Molecular and Cellular Neuroscience 75 (2016) 81–92

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Calpain-dependent truncated form of TrkB-FL increases in neurodegenerative processes

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ARTICLE INFO

Article history:
Received 12 April 2016
Revised 24 June 2016
Accepted 19 July 2016
Available online 20 July 2016

Keywords:
Status epilepticus
Neuronal death
TrkB-FL
Truncated TrkB-FL
Calpain
Neurite degeneration

ABSTRACT

Recent findings indicate that the mechanisms that drive reshaping of the nervous system are aberrantly activated in epilepsy and several neurodegenerative diseases. The recurrent seizures in epilepsy, particularly in the condition called *status epilepticus*, can cause permanent neurological damage, resulting in cognitive dysfunction and other serious neurological conditions. In this study, we used an in vitro model of *status epilepticus* to examine the role of calpain in the degeneration of hippocampal neurons. We grew neurons on a culture system that allowed studying the dendritic and axonal domains separately from the cell bodies. We found that a recently characterized calpain substrate, the neurotrophin receptor TrkB, is cleaved in the dendritic and axonal domain of neurons committed to die, and this constitutes an early step in the neuronal degeneration process. While the full-length TrkB (TrkB-FL) levels decreased, the truncated form of TrkB (Tc TrkB-FL) concurrently increased, leading to a TrkB-FL/Tc TrkB-FL imbalance, which is thought to be causally linked to neurodegeneration. We further show that the treatment with *N*-acetyl-Leu-Leu-norleucinal, a specific calpain activity blocker, fully protects the neuronal processes from degeneration, prevents the TrkB-FL/Tc TrkB-FL imbalance, and provides full neuroprotection. Moreover, the use of the TrkB antagonist ANA 12 at the time when the levels of TrkB-FL were significantly decreased, totally blocked neuronal death, suggesting that Tc TrkB-FL may have a role in neuronal death. These results indicate that the imbalance of these neurotrophins receptors plays a key role in neurite degeneration induced by seizures.

1. Introduction

Most neurons initially develop an extensive set of neuritic processes that are subsequently pruned, so that only those that are part of the mature neuronal network are retained. In addition, significant sculpting of neuritic processes can occur throughout the neuron's lifespan (Pease and Segal, 2014). The changes in the morphological structure of neurites are mediated by conserved signaling mechanisms that are now beginning to be understood. Importantly, recent findings indicate that the mechanisms driving the reshaping of the nervous system are aberrantly activated in neurodegenerative diseases (Liu et al., 2008; Ma, 2013). The term "neurodegeneration" is defined as the progressive loss of structure and function of neurons, which ultimately leads to neuronal death (Yildiz-Unal et al., 2015).

Epilepsy is a disease of the central nervous system (CNS) characterized by recurrent seizures, and affects 1–2% of the population worldwide (Harvey and Sloviter, 2005). A seizure is a clinical manifestation of abnormal, disordered and synchronized high-frequency firing of neuronal populations of the CNS (De Lorenzo et al., 2005). In some circumstances, the seizures do not stop spontaneously. If their duration exceeds 30 min they are classified as *status epilepticus* (SE), which can cause permanent neurological damage, resulting in cognitive dysfunction and other serious neurological deficits (Liu et al., 2013).

The neuronal populations engaged in SE characteristically show an increase in intracellular calcium levels ($[Ca^{2+}]^i$) (DeLorenzo et al., 2005) resulting from massive calcium entry. It has been demonstrated that this calcium inflow induces changes ranging from an increase in the expression levels of several genes to the activation of proteases (Hardingham and Bading, 2010), calpains among them. Calpains are a family of Ca^{2+}-dependent non-lysosomal cysteine proteases (Perlmutter et al., 1990; Goll et al., 2003; Ma, 2013), whose substrates include cytoskeletal proteins (i.e. α-spectrin, neurofilaments), membrane receptors (e.g. the NMDA receptor) (Gladding et al., 2012), the neurotrophin receptor TrkB (Vidaurre et al., 2012; Gomes et al., 2012;

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http://dx.doi.org/10.1016/j.mcn.2016.07.002
1044-7431/

Xie et al., 2014; Jerónimo-Santos et al., 2015), and other proteases, such as caspases (Blomgren et al., 2001; Graham et al., 2006). The cleavage products of calpains action on their substrate may show biological activity (Ma, 2013).

One of the most distinctive features of SE is that it induces an increase in the levels of BDNF and proBDNF, as well as an imbalance in the expression of their receptors, TrkB and p75ntr, respectively, before the onset of neuronal death, a feature shared with other neurodegenerative diseases (Unsain et al., 2008, 2009; Brito et al., 2013). In this regard, it has been demonstrated in models of ischemia, Alzheimer disease (AD) and SE, that calpain can process TrkB full-length (TrkB-FL), producing a cleaved receptor, the truncated TrkB-FL (Tc TrkB-FL), which is similar to the splice-variant TrkB T1 in that both receptors lack most of the intracellular domain. The biological consequence of this processing is still a matter of debate (Vidaurre et al., 2012; Gomes et al., 2012; Xie et al., 2014; Jerónimo-Santos et al., 2015). Because neurite degeneration is a common characteristic of these conditions, it is possible that TrkB-FL decreased, and Tc TrkB-FL increased initially in dendritic and axonal domains of neurons committed to die.

Our results show that the rise in intracellular calcium levels during SE activates calpain, which in turn processes TrkB receptors in dendrites and axons, and suggest that this phenomenon contributes to neuronal degeneration during SE in hippocampal neurons.

2. Materials and methods

2.1. Primary neuronal cultures

Cultures of rat hippocampal neurons were prepared from E17–18 Wistar embryos as previously described (Kaech and Banker, 2006), with minor modifications. Cells were cultured at a density of 50,000 cells/cm^2 on poly-L-lysine-coated coverslips or on cell culture filter inserts (1 μm pore size, BD Falcon) (for immunocytochemistry) and on Corning plate dishes or on cell culture filter inserts (1 μm pore size, BD Falcon) (for Western Blot, WB). Hippocampal cultures were kept in maintenance medium composed of Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), glutamine (0.5 mM), and Pen/Strep 0.5%. The neurons were kept at 37 °C in a humidified incubator with 5% CO_2/95% air for 10–11 days. After the second day in vitro (DIV), AraC or FDU was added to the medium to prevent glial cell proliferation (at 10 DIV, GFAP-positive cells represented about 5% of the cell population, data not shown). The inhibitor calpain ALLN (Calbiochem) and ANA12 (Cazorla et al., 2011) were added at 10uM in the indicated experiments. The animals used in the preparation of cell cultures were handled according to the National Institutes of Health guidelines for the care and use of laboratory animals.

2.2. In vitro model of SE protocol

The SE protocol consisted of exposing the culture to a $MgCl_2$-free buffer solution (Sombati and Delorenzo, 1995). On the 10/11th DIV, the maintenance medium was replaced by control or $MgCl_2$-free buffer solution containing (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 2 $CaCl_2$, 10 glucose, 0.002 glycine, plus or minus 1 $MgCl_2$, pH 7.3. Three washes (3 × 1.5 ml of buffer solution) were performed to remove all traces of Neurobasal medium. The cells were then incubated in the appropriate buffer solution at 37 °C in an atmosphere of 5% CO_2/95% air for 3 h. The neurons were incubated again in maintenance medium under the same temperature and atmosphere conditions. We analyzed and quantified the data at the following time points after the end of the SE stimulus: 0 (immediately after), 6, and 12 h for assessing cell survival over time and the pattern of neurodegeneration, and 0, 3 and 6 h for the WB experiments.

2.3. Validation of the in vitro model of SE: calcium images

Fluo4-AM, a well-characterized calcium fluorescent marker, was used to quantify the intracellular concentration of this ion (Gee et al., 2000). On the 10th DIV, the maintenance medium was replaced with control buffer (i.e. with Mg^{2+}), and Fluo4-AM (5 mM) was subsequently added to "load" the neurons with the dye. The neurons were incubated for 30 min in the presence of the calcium dye, followed by two washes with control buffer to remove the excess of Fluo4-AM. Following this, the cells were filmed during 35 min. The test was repeated three times. We used the following formula to quantify the average concentration of intracellular calcium during the SE in vitro: Calcium concentration = (fluorescence intensity of calcium / calcium average intensity over the basal time period of 5 min) × 100.

