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BDNF mRNA splice variants display activity-dependent targeting to distinct hippocampal laminae

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Brain-derived neurotrophic factor (BDNF) may exert contrasting effects depending on its different subcellular sites of action (soma, dendrites, axons). These contrasting effects may explain contradictory findings, for example that BDNF may favour or oppose epileptogenesis. We determined the distribution of five BDNF splice variants in the soma and dendrites of rat hippocampal principal neurons, after application of stimuli that prompt BDNF mRNA accumulation in dendrites (epileptogenic seizures). Under basal conditions, no BDNF mRNA splice variant was detectable in dendrites, while specific splice variants were found in dendrites in response to epileptogenic seizures. Three hours after pilocarpine administration, exon VI and exon II splice variants were found in dendrites, while exons I and IV transcripts displayed a strictly somatic localization. Three hours after kainate administration, only exon VI was found in dendrites. These data suggest that the regulated expression of different splice variants may provide a spatial code to ensure the delivery of BDNF to precise destinations in the cell soma or along the dendrites.

Keywords: Epilepsy; Hippocampus; Neurotrophic factors; mRNA trafficking

Introduction

Epileptogenesis, i.e. the transformation of a normal brain area into epileptic, is associated with rearrangements of neuronal circuits (including sprouting of neuronal processes, neuronal cell death, neurogenesis), ultimately leading to hyperexcitability and spontaneous seizures (Pitkanen and Sutula, 2002). Increasing evidence supports the role of neurotrophic factors in controlling long-term synaptic plasticity as well as epileptogenesis (Schuman, 1999; Simonato et al., 2006). In particular, Brain Derived Neurotrophic Factor (BDNF) may contribute to all cellular effects observed in epileptogenesis (Casaccia-Bonnefil et al., 1999; Bibel and Barde, 2000), with contrasting (pro- and anti-epileptogenic) implications (Simonato et al., 2006). One mechanism that may underlie these contrasting effects may be the precise regulation of its local availability, because BDNF may induce different and in part opposite cellular effects at a very local scale (soma or dendrites; different dendritic domains) (Horch and Katz, 2002; Kanhema et al., 2006).

We have recently proposed that these phenomena may depend upon a differential regulation of the local, subcellular availability of the different BDNF mRNA splice variants (Simonato et al., 2006; Tongiorgi et al., 2006). As many as 11 different BDNF transcripts can be generated in both humans and rodents by alternative splicing (Aid et al., 2007). These transcripts differ only in their 5′ untranslated region (UTR, exons I-IXA) but share a common coding region and 3′ UTR (exon IX, see Fig. 1A). All these BDNF mRNAs are expressed in the brain at very different levels and not in all neuronal populations, generating a spatial diversity of expression (Timmusk et al., 1993; Liu et al., 2006; Aid et al., 2007). In addition, in experimental animal models of epilepsy a differential regulation of the transcription of the different splice variants has been reported to occur (Metsis et al., 1993; Timmusk et al., 1993).

Based on analysis of the sub-cellular distribution of the common coding region (exon IX), we have previously shown that BDNF mRNA can be targeted to distal dendrites *in vitro* in response to electrical activity and to BDNF (Tongiorgi et al., 1997; Righi et al., 2000), and *in vivo* in response to visual stimuli (Capsoni et al., 1999) as well as epileptogenic seizures (Tongiorgi et al., 2004). More recently, we have shown that, in cortical neurons, transcripts containing the exon IV have a somatic localization, while exon VI transcripts can be targeted to distal dendrites *in vivo* (Pattabiraman et al., 2005). These data suggest that the alternatively spliced 5′ exons dictate the sub-cellular distribution and the local synthesis of BDNF. In the present study, we sought to identify if specific BDNF mRNA splice variant(s) are targeted to the dendrites following epileptogenic seizures. Therefore, we investigated the subcellular distribution in the hippocampus of rats treated with kainate or pilocarpine, two models of

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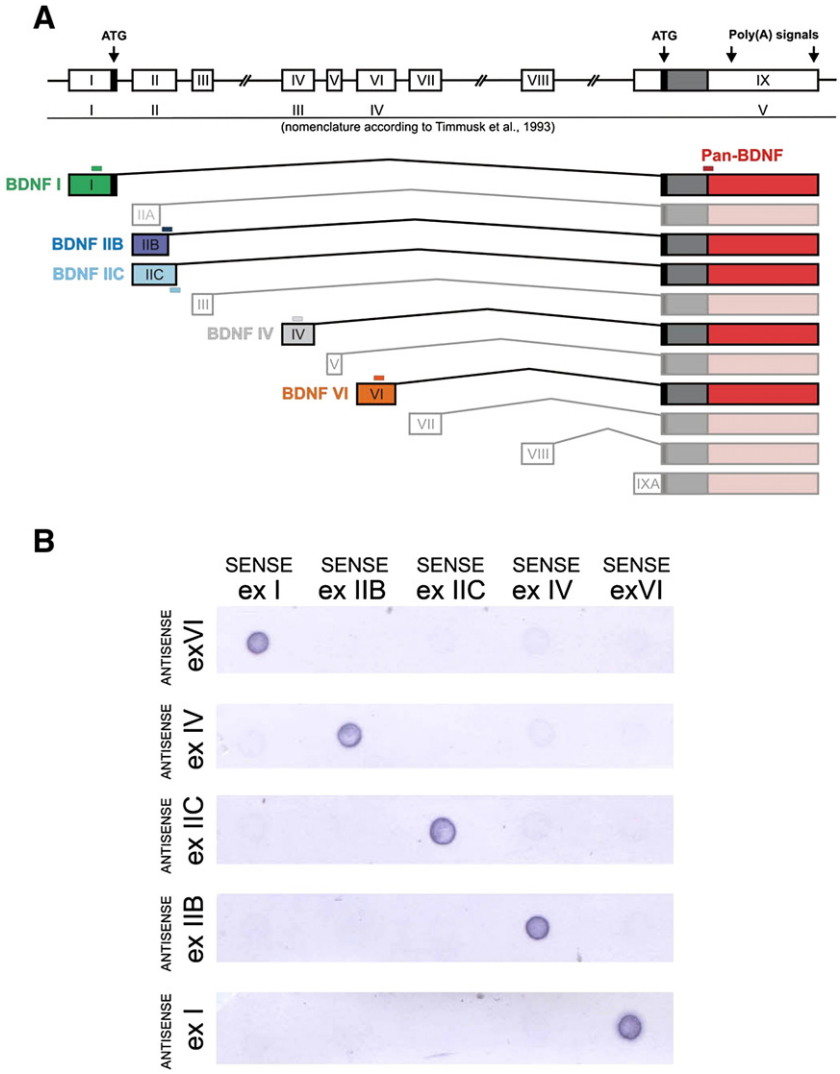

