Localization of Brain-Derived Neurotrophic Factor to Distinct Terminals of Mossy Fiber Axons Implies Regulation of Both Excitation and Feedforward Inhibition of CA3 Pyramidal Cells

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Hippocampal dentate granule cells directly excite and indirectly inhibit CA3 pyramidal cells via distinct presynaptic terminal specializations of their mossy fiber axons. This mossy fiber pathway contains the highest concentration of brain-derived neurotrophic factor (BDNF) in the CNS, yet whether BDNF is positioned to regulate the excitatory and/or inhibitory pathways is unknown. To localize BDNF, confocal microscopy of green fluorescent protein transgenic mice was combined with BDNF immunohistochemistry. Approximately half of presynaptic granule cell–CA3 pyramidal cell contacts were found to contain BDNF. Moreover, enhanced neuronal activity virtually doubled the percentage of BDNF-immunoreactive terminals contacting CA3 pyramidal cells. To our surprise, BDNF was also found in mossy fiber terminals contacting inhibitory neurons. These studies demonstrate that mossy fiber BDNF is poised to regulate both direct excitatory and indirect feedforward inhibitory inputs to CA3 pyramidal cells and reveal that seizure activity increases the pool of BDNF-expressing granule cell presynaptic terminals contacting CA3 pyramidal cells.

Key words: astrocyte (astroglia); epilepsy; granule cell; hippocampus; long-term potentiation; mossy fiber

Introduction

Hippocampal dentate granule cells convey neocortical information to the CA3 region via their mossy fiber axons. Brain-derived neurotrophic factor (BDNF) and its receptor, TrkB, may play key roles in regulating information flow through this circuit. Indeed, the hippocampal mossy fiber pathway—the projection region of granule cell axons—contains the highest levels of BDNF protein in the CNS (Conner et al., 1997; Yan et al., 1997), and BDNF has been shown to regulate synaptic efficacy (Korte et al., 1995; Patterson et al., 1996; Korte et al., 1998). Furthermore, BDNF in the mossy fiber pathway has been implicated in regulating granule cell morphology (Patel and McNamara, 1995; Lowenstein and Arsenault, 1996; Danzer et al., 2002; Tolwani et al., 2002) and susceptibility to temporal lobe seizures (Kokaia et al., 1995; Binder et al., 1999a,b; Croll et al., 1999; Lähtinen et al., 2002; Danzer et al., 2004a; He et al., 2004).

The mossy fiber axons are unique among CNS principle neurons in that they exhibit three distinct presynaptic specializations,

mossy fiber boutons, en passant terminals, and filipodial extensions (Amaral and Dent, 1981; Acsády et al., 1998). Interestingly, these distinct specializations innervate distinct targets. The combination of intracellular labeling of granule cells *in vivo* followed by immunochemistry and electron microscopy led to the demonstration that the giant mossy fiber boutons almost exclusively innervate CA3 pyramidal cells, whereas the filipodia and en passant terminals primarily innervate interneurons (Acsády et al., 1998). Mossy fiber boutons, by exciting CA3 pyramidal cells, make up one part of the classic hippocampal “trisynaptic circuit.” Filipodial and en passant terminals, on the other hand, constitute a more recently identified albeit critical feedforward inhibitory pathway wherein granule cells excite inhibitory neurons, which in turn inhibit CA3 pyramidal cells (for review, see Lawrence and McBain, 2003). The synapses formed by these distinct mossy fiber specializations also exhibit strikingly different properties; for example, a high-frequency stimulus of the mossy fiber pathway induces long-term potentiation (LTP) of the giant mossy fiber bouton synapses on CA3 pyramidal cells but not of the filipodial and en passant synapses on interneurons (Maccaferri et al., 1998). Elucidating the distribution of BDNF among the different synaptic specializations of the mossy fiber pathway is critical to understanding how BDNF regulates synaptic control of CA3 pyramidal cells by the granule cells.

To determine whether BDNF might regulate the efficacy of different synapses formed by mossy fiber terminals, a logical first step is to define whether BDNF is in fact present at these synapses

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and, if so, whether it is differentially distributed among the different specializations. The light microscopic pattern of BDNF immunoreactivity in the mossy fiber pathway and the ability of these synapses to undergo LTP led us to hypothesize that BDNF is localized to the giant boutons of the mossy fibers (Fawcett et al., 1997; Maccaferri et al., 1998; Danzer et al., 2004b). To test this hypothesis, BDNF immunostaining of sections from green fluorescent protein (GFP) transgenic mice was combined with advanced confocal microscopy of synaptic terminals.