2.4. Neuronal death assay

Neuronal death was evaluated by two different methods: calcein/ethidium staining, and assessing the presence/absence of the nuclear protein NeuN (Liu et al., 2013). To perform these assays, six random fields in each coverslip were selected for analysis. The number of calcein-positive neurons was divided by the number of those labeled with ethidium and calcein (total of neurons), taking the control as 100%. The percentage of neuronal death was calculated as the number of neurons labeled with NeuN, with respect to number of labeled neurons in the control condition, taken as 100%. At least two coverslips with cultured neurons were evaluated for each condition, and each experiment was repeated three times.

2.5. Immunocytochemistry

Hippocampal neurons grown on cell culture filter inserts (1 μm pore size, BD Falcon) or bulk cultures were fixed with 4% paraformaldehyde for 10 min at room temperature, and then blocked with blocking solution containing TBS-T, 5% skim milk and 0.3% Triton X-100. Hippocampal neurons were then incubated overnight at 4 °C with antibodies against β-III tubulin (1:20,000, MAB 5564), MAP2 antibody (Sigma M1406, diluted 1:10,000 in blocking solution), TrkB 8316 Santa Cruz (1/750), pTrkB-Y817 rabbit Epitomics 2149-1 (1/1000), NeuN (1:500, MAB 377) and NF-M (1:1000, Millipore AB 1987). Filter inserts or bulk cultures were subsequently incubated with Alexa 488- or 555-conjugated goat anti-mouse/rabbit secondary antibodies for 1 h at room temperature. The filters were removed from the insert, placed in fluorescent mounting medium, and mounted on Superfrost Plus slides (Fisher Scientific).

2.6. Western blot

Hippocampal neuronal cultures were washed twice with ice-cold PBS, followed by a wash with PBS buffer. The cells were then lysed with RIPA buffer supplemented with the mixture of protease and phosphatase inhibitors. After centrifugation at 16,000 × *g* for 10 min, proteins in the supernatants were quantified, and the samples were denatured with concentrated Laemmli denaturing buffer at 95 °C for 5 min. Protein samples were separated by SDS-PAGE, in 10% polyacrylamide gels (α-spectrin, TrkB, pTrkB, NF-M and p75ntr) and in 15% (procaspase-3), transferred to nitrocellulose membranes (Millipore). The blots were incubated with primary antibodies (overnight at 4 °C), washed, and reincubated with the secondary HRP conjugated antibody (1:2000 dilution for anti-rabbit and anti-mouse IgG; 1 h at room temperature). Peroxidase activity was visualized by enhanced chemiluminescence on the ECL and blot imaging system, and quantified with the FIJI program (From NIH). The following primary antibodies were utilized: anti-TrkB (1:750, Milipore 07-225), anti-spectrin (1:1000, MAB1622; Millipore Bioscience Research Reagents), pTrkB-Y817 rabbit Epitomics 2149-1 (1/1000), Rex antibody (1:1000), Anti NF-M (1:1000, Millipore AB

1987), anti procaspase-3 (1:1000, Cell Signaling 9662). The anti-β-III tubulin (1:20,000) antibody was used as loading control.

2.7. Co-immunoprecipitation assay

At 0, 6 and 12 h following the end of SE in vitro, the hippocampal neuron cultures were homogenized with RIPA-modified buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, and 1% Triton X-100) supplemented with protease and phosphatase inhibitors. The homogenates were centrifuged at 14,000 ×*g* for 10 min, and 500 μg of total protein from the supernatants was pre-cleared with 8 μl of Protein A/G PLUS-agarose (Santa Cruz Biotechnology) for 2 h at 4 °C. After centrifugation, the pre-cleared supernatants were incubated with either α-BDNF (sc-546, 2 μg, Santa Cruz Biotechnology) or α-proBDNF (ANT-006, 2 μg Alomone Laboratories Ltd.) at 4 °C for 13 h. Then 10 μl protein A/G PLUS-agarose was added, pre-blocked in 1% BSA and incubated at 4 °C for 4 h. The immunoprecipitates were washed four times with ice-cold RIPA-modified buffer, eluted with SDS sample buffer, and analyzed by WB. Membranes where then probed with Rex antibody. Because we used a primary antibody from rabbit, and a secondary α-rabbit-IgG, we first incubated the membranes with the secondary antibody alone to control for equal protein loading between wells (due to the presence of α-BDNF/α-proBDNF IgG in the membranes) and to interpret correctly the positive bands when using the α-p75ntr antibodies. At least two culture dishes were evaluated for each condition and each experiment was repeated three times.

2.8. Immunocytochemistry and quantification of axonal degeneration

Hippocampal neuron cultures grown on filter inserts or on coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature and then blocked with blocking solution containing PBS, 5% BSA, and 0.3% Triton X-100. The hippocampal neurons were then incubated overnight at 4 °C with antibodies against β-III tubulin (diluted 1:10,000), NF-M (diluted 1:1000) and MAP2 (diluted 1:1000) in blocking solution. Filter inserts or coverslips cultures were incubated with Alexa488/Alexa545-conjugated goat anti-mouse/anti-rabbit secondary antibodies (The Jackson Laboratory) for 1 h at room temperature. Before the last wash, the filters/coverslips were also incubated with Hoescht 33,258 (Invitrogen). The filters removed from the inserts or coverslips were placed in fluorescent mounting medium (Dako) and mounted on slides. β-III tubulin, NF-M or MAP2-stained coverslips/filters were imaged with a systematic random sampling approach, to obtain a total of 12–16 images per filter/bulk culture. The degeneration of neuronal processes was quantified by using the software WIS-NeuroMath (Galun et al., 2007; Rishal et al., 2012). At least two filters/coverslips culture were evaluated for each condition, and each experiment was repeated three times.

2.9. Statistical analysis

The results are expressed as percentages of the control values (mean ± SEM). The ANOVA was followed by Tukey post hoc comparisons, with $p \leq 0.05$ considered significant. Protein levels were quantified in a minimum of 2 different cultures per group. A one-way ANOVA was used to compare relative protein levels between groups. The ANOVA was followed by Tukey post hoc comparisons, with $p \leq 0.05$ considered significant. When necessary, the Kruskal–Wallis test was used for non-normal distributions. In the quantification of calcein-ethidium- or NeuN-positive cells, an ANOVA test with a nested model was used, considering each field of the same dish as a pseudo-replica.

3. Results

3.1. Mg^{2+} free media induces an increase in intracellular calcium

Elevated concentration of intracellular calcium is a common feature of most neurodegenerative diseases (DeLorenzo et al., 2005; Hardingham and Bading, 2010). Previous work by others has demonstrated that Mg^{2+}-free medium induces in hippocampal neuronal cultures a rise in $[Ca^{2+}]^i$ and persistent neuronal activity, which has been proposed as an in vitro model of *status epilepticus* (SE) (Sombati and DeLorenzo, 1995; DeLorenzo and Pal, 1998; Xie et al., 2014). As expected from those reports, we show here that shortly after exposing neurons to a Mg^{2+}-free media (a condition herein referred to as SE), $[Ca^{2+}]^i$ increased and remained elevated until Mg^{2+}-containing media was reintroduced (Fig. 1B and D). This increase in $[Ca^{2+}]^i$ was not induced by the buffer exchange per se (Fig. 1B and C). The levels of $[Ca^{2+}]^i$ persistently increased in almost 80% of hippocampal neurons during SE, returning to higher levels than baseline immediately after the reintroduction of the maintenance medium. Only in a small fraction of neurons $[Ca^{2+}]^i$ did not increase during SE (Fig. 1E and F).

3.2. SE in vitro induce hippocampal neuronal death

SE in vivo can cause neuronal degeneration and cell death in different areas of the CNS, most notably in subpopulations of the hippocampus and the entorhinal cortex (Fujikawa, 1996; von Bohlen und Halbach et al., 2004; Unsain et al., 2008). In order to study the molecular mechanisms underlying SE-induced neuronal death, we first determined the time course of neuronal death in hippocampal cultures. This was quantified with two complementary methods: calcein-ethidium staining, and the presence/absence of NeuN protein. We found that 3 h of SE induced neuronal death that became statistically significant 12 h after the end of the SE (Fig. 2B, C, D and E).

3.3. Neurite degeneration precedes hippocampal neuronal death

To assess if SE induces a loss of neuronal processes prior to neuronal death in this in vitro model, hippocampal neurons were stained with antibodies against β-III tubulin and MAP2 at 0 h, and 6 h after the SE. The induction of SE caused the loss of cell structure after 6 h. At this time, there was an increased in MAP2 degradation (Fig. 2F and G). At the same time, β-III tubulin staining showed varicosities across the culture dish with very few process-like structures (Fig. 2H and I). The dystrophic morphology evident in dendritic and axonal domains is similar to the structural early signs observed in several neurodegenerative diseases (Luo and O'Leary, 2005; Liu et al., 2008; Bevers and Neumar, 2008; Pease and Segal, 2014).

