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Brain-derived neurotrophic factor superinduction parallels anti-epileptic–neuroprotective treatment in the pilocarpine epilepsy model

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Abstract

Antiepileptic drugs provide neuroprotection in several animal models of brain damage, including those induced by status epilepticus (SE). The mechanisms involved in this action are unknown, but neurotrophic factors such as brain-derived neurotrophic factor (BDNF) may play a role. In this study we investigated the changes in BDNF levels in rats in which SE had been induced by pilocarpine injection (400 mg/kg i.p.) and continued for several hours (unprotected group). In other animals (protected groups), SE was suppressed after 30 min by intraperitoneal injection of either diazepam (10 mg/kg) + pentobarbital (30 mg/kg) or paraldehyde (0.3 mg/kg). In diazepam + pentobarbital-treated rats the hippocampal damage caused by SE was significantly lower ($p < 0.05$) than in unprotected animals. In addition, 2 and 24 h after pilocarpine injection, the levels of BDNF mRNA were moderately increased in the unprotected group, but 'super-induced' in protected animals, especially in the neocortex and hippocampus. A time-dependent increase in BDNF immunoreactivity was also found by western blot analysis in rats treated with diazepam + pentobarbital. In contrast, a decrease of BDNF immunoreactivity occurred in the unprotected group. In conclusion, these results show that neuroprotection induced by anti-epileptic drugs in pilocarpine-treated rats is accompanied by strong potentiation of BDNF synthesis in brain regions involved in SE.

Keywords: anti-epileptic drugs, hippocampus, neuroprotection, neurotrophic factors, status epilepticus.

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Anti-epileptic drugs can exert neuroprotective effects in animal models of status epilepticus (SE) (Turski *et al*. 1987; Lemos and Cavalheiro 1995), as well as in other models of brain damage (Schwartz *et al*. 1995; Wallis and Panizzon 1995). In rats treated with pilocarpine, only anti-epileptic drugs that prevent or abort SE are effectively protective (Morrisett *et al*. 1987; Turski *et al*. 1987). However, these agents must be administered rapidly to be effective. Neuronal damage occurs within 20–40 min of SE (Fujikawa 1996; Motte *et al*. 1998), and after 2 h it becomes irreversible, leading to a chronic epileptic condition that is manifested clinically 2–3 weeks later (Soderfeldt *et al*. 1983; Ingvar *et al*. 1988; Lemos and Cavalheiro 1995; Motte *et al*. 1998; Fujikawa *et al*. 1999). Hence, the therapeutic time window for anti-epileptic drug treatment is extremely narrow. In fact, it is on damaged cells committed to die with prolonged seizure activity that anti-epileptic drugs could produce an effective therapeutic intervention, resulting in neuroprotection.

It is not known how the protective effects induced by anti-epileptic drugs are implemented. However, the expression of several trophic factors is modulated and, indeed, increased by drugs that afford neuroprotection (Biagini *et al*. 1993, 1994; Seniuk *et al*. 1994; Semkova *et al*. 1996; Culmsee *et al*. 1999; Labie *et al*. 1999; Semkova and Krieglstein

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Abbreviations used: BDNF, brain-derived neurotrophic factor; EEG, electroencephalograph; NT-3, neurotrophin-3; PC1, pro-protein convertase 1; SE, status epilepticus.

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1999). Therefore, the protective action exerted by anti-epileptic drugs may be mediated by one or more growth factors. Brain-derived neurotrophic factor (BDNF) may play an important role because its expression is increased in various models of epilepsy (reviewed by Gall *et al.* 1997). For example, following a convulsive dose of pilocarpine, BDNF mRNA levels increase in several brain regions, including neocortex, hippocampus, piriform cortex, amygdala and some thalamic and hypothalamic nuclei (da Penha Berzaghi *et al.* 1993; Metsis *et al.* 1993; Schmidt-Kastner *et al.* 1996; Marcinkiewicz *et al.* 1997). This phenomenon is transient, beginning 1 h after pilocarpine injection and declining 12 h later, recovering to basal levels within 3 days (Marcinkiewicz *et al.* 1997). Interestingly, the increase in BDNF mRNA synthesis is gradual and commensurate with the intensity of the stimulus, indicating the existence of a direct relationship between neuronal activity and BDNF expression (Zafra *et al.* 1990; Patterson *et al.* 1992).

