequencing assays (ChIP-seq). Furthermore, Spatial conditional knockout and/or knock-in mice models will be valuable tools for further understanding Spatial's function in neuronal morphogenesis and synaptic plasticity through its interaction with the kinesin motor KIF17.

One common and best characterized group of growth factors that influences nervous system development is the neurotrophin family. The most famous member is the NGF, which can act as an extrinsic factor to influence dendritic morphology of cortical neurons (McAllister et al., 1995). Through TrkA receptors, NGF promotes transcription factor phosphorylation, triggering thus G1/S arrest and promoting differentiation (Tedeschi and Di Giovanni, 2009). It also elicits a phosphorylation cascade that activates CREB transcription factor which is critical for the transcriptional regulation of dendritic complexity (Wayman et al., 2006). Loss of CREB function has been shown to impair dendrite growth and arborization of newborn hippocampal neurons (Jagasia et al., 2009). NGF has always been described to act on responsive neurons by binding to its specific receptors on axonal termini activating thus the retrograde transport to the cell body (Johnson et al., 1987). In the hippocampus, NGF is synthesized in hippocampal neurons and retrogradely delivered to promote neuron survival (Gustilo et al., 1999). Interestingly, a recent study has shown that NGF synthesized in the hippocampus could signal in an anterograde fashion to modulate synaptic transmission in neurons (Guo et al., 2012). In line with these reports, we have found that NGF increases Spatial expression in primary hippocampal neurons. In addition, Spatial siRNA knockdown in NGF-induced neurons showed a decrease in neurite number. Thus, Spatial can enhance NGF extracellular signals and enhances neurite outgrowth from primary hippocampal cells. Indeed, these results will add to our understanding of the pathway controlling Spatial expression during cell morphology and dendrite patterning.

Recent studies have shown that Spatial mediates cell cycle arrest via its interaction with Uba3, the catalytic subunit of the activating enzyme complex E1 (Flomerfelt et al., 2010). Spatial/Uba3 interaction inhibited the neddylation pathway and promoted the degradation of several cell cycle inhibitors via the ubiquitin mediated proteasome pathway (Bloom et al., 2003). The ubiquitin proteasome system has been shown to be implicated in various aspects of neural development including: synaptogenesis, neurite guidance and pruning (Liao et al., 2004). In Drosophila mushroom body neurons Cul3 (neddylation) has been shown to be involved in axonal arborization and dendritic elaboration (Zhu et al., 2005). These observations suggest that common mechanisms of neddylation may be regulated by Spatial to control neurite outgrowth. Confirming the conservation of this mechanism between cell cycle inhibition and induction of neurogenesis promises to help elucidate the molecular mechanisms underlying brain development.

Experimental methods

Animals

CD1 mice were maintained under specific pathogen-free conditions. Experimental and surgical procedures were approved by the veterinary office of the Ministry of Agriculture (authorization number: 13–27).

Mouse primary hippocampal neuron cultures

Neuronal cultures were prepared from E16.5 CD1 mice hippocampi, as described in Chauvet et al., 2007 (Chauvet et al., 2007). Briefly, hippocampi were dissected from E16.5 mouse brain embryos and dissociated using 0.05% Trypsin-EDTA (Invitrogen, cat. no. 25300-054). Dissociated cells (25,000–75,000) were seeded into a 4-well chamber with coverslips (Marienfield) coated with poly-L-lysine (Sigma, 1 mg/ml; (Kaech and Banker, 2006; Chauvet et al., 2007) and laminin (Invitrogen, 10 μg/ml) in Neurobasal/B27/200 mM glutamine (Invitrogen). Cells were grown under 5% CO_2 at 37 °C. For NGF treatment: after 7 days in culture, cells were washed and switched to different concentrations of NGF (10, 50, 100 ng/ml).

SK-Br-3 cell culture

Human breast cancer SK-Br-3 cells (ATCC) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) containing 10% (v/v) fetal bovine serum (FBS), 1% Pyruvate (Invitrogen) and 1% Penicillin-Streptomycin, in humidified 5% CO_2 at 37 °C.

Spatial variants cloning

Spatial-α, -β, -γ, -ε, -δ isoforms (CDS region) were cloned in pENTR-D-TOPO entry vectors (Invitrogen). LR recombination reaction was performed to generate expression clones in pDEST-26 destination vectors (6xHis tag). SK-Br-3 cells were used to express each Spatial variant. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

