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# Brain-derived neurotrophic factor facilitates TrkB down-regulation and neuronal injury after status epilepticus in the rat hippocampus

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**Abstract**

Brain-derived neurotrophic factor (BDNF) is involved in many aspects of neuronal biology and hippocampal physiology. Status epilepticus (SE) is a condition in which prolonged seizures lead to neuronal degeneration. SE-induced in rodents serves as a model of Temporal Lobe Epilepsy with hippocampal sclerosis, the most frequent epilepsy in humans. We have recently described a strong correlation between TrkB decrease and p75ntr increase with neuronal degeneration (*Neuroscience* 154:978, 2008). In this report, we report that local, acute intra-hippocampal infusion of function-blocking antibodies against BDNF prevented both early TrkB down-regulation and neuronal degeneration after SE. Conversely, the infusion of recombinant human BDNF protein after SE greatly increased neuronal degeneration. The inhibition of BDNF mRNA translation by the infusion of antisense oligonucleotides induced a rapid decrease of BDNF protein levels, and a delayed increase. If seizures were induced at the time endogenous BDNF was decreased, SE-induced neuronal damage was prevented. On the other hand, if seizures were induced at the time endogenous BDNF was increased, SE-induced neuronal damage was exacerbated. These results indicate that under a pathological condition BDNF exacerbates neuronal injury.

**Keywords:** BDNF, hippocampus, infusion, neurotrophin receptors, pilocarpine, seizures.

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Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family that plays an important role in the survival, differentiation and growth of many types of neurons (Bibel and Barde 2000; Chao 2003). BDNF effects on survival and differentiation have been associated with activation of the TrkB receptor, implying receptor autophosphorylation after ligand binding (Barbacid 1994). On the other hand, it has been demonstrated that the activation of p75ntr by BDNF induces cell death, which in most cell types analyzed only takes place in the absence of TrkB signaling or when it is diminished (Davey and Davies 1998; Friedman 2000). The differential maturation of secreted neurotrophins adds another level of complexity to the system. All neurotrophins are synthesized as precursor forms (proneurotrophins), which dimerize after translation (Kolbeck *et al.* 1994). Proneurotrophins, including the precursor of BDNF (proBDNF), can be secreted from cells (Yang *et al.* 2009) or cleaved intracellularly by furin or proconvertases to yield C-terminal mature neurotrophin dimers (Mowla *et al.* 2001).

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*Abbreviations used*: ASO-*bdnf,* anti-sense oligonucleotides against BDNF mRNA; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; FJB, Fluoro Jade B; IP, immunoprecipitation; mBDNF, mature brain-derived neurotrophic factor; MSO-bdnf, miss-sense oligonucleotides with the same nucleotides, but not same sequence, as ASO-*bdnf*; p75ntr, p75 neurotrophic receptor; PBS, phosphate-buffered saline; proBDNF, immature (pro) brain-derived neurotrophic factor; rhBDNF, recombinant human BDNF protein; RIPA buffer, radio-immunoprecipitation assay buffer; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SE, status epilepticus; TrkB, tropomyosin receptor kinase B; TrkB-fl, full-length TrkB; TrkB-IR, TrkB immunoreactivity; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick-End Labeling.

 

Until recently, only mature BDNF was considered as the biologically active form that elicited TrkB- or p75ntr-dependent signaling. However, recent studies indicate that proBDNF can be secreted by cells and act via a dual receptor system of p75ntr and the type I transmembrane protein sortilin to mediate cell apoptosis (Teng *et al.* 2005). This phenomenon was also observed for proNGF (Nykjaer *et al.* 2004). Despite our recent knowledge on the capacity of neurotrophins to elicit neuronal apoptosis, the understanding of their physiological relevance is still poor, and interestingly it could be related with pathological conditions affecting human health.

The administration of pilocarpine to rats pre-treated with lithium leads to a prolonged-seizures condition called status epilepticus (SE), and results in both necrotic and apoptotic cell death in various brain areas (Fujikawa 1996). At the same time, SE induces an increase in BDNF protein expression in the same brain regions (Schmidt-Kastner *et al.* 1996; Rudge *et al.* 1998). For many years, this rise in the neurotrophin was postulated as playing a protective role against seizure-mediated excitotoxicity (Gall 1993; Schmidt-Kastner *et al.* 1996). However, we have recently shown that SE induces a rapid down-regulation of TrkB that accompanies neuronal injury in the hilus and precedes that of the CA1 (Unsain *et al.* 2008) and others have found that p75ntr is up-regulated after SE in TUNEL-positive neurons (Roux *et al.* 1999). These results open the possibility for a potential apoptotic function of BDNF and/or proBDNF after seizures. Also, studies *in vitro* and *in vivo* have determined that the infusion of exogenous BDNF induced the down-regulation of TrkB (Frank *et al.* 1996, 1997; Knusel *et al.* 1997), although it is not known if this can occur *in vivo* with endogenous BDNF. All these evidences suggest that the exacerbated release of BDNF after SE is the possible signal inducing both TrkB down-regulation and neuronal death.

Previous studies have shown evidence for the role of BDNF in SE (Larmet *et al.* 1995; Rudge *et al.* 1998; Tandon *et al.* 1999; Lahteinen *et al.* 2002, 2003; He *et al.* 2004; Xu *et al.* 2004). However, its role during or shortly after SE onset is not known. Hence, the role of BDNF on SE-induced neuronal damage and the TrkB down-regulation was directly assessed by the acute intrahippocampal infusion (into the CA1) of antibodies, antisense oligonucleotides, and recombinant proteins.

## Experimental procedures

### Animals and induction of status epilepticus

Adult male Wistar rats (Instituto Ferreyra, Córdoba, Argentina), aged 2–2½ months and weighing 200–300 g, were used, housed under environmentally controlled conditions. Animals received water and food *ad libitum* and were maintained in a 12 h light/dark cycle (light period from 7 AM to 7 PM). The experimental protocol for this study followed the guidelines of the USA National Research Council Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and was approved by the Animal Care and Use Committee of the National University of Córdoba. Status epilepticus was induced by the i.p. administration of lithium chloride 3 meq/kg 12–16 h before pilocarpine hydrochloride 45 mg/kg (Sigma, St Louis, MO, USA). In all experiments, pilocarpine was administered between 8 AM and 9 AM. Diazepam (10 mg/kg, i.p.; Hoffmann-La Roche Ltd, Basel, Switzerland) was injected 3 h after the onset of SE to stop seizure activity. In the experiments with the infusion of recombinant human BDNF, when indicated, diazepam administration was performed 1.5 h after the onset of SE. Control animals were treated identically to the experimental group, except that they received saline instead of pilocarpine. Following the initial pilocarpine injection, the animals were closely monitored to determine the onset of SE. The validity of relying on behavioral monitoring to assess seizure activity has been demonstrated previously by studies correlating electrophysiological measures with observed seizure behaviors (Tremblay *et al.* 1984). By a close observation of seizure behavior we were able to identify two clear-cut groups. One of them was named 'R2' and the other 'SE'. Behavioral patterns of each group were described before (Unsain *et al.* 2008). Briefly, R2 animals (20% of injected animals) failed to enter SE, but continuously or intermittently in the observation period manifested typical behaviors of stage 2 of Racine's scale (Racine 1972).