BDNF protein levels also increase in models of epilepsy, indicating active neosynthesis (Wetmore *et al.* 1994; Nawa *et al.* 1995; Vezzani *et al.* 1999). This newly synthesized BDNF is presumably released by the activated cells, as suggested by the shift in BDNF immunoreactivity from inside the neuron to the surrounding neuropil within 2–3 h after seizure onset (Wetmore *et al.* 1994). Moreover, after release, BDNF might influence neuronal survival and fibre growth, thus preserving the organization of neuronal networks in brain areas involved in the epileptic activity (Lindvall *et al.* 1994). Data supporting such a protective role have been obtained in juvenile rats (Tandon *et al.* 1999). However, in adult animals this evidence is lacking, and stimulation of BDNF synthesis during seizures coexists with widespread neuronal damage followed by the development of a chronic epileptic condition. Hence, the changes in BDNF may reflect a failing mechanism of neuroprotection (Gall *et al.* 1997).

Contrary to this hypothesis, other authors have demonstrated a role of exogenous BDNF in the prevention of epilepsy developed in kindled adult rats (Larmet *et al.* 1995). These contradictory findings encourage a re-appraisal of the role that BDNF plays in the degenerative/regenerative events accompanying epilepsy. In particular, our aim was to investigate whether BDNF expression could be modulated by drug treatments able to rescue neuronal cells during the therapeutic time window of SE. For this purpose, we used different techniques to understand whether and how anti-epileptic drugs modify BDNF mRNA and protein levels in relation to neuronal damage in the rat pilocarpine model of SE.

Materials and methods

Animals and treatments

A total of 128 Sprague–Dawley male rats (225–250 g) was used. They were housed two per cage, under controlled temperature (23 ± 1°C), humidity (40–60%) and daylight cycle (light from 07.00 to 19.00). Ethics approval was obtained from the animal care committee, and we followed the guidelines of the Canadian Council on Animal Care.

A first experiment aimed to study the modulation of BDNF mRNA levels by means of an anti-epileptic drug protocol demonstrated to be neuroprotective in the pilocarpine model of SE (Lemos and Cavalheiro 1995). Hence, changes in BDNF expression were studied by *in situ* hybridization, 2 and 24 h after the following drug treatments:

(i) pilocarpine at convulsive dose (400 mg/kg) and, after 30 min of SE, saline (unprotected group, $n = 16$);
(ii) pilocarpine (400 mg/kg) and, after 30 min of SE, diazepam (10 mg/kg) + pentobarbital (30 mg/kg) (protected group, $n = 16$);
(iii) diazepam (10 mg/kg) + pentobarbital (30 mg/kg) ($n = 8$);
(iv) pilocarpine at subconvulsive dose (200 mg/kg) ($n = 8$);
(v) saline (control rats) ($n = 8$).

All treatments were administered intraperitoneally. To prevent discomfort caused by stimulation of peripheral muscarinic receptors by pilocarpine, animals were pretreated with subcutaneous scopolamine methylnitrate (1 mg/kg 30 min before pilocarpine injection).

In a second experiment, the same protocol was replicated to study BDNF, neurotrophin-3 (NT-3) and pro-protein convertase 1 (PC1) mRNA levels with northern blot hybridization 2 h after treatment ($n = 2$–4 rats per group).

A third experiment ($n = 3$–6 rats per group) was aimed at studying the time course of BDNF protein levels at 2, 4, 8 and 16 h after either pilocarpine (400 mg/kg) and saline (unprotected animals) or pilocarpine (400 mg/kg) and diazepam (10 mg/kg) + pentobarbital (30 mg/kg) (protected animals) injections.

A fourth experiment was instead designed to study the effects of the anticonvulsant paraldehyde on BDNF mRNA levels by northern blot analysis 2 h after injection. To this aim, five groups of treatment were considered ($n = 3$–6 per group): the first was treated with a convulsive dose of pilocarpine and post-treated with saline (unprotected group); the second was treated with a convulsive dose of pilocarpine (400 mg/kg) and post-treated with paraldehyde intramuscularly (0.3 mg/kg) (protected group); the third was treated with paraldehyde (0.3 mg/kg); the fourth was treated with pilocarpine at a subconvulsive dose (200 mg/kg); and finally, the fifth was treated with saline (controls). Every post-treatment was administered 30 min after the beginning of SE, as already described.