3.4. SE in vitro induces significant changes in TrkB and p75ntr in neurons undergoing degeneration

We have previously shown that SE in vivo induced modifications in the interaction between BDNF/proBDNF and their receptors (Unsain et al., 2008, 2009). To investigate if these changes also occur in neurons in this in vitro model of SE, we determined the levels of TrkB and p75ntr following SE.

To determine the SE-induced changes in TrkB receptors at the cell surface level, we evaluated TrkB by immunocytochemistry without permeabilization. We observed that TrkB immunoreactivity increased in the neuritic processes immediately after (0 h) and 6 h after the conclusion of SE (Fig. 3A). Because the antibody used is directed to the TrkB extracellular domain, it was not possible to differentiate between TrkB Fl and Tc TrkB-Fl. To analyze which of the two proteins were modified we performed a WB using the same antibody. To analyze the changes in these two proteins, we examined their levels by WB using the same antibody. Immediately after SE, there was an imbalance between

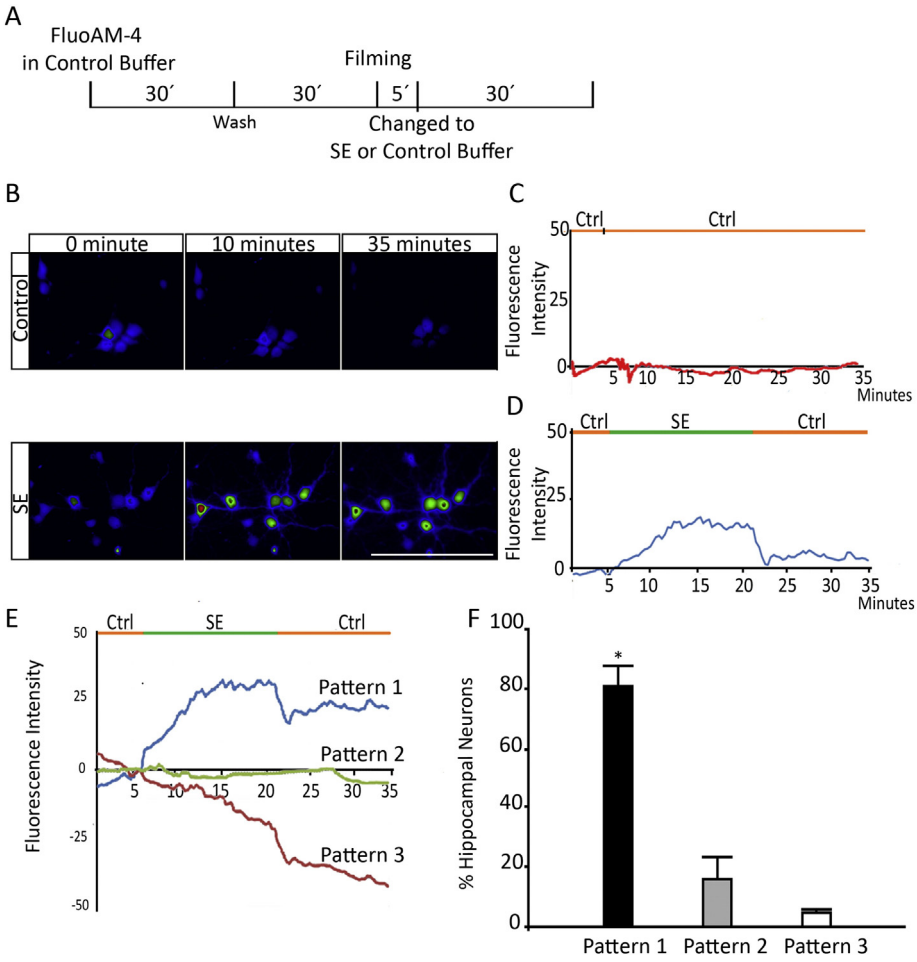

Fig. 1. SE in vitro induces an increase of intracellular calcium. A) Protocol for the measurement of the changes in $[Ca^{2+}]^i$; B) Representative micrographs showing the changes in $[Ca^{2+}]^i$ in the control and in the SE group at different time points; C) No calcium changes were observed in the control experiments; D) Mg^{2+}-free buffer induced an increased in $[Ca^{2+}]_i$; E) Following exposure to the Mg^{2+}-free buffer, 3 patterns of calcium change were observed; pattern 1; pattern 2; pattern 3; F) Frequency of neurons corresponding to the 3 different patterns of calcium change observed in the total population. Eighty percent of the neurons showed a sustained intracellular calcium increase during the entire experimental time. Scale bar 200 μm. (*) Shows significant difference, with a $p < 0.05$.

these TrkB receptor variants, evidenced by a significant decrease in the levels of TrkB-FL and, at the same time, an increase in the levels of the Tc TrkB-FL (Fig. 3B and C). These results demonstrated that SE in vitro induced an increase in Tc TrkB-FL.

It was important to analyze whether SE also induces TrkB phosphorylation (pTrkB). We found that 3 h of SE induced an increase in the levels of pTrkB, which returned to baseline levels 6 h after the end of SE (Fig. 3E and F). Similar results were observed by immunocytochemistry (Fig. 3D). At 6 h after SE there was an increase in the levels of p75ntr, while a significant drop of this protein surprisingly occurred 0 h after SE (Fig. 3G and H). An increase in BDNF and proBDNF binding to p75ntr was also observed, a common characteristic of SE (Unsain et al., 2008) (Fig. 3I and J).

3.5. SE in vitro induces an increase of Tc TrkB-FL in dendritic and axonal domains prior to the onset of neuronal death

Neuronal degeneration is a common feature of several neurological diseases (Luo and O'Leary, 2005; Liu et al., 2008; Bevers and Neumar, 2008; Pease and Segal, 2014). Also, it has been demonstrated that the decrease in TrkB-FL is a key early event preceding neuronal death (Unsain et al., 2008; Vidaurre et al., 2012; Jerónimo-Santos et al., 2015). Thus, it is possible that this event occurs first in the dendritic and axonal domains. To test this hypothesis, we took advantage of the filter insert culture system, which allows to study the neurites in isolation (Fig. 4B). As illustrated in Fig. 4C, D and E, TrkB-FL levels decreased both in the top fraction (cell bodies/processes) and bottom fraction (dentritic and axonal domains) of the filter insert culture system. Surprisingly, Tc TrkB-FL levels increased in the dendritic and axonal domains at the first hour after the beginning of the SE and lasting until 6 h after the end of the SE (Fig. 4E and F).

3.6. Differential rates of calpain activity in somatic and neuritic domains following SE in vitro

Recent reports show that calpain can process TrkB-FL to produce a degradation product that migrates at around 95 kDa, known as the truncated form of TrkB-FL (Tc TrkB-FL) (Vidaurre et al., 2012). Hence, we tested if calpain was activated by SE in vitro by examining the degradation pattern of several of its substrates, namely α-spectrin, neurofilament-M, and procaspase-3 (Ma, 2013).

SE induced the cleavage of α-spectrin in both fractions, cell bodies-processes, (top fraction) and dentritic-axonal domains, (bottom fraction), reaching a maximum 6 h after the excitotoxic insult (Fig. 5B, C and D).

We also evaluated the effect of SE on neurofilaments (NF), another type of cytoskeletal proteins. Of the three NF isoforms (NF-L, NF-M, and NF-H), NF-M is the most susceptible to proteolysis in the hippocampus (Ma, 2013). The exposure to 3 h of SE induced the degradation of the NF-M. The rate of degradation differed between the top and bottom fractions grown in the filter insert culture system, being faster in the bottom fraction (Fig. 5B and E).