### Surgery and general infusion procedure

Male Wistar rats were implanted under deep ketamine : xylazine (100 : 15 mg/kg) anesthesia with 22 gauge guide cannulas in the dorsal CA1 region of the left hippocampus at the coordinates of Paxinos and Watson's atlas (Paxinos and Watson 2007), anterior:−3.2, lateral:+2.7, ventral:−1.2. A screw was placed in the skull and the cannulae were fixed to the skull with dental acrylic. After 4–5 days of recovery from the surgery, the animals were subjected to SE, before or after receiving the infusions. Infusion cannulae (30 gauge) passed the end of the guide cannulae by 1.8 mm, making the final ventral coordinate of the infusion −3 mm. Infusions were in all cases performed at a rate of 1 μL/min with a Hamilton precision pump. When the desired volume was infused, the infusion cannulae were left *in situ* 1–2 min in order to allow the liquid to penetrate the tissue. Histological examination of infusion cannulae placements was performed by analyzing serial, Nissl-stained sections. Only the data from animals with the cannulae located in the intended site were included in the final analysis. Infusion sites of each animal analyzed are shown in Fig. S1b. At least 4–5 animals were considered in each experimental condition.

### Infused Molecules

In the experiments with function-blocking anti-BDNF antibody, cannulated rats, either 3 h before pilocarpine or immediately after diazepam, received 3 μL of sterile saline or rabbit purified IgGs (0.8 μg/μL; Chemicon Int., Temecula, CA, USA) or function-blocking anti-BDNF antibody (0.8 μg/μL; Chemicon Int.; AB1779SP). Infusion of antibodies against proBDNF was 3 μL (0.8 μg/μL; Alomone Labs. (Jerusalem, Israel) ANT-006).

In the experiments with recombinant human BDNF protein, hrBDNF (Chemicon Int., GF029) was prepared in sterile saline

 

(final concentration 0.25 μg/μL). The volume infused was 1–1.25 μL. As a control infusion we used Cytochrome-*c* (Sigma) at the same concentration.

In the experiments with oligodeoxinucleotides (ODN), the infusions were very similar to that used by others (Bekinschtein *et al.* 2007; Barnes and Thomas 2008). Briefly, ODN (Genbiotech S.R.L., Argentine) were HPLC-purified phosphorothioate end-capped 18-mer sequences, resuspended in sterile saline to a concentration of 2 nmol/μL and a volume of 1–1.25 μL was infused. Both ODNs were phosphorothioated on the three terminal bases of both 5′- and 3′-ends. This modification results in increased stability and less toxicity of the ODN. BDNF ASO, 5′-TC-TTCCCCTTTTAATGGT-3′; BDNF MSO, 5′-ATACTTTCTGTT-CTTGCC-3′.

### Western blot analyses

Animals were killed by decapitation at the indicated time points following diazepam administration in SE-induced animals or immediately after diazepam in saline-treated controls. The hippocampus was immediately removed from the skull and homogenized in radio-immunoprecipitation assay buffer or in radio-immunoprecipitation assay-modified buffer containing protease and phosphatase inhibitors (protease inhibitor cocktail Sigma P8340 and phosphatase inhibitor cocktail Sigma P5726). In some experiments, as indicated in the text, left and right hippocampus were homogenized separately. Homogenates were cleared by centrifugation at 500 *g* twice for 7 min and protein concentration was determined using the Bradford protocol (Bradford 1976); samples were then boiled in gel-loading buffer and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% for TrkB, 15% for BDNF and proBDNF). Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories Inc., PA, USA) and blocked with 5% non-fat milk in Tris-buffered saline with 0.05% Tween, for 3 h at 23°C. Membranes were then incubated with the primary antibody of interest. Rabbit polyclonal antibodies against TrkB (sc:8316) and BDNF (sc:546) were from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Membranes were then incubated with appropriate biotinylated secondary antibodies, followed by incubation with streptavidin-conjugated peroxidase and enhanced chemiluminescence detection (ECL), and then exposed to Kodak X-OMAT films (Kodak, Rochester, NY, USA). Membranes were re-probed with a monoclonal antibody against anti-tubulin (Santa Cruz Biotechnology) to control protein loading. Images were scanned and gel bands were analyzed using gel-scanning integrated optical density software (Scion Imaging 4.0.2., NIH, USA). Values for treatments were expressed as a percentage of control animals.

### Fluoro Jade B staining

The number of dying neurons after pilocarpine-induced SE was assessed by labeling with Fluoro Jade B according to the published protocol (Schmued *et al.*, 1997; Schmued and Hopkins, 2000). Epifluorescent images were captured digitally. In each section, square fields were analyzed for each of the following areas: hilus (neurons within the polymorphic layer of the dentate gyrus), CA1 (cells within the stratum piramidale) and piriform cortex. Square fields are equivalent to those shown in Figs 2 and 3. The quantification performed did not aim to estimate the total number of injured neurons in a given brain area, but to quantitatively compare the degree of injury between hippocampi from both hemispheres within each section.

### Cresyl violet staining

Briefly, 50 μm-sections were mounted in alcohol–gelatin solution on gelatinized slides, dried overnight at 37°C, defatted in xylene and hydrated through a gradient of alcohols to distilled water. Slides were then incubated in Cresyl violet acetate 0.5% for 2 min, differentiated in 70% alcohol, dehydrated, cleared in xylene and coverslipped.

### Neuronal death analyses

To make sure that equivalent sections were analyzed in all animals, consecutive, 1 out of 5, 50 μm sections from all the hippocampi were collected in five different series. In one series chosen at random, 4–6 consecutive sections around the infusion site ('near' sections, ranging from −2.45 to −3.95 mm from Bregma) were analyzed to calculate ipsilateral damage compared with the contralateral hippocampus of each section. Also, a similar group of sections posterior to the first one was analyzed independently ('far' sections, ranging from −4.2 to −5.7 mm from Bregma) to determine possible differential effects as a consequence of less concentration due to limited diffusion of the molecules.

We calculated the percentage of Fluoro Jade B-positive neurons (FJB+) in the infused side (ipsilateral) compared with those in the right side (contralateral). Finally the mean percentage from 4–6 sections per animal was the value for that animal. By doing this, we were able to compare the effect of the infused molecules even between animals with variable extents of neuronal injury, as is common after SE.

In some experiments (for example with the infusion of rhBDNF) and in some brain areas (such as piriform cortex or hippocampus), we had to compare a wide array of neuronal injury features, including sections with no damage and sections with wholes and fractures due to extensive neuronal death. Hence, the simple counting of FJB+ neurons was not a good tool in these cases. For that reason, we designed and used a semi-quantitative Neuronal Injury Scale. Each side (infused or non-infused) of a given area in a given section was assigned a number from 0 to 6 according to the description of each damage level, after examining Nissl- and FJB-stained sections. The levels were as follows: 0, No evidence for neuronal damage. 1, less than 10 FJB-positive neurons. Injury not detected by Nissl staining. 2, little more than 10 FJB-positive neurons. Some intensively Nissl-stained bodies are seen; 3, Almost half of neurons are FJB-positive. By Nissl, equal parts of normal, condensed and blank spaces are found (probably due to neuronal loss); 4, more than half of neurons are FJB-positive. In Nissl stains, non-stained areas star to be predominant. 5, Almost all neurons are FJB-positive. Important decrease in Nissl stain. 6, Not much FJB due to important neuronal loss. Tissue brakes.