Animals receiving the convulsive dose of pilocarpine were monitored for at least 2 h to determine the convulsion score and the effects of the anticonvulsive treatments according to the classification of Golarai *et al.* (1992) in five categories. In particular, hypoactivity corresponded to stage I; monoclonic jerks of the head, head bobbing and facial automatism to stage II; whole body bilateral activity resembling wet dog shakes to stage III; rearing of forelimbs to stage IV; and generalized clonic–tonic activity and loss of posture to stage V. At an appropriate time following the injection of pilocarpine, animals were killed by decapitation and brains were rapidly extracted. For *in situ* hybridization and histology, brains were frozen in liquid isopentane at −35°C and cut on a cryostat into 6–8-μm sections. For northern and western blot

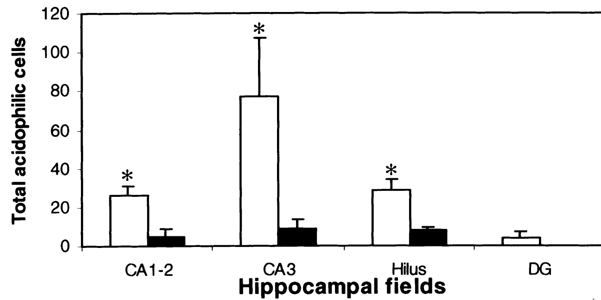

Fig. 1 Quantitative evaluation of acidophilic cells in the CA1-2, CA3, hilus and dentate gyrus (DG) of rats injected with pilocarpine (open bars) or with pilocarpine followed by diazepam + pentobarbital treatment (solid bars). Values are mean ± SEM; *$p \leq 0.05$.

analysis, cerebral cortices and hippocampi were rapidly dissected out and immediately frozen with dry ice powder at −80°C.

Histological procedures

The damage caused by SE was evaluated in the hippocampus 24 h after pilocarpine injection. Haematoxylin–eosin staining was performed on 6-μm-thick sections fixed for 60 min in 4% phosphate-buffered formaldehyde and washed overnight in water. According to Fujikawa (1996), acidophilic neurons were considered to be irreversibly damaged cells. They were counted in each hippocampal region by a blinded collaborator.

In situ hybridization

The procedure of *in situ* hybridization has been described previously (Marcinkiewicz *et al.* 1997). Briefly, 6-μm sections were fixed for 1 h with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2), washed with phosphate-buffered saline (PBS), and reacted with acetic anhydride in triethanolamine 0.1 M. Sections were hybridized overnight at 55°C using a [^{35}S]-UTP-labelled cRNA probe corresponding to cDNA mBDNF 785–210 bp equivalent (Marcinkiewicz *et al.* 1997). After extensive washing, sections were dried and exposed to X-ray film (exposure time = 1 or 2 days). The sections were dipped in photographic emulsion (NTB-2 Kodak) and exposed for 6–12 days at 4°C before development with the D19 solution (Kodak). Image analysis of the *in situ* hybridization was performed on emulsion-dipped slides by counting silver grains/μm^2 in selected areas, using the NORTHERN ECLIPSE software set at 0.2 μm diameter length as maximal limit of detection. Specific hybridization values of silver grains/μm^2 were calculated as total values minus background values measured in slides hybridized with sense riboprobes.

Northern blot hybridization

As described previously (Meyer *et al.* 1996), after pooling the brains obtained from the animals in a given treatment group, total RNA was extracted from cerebral cortices and hippocampi using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). Approximately 10 μg of total RNA and RNA ladder (GIBCO/BRL, Burlington, ON, Canada), were separated by electrophoresis in 1.2% agarose gel, and then transferred to a 0.45-μm pore nylon membrane (Nytran Plus, Schleicher & Schuell, Keene, NH, USA) by capillarity. The filters were hybridized overnight at 65°C in the presence of a previously described [^{32}P]-UTP cRNA probe. After hybridization, filters were washed and exposed to X-ray film (Kodak) with intensifying screen at −70°C for 1–5 days. The same blots were stripped by boiling and were reprobed for 18S rRNA as described previously (Marcinkiewicz *et al.* 1998).

Western blot procedure

BDNF immunoblotting was performed as described previously (Marcinkiewicz *et al.* 1998). Briefly, proteins of the hippocampi were extracted in extraction buffer (Tris–HCl 50 mM, pH 7.4, EDTA 2.5 mM and NaCl 150 mM) containing freshly dissolved protease inhibitors (leupeptin 2 μg/mL, aprotinin 2 μg/mL, pepstatin 100 μM, A-PMSF 100 μg/ml) and β-mercaptoethanol 2 mM. Tissues were homogenized with polytron, centrifuged at 13 000 ***g*** for 30 min at 4°C. Fifty micrograms of proteins were applied on 8% Tris–Tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After migration proteins were transferred onto polyvinylidene difluoride protein transfer membranes (Schleicher & Schuell). The membranes were blocked for 1 h at room temperature with 4% skim milk in Tris-buffered saline (Tris–HCl 0.05 M and NaCl 0.15 M, pH 7.5) and incubated overnight at 4°C with a primary antibody to BDNF (Catalogue # SC-546, Santa Cruz Biotechnology, Santa Cruz, CA, USA) dissolved 1 : 750. The membranes were later incubated with donkey antirabbit IgG-peroxidase (Jackson Laboratories, West Grove, PA, USA) diluted 1 : 10 000 and the product of peroxidase activity was revealed with the BM Chemiluminescence blotting substrate POD (Boehringer–Mannheim) according to the manufacturer's instructions. As a control for specificity, antiserum was preadsorbed with synthetic peptide (human BDNF 128–147 equivalent, catalogue # SC-546 Santa Cruz Biotechnologies). In order to obtain an index of BDNF/proBDNF levels, the immunoblots were acquired by means of a high-definition scanner and the area of immunoreactivity corresponding to each band was measured using the Adobe photoshop computer program.