Fig. 1. Alternative transcripts of the BDNF gene and specificity of the riboprobes used for this study. In (A) is shown a schematic representation of the rat splice variants according to the novel nomenclature proposed by Aid et al. (2007). The splice variants analyzed in the present study are highlighted. This colour code will be used in the subsequent figures. The position of the probes used for in situ hybridization is also shown. (B) Specificity of the probes used for this study, as verified on a dot-blot assay. The digoxigenin labelled antisense riboprobes were hybridized with a stripe of nylon on which the sense strand of each exon was spotted. Note the strong hybridization of each probe to its own sense sequence and the absence of any cross-reactivity with other BDNF splice variants.

epileptogenesis, of the five major BDNF splice variants corresponding to the originally described transcripts: exons I, IIB, IIC, IV and VI (Timmusk et al., 1993). These transcripts were recently confirmed to be the most abundant in rat and mouse hippocampus (Liu et al., 2006).

Results

In this study, we analyzed the laminar distribution of five BDNF splice variants (highlighted in Fig. 1A) in the rat dorsal hippocampus, by means of in situ hybridization. The specificity of the probes was tested in a reverse dot blot assay. Stripes of nylon membrane spotted with the sense strands for exon I, IIB, IIC, IV and VI splice variants were hybridized with each antisense probe at the same hybridization conditions used for the in situ hybridization experiments. Results indicate a high specificity of the probes (Fig. 1B).

In situ hybridization experiments on adult rat brain sections confirmed the absence of any detectable amount of exon I-containing BDNF splice variant in the dorsal hippocampus of untreated and pilocarpine-injected rats (Fig. 2, top). In contrast, expression of exon I transcripts was induced by kainate in the dentate gyrus (DG). The signal was confined to the granule cell layer, without any labeling in the molecular layer (Fig. 2, top, row DG), a clear indication of a strictly somatic localization. Because of the presence of two internal cryptic splice sites, rat exon II is known to produce three different transcripts, the very short exon IIA (described only recently) and the principal two, exon IIB and exon IIC. Exon IIB- and exon IIC-containing splice variants were markedly up-regulated in all hippocampal areas 3 h after kainate and pilocarpine (Fig. 2 mid, bottom). Additionally, we found that, in animals treated with pilocarpine, both exon IIB and IIC variants were targeted to the proximal CA1 stratum radiatum and, to a much lesser extent, to the CA3 stratum lucidum/radiatum, while they remained somatic in the kainate model (Fig. 2, rows CA1). Non-specific hybridization, estimated using the BDNF sense probes for