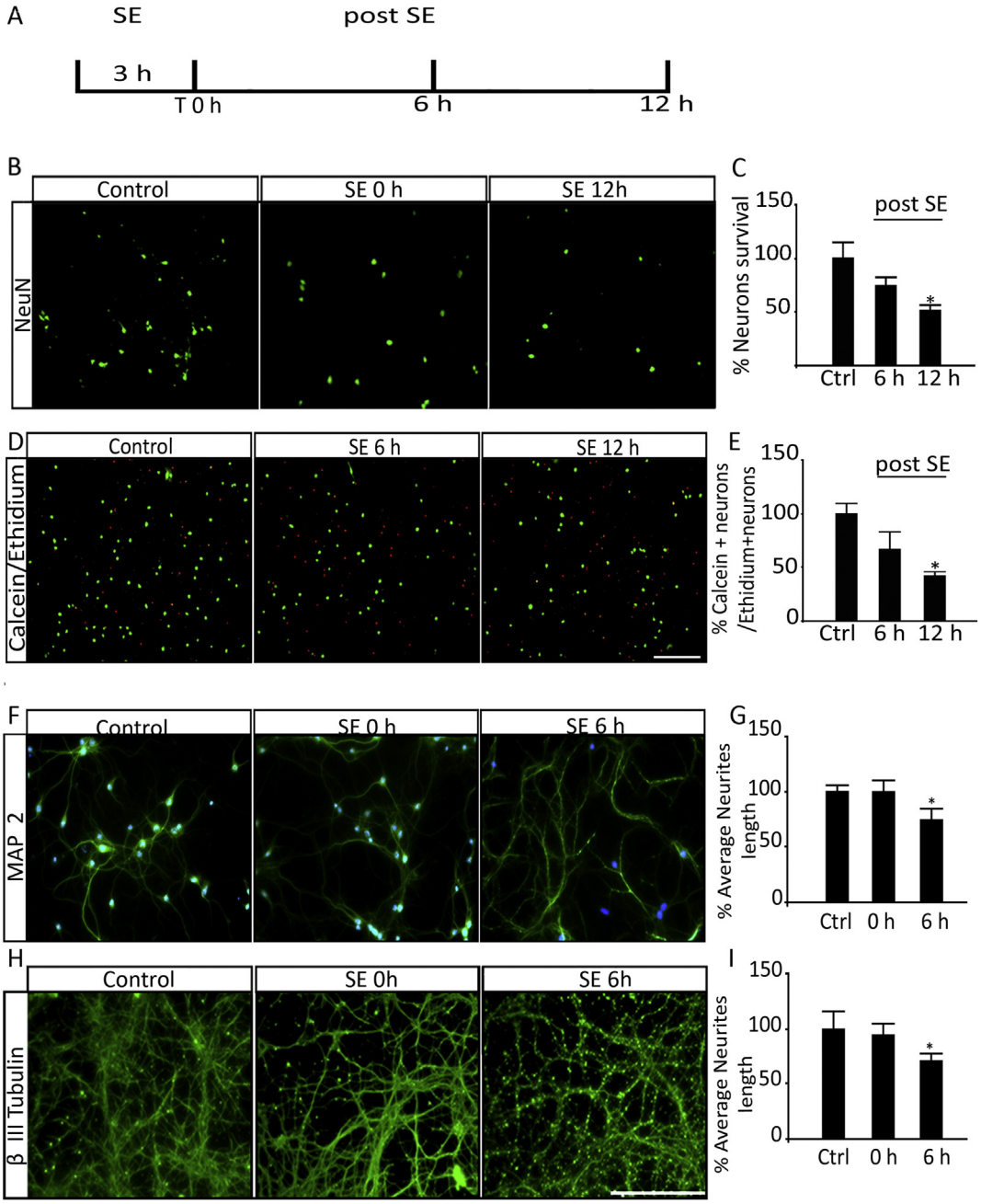

Fig. 2. SE in vitro induces hippocampal neuronal death. A) Time points analyzed following SE; B) Representative micrographs showing NeuN positive neurons in the control and SE groups at different time points. C) Twelve hours after SE there was a significant decrease in NeuN positive neurons. D) Representative micrographs showing hippocampal neurons stained with calcein/ethidium at different time point; E) Twelve hours after SE there was a significant decrease in the percentage of calcein positive neurons; F) Six hours after the SE, and before the onset of hippocampal neuronal death, there was an increase in the MAP2 degradation pattern; G) Quantification of neurite length based on MAP2 labeling; H) Six hours after the SE, and before the onset of hippocampal neuronal death, there was an increase in the β-III tubulin degradation pattern; I) Quantification of neurite length based on β-III tubulin labeling. In F and H, the micrographs shown are representative of all evaluated hippocampal cultures; however, each determination was performed in consecutive fields of each culture dish. Scale bar 200 μm. Means ± SEM are shown. Asterisks indicate significant differences compared with control. (*) shows significance ($p < 0.05$).

It has been demonstrated that calpain cleaves procaspase-3 to yield a procaspase-3 isoform of ~29 kDa (Blomgren et al., 2001). Three hours after the SE was completed, there was an increase in the level of this isoform. In this case, no differences were observed in the degradation rate between the top and bottom fraction (Fig. 5B and F).

It was important to determine whether the inhibition of calpain could prevent the protein degradation pattern observed for α-spectrin, NF-M, TrkB, and procaspase-3. To analyze this, we used the well-known calpain activity blocker *N*-acetyl-Leu-Leu-nor leucinal (ALLN). The entire protein degradation pattern was completely abolished in the presence of this inhibitor (Fig. 6). These results confirmed that SE in vitro induces an increase in calpain activity.

3.7. Calpain activation induces neuronal degeneration

To assess whether the inhibition of calpain activity prevents the neurodegeneration of hippocampal neuritic processes, we evaluated β-III tubulin immunoreactivity as a marker of structural damage, and NF-M and TrkB-FL degradation as indicator of calpain activity. As shown earlier, the changes in calpain substrates occur first in the dendritic and axonal domains; therefore, we assessed the effect of calpain inhibition on

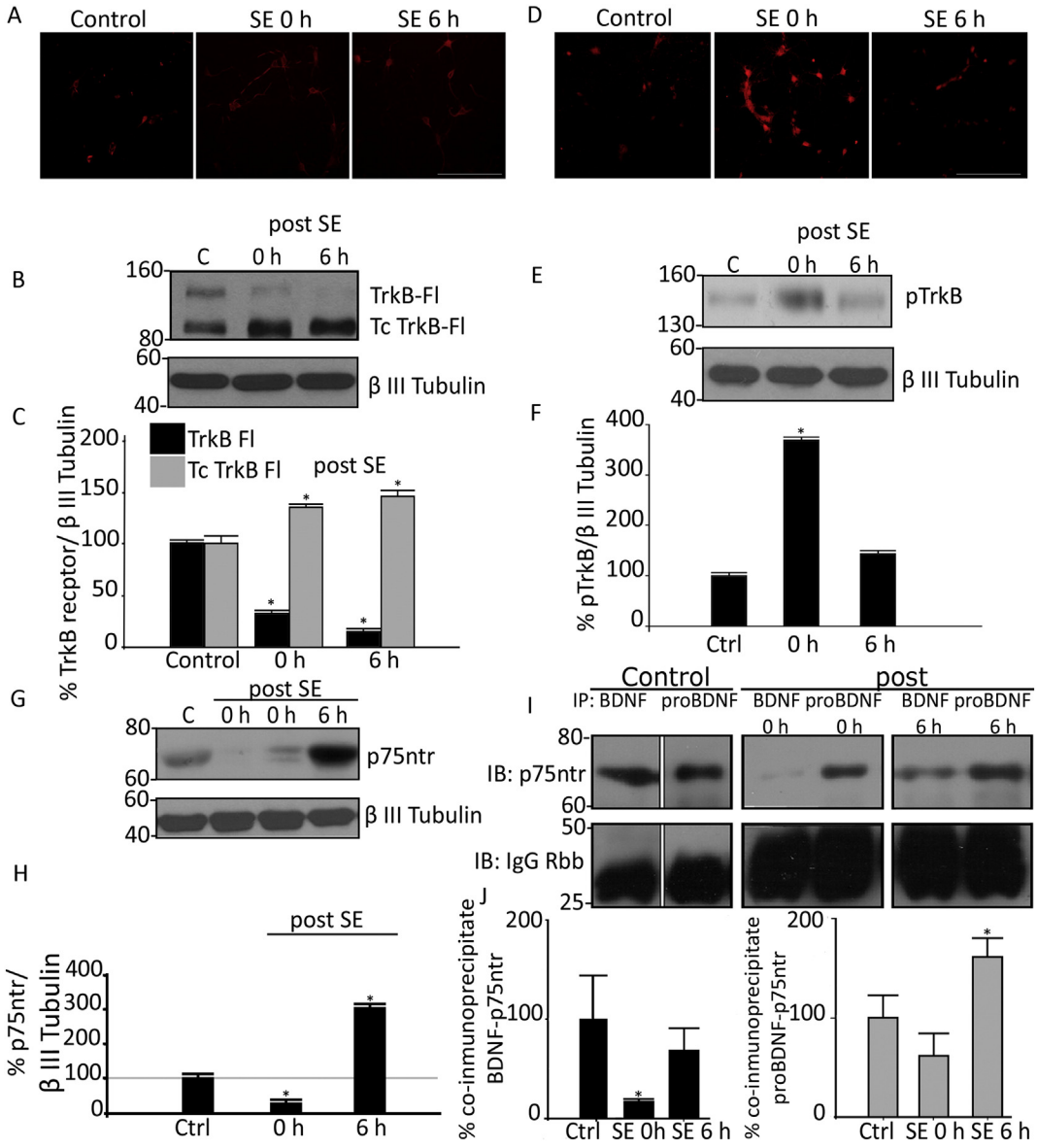

Fig. 3. SE in vitro induces modifications in the levels of TrkB, pTrkB, and p75ntr. The changes were measured at 0 h and 6 h after SE. A) Representative micrographs showing cell surface TrkB levels; B) WB assessment of TrkB-FL and Tc TrkB-FL levels; C) Quantification of TrkB-FL and Tc TrkB-FL over β-III tubulin. D) Representative micrographs showing the pTrkB levels in neurons; E) WB assessment of pTrkB levels; F) Quantification of pTrkB over β-III tubulin; G) WB assessment of p75ntr levels; H) Quantification of p75ntr over β-III tubulin; I) Levels of p75ntr co-immunoprecipitated with total BDNF and proBDNF; J) When proBDNF was specifically immunoprecipitated, the levels of co-immunoprecipitated p75ntr were significantly increased at 6 h. Means ± SEM are shown. Asterisks indicate significant differences compared with control. Scale bar 200 μm. (*) shows significance ($p < 0.05$).