Results

Behavioural analysis

After pilocarpine injection, animals entered stage I within 3 min and progressed rapidly through the other stages developing generalized clonic–tonic seizures (23.6 ± 2.5 min, mean ± SEM). SE was defined as uninterrupted stage V activity for at least 30 min. The few animals ($n = 14$) that did not enter stage V were discarded. Rats receiving diazepam + pentobarbital or paraldehyde treatments stopped having convulsions within 8–30 min of the injections (17.1 ± 2.6 min, pooled data from 27 rats). Although we have not provided electroencephalographic (EEG) evidence of seizure cessation, the effects of paraldehyde or diazepam + pentobarbital treatments on convulsions were previously shown to be associated with significant depression of EEG recordings (Morrisett *et al.* 1987; Lemos and Cavalheiro 1995). Mortality was ≈ 20%, as reported previously by Liu *et al.* (1994).

Neuropathological score

To determine the extent of damage caused by pilocarpine-induced SE, acidophilic cells in CA1-2, CA3, hilus and dentate gyrus were counted (Fujikawa 1996). No acidophilic cells were observed in control animals, or in diazepam + pentobarbital or pilocarpine 200 mg/kg treated rats. Figure 1 shows that in rats presenting SE, cell damage was seen in various hippocampal areas, mainly in CA3, whereas the less damaged area was the dentate gyrus. The CA1 and CA2 fields were also less injured than the CA3 area. Acidophilic neurons were also found in animals receiving the neuroprotective treatment, but to a significantly lower ($P < 0.05$, Mann–Whitney test) extent (Fig. 1).

Changes in BDNF mRNA levels

As reported in previous studies performed in the pilocarpine model of epilepsy (da Penha Berzaghi *et al.* 1993; Marcinkiewicz *et al.* 1997), *in situ* hybridization revealed an increase in BDNF mRNA that occurred in several brain areas including the neocortex, hippocampus, piriform cortex and amygdala, 2 h after the convulsive dose (i.e. 400 mg/kg) of pilocarpine (Fig. 2a). Increases in BDNF mRNA with a similar pattern of distribution were also seen in rat brains 2 h after pilocarpine injection followed by diazepam + pentobarbital treatment. However, these increases in BDNF mRNA were dramatically more robust (Fig. 2b). As shown in Figs 2(d and e), only small changes in BDNF mRNA levels, compared with saline-treated rats (Fig. 2c), were seen in the hippocampal areas of rat brains obtained 2 h after the injection of either diazepam + pentobarbital or subconvulsive pilocarpine dose.

Using silver-grain counting we also quantified the changes in BDNF mRNA, 2 and 24 h after different pharmacological treatments. To this end we calculated the ratio between silver grain counts in drug-treated rats and those obtained in the saline-treated control group. As shown in Fig. 3, 2 h after a convulsive pilocarpine dose the highest increase in BDNF mRNA was found in the CA1-2 hippocampal fields where it amounted to an $\approx$10-fold increase of that seen under control conditions. In the other brain regions, the increase in BDNF levels varied between twofold (hypothalamus) and sixfold (dentate gyrus and amygdala). Quantitative analysis of BDNF mRNA levels in the brains of rats treated with pilocarpine and later post-treated with diazepam + pentobarbital, revealed increases in the silver grain ratio at 2 h that were consistently larger than those seen in animals treated with pilocarpine only (Figs 4a and c). In the CA1-2 fields, they approximated 32-fold those seen under control conditions, whereas in the other regions it ranged between sixfold (hypothalamus) and 22-fold (dentate gyrus and amygdala). The other treatments had milder stimulatory effects (with one to fourfold increases of the BDNF basal levels).