Materials and Methods

Thy1 GFP-expressing mice. Mice expressing GFP under control of the Thy1 promoter were bred from the M line, which has been described previously (Feng et al., 2000). Thy1 belongs to the Ig superfamily and is expressed in many neuronal as well as some non-neuronal cells. Importantly, the dentate granule cells labeled by GFP in the M line in our study appear to represent typical granule cells in that their somatic, dendritic, and axonal architectures replicate patterns revealed by other techniques (Ramon y Cajal, 1911).

Status epilepticus induction. All procedures conformed to National Institutes of Health and institutional guidelines for the care and use of animals. Mice were maintained on a 12 hr light/dark cycle. Eleven male GFP-expressing mice 2–3 months old were injected with 1 mg/kg methyl scopolamine nitrate intraperitoneally (Sigma, St. Louis, MO). Fifteen minutes later, six mice were injected intraperitoneally with 340 mg/kg pilocarpine (Sigma), and five littermates were injected with saline. Pilocarpine was administered between 10 A.M. and noon. Mice were observed after the injections for the appearance of seizure activity and onset of status epilepticus. Status epilepticus was defined as occasional or frequent myoclonic jerks, partial- or whole-body clonus, shivering, loss of posture, and/or rearing and falling that was not interrupted by periods of normal behavior. Animals were allowed to remain in status epilepticus for 3 hr before the condition was terminated by injecting 10 mg/kg diazepam. Control animals also received diazepam injections.

Forty-eight hours after the termination of seizure activity, mice were anesthetized with 100 mg/kg pentobarbital intraperitoneally and perfused through the ascending aorta at 10 ml/min for 30 sec with ice-cold PBS with 1 U/ml heparin followed by room temperature 2.5% paraformaldehyde and 4% sucrose, pH 7.4, for 10 min. The brains were removed and postfixed in ice-cold 2.5% paraformaldehyde and 4% sucrose, pH 7.4, for 1 hr, after which they were cryoprotected in 10% sucrose in PBS overnight, for 24 hr in 20% sucrose in PBS, and finally for 48 hr in 30% sucrose in PBS. After cryoprotection, brains were snap-frozen in isopentane cooled to -25°C with dry ice and stored at -80°C until cryosectioning. Sections were cut at 40 μm , wet-mounted to Superfrost Plus slides (Port City Diagnostics, Wilmington, NC), and stored at -80°C until immunostaining.

BDNF and GFP immunohistochemistry. The BDNF primary antibody (generously donated by Amgen, Thousand Oaks, CA) was used at a concentration of 1 $\mu\text{g}/\text{ml}$. This polyclonal rabbit antibody has been extensively characterized, and its specificity for BDNF has been established (Conner et al., 1997; Yan et al., 1997). Antibody specificity was further confirmed in our hands by preincubating the primary antibody with BDNF peptide (Danzer et al., 2004a) and by using conditional BDNF knock-out animals (Danzer et al., 2004b; He et al., 2004). GFP expression was simultaneously enhanced by incubating the sections with mouse anti-GFP monoclonal antibodies (1:500; Chemicon, Temecula, CA). After a 1 hr incubation at room temperature in PBS, 5% NGS, and 0.5% Igepal (Sigma), both primary antibodies were applied simultaneously at 4°C for 24 hr in PBS and 5% normal goat serum. Alexa Fluor 594 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse secondary antibodies were used at concentrations of 1:750 (Molecular Probes, Eugene, OR). Adjacent sections not treated with the BDNF primary antibody were also run for each animal. In all cases, sections from pilocarpine-treated mice were processed simultaneously in the same incubation dishes with their littermate controls so that valid comparisons could be made.