### Immunohistochemistry

This technique was used to detect the antibodies infused in the rat brain. Animals were anesthetized at the indicated time points by injection of chloral hydrate (16%, 400 mg/kg, i.p.; MTC Pharmaceuticals, ON, Canada), and perfused transcardially with physiological solution (0.8% NaCl, 0.4% glucose and 0.8% sucrose)

 

followed by 4% paraformaldehyde in borate buffer. After perfusion, brains were left *in situ* for 24 h, then removed and cryoprotected in 30% sucrose at 4°C for 3 days before sectioning. When frozen, they were sectioned with a microtome and 40 μm sections were stored in cryoprotectant solution at −20°C (30% sucrose and 30% ethylene glycol in phosphate-buffered saline, PBS) and assayed within 2 s of sectioning. Free-floating sections were incubated for 1 h at 23°C in 0.3% hydrogen peroxide to block endogenous peroxidase, washed and blocked for 2 h at 23°C in 5% bovine serum albumin (BSA) and 5% of normal goat serum. Afterwards, sections were incubated with biotinylated anti-rabbit antibody as a primary antibody that will bind to the antibodies infused (1 : 100 in PBS, 1% BSA and 1% triton X-100) for 60 min at 23°C. After 3 washes in PBS 0.01 M for 5 min, sections were incubated with Streptavidin-conjugated Peroxidase for 90 min at 23°C, and finally exposed to a 3-3′-diamninobenzidine (Sigma) solution containing 0.03% $NH_4Cl$, 0.0075% $Cl_2Ni$, 0.0095% $Cl_2Co$, 0.0375% 3-3′-diamninobenzidine and 0.6 μL/mL $H_2O_2$. After 10 min incubation, sections were washed four times in PBS at 23°C, mounted in alcohol-gelatin solution on gelatinized slides, dried overnight, cleared in xylene for 1 h and coverslipped with Canadian balsam.

### Statistical analysis

Results are expressed as percentage of control (mean + SEM). Protein levels were quantified in a minimum of three animals 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 $p < 0.05$ considered significant. When necessary, the Kruskal–Wallis test was used for non-normal distributions. In the quantification of Fluoro Jade B-positive cells, an ANOVA test with a nested model was used, considering each slice of the same animal as a pseudo-replicate.

## Results

### Antibodies against BDNF prevent the early decrease of TrkB after status epilepticus

We have recently shown that SE induces a down-regulation of TrkB, which accompanied hilar degeneration and preceded CA1 injury (Unsain *et al.* 2008). Also, it has been shown by others that the exogenous infusion of BDNF in the hippocampus (or in hippocampal neurons in culture) decreased TrkB protein (Frank *et al.* 1996, 1997; Knusel *et al.* 1997; Xu *et al.* 2004). Moreover, we have recently reported that BDNF protein increases in our model of SE (Unsain *et al.* 2008). Then, we asked if the decrease observed after SE was due to increased BDNF release induced by seizures. To answer this question, we infused antibodies known to block BDNF activity into the left, dorsal hippocampal CA1 in animals injected with saline, or with pilocarpine. From animals injected with pilocarpine, we analyzed those developing SE and those which failed to enter SE (called R2 because of their behavior after pilocarpine injection, see the Experimental procedures). The anti-BDNF antibody and dose used was the same that was able to inhibit BDNF-dependent physiological processes in the intact hippocampus, such as learning and memory processes (Alonso *et al.* 2002; Bekinschtein *et al.* 2007).

We first analyzed if implanted and non-implanted animals shows similar values of relevant seizure parameters, such as time to onset, chance to enter SE, animal survival after/during SE and the degree of injury produced in the hilus and piriform cortex. No significant differences were found in any of the parameters evaluated (Table 1).

As another preliminary, control experiment, we determined the distribution of the infused antibodies anti-BDNF 6, 12 and 24 h after their unilateral administration in the hippocampus (Fig. S1a). The detection of the infused antibodies was performed by the immuno-peroxidase method using a anti-rabbit IgG as the primary antibody. We found that the maximal concentration and distribution of the antibodies is achieved 6 h after the infusion, with a strong decrease by 12 h. At 24 h, the antibodies could hardly be observed. This determination clearly indicates a relatively acute effect in a restricted area. Figure S1b shows the infusion sites of all animals used in the present paper.

In order to evaluate the effect of anti-BDNF antibody on the level of TrkB protein, a group of animals were

**Table 1** Relevant seizure parameters on implanted and non-implanted animals

| | | Implants without infusions | | Infusions before SE | | | |
|---|---|---|---|---|---|---|---|
| Parameters | Area | No (30)[a] | Yes (34) | Rabbit IgG | Anti-BDNF | MSO-bdnf | ASO-bdnf |
| Minutes to SE[b] | | 45.28 ± 7.38 | 41.54 ± 5.3 | 41.75 ± 3.94 | 37.5 ± 10.36 | 55.0 ± 2.89 | 60.83 ± 3.27 |
| % of animals entering SE | | 62.33 ± 17.29 | 79.63 ± 8.02 | 57.1 (4/7) | 54.6 (6/11) | 75.00 (6/8) | 84.2 (16/19) |
| % of animals surviving after pilocarpine[c] | | 50.0 ± 12.12 | 75.88 ± 7.03 | 85.7 (6/7) | 100.0 (11/11) | 62.50 (5/8) | 84.2 (16/19) |
| Degree of injury 24 h after SE | Hilus[d] | 70.7 ± 10.8 | 76.13 ± 2.65 | 93.01 ± 7.35 | 90.18 ± 2.23 | 95.57 ± 8.77 | 83.66 ± 6.18 |
| | Pir ctx[e] | 4.6 ± 0.24 | 3.68 ± 0.21 | 3.67 ± 0.93 | 4.0 ± 0.81 | 3.4 ± 1.05 | 4.17 ± 0.73 |

[a]Number of animals at the beginning of the experiment is shown between brackets. [b]Minutes to first Racine 3-like convulsion from pilocarpine administration. [c]For a survival time of 24 h. [d]Number of cells per image taken from the hilus, as indicated in the material and methods section. [e]Piriform cortex damage using the Neuronal Injury Scale (a.u.).

 

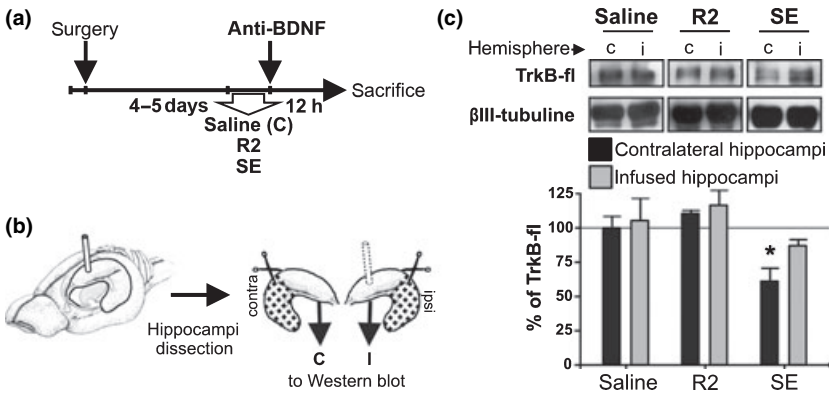


**Fig. 1** Antibodies against BDNF prevent the early decrease of TrkB after status epilepticus. (a) Control, SE or R2 animals received an infusion of anti-BDNF after diazepam and hippocampus homogenates were analyzed 12 h later. (b) The septal halves of both hippocampus were homogenated separately and analyzed by western blot for the determination of TrkB levels. (c) SE induced the decrease of TrkB in the non-infused side (black bar). However, inhibiting BDNF in the infused hippocampus prevented such decrease (gray bar). Hippocampal TrkB was not modified in any hemisphere in control or R2 animals. Mean + SEM are indicated. Asterisks indicate significant differences compared with control animals, $p < 0.05$.

unilaterally infused with the antibodies in the hippocampus immediately after 3-h SE (i.e. immediately after diazepam administration, time '0 h', Fig. 1a). Twelve hours later, infused (left) and non-infused (right) septal halves of the hippocampus were homogenated separately to compare the levels of TrkB protein by western blot (Fig. 1b). Confirming our previous result, status epilepticus induced a rapid down-regulation of TrkB protein level in the non-infused control hippocampus (Fig. 1c, black bars (Unsain *et al.* 2008). However, blocking of BDNF in the infused side prevented such decrease (Fig. 1c, gray bars). These results strongly suggest that BDNF released after SE is involved in TrkB down-regulation. On the other hand, the infusion of anti-BDNF antibody in R2 or control animals did not modify the levels of TrkB protein.