RNA isolation and quantitative RT-PCR

RNA was isolated using TRIzol® reagent (Invitrogen) and quality was monitored with RNA Nano-6000 Chips and 2100-BioAnalyzer (Agilent). Two-step quantitative RT-PCR was performed using the ABI-PRISM-7000 Sequence Detection System. Oligo(dT)25 and the reverse transcriptase SuperScript II (Invitrogen) were used for RNA reverse transcription. PCR was performed with Syber® Green PCR Mix (Applied Biosystem). Primers used to specifically detect Spatial short and long isoforms were: forward: 5′-AGGTGACTAGTCCAAAGGCAGAG-3′, reverse: 5′-CCCGTGGCTCCTT GGAA-3′ and forward: 5′-GTCACCACCTCT ACCCAAAACAG-3′, reverse: 5′-GGATGCACCAGGAGGACTTG-3′. β-actin or 18S-rRNA was used as housekeeping gene to normalize expression between different samples and to monitor assay reproducibility. Primers used to amplify 18S-rRNA were: forward: 5′-ACCGCG GTT CTATTTTGTTG-3′ and reverse: 5′ CCCTCTTAATCATGGCCTCA-3′. For β-actin, forward: 5′- TTGCTGACAGGATGCAGAAG-3′ and reverse: 5′-ACATCTGCTGGA AGGTGGAC-3′. Relative quantification of all targets was calculated by using the comparative cycle threshold method (Livak and Schmittgen, 2001).

Kinesins dominant negative (DNKif), siRNA and Spatial-pDEST26 transfections

DNKif expression vectors were a kind gift of Dr. Don B. Arnold (Department of Biology, University of Southern California, Los Angeles). DNKifs were generated by replacing the motor domains of the respective kinesin molecules with GFP or YFP (Tsunoda et al., 1997). For DNKif17, 21B, 5B, 5A and C2, amino acids 2–323, 2–530, 2–366, 2–373, and 370–792 were deleted (Chu et al., 2005). Dissociated hippocampal neuron cultures at 11 days in vitro were transfected using Lipofectamin 2000 (Gartner et al., 2006).

For SiRNA transfection: primary hippocampal neurons, were directly electroporated after dissociation, with a GFP-pCAGGS vector alone, or together with siRNA control, siRNA1 designed in the common Exon 5 (siRNA-Ex5: 5′-GGAAGAAACUGAAGGACC-3′ and 5′-AGGUCCUUCAGU UUCUUC-3′), or siRNA2 specific to 3′UTR regions (siRNA-3′UTR: 5′-CCCAGAAACACCCUCUAAA-3′ and 5′-UUUAGAGGGUGUUUCUG GG-3′; Eurogentec). After 24 h treatment with B27 supplement, quantitative RT-PCR and cell morphology analyses were assessed.

Western-blot

Cell lysates were electrophoresed, transferred and incubated with the rabbit polyclonal antibody anti-Spatial (1:1000; (Irla et al., 2004)) or mouse anti-β-actin (Santa Cruz biotechnology). Proteins were visualized using horseradish peroxidase-conjugated secondary antibody (1:1000; Amersham Pharmacia Biotech) and the enhanced chemiluminescence detection system (Pierce).

Immunofluorescence

Cells were post-fixed in 4% PFA, permeabilized with 0.1% Triton, blocked and incubated with rabbit anti-Spatial (1:100), mouse anti-MAP2 (1:500; clone AP20; Millipore), antiphosphorylated NF-H (1:900, clone SMI31, Chemicon) or rat anti-GFP (1:500) antibodies. They were then incubated with Alexa fluorescent secondary antibodies (Molecular Probes). Cells' nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI) and mounted with Mowiol (Calbiochem). Fluorescent images were acquired with the Zeiss LSM 510 confocal microscope.

Fluorescence analyses and neurite outgrowth determination

Time interval parameters and gain settings of the camera were adjusted so the bright areas did not reach saturation. Same conditions were applied to capture all images. The immunofluorescence was measured using ImageJ software. Expression levels were determined by calculating the total amount of fluorescence associated with a given protein in the entire cell. Only expression levels of proteins detected by the same antibodies were compared. The ratio of the expression level of Spatial in the dendrites versus the cell body was calculated as follows. The average intensity of fluorescence associated with Spatial staining was measured in the cell body and at points 20–35 μm from the cell body on at least three dendrites for each neuron. The ratio of the dendritic value to that of the cell body was calculated for individual neurons. For DN transfection data, results for each experimental condition are from three independent preparations and 20 cells were evaluated each time. Neurite outgrowth was determined using ImageJ: the number of neurite and the total neurite length per neuron were measured and analyzed with the NeuronJ plugin (Meijering et al., 2004). The values were then averaged for each experiment and the data were pooled from three independent experiments. 100 transfected cells were evaluated for each condition. Statistical differences were determined with Student's *t*-test (while comparing 2 groups) or ANOVA (Analysis of variance) followed by the Student–Newman–Keuls test (while comparing more than 2 groups). Statistical significance was considered at p value (p) < 0.05. All experiments were repeated at least three times.

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Competing interests

The authors declare no financial or commercial conflict of interest.

Author contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: CN SC MY. Performed the experiments: MY MS. Analyzed the data: MY MS. Contributed reagents/materials/analysis tools: SC MS. Wrote the paper: MY CN MS SC.

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