Microscopy and data collection. All data collection and analysis were conducted with the experimenter blinded to treatment group. GFP-

expressing dentate granule cell terminals in the stratum lucidum of CA3b were imaged using a Leica (Nussloch, Germany) TCS SL confocal system set up on a Leica DMIRE2 inverted microscope equipped with epifluorescent illumination and a $63\times$ oil immersion objective (numerical aperture, 1.4). CA3a and CA3b pyramidal cell thorny excrescences were also imaged. Images were captured using $6\times$ optical zoom. GFP (Alexa Fluor 488) and BDNF (Alexa Fluor 594) immunoreactivity signals were captured using sequential line scanning. Z-series “stacks” of neuronal processes were collected at 0.4 μm increments with the pinhole set to 0.7 Airy units. Four times line averaging was used to improve image quality. Neuronal structures were identified from the z-series stacks and scored as either BDNF-negative or -positive. Colocalization was confirmed in x, y, and z dimensions. The thickness of the focal plane of the microscope becomes significant for colocalization in the z dimension. For the objective used in the present study, z resolution is stated to be 235 nm (Leica). (This assumes ideal conditions and 488 nm light, and, in practice, z resolution will be somewhat larger.) Maximum resolution in the x–y plane is 180 nm.

Mossy fiber boutons, en passant terminals, and thorny excrescence spine heads were scored as positive for BDNF immunoreactivity if at least 50% of the structure was immunoreactive and if the immunoreactive signal was at least twice as intense as the area immediately surrounding the terminal. Filipodia were scored as positive if they either met the above criteria or if three regions of a filipodium were twice as intense as background. These criteria for filipodia were used because BDNF-immunoreactive particles $<1\ \mu\text{m}$ in diameter were often seen to “track” the path of the filipodia (see Fig. 3, right column), indicating that the filipodium was almost certainly BDNF-immunoreactive but not always meeting the 50% filled criterion. The experimenter’s ability to consistently distinguish structures as twice as intense as background was confirmed with the Leica software channel dye separator, which can quantify BDNF and GFP signals from captured images. To confirm that the settings used to capture the signal for BDNF immunoreactivity did not also capture the GFP immunoreactivity, sections immunostained as above but with the BDNF antibody excluded from the immunostaining procedure were also examined. GFP immunoreactivity did not interfere with the BDNF signal. Finally, we note that the criteria used for determining whether a structure is immunopositive are deliberately conservative. Because this is the first study to directly examine BDNF immunoreactivity in many of these structures, we did not want to falsely identify a structure as containing BDNF. A consequence of this approach, however, is that our values likely underestimate the number of immunoreactive structures. We also recommend caution when comparing the different percentages of BDNF-immunopositive structures (e.g., boutons vs filipodia). The different sizes and shapes of these terminal structures may differentially affect our ability to detect BDNF immunoreactivity in them.

Identification of dentate granule cell giant mossy fibers boutons was based on their location in the stratum lucidum and their unique structure: their unusually large size ($8\text{--}17\ \mu\text{m}^2$) and their continuity with the mossy fiber axon (Amaral and Dent, 1981). En passant terminals were distinguished from giant mossy fiber boutons by their smaller size ($0.5\text{--}2\ \mu\text{m}$) (Acsády et al., 1998) and smoother appearance (see Fig. 2). Expansions had to be at least twice the diameter of the parent axon to be counted as an en passant terminal (Claiborne et al., 1986). CA3 pyramidal cell thorny excrescences, which are located on the proximal dendrite, were identified based on their unique structure: single or clusters of large postsynaptic spines attached to a single stalk (see Fig. 2).

Statistics. To avoid pseudoreplication, each animal was given a single percentage score for each structure assessed for BDNF immunoreactivity. Comparisons used Mann–Whitney rank–sum tests run on SigmaStat software (Jandel Scientific, San Rafael, CA).

Results

To determine the cellular and subcellular localization of BDNF in stratum lucidum of hippocampus, BDNF immunohistochemistry was conducted on coronal sections from GFP-expressing mice. In these animals, numerous GFP-labeled dentate granule cells and occasional GFP labeled CA3 pyramidal cells were

Figure 1. Confocal reconstruction showing GFP immunoreactivity in the hippocampus of the Thy1 GFP-expressing mouse. In these sections, numerous dentate granule cells are labeled. Their axons can be seen projecting into the mossy fiber pathway (hilus, stratum lucidum over CA3). A single labeled CA3 pyramidal cell is also pictured. Scale bar, 250 μ m.