## Antibodies against BDNF decrease neuronal death after status epilepticus

It has been suggested that p75ntr mediates neuronal death after SE (Roux *et al.* 1999; Troy *et al.* 2002; Volosin *et al.* 2006, 2008) and we have recently shown evidence suggesting that BDNF might be the activating ligand (Unsain *et al.* 2008). It was important, then, to test the hypothesis that BDNF released during SE plays an essential part in neuronal injury. Cannulae-implanted animals were infused in the left dorsal hippocampal CA1 with blocking-function anti-BDNF antibody 3 h before SE and 24 h after, and brains were analyzed for neuronal injury by Nissl and stains Fluoro Jade B (Fig. 2a). By infusing 3 h before SE we aimed to let the antibody diffuse in a considerable volume by the time seizures began (Fig. S1a). FJB positive neurons counted in the left, infused side were expressed as a percentage of those found in the right, non-infused hippocampus (For more details on sectioning, sample selection and other issues, see Experimental procedures). Six sections per animal were analyzed ('near' sections, ranging from −2.45 to −3.95 mm from Bregma).

Given that BDNF is well-known to modulate synaptic responses, we determined whether the infusion of antibodies modified relevant seizure parameters, such as time to onset, chance to enter SE, survival after/during SE and degree of injury produced. There were no significant differences in any of the parameters analyzed (Table 1).

The infusion of anti-BDNF 3 h before SE significantly ($p \leq 0.05$) decreased neuronal death to 55 ± 1% hippocampus relative to the contralateral hippocampus (Fig. 2b and c). Control animals receiving a infusion of purified rabbit IgGs showed no differences between hemispheres.

In view of the abundant literature describing pro-excitatory properties of BDNF (for a revision see Poo 2001) it was important to rule out the possibility for diminished excitation in the infused hippocampus and thus decreased excitotoxicity. Then, to exclude that possibility, antibodies were infused immediately after SE, so that antibodies would not interfere with SE but only with processes as consequences of seizures. By doing so we found a similar neuroprotection, decreasing neuronal death to 40 + 7% (Fig. 2b and c).

No difference was observed in neuronal injury in the hilus of the dentate gyrus between both hippocampi in all treatments (Fig. S2a). Thus, this result was useful as an internal control for differences in neuronal damage due to the infusions. The lack of neuroprotection in the hilus might be due to the limited diffusion of the antibodies that were directed to the CA1 area (Fig. S1a) or because different processes determined neuronal death in this area.

Marginal or no neuroprotective effect was found in CA1 in sections far away from the infusion site (ranging from −4.2 to −5.7 mm from Bregma, 'far' sections, Fig. S2b).

 

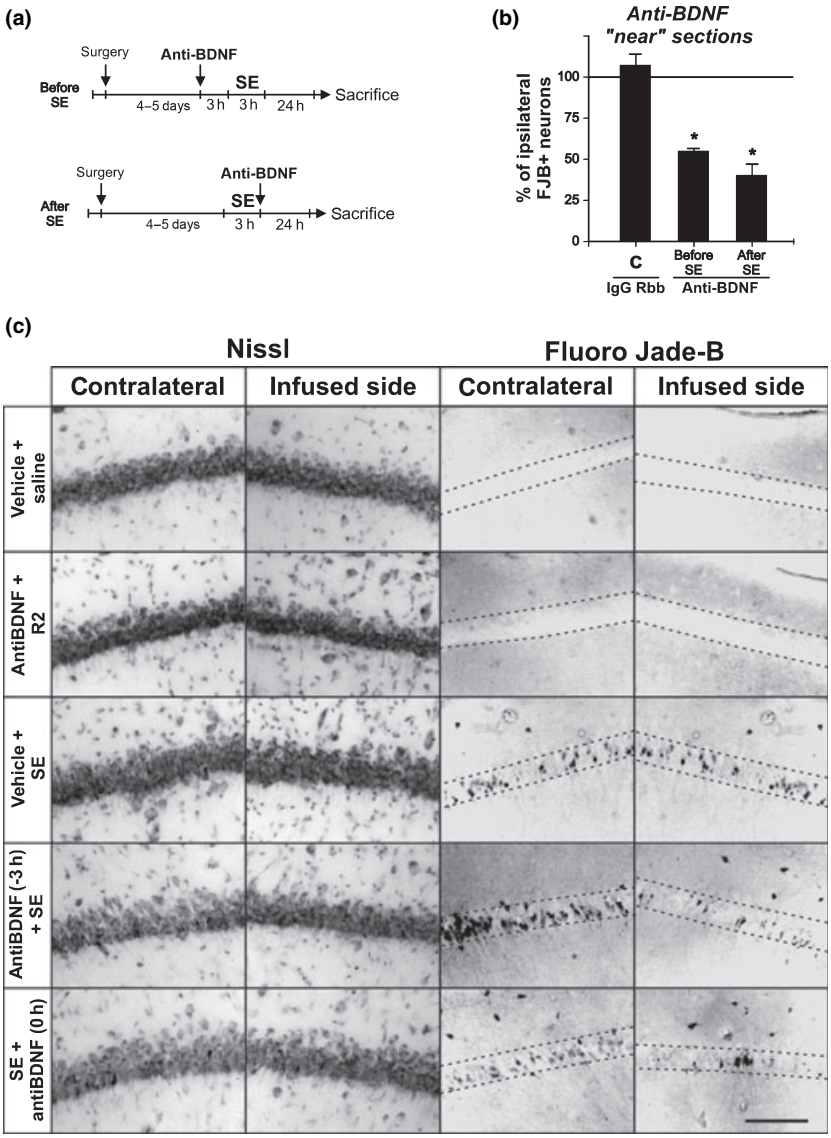


**Fig. 2** Antibodies against BDNF decrease neuronal death after Status Epilepticus. (a) Animals received the infusion of anti-BDNF before or after SE and neuronal damage was compared between hemispheres 24 h later. (b) The infusion of anti-BDNF before or after SE similarly decreased the number of FJB+ neurons to ~50% in the infused side. Mean + SEM are indicated. Asterisks indicate significant differences compared with control animals, $p < 0.05$. (c) Micrographs of hippocampal CA1 stained with Nissl (left) and with FJB (right) show representative animals from each treatment. Scale bar: 50 μm.