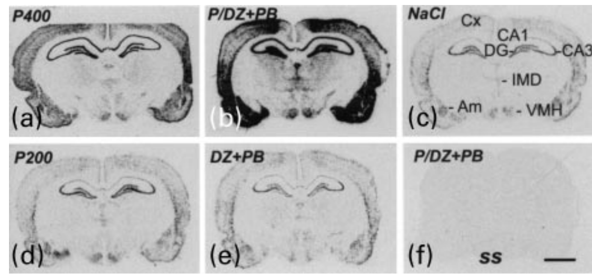

Fig. 2 Brain-derived neurotrophic factor (BDNF) *in situ* hybridization. X-Ray autoradiography showing BDNF mRNA distribution patterns at the brain coronal plane. The *in situ* hybridization procedure was carried out with antisense (a–e) and control sense (f) [^{35}S]-labelled cRNA probes, as described in Materials and methods, 2 h after treatment (a) with pilocarpine 400 mg/kg (P400), (b) diazepam 10 mg/kg and pentobarbital 30 mg/kg in animals experiencing 30 min of status epilepticus (P/DZ + PB), (c) saline (NaCl), (d) half dose of pilocarpine (P200), or (e) diazepam + pentobarbital (DZ + PB). A significant increase of BDNF mRNA expression levels was observed in several structures including cortex (Cx), hippocampal CA1 and CA3 regions and dentate gyrus (DG). Am, amygdala; IMD, intermediodorsal thalamic nucleus; VMH, ventromedial hypothalamic nucleus. Scale bar (in f) = 3 mm.

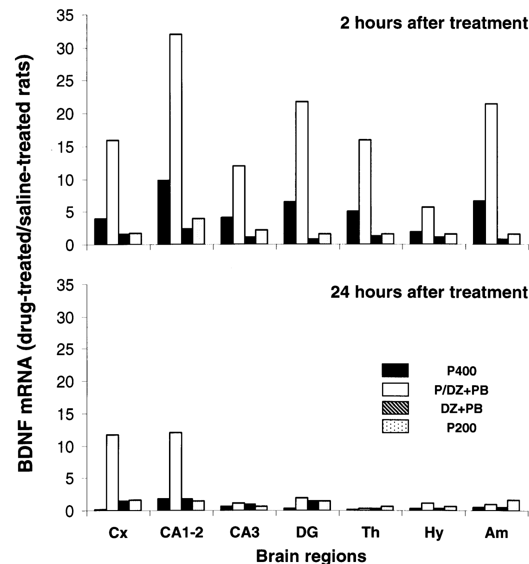

Fig. 3 Semiquantitative evaluation of changes in BDNF mRNA levels determined by counting silver grain number per μm^2 area in several brain regions following treatment with pilocarpine 400 mg/kg (P400), pilocarpine followed by diazepam 10 mg/kg + pentobarbital 30 mg/kg (P/DZ + PB), diazepam + pentobarbital only (DZ + PB), pilocarpine 200 mg/kg (P200), 2 h (a) and 24 h (b) following the first treatment. Data are presented as the ratio between treated and control saline-treated mean values. Am, amygdala; CA1-2 and CA3, hippocampal region; Cx, cortex; DG, dentate gyrus; Hy, hypothalamus; Th, thalamus.

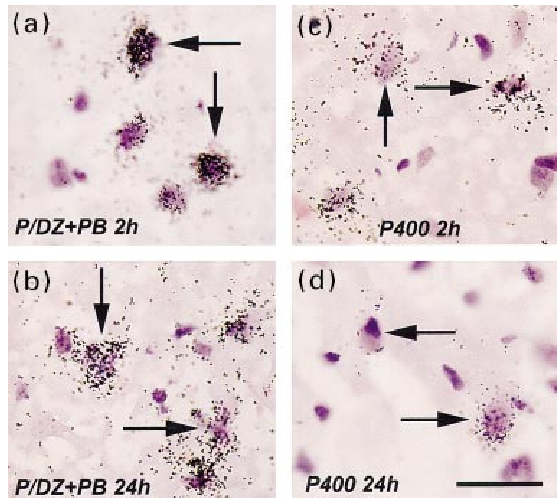

Fig. 4 Brain-derived neurotrophic factor (BDNF) *in situ* hybridization (dark silver grains) at cellular resolution (arrows) in the frontoparietal cortex motor area following treatment with pilocarpine and diazepam + pentobarbital (P/DZ + PB) and pilocarpine (P400) shown 2 h (a, c) and 24 h (b, d) later. Scale bar (in d) = 25 μm.