Figure 2. Composite confocal image showing a dentate granule cell giant mossy fiber bouton (white arrow), a dentate granule cell MFB filipodia (green arrowhead), dentate granule cell en passant terminals (yellow arrowheads, EPT), a CA3b pyramidal cell apical dendrite with thorny excrescences (blue arrows, Th. Exc.), and one of the individual spine heads that makes up an excrescence (blue arrows, Th. Exc. Spine head). Scale bar, 3 μ m.

present (Fig. 1). GFP fills all of the processes of these neurons, revealing dentate granule cell giant mossy fiber boutons, filipodia, and en passant terminals (Fig. 2). CA3 pyramidal cell apical dendrites, spines, and thorny excrescences in the stratum lucidum were also revealed (Fig. 2). Importantly, the unique morphology of these axonal and dendritic terminal processes, as described by Ramon y Cajal (1911), permits their unequivocal identification and establishes the utility of this technique for colocalization studies.

BDNF immunostaining

The pattern of BDNF immunostaining in mice was similar to that seen for rats with this antibody (Conner et al., 1997; Yan et al., 1997; Danzer et al., 2004a). Within the hippocampus, staining of the mossy fiber pathway was prominent (results not shown). Within the stratum lucidum, BDNF immunostaining was organized into clusters, as described previously (Fawcett et al., 1997; Danzer et al., 2004b). These BDNF-immunoreactive clusters are $\sim 5 \mu$ m in diameter (ranging from 3 to 10 μ m) and represent accumulations of BDNF protein as confirmed using BDNF con-

Figure 3. BDNF-immunoreactive giant mossy fiber boutons and mossy fiber bouton filipodia. Top, GFP in granule cell giant mossy fiber boutons (arrows) and mossy fiber bouton filipodia (arrowheads). Middle, BDNF-immunoreactive clusters that colocalize with giant boutons are denoted with arrows. Arrowheads denote the regions of the filipodia that are immunoreactive for BDNF (middle panel). Bottom, Merged GFP and BDNF images with double-labeled regions showing up as blue. Images are maximum projections (showing the regions of highest intensity) of several confocal scans through the pictured MFB but excluding the regions above and below the bouton. Scale bar, 3 μ m.

ditional knock-out mice (Danzer et al., 2004b). In addition to these clusters, smaller BDNF-immunoreactive particles, typically $< 1 \mu$ m in diameter, were also noted between clusters. The intensity of the particle staining was notably greater within the stratum lucidum compared with the adjacent stratum radiatum. The staining of these smaller particles was also eliminated in BDNF conditional knock-out mice (Danzer et al., 2004b), indicating that they also represent BDNF protein.

Dentate granule cell giant mossy fiber boutons

The size ($\sim 5 \mu$ m in diameter) and distribution (the mossy fiber pathway) of immunoreactive clusters observed in BDNF-immunostained sections led us to hypothesize that these clusters reflect accumulation of BDNF protein in dentate granule cell giant mossy fiber boutons (Danzer et al., 2004b). To test this hypothesis, regions of the stratum lucidum of CA3b containing mossy fiber boutons were identified by examining GFP immunostaining in sections from the GFP-expressing mouse line (Fig. 3, top, arrows). BDNF immunostaining in these sections was not examined during the selection process to avoid biasing the results. Confocal stacks [consisting of series of images of single focal planes taken at 0.4 μ m increments through the depth (z-axis) of a mossy fiber bouton] were captured of both GFP (Fig. 3, top,

arrows) and BDNF (Fig. 3, middle, arrows) immunoreactivity. Captured images were analyzed, and mossy fiber boutons were scored as either BDNF-negative or -positive. Approximately half of 87 GFP-labeled mossy fiber boutons from five control GFP-expressing mice were clearly BDNF-immunoreactive. In BDNF-positive mossy fiber boutons, BDNF immunoreactivity frequently filled the entire bouton and always appeared as conglomerations of many small particles $<1\ \mu\text{m}$ in size (Fig. 3, middle). Heterogeneity in the amount of BDNF immunoreactivity within a bouton was striking. Boutons completely filled with intense signal were found in close proximity (within 5–10 μm) to boutons on different mossy fiber axons with no detectable immunoreactivity.

BDNF-immunoreactive clusters were only observed colocalizing with mossy fiber boutons. Clusters were never seen to colocalize with any other structure, including CA3 pyramidal cell somas and dendrites, or astrocytic processes. Whether a small number of clusters represent BDNF expression in interneurons rather than mossy fiber giant boutons could not be excluded because the absence of GFP expression in interneurons precludes testing this possibility. The restriction of BDNF-immunoreactive clusters to the mossy fiber pathway and the orientation of clusters within this pathway, however, are entirely consistent with mossy fiber localization. In contrast, clusters did not follow the pattern of interneuron dendrites, which frequently project outside of the mossy fiber pathway.