## Exogenous BDNF infused after SE increases neuronal damage

The function-blocking experiments shown above suggest that after SE, released, endogenous BDNF is involved in the induced neurodegeneration. In order to test this suggestion we designed a gain-of-function approach under the hypothesis that the infusion of exogenous BDNF protein after SE would increase neuronal damage. Recombinant human BDNF (rhBDNF) was infused in the left hippocampus after SE. Twenty four hours later, damage was compared between the infused and non-infused hippocampi CA1. In order to control for the effects of protein infusion alone, a group of animals received an equivalent infusion of cytochrome *c* after SE, which has a similar molecular weight and charge as BDNF (Fig. 3a). In order to evaluate a possible increase in cell damage due to the infusion of BDNF the duration of SE was only for 1.5 h rather than 3 h. Decreasing SE duration will result in less damage and it will then be possible to obtain an increase in cell death due to the infusion. The wide range of neurodegeneration found in the different treatments of this experiment (from no damage to neuronal loss and even tissue rupture), prompted us to design a semi-quantitative scale that could account for all ranges in this wide spectrum. Thus, the scale ranges from 0 (no damage) to 6 (tissue breaking and almost complete neuronal loss) and is detailed in Experimental procedures.

To corroborate the BDNF isoforms present in recombinant human BDNF vial from a commercial source (Chemicon Int.), we determined both pro- and mBDNF by WB using antibodies that recognizes both proteins. We found that the vial was mostly composed of mBDNF, with a minor concentration of proBDNF (Fig. 3b).

 

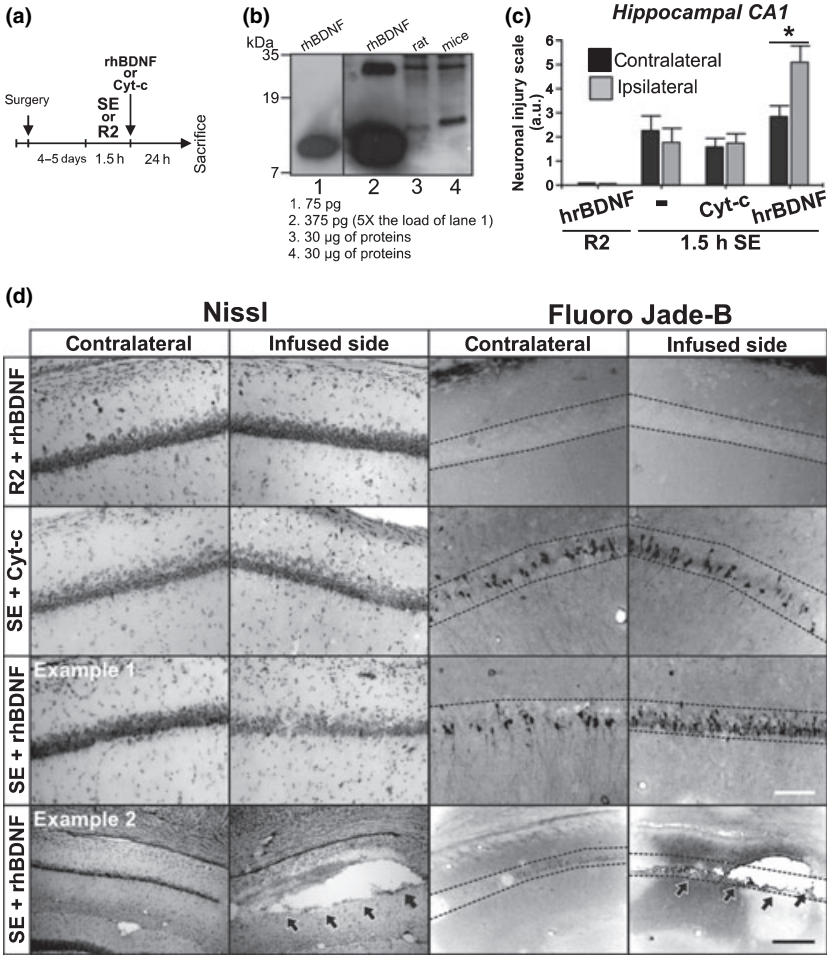


**Fig. 3** Exogenous BDNF infused after SE increases neuronal damage. (a) Implanted animals received an infusion of recombinant human BDNF (or a control infusion of cytochrome-*c*) after 1.5 h-SE (or after 1.5 h-R2 or in saline-treated animals). Twenty-four hours later, we compared neuronal death between infused and non-infused hippocampi using the Neuronal Injury Scale. (b) western blots describing the infused molecules. The infused molecules (lanes 1 and 2) are a mixture in which mBDNF predominates and proBNF is at a very low concentration. The molecular weights of the bands of rhBDNF detected using anti-BDNF are comparable to those found in rat and mice hippocampi (lanes 3 and 4, respectively). (c) Exogenous rhBDNF infused after SE dramatically increased neuronal damage, but had no effect in R2 animals. The control infusion of cytochrome-*c* did not alter the normal neuronal damage taking place after SE. (d) Micrographs of the hippocampal CA1 stained with Nissl (left) and with FJB (right) show representative animals from each treatment. Two examples of the infusion of rhBDNF are shown to better illustrate the effect of the infusion. Scale bar: 50 µm (white) and 200 µm (black).

As it was demonstrated that a high concentration of BDNF induces neuronal death in hippocampal neurons in culture (Friedman 2000), we first aimed to rule out the possibility that the *in vivo* infusion of rhBDNF *per se* would induce neuronal death in R2 animals, which normally do not show damage. The infusion of rhBDNF, in the doses used, did not induce any neuronal damage or cell morphology alterations in control or in R2 animals as assessed by Nissl and FJB stains (Fig. 3c and d), indicating that the rhBDNF *per se* (in the dose used) is not neurodegenerative. On the contrary, infusion of rhBDNF after SE drastically exacerbated neuronal damage in the infused side of SE animals, as compared with the non-infused side of the same animal (Fig. 3c and d). In two out of four animals infused with rhBDNF after SE we found that the tissue was broken at the level of the stratum piramidale of the CA1, together with massive loss of neurons (as observe by Nissl) and a substantial amount of FJB-positive neurons (bottom row in Fig. 3d). That extent of lesion in the CA1 was not observed in SE-induced animals, but it was present in the piriform cortex of SE-induced animals, where also shows evidences of massive neuronal damage. Unilateral infusion of Cyt-c after SE did not modify the normal damage induced by SE alone (Fig. 3c and d). The infusion of rhBDNF did not modify the regular cell damage induced by SE in the hippocampal hilus (Fig. S3a) or in the piriform cortex (Fig. S3b).

 

These results indicate that availability of BDNF in the extracellular space (in the concentrations used) facilitates neuronal damage after SE, but it is not sufficient to induce neuronal damage by itself. One of those effects induced by SE could be the BDNF receptor's switch previously published (Unsain *et al.* 2008).

### Preventing BDNF synthesis de novo by the infusion of antisense oligonucleotides before SE

Inhibiting BDNF mRNA translation to decrease basal BDNF protein levels has been previously achieved *in vivo* by the infusion of antisense oligonucleotides targeted to BDNF mRNA [ASO-*bdnf* (Bekinschtein *et al.* 2007, 2008; Barnes and Thomas 2008; Yang *et al.* 2004)]. So we made use of this approach to further investigate the role of BDNF in SE-induced damage to the hippocampal CA1 neurons. To control for possible interferences of the infusion with SE, we compared important parameters of SE between animals receiving ASO-*bdnf* with animals receiving a control oligonucleotide consisting of the same nucleotides of the ASO-*bdnf* but in a random sequence (miss-sense oligonucleotides, MSO-*bdnf*, Table 1).