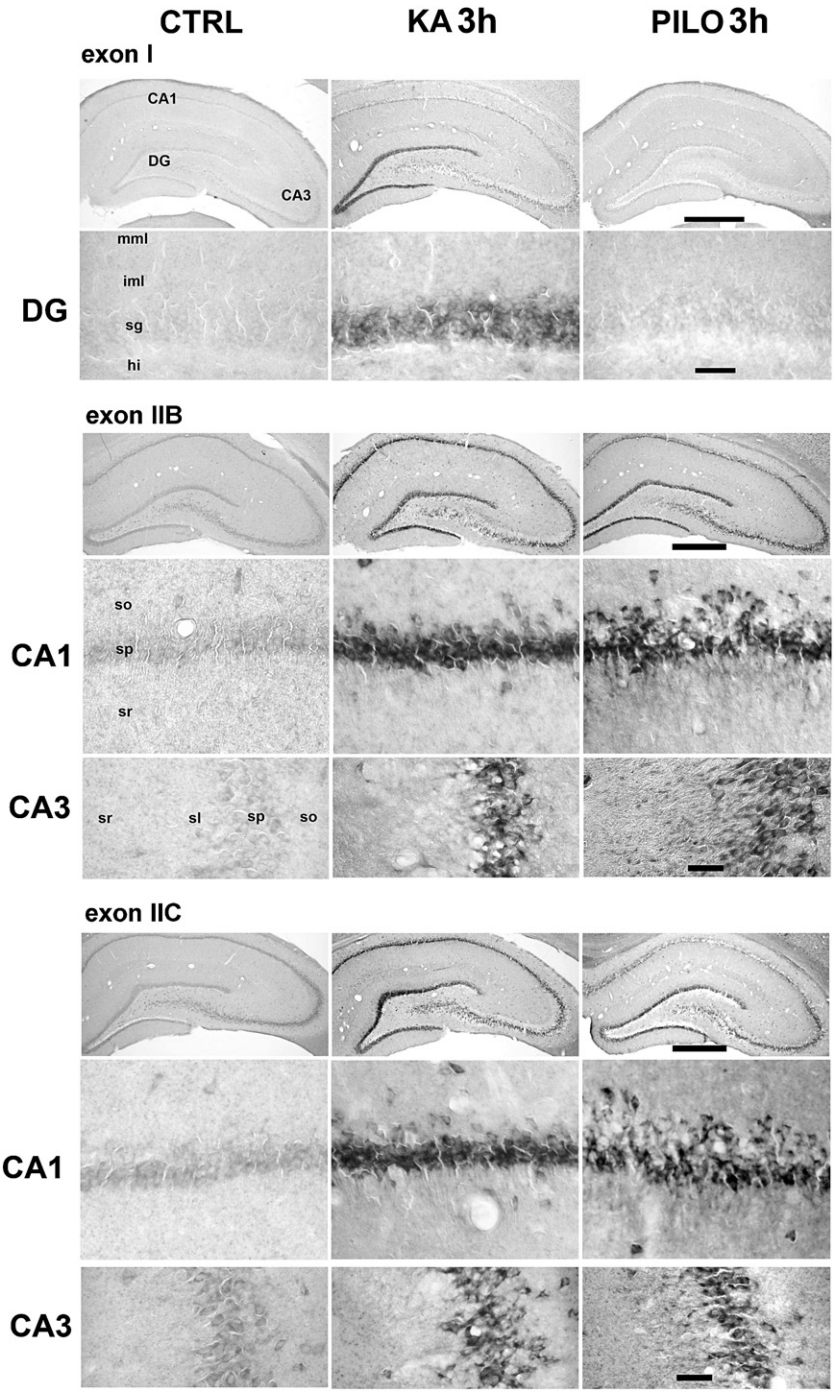

Fig. 2. Localization of exon I and IIB and IIC BDNF splice variants after seizures. Shown are representative coronal brain sections at the level of the dorsal hippocampus (plates 47–48, Pellegrino and Altman, 1979), exhibiting in situ hybridization with the indicated digoxigenin-labeled riboprobes. Exon I-containing BDNF mRNA is not found in the hippocampus under control conditions (CTRL) or 3 h after pilocarpine administration (PILO 3 h), but it is highly expressed in the granule cell layer of the dentate gyrus (DG) 3 h after kainate injection (KA 3 h, top). In contrast, both exon II BDNF splice variants [exon IIB and exon IIC, mid, bottom] are up-regulated after seizures (KA 3 h and PILO 3 h). However, while these splice variants are targeted to CA1 dendrites after pilocarpine, their expression remains strictly somatic after kainate. These representative sections will not fully correlate with the mean changes in dendritic BDNF mRNA levels shown in Fig. 4, because of slight differences among the 4–6 animals of each group. Scale bars = 850 μm for the whole hippocampus, = 50 μm for CA1, CA3 and DG. Mml = medial molecular layer; iml = inner molecular layer; sg = stratum granularis; hi = hilus; so = stratum oriens; sp = stratum pyramidalis; sl = stratum lucidum; sr = stratum radiatum.

the three splice variants analyzed, did not produce any detectable signal (not shown).

Exon IV was constitutively expressed in all principal hippocampal neurons and was up-regulated by kainate but not by pilocarpine (Fig. 3, top). A careful inspection of the CA1, CA3 and DG areas supports a strictly somatic localization of this transcript in both control and epileptic rats (Fig. 3, top). Finally, exon VI transcripts were also constitutively expressed in all principal hippocampal neurons (Fig. 3,

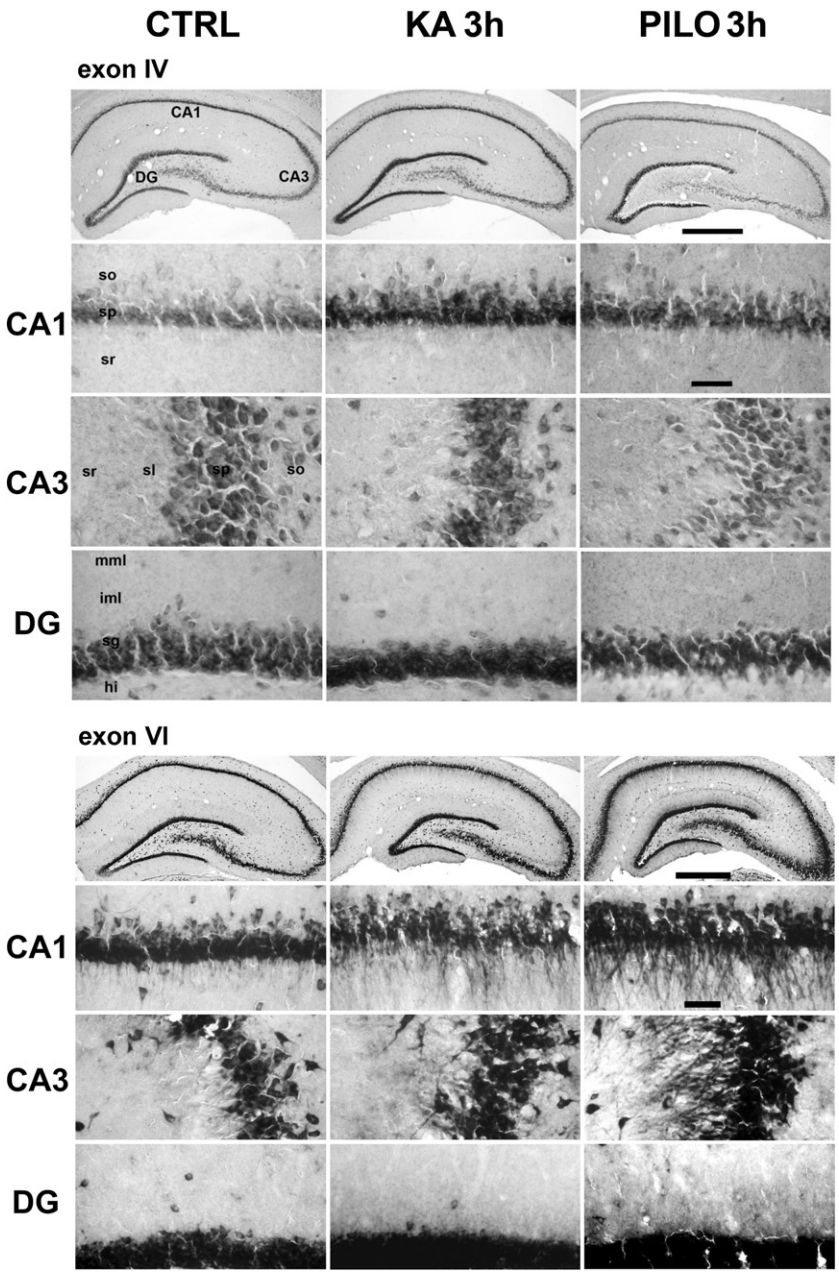

Fig. 3. Localization of exon IV and VI BDNF splice variants after seizures. Shown are representative coronal brain sections at the level of the dorsal hippocampus exhibiting in situ hybridization with the indicated digoxigenin-labeled riboprobes. The exon IV BDNF variant is constitutively expressed in all main hippocampal neurons of control rats (CTRL, top), and its levels increase after kainate, but not pilocarpine, administration. Higher magnification in the CA1, CA3 and DG areas show that exon IV localization is strictly somatic, even after kainate. The exon VI BDNF variant is constitutively expressed in the entire hippocampus (bottom). Higher magnification of CA1 shows that these transcripts are localized in the proximal part of the dendrites in control animals (CTRL, bottom), while the localization extends to distal dendrites after seizures (KA 3 h and PILO 3 h, bottom), especially after those induced by pilocarpine. Dendritic localization of exon VI transcripts is also detectable in CA3 apical dendrites and in the inner third of the DG molecular layer, only after pilocarpine seizures (DG, bottom). These representative sections will not fully correlate with the mean changes in dendritic BDNF mRNA levels shown in Fig. 4, because of slight differences among the 4–6 animals of each group. Scale bars = 850 μm for the whole hippocampus, = 50 μm for CA1, CA3 and DG. Mml = medial molecular layer; iml = inner molecular layer; sg = stratum granularis; hi = hilus; so = stratum oriens; sp = stratum pyramidalis; sl = stratum lucidum; sr = stratum radiatum.

bottom). Both kainate and pilocarpine induced a dramatic accumulation of this splice variant in the stratum radiatum of CA1 (Fig. 3, CA1) and, for pilocarpine treatment only, in the strata lucidum and radiatum of CA3 (Fig. 3, CA3) and in the inner third of the DG molecular layer (Fig. 3, DG). The corresponding BDNF sense probes did not produce any detectable signal (not shown).