Filipodia extensions

The stratum lucidum also contains BDNF immunoreactivity not organized into obvious clusters. Because the axons of the granule cells exhibit presynaptic specializations in addition to mossy fiber boutons, we first queried whether nonclustered BDNF immunoreactivity was expressed in these specializations. The first of these, termed *filipodial extensions*, are fiber-like protrusions of the giant mossy fiber boutons that range up to 50 μm in length (Fig. 2) (Amaral, 1979; Acsády et al., 1998). Previously captured mossy fiber bouton image stacks were reviewed, and mossy fiber boutons possessing filipodia were identified (47 of the 87 boutons had at least one filipodium). These filipodia were almost equally split in their origination from BDNF-positive or -negative mossy fiber boutons. Filipodia were scored as BDNF-positive or -negative. In Figure 3, the arrowheads denote BDNF-immunoreactive mossy fiber bouton filipodia, which can be seen originating from their parent mossy fiber boutons. Fifteen percent of the 47 filipodia examined were BDNF-immunoreactive. To avoid biasing these results toward mossy fiber boutons with multiple filipodia, only one filipodium per mossy fiber bouton was analyzed. We do note, however, that among boutons with multiple filipodia, all of the filipodia tended to be either positive or negative. In addition, positive filipodia invariably belonged to BDNF-positive mossy fiber boutons, whereas negative filipodia extended from both positive and negative boutons.

En passant terminals

Another presynaptic terminal specialization of the mossy fiber axons is the *en passant terminal*, which is characterized by the more typical small size of CNS presynaptic terminals (0.5–2 μm in diameter). Again, the image stacks of mossy fiber boutons captured previously were reexamined to identify *en passant* terminals, and these terminals were then scored for BDNF immunoreactivity. Sixty-three GFP-expressing *en passant* terminals were identified in these image stacks, and 13% were clearly BDNF-immunoreactive (Fig. 4A, top arrowhead). This percent-

Figure 4. *A*, BDNF-immunoreactive mossy fiber *en passant* terminals. Top, GFP-expressing giant mossy fiber bouton (arrow) and *en passant* terminals (arrowheads) in the stratum lucidum. Circled regions denote BDNF-immunoreactive particles within axons. Middle, BDNF immunoreactivity in the same fields as shown in the top panels. The mossy fiber bouton (arrow) and the top *en passant* terminal (arrowhead) are BDNF immunopositive. The bottom *en passant* terminal is negative. Bottom, Merged GFP and BDNF images with double-labeled regions showing up as blue. Images are maximum projections (showing the regions of highest intensity) of several confocal scans through the pictured *en passant* terminals but excluding the regions above and below the terminal. *B*, BDNF-immunoreactive particles are found in dentate granule cell mossy fiber axons. Circled regions denote BDNF-immunoreactive particles within axons. The GFP image (top) is a maximum projection of the axon (showing the regions of highest intensity), and the BDNF image (middle) is a projection of three focal planes showing the BDNF particles within the axon. Scale bars: *A*, 3 μm ; *B*, 1 μm .

age likely underestimates the actual number of immunoreactive terminals because some of the small expansions identified with light microscopy lack synaptic release sites when examined with electron microscopy (Acsády et al., 1998). As seen with giant mossy fiber boutons, BDNF immunoreactivity in *en passant* terminals also appeared as conglomerations of many small particles $<1\ \mu\text{m}$ in size, except that the conglomerations contained many fewer particles than did mossy fiber boutons (Fig. 4A, compare the *en passant* terminal, top arrowhead, with the mossy fiber bouton, arrow).