In order to determine the effective decrease of BDNF protein levels by the infusion, sham control and 6 h- and 12 h-after -infusion animals were killed. The levels of BDNF proteins from the dorsal hippocampus were compared between hemispheres by western blot (Fig. 4a). The protein levels of BDNF isoforms were not modified in the contralateral side at any time. On the contrary, the levels of proBDNF and mBDNF decreased by ~30% 6 h after the infusion of *ASO-bdnf.* Surprisingly, at 12 h, the levels of mBDNF increased by ~25% and proBDNF returned to baseline (Fig. 4b and c).

Taking into account this temporal profile and in order to get the greatest decrease in BDNF protein levels at the beginning of SE, oligonucleotides were infused 6 h before SE (Fig. 5a). A remarkable decrease was found in neuronal damage in all animals tested (Fig. 5b and c). Interestingly, the neuroprotection was found both in the infused side and in the contralateral hippocampus. On the other hand, the infusion of MSO-*bdnf* did not modify the normal SE-induced neuronal damage.

To induce a gain-of-function approach we took advantage of our finding that the levels of endogenous BDNF protein are up-regulated 12 h after the infusion of ASO-*bdnf* (Fig. 4b and c). Animals were therefore infused with ASO-*bdnf* or MSO-*bdnf* 12 h before pilocarpine administration. Interestingly, with this approach, a remarkable ipsilateral increase was found in neuronal injury (Fig. 5b and d), resembling our finding with the infusion of exogenous rhBDNF. In the same animals, the treatment did not modify the degree of neuronal injury normally found after SE in the hippocampal hilus or in the piriform cortex after SE (Fig. S4a and b).

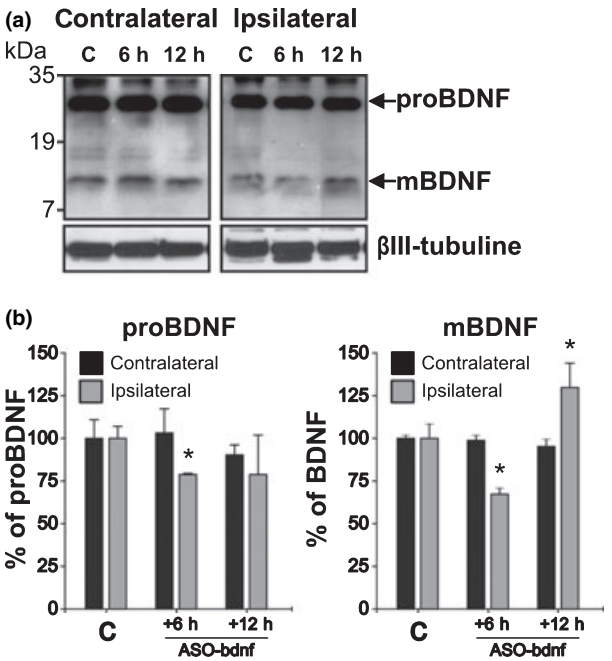


**Fig. 4** The effect of the infusion of antisense oligonucleotides against the mRNA of bdnf on the levels of BDNF proteins. (a) Representative western blot analyses on proBDNF and mBDNF in samples from the dorsal contralateral (left blot) and ipsilateral (right blot) hippocampus 6 or 12 h after the infusion of ASO-*bdnf*. (b) Six hours after the infusion of ASO-*bdnf* the endogenous levels of proBDNF and mBDNF proteins decreased by 25% and 30%, respectively. By 12 h, proBDNF protein levels returned to control values, but mBDNF protein levels increased by 25% respect to controls ($n = 4$ per treatment). Mean + SEM are indicated. Asterisks indicate significant differences compared with control animals, $p < 0.05$.

Taken together, these results show that BDNF facilitates SE-induced neuronal injury.

### Anti-proBDNF also prevents neuronal death

It has been shown in cell cultures that released, unprocessed BDNF is a potent inducer of neuronal death (Lee *et al.* 2001; Teng *et al.* 2005), so we aimed to investigate the relative role of proBDNF in neuronal death *in vivo* after SE. The anti-BDNF used so far does not differentiate between mature and proBDNF. So we sought to determine exclusively the role of proBDNF by the infusion of an antibody that recognizes only pro-BDNF and does not interact with other pro-neurotrophins or with mature BDNF. For that purpose, animals were subjected to SE for 3 h, administered diazepam and then received an infusion of anti-proBDNF. Twenty-four hours later, animals were perfused and the neuronal injury was compared (Fig. 6a). To corroborate that the infused antibody anti-proBDNF only recognizes that BDNF form, we made a western blot analysis where it can be observed that only one band with the expected molecular weight is recognized by the antibody (Fig. 6b, left blot). On

 

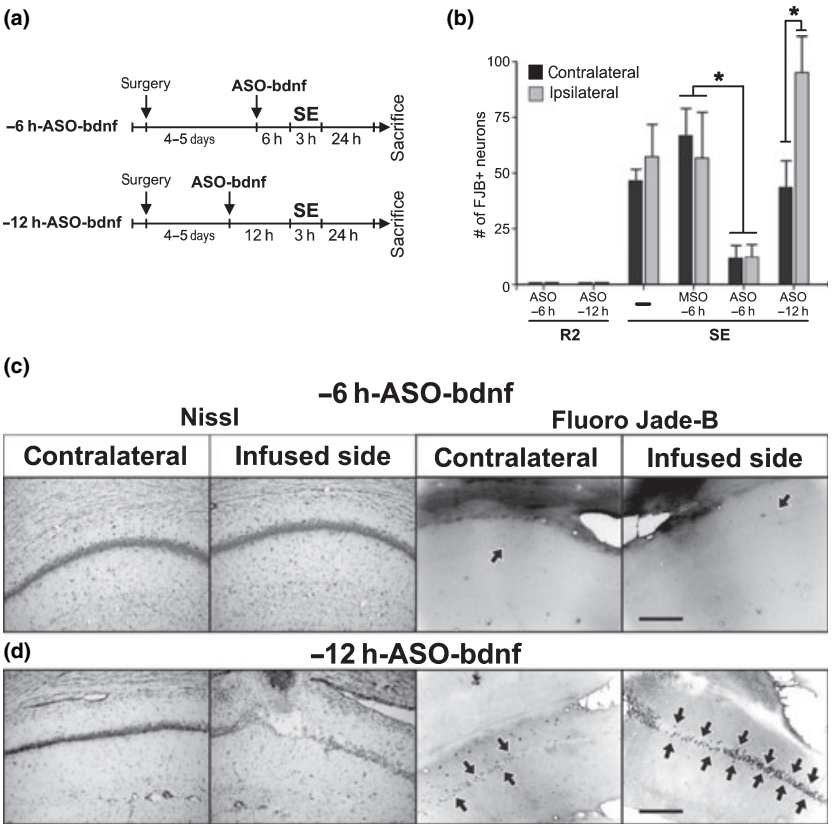


**Fig. 5** Effects of ASO-*bdnf* in SE-induced neuronal damage. (a) Animals received a infusion of ASO-*bdnf* (or a control infusion) 6 or 12 h before SE. Neuronal damage was analyzed 24 h later. (b) The infusion of ASO-*bdnf* 6 h before SE had a dramatic impact in neuronal survival after SE, given that it prevented neuronal death in the infused hippocampus but also in the contralateral hippocampus. On the other hand, the infusion 12 h before SE had the opposite effect: it increased neuronal damage. Mean + SEM are indicated. Asterisks indicate significant differences between the indicated groups, $p < 0.05$. (c and d) Micrographs of the hippocampal CA1 stained with Nissl (left) and with FJB (right) show the non-infused and infused sides of representative animals receiving ASO-*bdnf* 6 h before SE (c) or 12 h before SE (d). Arrows depict some individual FJB+ cells in each micrograph. Scale bar: 200 μm.

the other hand, the same hipocampal sample analyzed with a anti-BDNF shows other reactive bands, including mBDNF (Fig. 6b right blot).