We determined the exact laminar distribution of the transcripts by performing a densitometric analysis of the dendritic labelling in comparison with the distribution of a pan-BDNF (exon IX) probe under control conditions and 3 h after pilocarpine or kainate administration (Fig. 4). This quantitative analysis confirmed the results described above. In summary (see the schematic drawing in Fig. 5): in

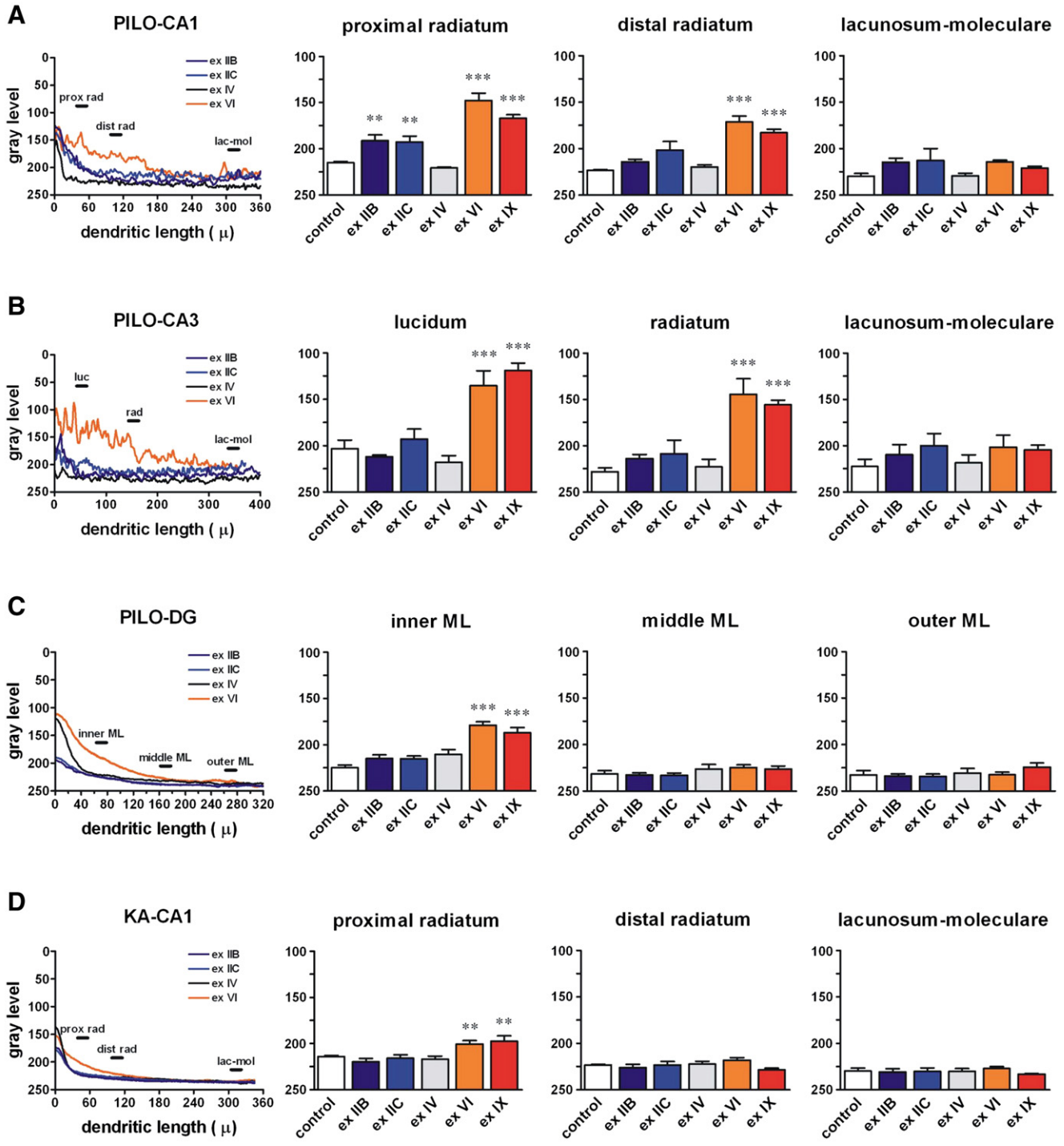

Fig. 4. Densitometric analysis of pilocarpine and kainate seizure effects on BDNF splice variants. Densitometric analysis of the dendritic labelling of BDNF splice variants in CA1 (A), CA3 (B) and DG (C) areas after pilocarpine seizures, and in CA1 after kainate seizures (D), expressed as grey level as a function of the distance from the cell soma (in microns). Analysis was performed as described in Experimental methods. Control (white columns): pan-BDNF [exon IX] in control animals; ex IIB (dark blue line and columns): exon IIB in pilocarpine-treated rats; ex IIC (light blue line and columns): exon IIC in pilocarpine-treated rats; ex IV (grey columns): exon IV in pilocarpine-treated rats; ex VI (orange line and columns): exon VI in pilocarpine-treated rats; ex IX (red columns): pan-BDNF (exon IX) in pilocarpine-treated rats. The main dendritic splice variant contains exon IX. Data are the means ± S.E. of 4–6 animals per group. $^{**}P<0.01$, $^{***}P<0.001$ vs. control; ANOVA and *post-hoc* Newman–Keuls test.

the pilocarpine model, (i) exon II and VI transcripts contribute to the accumulation of BDNF mRNA in dendrites of CA1 neurons, while (ii) BDNF mRNA dendritic targeting in CA3 and DG neurons is accounted entirely by the exon VI splice variant; in addition, (iii) exon VI transcripts account entirely for the dendritic targeting of BDNF mRNA in the CA1 stratum radiatum in the kainate model. It should be remarked that in both the pilocarpine and kainate models, the densitometric values for exon VI transcripts are not statistically