NF-M and TrkB protein levels only in this fraction, using two different exposure patterns (Fig. 7A). Exposure to the inhibitor ALLN during the whole duration of the experiment (i.e. before, during and after SE) prevented the reduction of NF-M and TrkB levels. In contrast, if the inhibitor was added after SE, the degradation pattern of these proteins was similar as with SE alone (Fig. 7B, C and D).

We then evaluated the β-III tubulin degradation pattern only in the dendritic and axonal domains. The results show an absence of neuronal degeneration when calpain activity was inhibited throughout the experiment. However, if the inhibition was induced after the SE finished, β-III tubulin degradation occurred unimpeded (Fig. 7E and F).

3.8. Blocking calpain activity and Tc TrkB-FL prevents SE-induced neuronal death

To evaluate whether calpain activity induces neuronal death, we evaluated neuronal survival in the presence or absence of ALLN at 12 h after SE. Blocking calpain activity during the length of the experiment resulted in a remarkable neuroprotection. However, no neuroprotection occurred if ALLN was added after the induction of SE (Fig. 8A and B).

Similarly, to assess whether Tc TrkB-FL is involved in neuronal death, we analyzed the effect of the specific TrkB antagonist ANA12 (Cazorla et al., 2011). Because we previously observed that immediately after SE (0 h) there was a significant drop in TrkB-FL protein levels (Figs. 3 and 4), we assumed that ANA12 affected mainly Tc TrkB-FL. As can be seen in Fig. 8E and F, the addition of ANA12 immediately after the conclusion of SE totally blocked neuronal death.

4. Discussion

The degeneration of neuritic processes is a common early event in a wide range of neurodegenerative diseases and neurological conditions (Raff et al., 2002; Luo and O'Leary, 2005; Bevers and Neumar, 2008; Vickers et al., 2009; Neukomm and Freeman, 2014; Pease and Segal, 2014). We used a neuronal culture system that allows to evaluate the

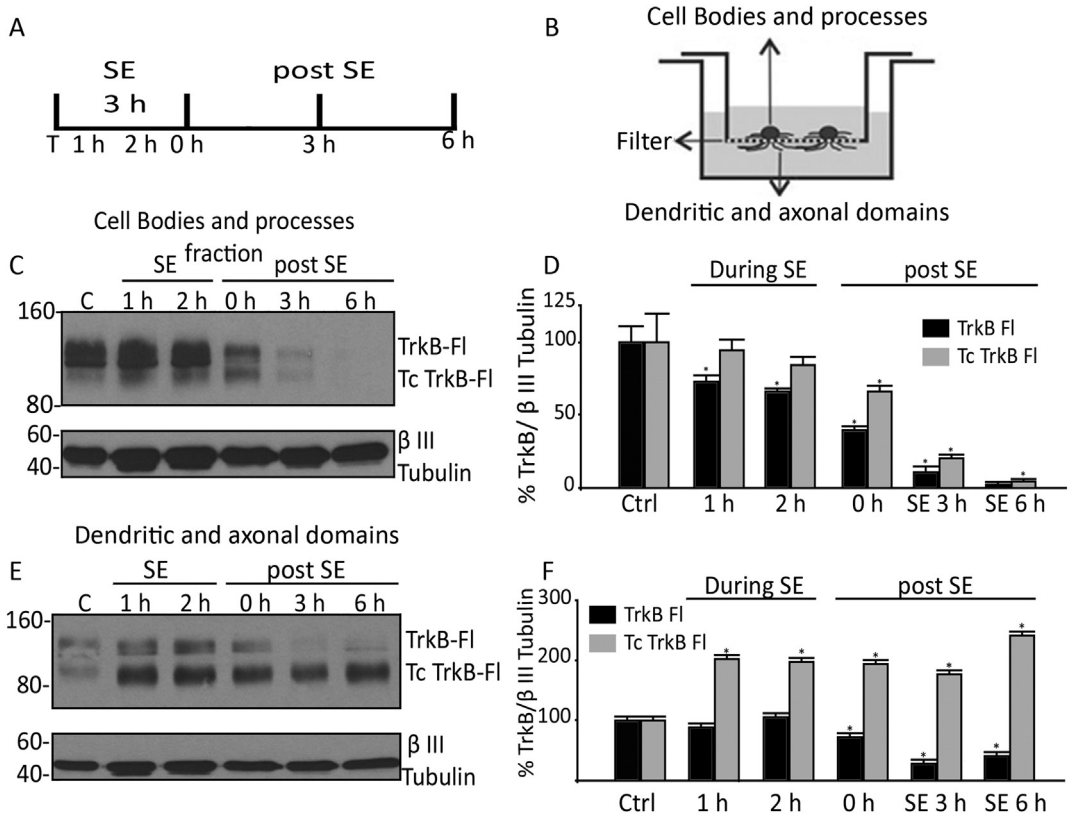

Fig. 4. SE in vitro induces an increase in Tc TrkB-FL levels in the dendritic and axonal fraction. A) Time points analyzed following SE; B) Schematic diagram of the filter insert system used to study the biochemical difference between top (cell bodies and processes) and bottom (dendritic and axonal domains) fractions; C) WB assessment of TrkB-FL and Tc TrkB-FL levels in cell bodies and processes fractions during and after SE (at 1, 2 h-during SE- and 0, 3 and 6 h after SE); D) Quantification of TrkB-FL and Tc TrkB-FL levels in the cell bodies and processes fractions; E) WB assessment of TrkB-FL and Tc TrkB-FL levels in the dendritic and axonal domains during and after SE (at 1, 2 h-during SE- and 0, 3 and 6 h after SE); F) Quantification of TrkB-FL and Tc TrkB-FL levels in the dendritic and axonal domains. Tc TrkB-Fl levels increased in the dendritic and axonal domains after 1 h of SE and remained elevated at 6 h. In contrast, TrkB-FL decreased in both fractions. Means ± SEM are shown. Asterisks indicate significant differences compared with control. (*) shows significance ($p < 0.05$).

changes that occur in different neuronal domains to determine the morphological and biochemical events that occur before the onset of neuronal death. Several studies have used the Mg^{2+}-free-media-induced activation of *N*-methyl-D-aspartate glutamate receptors (NMDA) paradigm to determine the mechanism underlying epileptogenesis in a scenario where no cell death is provoked (Sombati and Delorenzo, 1995; DeLorenzo et al., 2005, Xie et al., 2014). However, very few have studied the mechanisms that lead to neuronal death (Skaper et al., 1998). In this work, we found that 3 h of SE induced significant neuronal death 12 h after the end of the exposure to the Mg^{2+}-free media. One of the main differences with other studies is that we used an astrocyte-free-culture condition. It has been suggested that astrocytes exert a protective effect on neurons (Skaper et al., 1998; Vidaurre et al., 2012). Indeed, unpublished data from our laboratory indicate that, in this model of SE in vitro, astrocytes provide a remarkable protective effect, thereby enhancing neuronal survival.