We found an important decrease in the number of FJB-positive cells in the infused hippocampus as compared with the contralateral, non-infused hippocampus (Fig. 6c). Interestingly, the extent of neuroprotection found is similar to that obtained with the infusion of anti-BDNF, suggesting either that mature- and pro-BDNF are involved in the same cascade or that the whole effect is achieved only by proBDNF.

## Discussion

The present experiments were designed to test the hypothesis that BDNF is involved in neuronal injury after status epilepticus. First we demonstrated that BDNF is involved in the rapid TrkB down-regulation after SE. Then we found that BDNF facilitates neuronal injury after SE by a loss-of-function approach using blocking-function antibodies and antisense oligonucleotides and by a gain-of-function approach using rhBDNF. Finally, we demonstrated that the same neuroprotection is achieved by the infusion of antibodies recognizing only proBDNF, yielding the striking possibility that released, unprocessed BDNF is also one of the BDNF forms facilitating neuronal death after SE.

The role of the BDNF signaling system in hippocampal physiology has been widely studied. BDNF plays different, important activities in hippocampal physiology, including neuronal differentiation and maturation, LTP and LTD formation, learning and memory, neuroprotection and neurogenesis. Some works have tried to assess the role of BDNF signaling after status epilepticus, a condition in which excessive and generalized neuronal activity (seizures lasting 90–180 min) leads to neuronal death and epileptogenesis. Unfortunately, those attempts have yielded conflicting results, probably because of the wide variety of approaches used, from chronic infusions of antibodies or antisense oligonucleotides to regular knock-in and knock-out techniques. Those transgenic approaches might yield compensations in signaling networks that could mask the real contributions of the proteins being tested, thus leading to wrong interpretations. For example, by overexpressing TrkB truncated variants some studies have analyzed those mice as BDNF silent animals, without taking into account the possibility of p75ntr-mediated signaling after seizures (Lahteinen *et al.* 2002, 2003; He *et al.* 2004). For these reasons, we wanted to address the role of BDNF in neuronal degeneration after SE by the infusion of function-blocking antibodies, antisense oligonucleotides and recombinant proteins, all intended to locally and acutely block or enhance BDNF activity *in vivo*, before or after the excitotoxic insult.

 

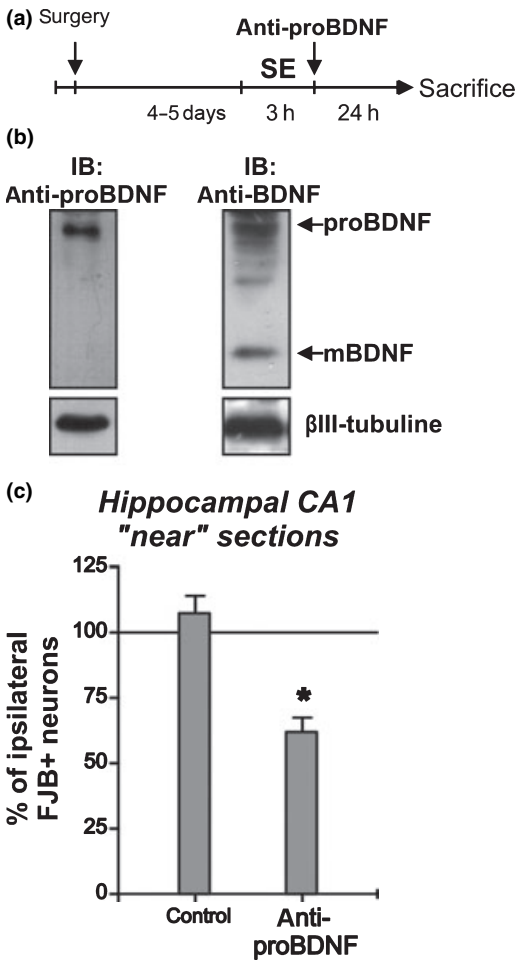


**Fig. 6** Antibodies exclusively against proBDNF also decrease neuronal death after status epilepticus. (a) Animals received the infusion of anti-proBDNF after SE and neuronal damage was compared between hemispheres 24 h later. (b) Western blot showing that the anti-proBDNF antibody infused in this experiment detects a unique band corresponding to proBDNF from rat hippocampus, whereas the anti-BDNF recognizes more isoforms. (c) The infusion of anti-proBDNF after SE decreased the number of FJB+ neurons in the infused side to ~50%. Mean + SEM are indicated. Asterisks indicate significant differences compared with control animals, $p < 0.05$.

### Released BDNF facilitates TrkB down-regulation after status epilepticus

We have recently shown that SE induces a down-regulation of TrkB, which accompanies hilar degeneration and precedes CA1 injury (Unsain *et al.* 2008). In that report, we raised the hypothesis that the overproduction and release of BDNF after prolonged seizures might be the signal leading to the down-regulation of hippocampal TrkB after SE and in this way be one of the signals driving to cell death. In support of this hypothesis, experiments in cell culture demonstrated that exposure to BDNF itself induces down-regulation of TrkB protein (Frank *et al.* 1996; Knusel *et al.* 1997; Sommerfeld *et al.* 2000) and the infusion *in vivo* of BDNF in retina, midbrain, olfactory bulb and hippocampus (Frank *et al.* 1996; Chen and Weber 2004; Xu *et al.* 2004) also down-regulates the TrkB receptor. In the present report, we directly tested that hypothesis, blocking BDNF *in vivo* after SE and quantifying TrkB levels 12 h later. We found that in the non-infused hippocampus SE significantly decreased TrkB levels, but that the decrease was prevented in the infused hippocampus.

### Released BDNF facilitates neuronal injury after status epilepticus

In the present report, we describe that BDNF is a positive modulator of neuronal injury after SE using three approaches: by the infusion of function-blocking antibodies, antisense oligonucleotides and recombinant human BDNF protein. The advantage of the acute and local interference of BDNF activities is that we were able to assess the role of this signaling protein in a restricted area, in a short time window and, most importantly, after a SE taking place in wild-type animals. These attributes are not present in approaches based on chronic infusions or genetically modified animals, in which SE develops in an abnormal scenario (due to homeostatic compensations) that yield to conclusions that cannot be transferred to explain the process taking place in intact animals.

By performing infusions of function-blocking antibodies before or after SE we found that BDNF released after SE is a positive modulator of neuronal injury. Moreover, we found that the neuroprotection induced by the antibody was sensitive to the distance from the canullae, given that the neuroprotección observed in 'near' sections was almost absent in 'far' sections. Interestingly, animals infused 3 h before SE showed a marginal ($0.1 > p > 0.05$) neuroprotection in far sections. One plausible interpretation for that slight difference in neuroprotection between infusions before or after SE is that for the latter there is no time for the antibodies to reach the 'far' sections before the death-promoting activity of BDNF takes place. On the other hand, in animals infused 3 h before SE the antibody had enough time to reach those sections, although not in the concentration needed for the greater neuroprotection observed in 'near' sections. Taken together, these data strongly suggest that the injury-promoting role of the BDNF released takes place in a short time window after SE.