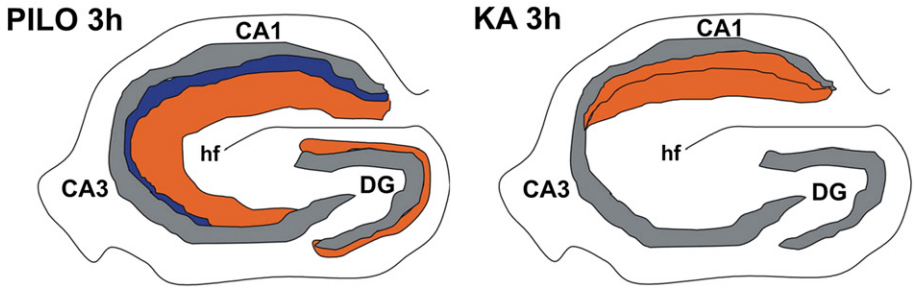

Fig. 5. Schematic illustration of the laminar localization of BDNF mRNA transcripts after epileptogenic seizures. Three hours after pilocarpine injection (PILO 3 h, left) exon II and VI splice variants localize in the proximal part of CA1 stratum radiatum (blue). Only exon VI localizes, together with exon IX, in the distal part of CA1 stratum radiatum, in the CA3 strata lucidum and radiatum and in the inner third of the DG molecular layer (orange). Kainate injection (KA 3 h, right) induces a weaker effect: dendritic localization of exon VI is detectable, together with exon IX, in the CA1 stratum radiatum (orange), while no dendritic staining is detectable in CA3 or DG. hf: hippocampal fissure.

different from those obtained with the pan-BDNF (exon IX) probes (Fig. 4). Therefore, apart from the contribution of exon II transcripts in the proximal lamina of the stratum radiatum after pilocarpine and although seizures up-regulate the somatic expression of several transcripts, it can be concluded that epileptogenic seizure-induced dendritic accumulation of BDNF mRNA in the hippocampus (Tongiorgi et al., 2004) is essentially dependent on exon VI-containing transcripts.

Discussion

This study provides the first evidence for a striking laminar segregation of five different BDNF mRNA splice variants in the rat hippocampus following epileptogenic status epilepticus. In animals treated with the muscarinic acetylcholine receptor agonist pilocarpine, exon VI splice variants were found in the hippocampal laminas containing the apical dendrites of CA1, CA3 and DG neurons, exon II splice variants were found only in the proximal CA1 and CA3 stratum radiatum and the other variants displayed a strictly somatic localization. In contrast, in animals treated with the glutamatergic AMPA/kainate receptor agonist kainate, only the exon VI variant was found in dendrites and only in CA1 neurons, while all other variants were restricted to the soma. The differential distribution of the different splice variants in response to different incoming stimuli appears to define a spatial code to control BDNF availability at different synapses through a segregated local regulation of its synthesis and/or trafficking. Thus, apart from and more broadly than epilepsy, these results provide a completely novel paradigm to re-interpret previous studies on the expression of different BDNF mRNA splice variants according to specific increases (or decreases) of BDNF protein availability near spatially segregated synaptic contacts, depending upon the exon(s) in use. Below, we will discuss the differences in the expression patterns and in the subcellular distribution of the BDNF mRNA splice variants, and the implications of these differences for BDNF protein localization and functional effects in terms of development of epilepsy.

Somatic expression levels of BDNF mRNA splice variants are differentially regulated by epileptogenic seizures

In agreement with previous studies, we found that, in the hippocampus of untreated rats, transcripts containing exon I are barely detectable while those containing either exons II, IV or VI are expressed at low to moderate level in all principal hippocampal neurons (Metsis et al., 1993; Timmusk et al., 1993). Moreover, in analogy with previous reports, we found that the different BDNF mRNA splice variants are up-regulated in the soma of principal hippocampal neurons in the pilocarpine and kainate models of epilepsy, with distinct expression patterns (Timmusk et al., 1993; Poulsen et al., 2004; Sathanoori et al., 2004). It should be noted, however, that the relative levels of each BDNF transcript and the time course of their up-regulation appear to differ in the different studies, particularly concerning exon I and exon VI transcripts. With reference to somatic expression levels, therefore, the results obtained in the present study for exon I, II and IV are similar to those obtained by Timmusk et al. (1993) while we observed relatively higher expression levels for exon VI transcripts. However, the exon VI expression pattern described here perfectly matches the one reported by others (Poulsen et al., 2004; Malkovska et al., 2006). Furthermore, we found that exon I was up-regulated in the DG, 3 h after kainate but not after pilocarpine, while others reported an up-regulation in all principal hippocampal neurons 6 h after pilocarpine (Poulsen et al., 2004) or kainate administration (Sathanoori et al., 2004). In sum, from the analysis of the literature on BDNF expression in the rodent hippocampus, it emerges that discrepancies concern basically only the expression of exon I in CA1 and CA3 (some laboratories report a strong, other a much weaker expression) and the expression levels of exon VI (some laboratories report this exon with a weak expression) while there is a general consensus on the expression of exons II and IV. Differences in time course, drug concentration, route or administration or model (intact animals vs. organotypic slices) may explain these discrepancies. Nonetheless, accurate real-time PCR assessment of the local concentration of each single BDNF transcript in laser micro-dissected hippocampal regions may help clarifying this issue in the future.

Epileptogenic seizures cause localization of selected BDNF mRNA splice variants to highly discrete dendritic laminas

The main finding of this study is that, following epileptogenic seizures, the different BDNF splice variants display a different laminar distribution in the dendrites. Accordingly, exon I and exon IV transcripts were always confined within the neuronal cell bodies. In contrast, exon II and exon VI splice variants accumulated in the apical dendrites of CA1, CA3 and DG neurons after epileptogenic seizures. Remarkably, the dendritic transcripts II and VI showed striking differences in their laminar localization: after pilocarpine,

the former localized to the most proximal portion of the CA1 stratum radiatum, while the latter was also found into the more distal part of the CA1 stratum radiatum, in the CA3 strata lucidum and radiatum and in the DG inner molecular layer. In the kainate model, only exon VI was found in the stratum radiatum of CA1.

Interestingly, the laminar expression pattern obtained with the probe for exon VI was identical to the one obtained with a probe made against the coding region encoded by exon IX, which recognizes all BDNF isoforms (pan-BDNF): both exon VI and pan-BDNF probes label exactly the same laminae 3 h after pilocarpine or kainate injection (see also Tongiorgi et al., 2004). This is a strong evidence that the main BDNF mRNA isoform targeted to the dendrites is made up by exons VI and IX. It remains to be determined which of the two possible short (320 nt) or long (3320 nt) 3′UTR variants is encoded by these dendritic transcripts.