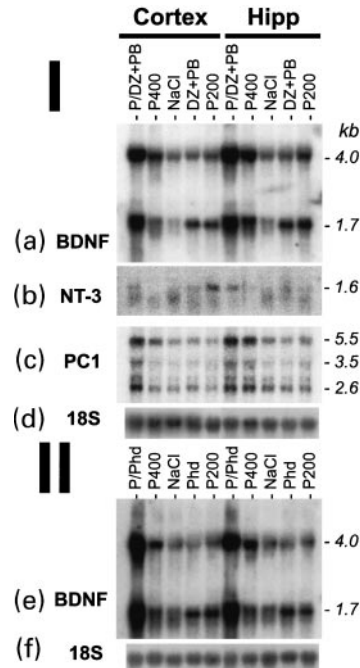

Fig. 5 Northern blot analysis showing the levels of brain-derived neurotrophic factor (BDNF) mRNA in rats after treatment with pilocarpine (400 mg/kg) and diazepam (10 mg/kg) + pentobarbital (30 mg/kg) (P/DZ + PB), pilocarpine (P400), saline (NaCl), diazepam + pentobarbital (DZ + PB) and pilocarpine half dose (P200) (Ia). For comparison, mRNA levels are also shown for NT-3 (Ib) and PC1 (Ic). Approximately 15 μg of total RNA extracted from cerebral cortex and hippocampus (Hipp) 2 h after beginning of each experiment were loaded in each lane. (II) Effects of paraldehyde (Phd in e) treatment (0.3 mg/kg) following a pilocarpine (400 mg/kg) injection (P/Phd) on BDNF mRNA levels. X-Ray film was exposed for 6 h (a and e) or 3 days (b and c). Molecular mass standards are indicated in kb. Hybridization to 18S ribosomal RNA is shown in (d and f).

Only a twofold increase in silver grain ratio was still present in the CA1-2 fields 24 h after pilocarpine injection, whereas at this time BDNF mRNA levels in other brain areas appeared similar to those seen in control saline-treated rats (i.e. ratio ≤ 1) (Fig. 3 and Fig. 4, compare a and b). In contrast, a 12-fold increase in silver grain ratio was still observed in the cerebral cortex (compare Fig. 4b with d) and CA1-2 fields of rats that were post-treated with diazepam + pentobarbital. In the cerebral cortex and hippocampus of animals treated with either the subconvulsive dose of pilocarpine or diazepam + pentobarbital, silver grain counts were also very close to basal levels assessed in saline-treated rats.

Comparison of changes in BDNF, PC1 and NT-3 mRNA levels

We also compared the changes in BDNF mRNA levels with those of other genes regulated by pilocarpine-induced SE (Marcinkiewicz *et al*. 1997). To this end we used Northern blot analysis to establish the changes in mRNA levels of convertase PC1 and neurotrophin NT-3. As shown in Fig. 5(Ia), treatment with 400 mg/kg pilocarpine only or pilocarpine followed by diazepam + pentobarbital, produced an induction of BDNF mRNA in cerebral cortex and hippocampus similar to that observed with *in situ* hybridization. NT-3 mRNA levels decreased following pilocarpine (400 mg/kg) treatment, as reported previously (Schmidt-Kastner and Olson 1995; Marcinkiewicz *et al*. 1997), but were unchanged in diazepam + pentobarbital post-treated group (Fig. 5Ib). The mRNA levels of PC1 also increased to some extent after the convulsive dose of pilocarpine (Marcinkiewicz *et al*. 1997), and a little further when diazepam + pentobarbital post-treatment was implemented (Fig. 5Ic). In rats receiving subconvulsive pilocarpine doses or diazepam + pentobarbital only, we observed a small increase in BDNF mRNA synthesis, but no changes in PC1 and NT-3 mRNA expression.

BDNF mRNA induction after treatment with diazepam + pentobarbital or paraldehyde

We compared the effects of diazepam + pentobarbital with those induced by paraldehyde. Paraldehyde shares neuroprotective effects with benzodiazepines and barbiturates in the pilocarpine model of SE, but it does not enhance $GABA_A$ receptor-mediated mechanisms (Morrisett *et al*. 1987). Also in this case, BDNF mRNA levels were analysed with Northern blot hybridization. The results shown in Fig. 5(IIe) show that BDNF mRNA superinduction was evident in neocortex and hippocampus when SE was arrested by injection of paraldehyde. Similar to diazepam + pentobarbital

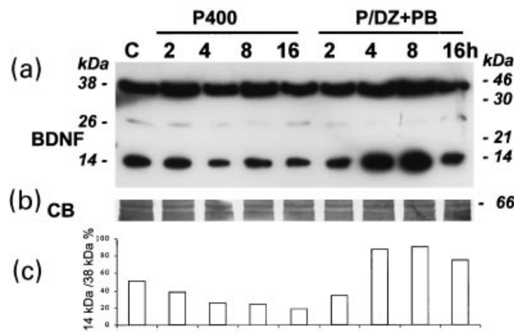

Fig. 6 Western blot analysis showing the levels of proBDNF (38 kDa) and BDNF (14 kDa) immunoreactivity 2, 4, 8 and 16 h following pilocarpine (P400) and pilocarpine followed by diazepam + pentobarbital (P/DZ + PB) administration, and in control (C) NaCl-treated animals, performed as described in Materials and methods (a). (b) Coomassie Brilliant Blue staining (CB) of proteins. (c) The index (%) of BDNF/proBDNF is reported for each time interval by calculating the area of each band. Molecular mass standards are indicated in kDa.

treatment, paraldehyde alone caused a small increase of BDNF mRNA.