A key characteristic of several neurodegenerative diseases is the occurrence of intracellular calcium overload, often the first step of the process leading to neuronal death. Ca^{2+} can enter neurons through different routes, and Ca^{2+} inflow via NMDA receptors has been identified as more important than others (Tymianski et al., 1993; Ouardouz et al., 2006). The activation of NMDA receptors causes Ca^{2+} influx and a brief transient increase in the intracellular concentration of this ion, which then returns to basal levels. However, when glutamate receptor activation levels are kept high at synapses (for example, in brain ischemia or SE), this causes a secondary rise in intracellular Ca^{2+} concentration, which remains elevated at higher levels than the initial Ca^{2+} elevation (Budd and Nicholls, 1996). This secondary Ca^{2+} increase has been called *delayed Ca^{2+} deregulation* (DCD) (Araújo et al., 2010). In line with these results, we confirmed that the exposure to Mg^{2+}-free media increased the intracellular calcium levels due to receptor hyperactivation (Sombati and Delorenzo, 1995; DeLorenzo and Pal, 1998; DeLorenzo et al., 2005). As can be observed in Fig. 1, we found different calcium response patterns to SE, although in the majority of neurons there was an increase in $[Ca^{2+}]_i$. Similar responses were observed in a model of ischemia (Jourdain et al., 2011).

Among the several events that occur between the increase in $[Ca^{2+}]^i$ and neuronal death, the activation of calpain, a calcium-regulated cysteine protease, seems to play a key role in several pathological conditions (O'Hanlon et al., 2003; Bevers and Neumar, 2008; Liu et al., 2008; Ma, 2013). It has been proposed that calpain activation helps maintain high cytoplasmic calcium levels as a result of the proteolytic inactivation of the NCX3 subtype of the Na^+–Ca^{2+} exchanger (NCX), a condition that impairs Ca^{2+} extrusion (Bano et al., 2005).

We detected that SE in vitro induces the degradation of TrkB-FL while increasing at the same time Tc TrkB-FL levels concurrently with p75ntr elevation. More importantly, this work shows that the imbalance in TrkB-FL/Tc TrkB-FL occurs first in the dendritic and axonal domains, and precedes the onset of neuronal death. Others authors have proposed that the degradation of TrkB-FL is caused by calpain, to produce the truncated protein form lacking the tyrosine kinase domain (Tc TrkB-FL). This protein is strikingly similar to the variant of TrkB isoform TrkB-T1 (Vidaurre et al., 2012). Here we show that the inhibition of calpain activity prevented this imbalance in the dendritic and axonal domains and, most importantly, it resulted in a complete absence of axonal degeneration and prevented neuronal death. Because calpain has an important role in the increase of Tc TrkB-FL levels, it is possible that this Tc TrkB-FL receptor has a biological role in neuronal death. Here we show that this is a plausible hypothesis, because ANA 12, a specific TrkB extracellular domain receptor antagonist (Cazorla et al.,

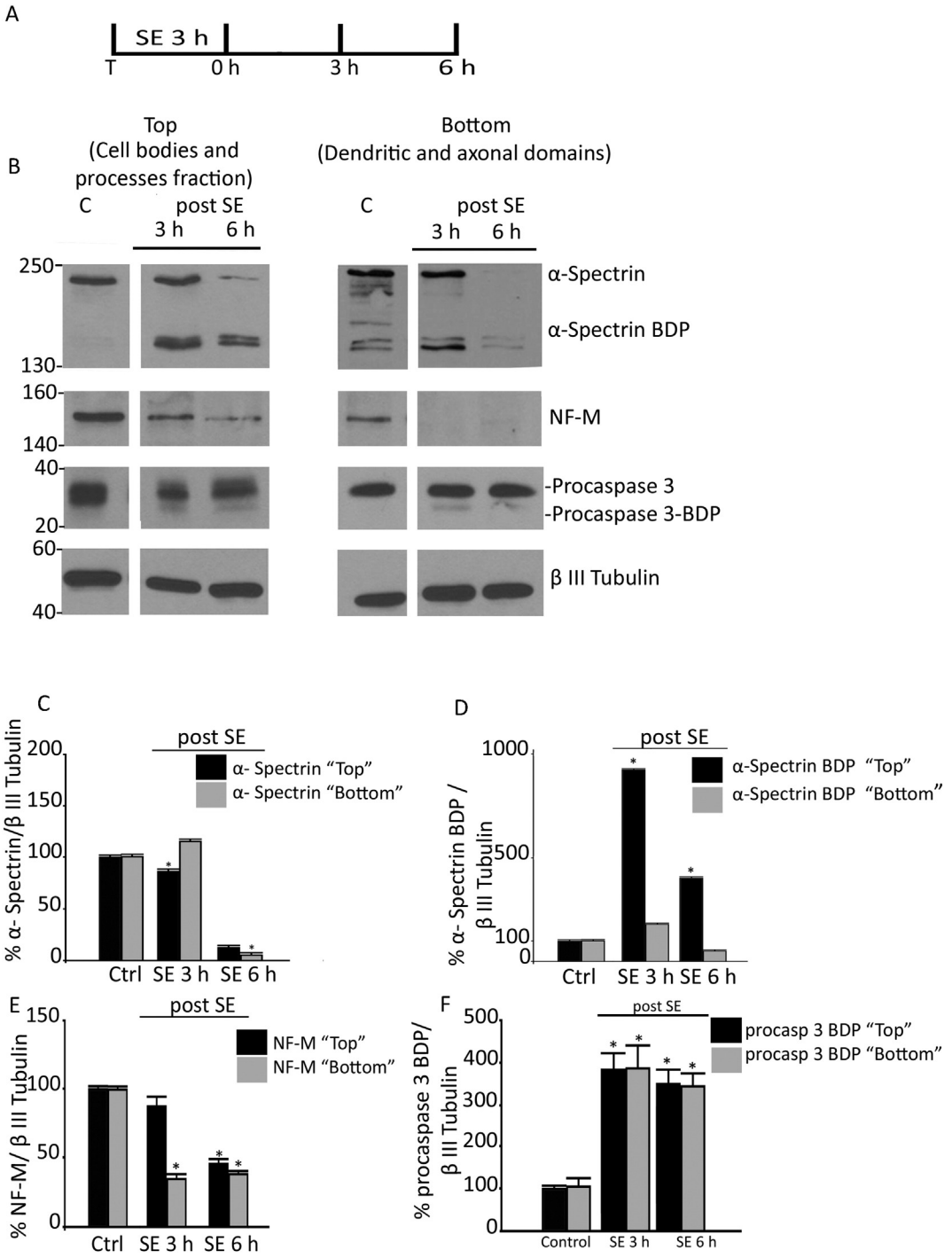

Fig. 5. SE in vitro increases the activity of calpain protease, measured by the breakdown of α-spectrin, NF-M and procaspase-3. A) Time points analyzed following SE; B) WB assessment of α-spectrin, NF-M and procaspase-3 levels in the cell bodies and processes fraction (top fraction) and in the dendritic and axonal fraction (bottom fraction); C-D) Quantification of α-spectrin and its breakdown product (BDP) in each fraction; E) Quantification of NF-M in each fraction; F) No difference was apparent in the degradation rate of procaspase-3 at 3 h and 6 h after SE between cell bodies/processes and dentritic and axonal domains. Means ± SEM are shown. Asterisks indicate significant differences compared with control. (*) shows significance ($p < 0.05$).

2011), completely blocked the SE-induced neuronal death. It will be important to further investigate the role of Tc TrkB-FL in this process. Because we added ANA12 to the neuronal culture (Fig. 8) at the time when a significant drop in the levels of TrkB-FL had already occurred (Fig. 3, Fig. 4), we believe that the observed effect of the antagonist did not result from TrkB-FL inhibition, but mostly from an effect on Tc TrkB-FL. Others have shown that following excitotoxic stimulation there is an increase in the synthesis of TrkB-T1 receptor, and that this event occurs prior to the onset of neuronal death (Vidaurre et al., 2012; Xie et al., 2014). However, the possibility that the ANA12 prevents neuronal death by inhibiting any signaling pathway downstream TrkB-T1 can be dismissed because blocking the synthesis of this protein did not alter neuronal death (Vidaurre et al., 2012). In addition to the TrkB-FL/Tc TrkB-FL, previous experiments in vivo from our laboratory have demonstrated that SE also induces an imbalance in TrkB-FL/p75ntr prior to the onset of neuronal death (Unsain et al., 2008, 2009). In agreement with those results, we found here the same phenomenon, as well as an increase in the interaction between BDNF and