It also remains unclear why the pattern of laminar distribution of BDNF mRNA is more widespread in the pilocarpine than in the kainate model, because both models are characterized by prolonged acute seizures followed, after a latent period, by spontaneous recurrent seizures. Based on our previous studies (Tongiorgi et al., 2004), we hypothesized that BDNF mRNA may be targeted to the most active synapses. Thus, highly active synapses during status epilepticus induced by kainate may be a subset of those most active during the one induced by pilocarpine. Indeed, the latter status epilepticus is more dramatic than the former, with more intense behavioural and EEG seizures.

Site of synthesis and functional effects of the BDNF protein after epileptogenic seizures

BDNF has been reported to exert contrasting effects in epilepsy. For example, BDNF signal reduction has been reported by many groups to retard epileptogenesis (Kokaia et al., 1995; Binder et al., 1999; Croll et al., 1999; Lahteinen et al., 2002; Xu et al., 2004; He et al., 2004) and, along the same line, BDNF has been reported to exacerbate seizure activity in epileptic hippocampi *in vitro* (Scharfman, 1997; Scharfman et al., 1999). However, BDNF may also exert beneficial effects at different times in the natural history of the disease (Palma et al., 2005). One mechanism that may underlie these contrasting effects may be the precise regulation of the local availability of BDNF (Tongiorgi et al., 2006). Indeed, other studies have provided evidence that BDNF has a very local effect on neuronal processes morphology (Horch and Katz, 2002). In organotypic cortical slices, BDNF supply promotes the dendritic arborization of pyramidal neurons (McAllister et al., 1995; Horch et al., 1999). The effects of BDNF are layer specific and only particular dendrites are affected, indicating that this molecule does not simply act to enhance neuronal growth but, rather, it acts instructively to modulate the patterns of dendritic arborization. In addition, BDNF may induce completely different effects depending on the cellular site of action or release: soma, axon, dendrites or different laminas of the dendritic tree receiving excitatory or inhibitory inputs (Lom and Cohen-Cory, 1999; Lom et al., 2002).

Based on the present data, we propose that generation of different splice variants with differential trafficking of the mRNAs represents a mechanism to deliver BDNF at distinct subcellular locations. According to this view, it is tempting to speculate that BDNF may exert local and, at least in part, different effects depending on its location. For instance, in analogy to sympathetic neurons where uncleaved precursor BDNF protein (pro-BDNF) has altered binding characteristics and distinct biological activity in comparison with mature BDNF protein (Lee et al., 2001; Teng et al., 2005), in hippocampal neurons somatic BDNF transcripts may synthesize “good” mature BDNF that may increase cell survival by activating an autocrine loop on somatic TrkB receptors, while dendritic mRNAs may produce uncleaved “bad” pro-BDNF, that may activate sortilin and p75NTR-mediated pro-apoptotic signalling cascades, and “bad” mature BDNF, that may potentiate excitatory synapses, increase synaptogenesis and increase dendritic arborization, thus favouring epileptogenesis (Lu et al., 2005; Simonato et al., 2006). Further studies are required to challenge this hypothesis.

Experimental methods

Preparation of the animals

Male Sprague–Dawley rats (280 g, Harlan, Italy) were used for all experiments. Procedures involving animals and their care were carried out in accordance with European Union and national laws and policies. All efforts were made to minimize animal suffering.

Pilocarpine, kainate or vehicle (saline) were administered i.p. (pilocarpine 300 mg/kg, kainate 10 mg/kg in saline). The rat's behaviour was observed for 3 h thereafter. Within the first hour after injection, all pilocarpine-treated and approximately 80% of kainate-treated animals developed seizures evolving into recurrent generalized convulsions (status epilepticus). These rats were sacrificed 3 h after injection of the chemoconvulsant [i.e. at peak time for accumulation of pan-BDNF mRNA (Tongiorgi et al., 2004)], and their brains used for in situ hybridization as described below. The remaining 20% of kainate-treated animals either did not seize or died within the first 3 h, and were not included in the study.

Riboprobes preparation

Probes for BDNF exons were synthesised with the DIG-RNA labelling kit (La Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. The probe for exon IX was described previously (Tongiorgi et al., 2004). The other probes corresponded to the following sequences: exon I nt 646–988 (Accession X67106; length = 342 nt), exon IV nt 703–901 (Accession X67107; length = 198 nt), exon VI nt 279–573 (Accession S71211; length = 294 nt). The probes for the two isoforms of the exon II (exonIIB and IIC) were not available and were cloned with a common forward primer named UPexonII that hybridizes with the segment 616–633 of the sequence S71201 (AATTCTCGAGCGGAGCGTTTGGAGAGCCAGCC) and two different reverse primers. The first reverse primer, used to amplify the sequence for the probe for exon IIB, hybridizes with the sequence 154–177 in exon IX (TTCCGCGGTGGGTAGGCCAAGTTGCCTTGTCC). The second reverse primer, used to amplify the sequence for the probe for exon IIC hybridizes with the sequence 112–134 in exon IX (TTCCGCGGATGGGCGCAGCCTTCATGCAACC). The underlined sequences correspond to the sequences for the *Xho*I and *Sac*II restriction sites for the cloning in pBSKS. The two probes, of the approximate length of 250 nt differ for an internal sequence of 84 nt which ensures, at high stringency conditions, a specific hybridization.

Reverse dot blot assay

Specificity of the riboprobes was tested in a reverse dot blot assay as follows. RNA probes sense and antisense were prepared as previously described (Tongiorgi et al., 1997). Sense probes were manually spotted (2 μl at 50 ng/μl concentration) on a nylon membrane (Hybond-N, Amersham), dried at 55 °C in oven and crosslinked for 5 min on a UV transilluminator. The membranes, placed in a revolving rotisserie hybridization oven, were prehybridized for 1 h at 60 °C in the prehybridization solution, containing: 20 mM Tris–HCl (pH 7.5), 1 mM EDTA (Sigma), Denhardt's solution, 300 mM NaCl, 100 mM dithiothreitol (Sigma), 0.5 mg:ml Salmon sperm

DNA (Sigma), 0.5 mg:ml polyadenylic acid (Sigma) and 50% formamide (Sigma). Hybridization with digoxigenin labelled antisense riboprobes (50 ng) was carried out overnight in the same buffer additioned with dextrane sulphate 10% at 60 °C. After hybridization, membranes were washed twice in 2× saline sodium citrate, 0.1% Tween 20 (SSCT):50% deionized formamide at 55 °C for 30 min, 20 min in 2× SSCT at 65 °C and twice in 0.1× SSCT at 65 °C for 30 min. Membranes hybridized with digoxigenin labeled riboprobes were processed for immunodetection with an anti-DIG antibody F(ab)2 fragment conjugated with alkaline phosphatase (Boehringer), diluted 1:1000 in PBST containing 5% fat free milk at RT for 1 h. After this incubation the membranes were washed four times in PBST at RT for 10 min, then incubated in developing buffer (0.1 M Tris–HCl buffer, 0.1 M NaCl, 0.05 M MgCl2, 1 mM Levamisol) at RT for 5 min, and finally incubated in a cromogen solution composed by the developing buffer containing nitro blue-terazolium (NBT) (Boehringer), and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Boehringer). The reaction was carried out for 15′ at RT or and stopped by rinsing the sections in stop-solution (10 mM Tris–HCl pH 8,1 mM EDTA).