BDNF protein levels following treatment with neuroprotective drugs

We also used western blot analysis to determine BDNF protein levels 2, 4, 8 and 16 h after administration of either pilocarpine (400 mg/kg) or pilocarpine (400 mg/kg) followed by diazepam + pentobarbital after 30 min of SE. As shown in Fig. 6(a), both precursor (38 kDa) and mature BDNF (14 kDa) isoforms were detected under any of the conditions studied. Taking into consideration the protein loading inconsistencies (Fig. 6b), these data demonstrate that BDNF immunoreactivity decreased over time to reach a minimum at 4 h, and remained depressed 16 h after pilocarpine injection. Conversely, in pilocarpine and diazepam + pentobarbital-treated animals, BDNF immunoreactivity increased overtime (up to 8 h after the pilocarpine injection) and later decreased toward basal values. By calculating the ratio between mature and precursor BDNF isoforms it was clear that in pilocarpine-treated, unprotected animals there was an over time depletion of the mature protein, whereas in pilocarpine and diazepam + pentobarbital rats there was an overactivity of BDNF neosynthesis. (Fig. 6c).

Discussion

Our findings are in line with the hypothesis that BDNF plays a role in the protective effects of anti-epileptic drugs in the CNS. The protective treatments we used were shown to arrest SE and prevent the development of spontaneous epilepsy due to pilocarpine-induced damage of limbic structures (Morrisett *et al.* 1987; Lemos and Cavalheiro 1995). Accordingly, we found that diazepam + pentobarbital treatment is able to counteract the loss of pyramidal neurons in hippocampus caused by pilocarpine-induced SE. This finding was associated with massive stimulation of BDNF synthesis which we defined as 'superinduction', to differentiate this phenomenon from the well-known induction of BDNF mRNA synthesis observed in the pilocarpine model of epilepsy (da Penha Berzaghi *et al.* 1993; Metsis *et al.* 1993; Schmidt-Kastner *et al.* 1996; Marcinkiewicz *et al.* 1997).

Moreover, our results suggest that during pilocarpine-induced SE, the stimulation of BDNF synthesis is insufficient to adequately rescue the injured neurons, mostly because of rapid depletion of the BDNF pool. This conclusion is supported by the presence of several necrotic cells in the hippocampus of pilocarpine-treated animals, in which stimulation of BDNF mRNA is not accompanied by a sufficient increase in protein synthesis. In line with this view, seizure termination by anti-epileptic drugs, when implemented within the limits of a therapeutic window (Lemos and Cavalheiro 1995), leads to superinduction of BDNF mRNA synthesis and the rescue of a large number of neurons located in vulnerable areas of the limbic system.

Alternatively, it can be argued that the larger lesion extent found in unprotected pilocarpine-treated animals could account for differences in BDNF expression. However, in line with other reports (da Penha Berzaghi *et al.* 1993; Metsis *et al.* 1993; Schmidt-Kastner *et al.* 1996; Marcinkiewicz *et al.* 1997), we showed that the maximal level of BDNF stimulation occurs 2 h after pilocarpine injection (i.e. ≈ 1.5 h after the beginning of SE), when neuronal cell damage is still largely reversible (Ingvar *et al.* 1988; Lemos and Cavalheiro 1995; Fujikawa 1996, 1999). Instead, 24 h after pilocarpine injection the lesion was clearly detectable, but BDNF mRNA levels in pilocarpine-treated and control rats were comparable. Our findings may indeed result from the concomitant occurrence of two otherwise unrelated phenomena (i.e. BDNF superinduction and neuroprotection) following anti-epileptic drug treatment. Nonetheless, several facts support a causal relationship. First, NT-3 mRNA levels were not induced by neuroprotective treatments, as may be expected if BDNF stimulation was nonspecific. Moreover, PC1 mRNA levels were increased by SE (Marcinkiewicz *et al.* 1997), but not superinduced by anti-epileptic drug treatment. Second, the CA3 field, which was the most damaged hippocampal area, showed the lowest increase in BDNF mRNA, both in control and rescued rats. Thus, as reported by Hicks *et al.* (1999), the expression of BDNF mRNA levels is directly correlated with neuronal survival, being higher in areas with lower neuronal damage. Third, based on western blot analysis, the ratio between mature and precursor BDNF was decreased in pilocarpine-treated rats in spite of the increase in mRNA levels. This finding suggests

that the decrease in BDNF protein levels could be strictly related to neuronal cell death in the hippocampus.