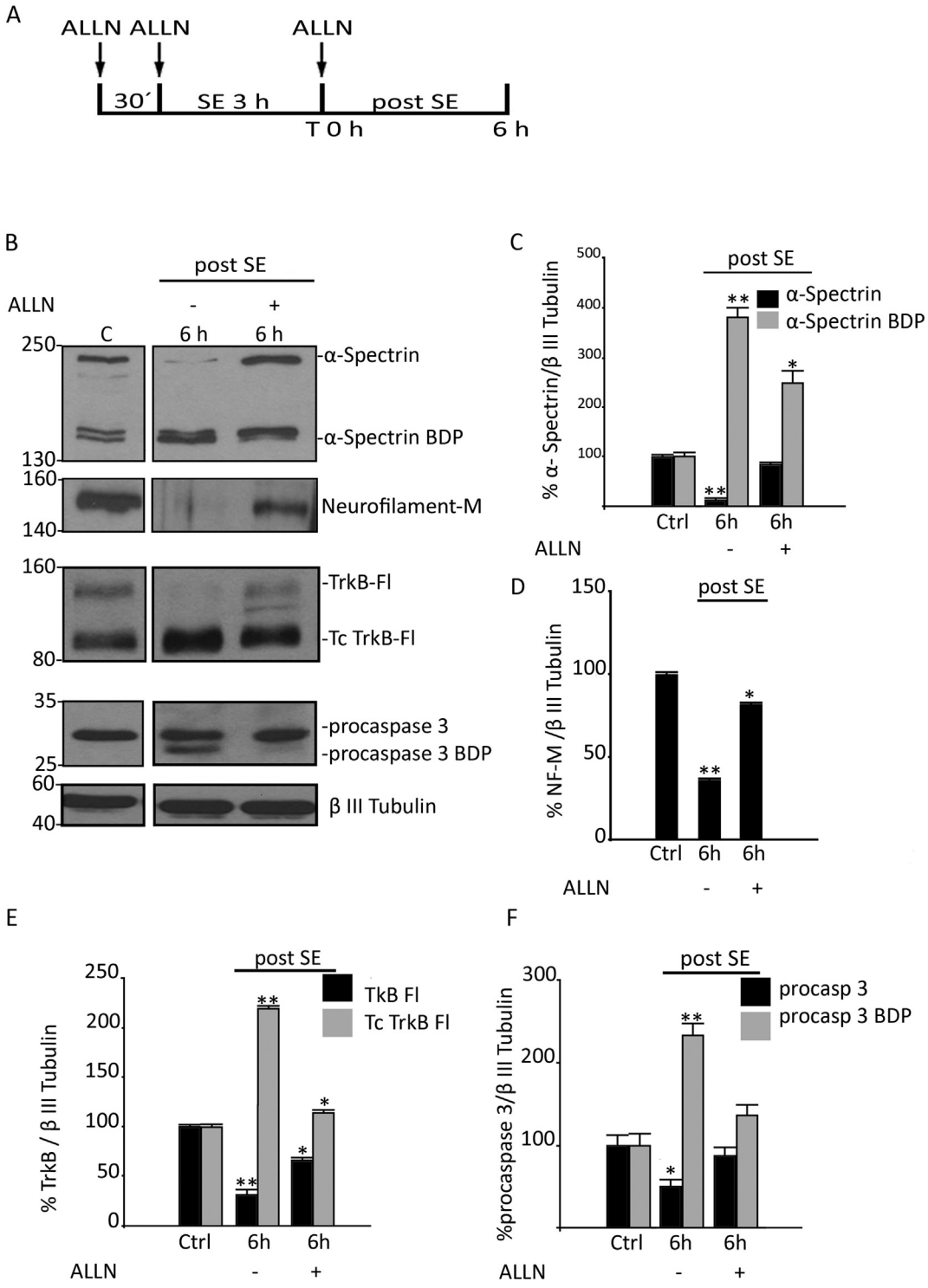

Fig. 6. The inhibition of calpain activity by ALLN abolishes protein degradation. A) Schematic representation of the experimental design for calpain activity inhibition, and time points analyzed following SE; B) WB showing that at 6 h after SE, inhibition of calpain by ALLN prevented the degradation of α-spectrin, NF-M, as well as the increase of Tc TrkB-FL and procaspase-3 BDP; C) Quantification of α-spectrin and its BDP; D) Quantification of NF-M levels; E) Quantification of TrkB-FL and Tc TrkB-FL levels; F) Quantification of procaspase-3 and its BDP. Means ± SEM are shown. (*) Asterisks indicate significance differences compared with control. (**) indicate significant compared with control and ALLN treated group. (*) shows significance ($p < 0.05$).

proBDNF with p75ntr before the onset of neuronal death. These findings help validate this in vitro model of SE.

Further evidence that calpain can process receptors is found in a Huntington disease (HD) model. Increase in calpain activity induced the cleavage of the C-terminal GluN2B subunits resulting in the pathological relocation of NMDA receptor (Gladding et al., 2012). The pharmacological inhibition of calpain completely inhibited the relocation of neuroprotective synaptic NMDA receptors to extrasynaptic sites, where they are thought to promote cell death instead (Hardingham and Bading, 2010).

Another event preceding the onset of neuronal death is dendritic and axonal degeneration. This involves a breakdown of components of the neuronal cytoskeleton (MAP2, α-spectrin and NF-M). Among several calpain substrates, α-spectrin seems to be the most studied. An