In situ hybridization

Rats were transcardially perfused with 4% paraformaldehyde under ketamine (100 mg/kg i.p.) anaesthesia, and their brains removed and kept in 4% paraformaldehyde/20% sucrose [in PBS 1×(PO_4^{2-} 0.01 M; NaCl 0.15 M), pH 7.3] at 4 °C for at least 3 days before sectioning.

In situ hybridization was performed as previously described (Tongiorgi et al., 1998; 2004) on free-floating, 40 μm coronal sections cut at the level of dorsal hippocampus (plates 47–48, Pellegrino and Altman, 1979). Hybridization temperature was 55 °C for exons I, II and IX probes and 60 °C for exons IV and VI. Final washes (2×30 min in 0.1× Sodium Saline Citrate buffer additioned with 0.1% Tween-20) were at 60 °C for exons II and IX probes and at 65 °C for exons IV and VI probes. To obtain reproducible and comparable results and to avoid saturation of the reaction, alkaline phosphatase development was always performed for 5 h at room temperature. All in situ hybridizations on epileptic brain sections have been conducted side-by-side with brain sections from control animals.

Quantification of in situ hybridization data

Quantification of in situ hybridization data was performed as previously described by a researcher unaware of the animal treatments (Tongiorgi et al., 2004). Images were acquired through a Nikon AX1200 camera mounted on a Nikon E800 microscope (Nikon SpA, Firenze Italy) and analyzed with the program Image Pro Plus® 4.0 for Windows (Media Cybernetics, Silver Spring, MD). Images were captured with a 10× objective for measures in CA3, and with a 20× for dentate gyrus and CA1. Illumination was adjusted to obtain optimal staining signal over the dendritic fields (this could imply signal saturation over the cell somas) and then was kept constant throughout all experiments. Densitometric analysis was carried out on a 400×315 μm frame under the 20× objective (which represents the entire frame observed through the camera) and on a 800×630 μm frame with the 10× objective, and both were subdivided in 721×569 pixels. For the densitometric analysis of the CA3, a 50×630 μm frame was chosen. For each animal and hippocampal subfield, therefore, we generated curves in which each data point represents the average grey level over a line of pixels at a given distance from the cell soma. Data obtained from each animal were normalized on the grey level in the white matter (callosum).

For statistical analysis, we used the average grey level measured in 20 μm intervals of the different layers in the different hippocampal subfields. The intervals chosen were (as a distance from the cell body layer): for CA1, 40–60 μm (proximal stratum radiatum), 100–120 μm (distal stratum radiatum) and 300–320 μm (stratum lacunosum-moleculare); for CA3, 50–70 μm (stratum lucidum), 150–170 μm (stratum radiatum) and 350–370 μm (stratum lacunosum-moleculare); for the dentate gyrus, 60–80 μm (inner molecular layer), 160–180 μm (middle molecular layer) and 260–280 μm (outer molecular layer). The data obtained were analyzed using one way-ANOVA and *post-hoc* Newman–Keuls test, using the GraphPad 4 software.

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References

Aid, T., Kazantseva, A., Piirsoo, M., Palm, K., Timmusk, T., 2007. Mouse and rat BDNF gene structure and expression revisited. J. Neurosci. Res. 85, 525–535.

Bibel, M., Barde, Y.A., 2000. Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev. 14, 2919–2937.

Binder, D.K., Routbort, M.J., McNamara, J.O., 1999. Immunohistochemical evidence of seizure-induced activation of trk receptors in the mossy fiber pathway of adult rat hippocampus. J. Neurosci. 19, 4616–4626.

Capsoni, S., Tongiorgi, E., Cattaneo, A., Domenici, L., 1999. Dark rearing blocks the developmental down-regulation of brain-derived neurotrophic factor messenger RNA expression in layers IV and V of the rat visual cortex. Neuroscience 88, 393–403.

Casaccia-Bonnefil, P., Gu, C., Chao, M.V., 1999. Neurotrophins in cell survival/death decisions. Adv. Exp. Med. Biol. 468, 275–282.

Croll, S.D., Suri, C., Compton, D.L., Simmons, M.V., Yancopoulos, G.D., Lindsay, R.M., Wiegand, S.J., Rudge, J.S., Scharfman, H.E., 1999. Brain-derived neurotrophic factor transgenic mice exhibit passive avoidance deficits, increased seizure severity and in vitro hyperexcitability in the hippocampus and entorhinal cortex. Neuroscience 93, 1491–1506.

He, X.P., Kotloski, R., Nef, S., Luikart, B.W., Parada, L.F., McNamara, J.O., 2004. Conditional deletion of TrkB but not BDNF prevents epileptogenesis in the kindling model. Neuron 43, 31–42.

Horch, H.W., Katz, L.C., 2002. BDNF release from single cells elicits local dendritic growth in nearby neurons. Nat. Neurosci. 5, 1177–1184.

Horch, H.W., Kruttgen, A., Portbury, S.D., Katz, L.C., 1999. Destabilization of cortical dendrites and spines by BDNF. Neuron 23, 353–364.

Kanhema, T., Dagestad, G., Panja, D., Tiron, A., Messaoudi, E., Havik, B., Ying, S.W., Nairn, A.C., Sonenberg, N., Bramham, C.R., 2006. Dual regulation of translation initiation and peptide chain elongation during BDNF-induced LTP in vivo: evidence for compartment-specific translation control. J. Neurochem. 99, 1328–1337.

Kokaia, M., Ernfors, P., Kokaia, Z., Elmer, E., Jaenisch, R., Lindvall, O., 1995. Suppressed epileptogenesis in BDNF mutant mice. Exp. Neurol. 133, 215–224.