A decrease in BDNF cellular immunoreactivity has been reported 3 h after convulsive treatment with return to basal levels within the following 24 h (Humpel *et al*. 1993; Wetmore *et al*. 1994). This finding suggests that an increased utilization of BDNF takes place during SE, thus overwhelming the cell capacity of protein neosynthesis leading to depletion of the BDNF reserve pool. Moreover, the cell capacity to *de novo* BDNF synthesis may be impaired during SE (Dwyer *et al*. 1986). Interestingly, the intensity of BDNF immunostaining is inversely related to the duration of the epileptiform activity (Vezzani *et al*. 1999). In contrast, in animals rescued by diazepam + pentobarbital treatment the relation between transcripted/ translated RNA might have been better preserved. Finally, in these rats the mature/precursor BDNF ratio seems to be rapidly restored and eventually increases over basal values, indicating adequate integration of the reserve pool. However, the changes in BDNF protein levels were not strictly correspondent to those found for BDNF mRNA. Discrepancies between mRNA and protein levels have been found even between brain regions of the normal rat, probably reflecting differences in translational and post-translational regulation of BDNF synthesis and/or axonal transportation (Nawa *et al*. 1995). However, this difference could possibly reflect a partial inhibition of protein synthesis (Dwyer *et al*. 1986) or an enhanced BDNF utilization in brain areas hit by the epileptic activity (Wetmore *et al*. 1994; Vezzani *et al*. 1999).

Our conclusions are at odds with those of Rudge *et al*. (1998) who have shown an increase in CA3 field damage in rats treated with intraventricular administration of BDNF for 5 days before and 1 week after kainic acid-induced seizure. However, other authors have reported that intraventricular BDNF administration reduces the size of ischaemic lesions in the hippocampus. Interestingly, in these experiments treatment was started concomitantly or slightly before the ischaemic insult (Beck *et al*. 1994; Schabitz *et al*. 1997). Hence, differences among the protocol used for BDNF treatment can be important for neuroprotection. In an *in vitro* model of glutamate excitotoxicity, BDNF antagonized protein kinase C inactivation when administered 8–16 h before glutamate treatment, but less effectively when longer periods were used (Tremblay *et al*. 1999). This effect may be linked to neuroprotection, because inactivation of protein kinase C plays a critical role in neuronal death mediated by activation of NMDA and AMPA receptors (Durkin *et al*. 1997). Moreover, BDNF administration for > 3 days produced structural effects leading to the potentiation of excitatory synapses consisting, for example, in alterations in the relative levels of NMDA receptor subunit distribution with increased NR2A and decreased NR2B expression (Small *et al*. 1998; Vicario-Abejon *et al*. 1998). Hence, exposition to BDNF for periods longer than 24 h can exert excitatory synapses potentiation (Scharfman 1997). As shown recently in transgenic mice overexpressing BDNF (Croll *et al*. 1999), entorhinal–hippocampal circuits are hyperexcitable and more responsive to convulsive agents, yet they do not present increased neuronal vulnerability.

It is unclear how the neuroprotective treatments used in this study stimulate BDNF synthesis. The mechanism of paraldehyde is unknown, whereas diazepam and pentobarbital modulate $GABA_A$ receptor function. In addition, pentobarbital also inhibits the glutamate AMPA and kainate receptors (Dildy-Mayfield *et al*. 1996). It has been shown that BDNF is enhanced by NMDA and AMPA receptor activation, and downregulated by $GABA_A$ receptor activation (Zafra *et al*. 1990; Wetmore *et al*. 1994). Moreover, benzodiazepines or barbiturates either prevent changes in BDNF expression when injected before the induction of seizures, or are ineffective when administered after the onset of convulsions (Zafra *et al*. 1990; Kornblum *et al*. 1997; Vezzani *et al*. 1999).

Our findings indicate that when diazepam and pentobarbital are combined they have a small stimulatory effect on BDNF expression, already under basal conditions. These findings are unexpected and suggest a different property of these drugs when combined, an effect that also is seen with paraldehyde. However, the stimulatory effects of these anti-epileptic drugs on BDNF expression in basal conditions are not quantitatively sufficient to interpret the superinduction of BDNF seen with SE. Thus, the superinduction is probably an endogenous reaction to seizures that is partially suppressed by SE and can be fully disclosed by forcing seizure activity to stop. Further experiments are required to clarify this point.

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