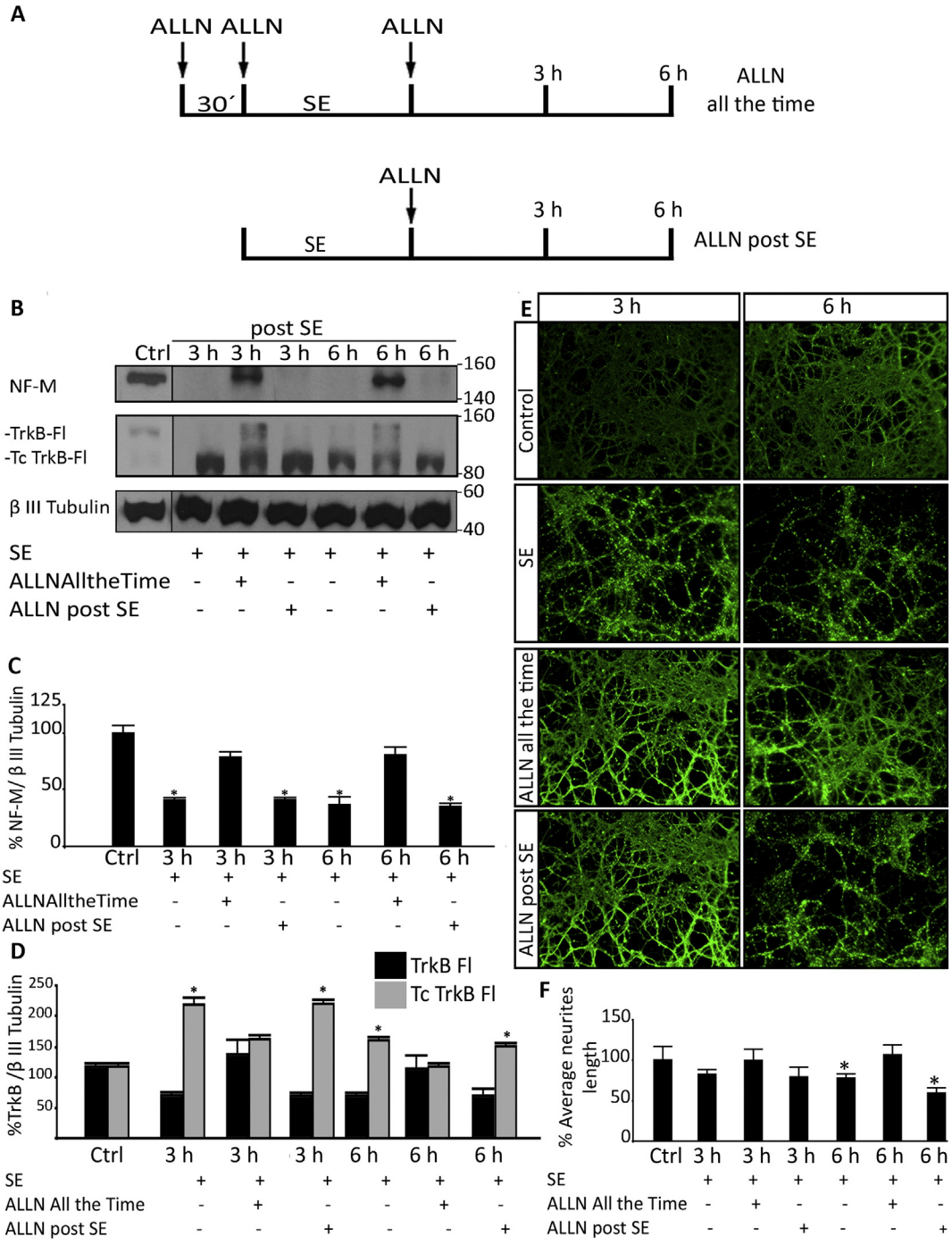

Fig. 7. Inhibition of calpain activity by ALLN prevents the dendritic and axonal degeneration, and the degradation of NF-M and TrkB in the dendritic and axonal fraction. A) Schematic representation of the two experimental designs for calpain activity inhibition, and time points analyzed; B) WB assessment of NF-M, TrkB-FL and Tc TrkB-FL levels in the dendritic and axonal fraction. C) Quantification of NF-M levels; D) Quantification of TrkB-FL and Tc TrkB-FL levels. The inhibition of calpain activity during and after SE (ALLN All time) prevented the degradation of NF-M and TrkB-FL, as well as the increase in Tc TrkB-FL. E) Micrographs showing the β-III tubulin degradation pattern in neuronal processes stained with anti-β-III tubulin antibody, at 3 and 6 h after SE in the following conditions: Control, SE, SE ALLN All time, and ALLN post-SE. Note the intense staining showing varicosities in the SE and ALLN post-SE groups. Calpain inhibition during the entire course of the experiment prevented the degradation of β-III tubulin. F) Quantification of β-III tubulin-labeled processes. Scale bar 200 μm. Means ± SEM are shown. The micrographs shown are representative of all evaluated hippocampal cultures; however each determination was performed in consecutive fields of each culture dish. Asterisks indicate significant differences compared with SE + ALLN (all the time). (*) shows significance ($p < 0.05$).

important function of spectrin is to provide a link between transmembrane proteins and cytoskeletal proteins, particularly actin filaments (Yan and Jeromin, 2012; Ma, 2013). A common feature of several neurological diseases is the proteolytic processing of α-spectrin, with a resulting increase in the amount of α-spectrin breakdown products (α-S-BDP) (Vidaurre et al., 2012; Yan and Jeromin, 2012). In this work, we used the α-spectrin degradation pattern to determine if SE induces an increase in calpain activity. In line with this, in ischemic-type lesions (such as the occlusion of the brain medial artery, MCAO), an early calpain-dependent-proteolysis of β IV-spectrin occurs, and it has been proposed as an earlier event damaging the axonal initial segment (Schafer et al., 2009; Komada, 2002). In addition, we showed that calpain can also process other cytoskeletal proteins, such as neurofilaments (NF). There are three different NF isoforms that are calpain substrates. Among them, the NF-M isoform seems to be more susceptible to calpain proteolysis (Chung et al., 2005; Ma, 2013). The loss of NF and α-spectrin results in

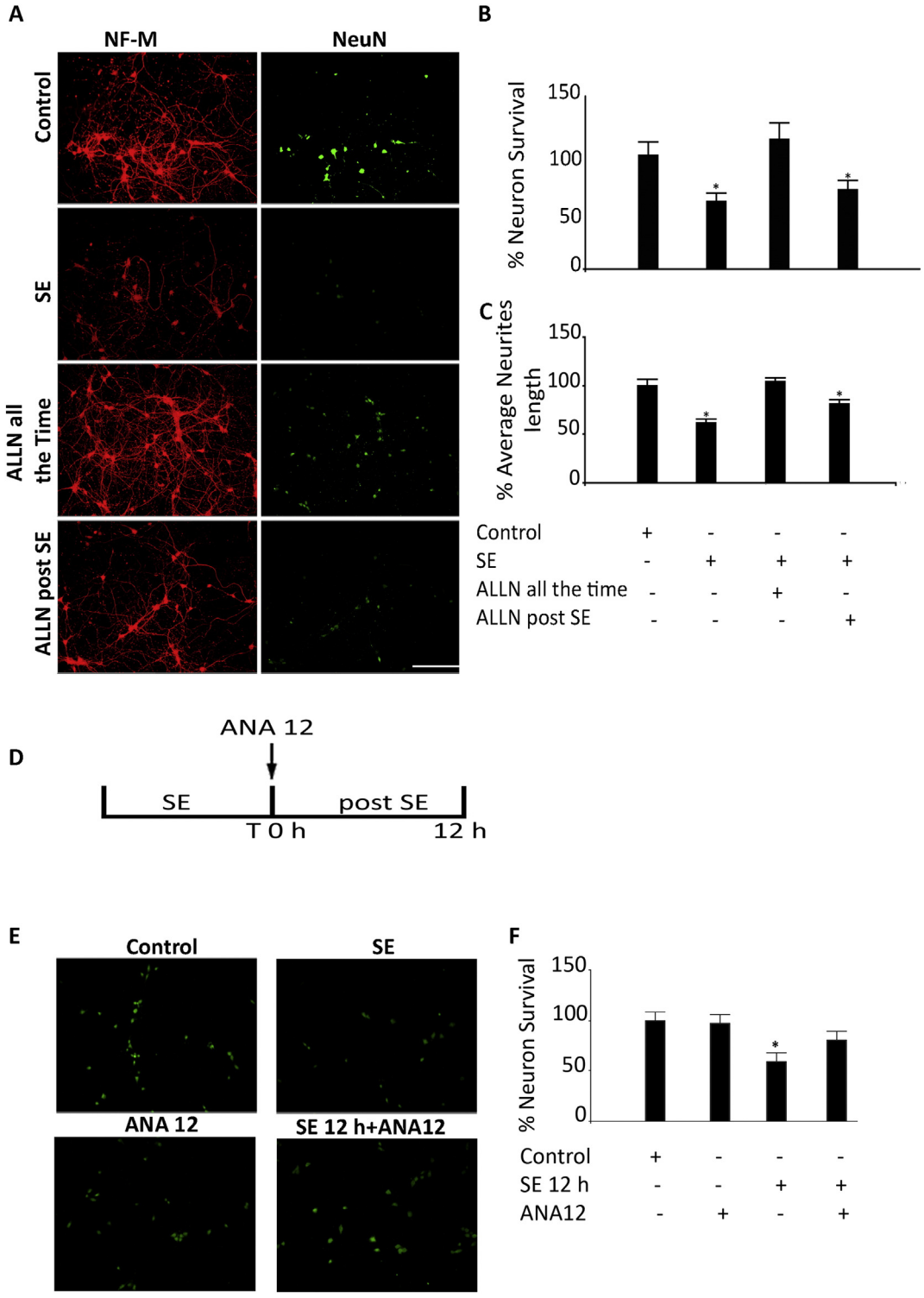

Fig. 8. Inhibition of calpain activity and Tc TrkB-FL provides neuroprotection. A) Micrographs showing NF-M (left) and NeuN (right) staining, assessed in Control, SE, SE + ALLN All time and ALLN post-SE; B) Quantification of the number of NeuN positive neurons. The inhibition of calpain activity during and after the SE prevented the neuronal death induced by SE; C) Quantification of NF-M positive neurites. D) Schematic representation of the experimental design for Tc TrkB-FL inhibition, and time points analyzed; E) Micrographs showing NeuN staining, assessed in Control, SE, SE + post-ANA12/ANA12 post-SE; F) Quantification of the number of NeuN positive neurons. The inhibition of Tc TrkB-FL after the SE prevented the neuronal death induced by SE. The micrographs are representative of all evaluated hippocampal cultures; however, each determination was performed in consecutive fields of each culture dish. Scale bar 200 μm. Means ± SEM are shown. Asterisks indicate significant differences compared with control. (*) shows significance ($p < 0.05$).

compromised axonal structure and is associated with the disruption of axonal transport (Posmantur et al., 1997).

It has been reported that the relationship between calpain and caspase-3 gives rise to a dynamic activation circuit resulting in cell death (Yildiz-Unal et al., 2015). It is known that cleavage of procaspase-3 by calpain leads to activation of caspase-3. On the other hand, caspases play a role in the degradation of a specific endogenous inhibitor of calpain, *calpastatin*, which accelerates calpain activation (Sharma and Rohrer, 2004; Strachan et al., 2005). This provides evidence that both proteolytic systems are involved in the progression of neuronal death.

Axon and dendrite degeneration may be initiated through a number of different mechanisms, with calpain activation and Tc TrkB-FL playing very important roles in this process. All the events described above occur before the onset of dendrite and axonal degeneration, a prominent common early characteristic of several neurodegenerative diseases.

Conflict of interest

All authors declare no actual or potential conflict of interest.

Acknowledgment

This work was supported by grants from CONICET, FONCYT 2012 # 2100 and SECyT-UNC. D.H.M. is a career member of CONICET; V.D. is recipient of a doctoral fellowship from CONICET. We thank The Company of Biologist and ISN-CAEN for the travel scholarships given to V.D. to perform some of the experiments of this work at Montreal Neurological Institute, McGill University, Montreal, Canada.

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