Lahteinen, S., Pitkanen, A., Saarelainen, T., Nissinen, J., Koponen, E., Castren, E., 2002. Decreased BDNF signalling in transgenic mice reduces epileptogenesis. Eur. J. Neurosci. 15, 721–734.

Lee, R., Kermani, P., Teng, K.K., Hempstead, B.L., 2001. Regulation of cell survival by secreted proneurotrophins. Science 294, 1945–1948.

Liu, Q.R., Lu, L., Zhu, X.G., Gong, J.P., Shaham, Y., Uhl, G.R., 2006. Rodent BDNF genes, novel promoters, novel splice variants, and regulation by cocaine. Brain Res. 1067, 1–12.

Lom, B., Cohen-Cory, S., 1999. Brain-derived neurotrophic factor differentially regulates retinal ganglion cell dendritic and axonal arborization in vivo. J. Neurosci. 19, 9928–9938.

Lom, B., Cogen, J., Sanchez, A.L., Vu, T., Cohen-Cory, S., 2002. Local and target-derived brain-derived neurotrophic factor exert opposing effects on the dendritic arborization of retinal ganglion cells in vivo. J. Neurosci. 22, 7639–7649.

Lu, B., Pang, P.T., Woo, N.H., 2005. The yin and yang of neurotrophin action. Nat. Rev., Neurosci. 6, 603–614.

Malkovska, I., Kernie, S.G., Parada, L.F., 2006. Differential expression of the four untranslated BDNF exons in the adult mouse brain. J. Neurosci. Res. 83, 211–221.

McAllister, A.K., Lo, D.C., Katz, L.C., 1995. Neurotrophins regulate dendritic growth in developing visual cortex. Neuron 15, 791–803.

Metsis, M., Timmusk, T., Arenas, E., Persson, H., 1993. Differential usage of multiple brain-derived neurotrophic factor promoters in the rat brain following neuronal activation. Proc. Natl. Acad. Sci. U. S. A. 90, 8802–8806.

Palma, E., Torchia, G., Limatola, C., Trettel, F., Arcella, A., Cantore, G., Di Gennaro, G., Manfredi, M., Esposito, V., Quarato, P.P., Miledi, R., Eusebi, F., 2005. BDNF modulates GABAA receptors microtransplanted from the human epileptic brain to Xenopus oocytes. Proc. Natl. Acad. Sci. U. S. A. 102, 1667–1672.

Pattabiraman, P.P., Tropea, D., Chiaruttini, C., Tongiorgi, E., Cattaneo, A., Domenici, L., 2005. Neuronal activity regulates the developmental expression and subcellular localization of cortical BDNF mRNA isoforms in vivo. Mol. Cell. Neurosci. 28, 556–570.

Pellegrino, L., Altman, J., 1979. A Sterotaxis Atlas of the Rat Brain. Plenum Press, New York, NY, USA.

Pitkanen, A., Sutula, T.P., 2002. Is epilepsy a progressive disorder? Prospects for new therapeutic approaches in temporal-lobe epilepsy. Lancet Neurol. 1, 173–181.

Poulsen, F.R., Lauterborn, J., Zimmer, J., Gall, C.M., 2004. Differential expression of brain-derived neurotrophic factor transcripts after pilocarpine-induced seizure-like activity is related to mode of Ca2+ entry. Neuroscience 126, 665–676.

Righi, M., Tongiorgi, E., Cattaneo, A., 2000. Brain-derived neurotrophic factor (BDNF) induces dendritic targeting of BDNF and tyrosine kinase B mRNAs in hippocampal neurons through a phosphatidylinositol-3 kinase-dependent pathway. J. Neurosci. 20, 3165–3174.

Sathanoori, M., Dias, B.G., Nair, A.R., Banerjee, S.B., Tole, S., Vaidya, V.A., 2004. Differential regulation of multiple brain-derived neurotrophic factor transcripts in the postnatal and adult rat hippocampus during development, and in response to kainate administration. Brain Res. Mol. Brain Res. 130, 170–177.

Scharfman, H.E., 1997. Hyperexcitability in combined entorhinal/hippocampal slices of adult rat after exposure to brain-derived neurotrophic factor. J. Neurophysiol. 78, 1082–1095.

Scharfman, H.E., Goodman, J.H., Sollas, A.L., 1999. Actions of brain-derived neurotrophic factor in slices from rats with spontaneous seizures and mossy fiber sprouting in the dentate gyrus. J. Neurosci. 19, 5619–5631.

Schuman, E.M., 1999. Neurotrophin regulation of synaptic transmission. Curr. Opin. Neurobiol. 9, 105–109.

Simonato, M., Tongiorgi, E., Kokaia, M., 2006. Angels and demons: neurotrophic factors and epilepsy. Trends Pharmacol. Sci. 27, 631–638.

Teng, H.K., Teng, K.K., Lee, R., Wright, S., Tevar, S., Almeida, R.D., Kermani, P., Torkin, R., Chen, Z.Y., Lee, F.S., Kraemer, R.T., Nykjaer, A., Hempstead, B.L., 2005. ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J. Neurosci. 25, 5455–5463.

Timmusk, T., Palm, K., Metsis, M., Reintam, T., Paalme, V., Saarma, M., Persson, H., 1993. Multiple promoters direct tissue-specific expression of the rat BDNF gene. Neuron 10, 475–489.

Tongiorgi, E., Righi, M., Cattaneo, A., 1997. Activity-dependent dendritic targeting of BDNF and TrkB mRNAs in hippocampal neurons. J. Neurosci. 17, 9492–9505.

Tongiorgi, E., Righi, M., Cattaneo, A., 1998. A non-radioactive in situ hybridization method that does not require RNAse-free conditions. J. Neurosci. Methods 85, 129–139.

Tongiorgi, E., Armellin, M., Giulianini, P.G., Bregola, G., Zucchini, S., Paradiso, B., Steward, O., Cattaneo, A., Simonato, M., 2004. Brain-derived neurotrophic factor mRNA and protein are targeted to discrete dendritic laminas by events that trigger epileptogenesis. J. Neurosci. 24, 6842–6852.

Tongiorgi, E., Domenici, L., Simonato, M., 2006. What is the biological significance of BDNF mRNA targeting in the dendrites? Clues from epilepsy and cortical development. Mol. Neurobiol. 33, 17–32.

Xu, B., Michalski, B., Racine, R.J., Fahnestock, M., 2004. The effects of brain-derived neurotrophic factor (BDNF) administration on kindling induction, Trk expression and seizure-related morphological changes. Neuroscience 126, 521–531.