Given that it is known that BDNF is important for the normal activity of the hippocampal circuitry, we asked whether the decrease in neuronal death observed in the infusion side was due to a prevention of epileptiform activity in this side. The fact that the infusion of BDNF antibody did not affect cell death in the hilus of both hemispheres and that SE output was the same whether the infusion was before or after the SE, indicate that the antibody did not compromise normal epileptiform neuronal activity. When antibodies were infused after SE (after the administration of i.p. of diazepam 10 mg/kg) we assume the infusion will not interfere with the development of SE. However, it could be expected that the infusion could interfere with post-SE neuronal activity

 

contributing to neuronal death (such as non-convulsive SE). Although non-convulsive SE is unlikely to produce detectable neurodegeneration in 24 h, is a subject that we cannot exclude with the present evidence. In that regard, then, it is possible that the facilitating role of BDNF in neuronal degeneration is in part due to the acute and local alteration in post-SE neuronal activity. Although we have performed some determinations to exclude that possibility (the quantification of neuronal death in the hilus, performing infusions after SE), it is still probable that the infusions blocks locally pro-excitatory activities of BDNF and protects by means of decreasing the insult. Altogether, that possibility is not in contradiction with a facilitating role of BDNF in neuronal injury after SE.

Another strategy used to test the hypothesis that BDNF modulates neuronal injury after SE was the infusion of antisense oligonucleotides to prevent the transcription of mRNA for BDNF. Taking into account our own preliminary results, in a first approach ASO-*bdnf* infusion was performed 6 h before SE in order to get the maximal decrease of BDNF by the beginning of SE. By doing so, we found that animals with a unilateral infusion of ASO-*bdnf* had a remarkable decrease in the number of FJB+ neurons and injury in CA1 in both contralateral and ipsilateral hippocampi. This intriguing bilateral decrease in neuronal injury was not observed in animals infused with MSO-*bdnf*. Although it is difficult to explain this phenomenon, still the effect is probably due to the infusion of ASO-*bdnf*, since the control infusion of MSO-*bdnf* did not alter SE-induced neuronal death. This finding is not totally unusual. For example, neurogenesis and 5HT1A mRNA increase in the dentate gyrus on both sides of the brain after unilateral BDNF infusions (Scharfman *et al.*2005; Pinnock and Herbert 2008). The distant effects are presumably due to indirect effects, because ASO-*bdnf* infusion did not decrease BDNF expression contralaterally and did not appear to spread contralaterally. What mediates this indirect action? At least two options can be considered: one likely candidate is decreased neuronal activity of hippocampal principal cells at the infusion site. In favor of this explanation is the abundant evidence that BDNF potentiates neurotransmission in hippocampus (for review, see Lu 2004). Second, the hippocampal pyramidal cells have robust commissural projections to the contralateral hippocampus so perturbing the ipsilateral side may influence the opposite hippocampus.

The results obtained by the infusion of ASO-*bdnf* 12 h, a treatment in which endogenous BDNF protein rose considerably by the time of SE onset, support the suggestion that BDNF facilitates neuronal death. As another way to increase BDNF activities after SE, the infusion of rhBDNF increased neuronal damage, but had no effect in control or R2 animals. All these evidences indicate that changes induced by SE modify the effects of BDNF. As was previously described (Unsain *et al.* 2008), the BDNF receptor's switch favoring p75ntr signaling after SE can be one of those changes. The effects on neuronal survival of increasing BDNF protein during/after SE (both by −12 h-ASO-*bdnf* or by rhBDNF) have to be analyzed also considering that BDNF exerts immediate effects on neuronal excitability. Most of the effects reported are pro-excitatory, such as increasing synaptic strength or increasing the frequency of spontaneous action potentials (Kang and Schuman 1995; McLean Bolton *et al.* 2000). Thus, it is possible that synaptic modulation of the infused exogenous BDNF (even after SE) could have exacerbated the excitotoxic damage *per se*. Moreover, those effects may add to other events found for endogenous BDNF, such as TrkB down-regulation and increased binding to p75ntr (Unsain *et al.* 2008).

### Released, unprocessed BDNF is also involved in neuronal injury after SE

In spite of the large amount of work suggesting or implying that proBDNF can be released from CNS neurons (Michalski and Fahnestock 2003; Peng *et al.* 2005; Schnydrig *et al.* 2007; Silhol *et al.* 2007; Ullal *et al.* 2007; Unsain *et al.* 2008), it was only a few months ago that this possibility was directly tested (Matsumoto *et al.* 2008; Yang *et al.* 2009). The first work appearing challenged the idea of proBDNF release (Matsumoto *et al.* 2008) and recently this result was challenged by new evidence showing its release and raised a number of methodological issues to explain why Matsumoto *et al.* have failed to reach the same conclusion (Yang *et al.* 2009). From other recent findings, it seems likely that the most common form being released from neurons is proBDNF, and the final proportion of each isoform is regulated by the extracellular conversion of BDNF from proBDNF (Nagappan *et al.* 2009).

In our study, the *in vivo* intra-hippocampal infusion of antibodies against proBDNF seems to decrease SE-induced neuronal death, indicating that proBDNF is actually being released from hippocampal neurons and is able to modulate neuronal injury in these circumstances. Apart from some *in vivo* evidence implying proNGF (unprocessed Nerve Growth Factor) in cellular injury (Harrington *et al.* 2004; Volosin *et al.* 2008), this is the first time that an injury-facilitating role for BDNF (and proBDNF) has been shown *in vivo*.

Taking into account the recent findings showing that modes of neuronal activity modulate the release and the subsequent processing of BDNF forms (Nagappan *et al.* 2009), it will be important in the future to investigate BDNF isoforms release (and their subsequent extracellular processing) in *in vitro* models of SE in hippocampal neurons (Deshpande *et al.* 2008).

The neuroprotective role of anti-proBDNF antibody at the same extent as the anti-BDNF (Fig. 6) suggests that the whole effect is achieved only by proBDNF. On the other hand, the infusion of exogenous BDNF (mostly composed of

 

mature BDNF) (Fig. 3b) increases neuronal damage, arguing that mBDNF also plays a role. This possible conflict can be explained taking into account the many regulatory points of BDNF isoformas. Briefly, released proBDNF could directly interact with p75ntr and/or be processed by plasmin to increase the extracellular levels of mBDNF. Extracellular mBDNF (both the released as such and the produced from extracellular proBDNF) would then participate in the binding and subsequent down-regulation of TrkB and could also interact with p75ntr. In the present manuscript we found that probably both mBDNF and proBDNF facilitates neuronal death and TrkB down-regulation after SE. Moreover, in a recent paper from our lab (Unsain *et al.*2008) we have shown that after SE the interactions of mBDNF and proBDNF with p75ntr increases while those with TrkB decreases after SE, supporting the possibility exposed.

In summary, this study, in which BDNF activity was affected by the acute intrahippocampal infusion of anti-BDNF antibodies, antisense oligonucleotides and recombinant BDNF, indicates a dual role of BDNF in SE-induced neuronal death.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Distribution of the infused antibodies and sites of infusion.

**Figure S2.** Effect of the antibodies against BDNF in the hilus and in CA1 from distant sections.

**Figure S3.** Effect of exogenous rhBDNF after SE in the hilus and the piriform cortex.

**Figure S4.** The infusion of ASO-*bdnf* does not alter the neuronal death taking place in the hilus and the piriform cortex.

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