Mossy fiber axons

In the process of examining BDNF immunoreactivity within the presynaptic terminals of the mossy fiber axons, BDNF immuno-

Figure 5. Confocal scan of a single focal plane reveals BDNF immunoreactivity surrounding CA3 pyramidal cell thorny excrescences. Left column, Thorny excrescence enveloping a BDNF-immunoreactive cluster. Right column, Thorny excrescence spine head surrounded by but not containing BDNF immunoreactivity (left of asterisk). GFP-immunoreactive thorny excrescences are shown in the top row; BDNF immunoreactivity in the same fields is shown in the middle row; and merged images are shown in the bottom row. Scale bar, 2 μ m.

reactivity was occasionally noted within portions of mossy fiber axons lacking presynaptic specializations. The BDNF immunoreactivity consisted of individual submicrometer-sized immunoreactive particles that were separated by micrometers to tens of micrometers within an axon (Fig. 4, circled regions). Given the clear presence of BDNF in mossy fiber terminals, that it would also be present in axons is not unexpected. Determining whether this immunoreactivity reflects retrograde or anterograde transport of BDNF, however, is beyond the scope of the present study.

BDNF immunoreactivity surrounds CA3 pyramidal cell thorny excrescences

The localization of BDNF to dentate granule cell giant mossy fiber boutons predicts that BDNF immunoreactivity will be found in direct apposition to CA3 pyramidal cell dendritic spines, known as thorny excrescences, because these excrescences are innervated by the mossy fiber bouton terminals. Consistent with this prediction, BDNF immunoreactivity was found intercalated among GFP-labeled thorny excrescences (Fig. 5, left column) and among the individual GFP-labeled spine heads of thorny excrescences (Fig. 5, right column, asterisk). BDNF immunostaining that surrounds but does not fill a thorny excrescence likely re-

Figure 6. Colocalization within scans from single focal planes reveals BDNF-immunoreactive CA3 pyramidal cell thorny excrescences. *A*, Maximum projection displaying the region of highest intensity from six focal planes taken at 0.4 μ m increments of the thorny excrescence shown in *B*. *B*, Three scans of the same thorny excrescence taken at three different focal planes, each plane being separated by 0.4 μ m. The scans show BDNF immunoreactivity colocalizing with the thorny excrescence spine heads. GFP immunoreactivity is shown in the top row; BDNF immunoreactivity in the same fields is shown in the middle row; and merged images are shown in the bottom row. Moving left to right, note how the GFP and BDNF signals from the spine denoted by the arrowhead disappear simultaneously in the right image. (In the right image, the GFP and BDNF signals from the spine become faint and "fuzzy" because they are no longer in the focal plane). Similarly, a second spine (arrow), which is just out of focus in the left image, appears simultaneously with BDNF immunoreactivity in the middle and right images. *d*, Dendrite. Scale bar, 2 μ m.

flects a GFP-negative, BDNF-immunopositive giant mossy fiber bouton making contact with the thorny excrescence.

To quantify the degree of possible contact between thorny excrescences and BDNF-immunoreactive particles, thorny excrescence spine heads were examined. CA3 pyramidal cell thorny excrescences are elaborate clusters of postsynaptic spines connected to the dendritic shaft by a thin neck (Blackstad and Kjaerheim, 1961; Amaral, 1979; Amaral and Dent, 1981; Chicurel and Harris, 1992; Gonzales et al., 2001). Spines terminate in a bulbous shaped "head" (Fig. 2). In the present study, 14 thorny excrescences from five control animals were examined. These thorny excrescences possessed between 6 and 41 spine heads (mean $\pm$ SEM, 17.3 ± 2.5). Excrescences with only a single head were excluded from the analysis to ensure that identification was unambiguous. Careful examination of 147 spine heads from these thorny excrescences revealed that 56% were in apparent contact with BDNF immunoreactivity. All 14 thorny excrescences examined possessed at least one spine head adjacent to BDNF immunoreactivity. Immunoreactivity adjacent to spine heads ranged from single, submicrometer-sized particles (Fig. 5, right column) to immunoreactive clusters (Fig. 5, left middle panel, probable GFP-negative, BDNF-positive mossy fiber bouton).

BDNF immunoreactivity in additional cell types within the stratum lucidum: CA3 pyramidal cell thorny excrescences

A subset of the thorny excrescences found in direct apposition to BDNF immunoreactivity were BDNF-immunoreactive themselves (Fig. 6), a finding consistent with studies localizing BDNF

to hippocampal spines in CA1 (Tongiorgi et al., 2004). A detailed analysis of 147 spine heads from control animals revealed that 7.6% were BDNF-immunoreactive. Typically, BDNF immunoreactivity was found within the spine heads and was excluded from the parent dendrite (Fig. 6). Occasional somatic labeling of cells likely to be CA3 pyramidal cells was observed in the present study (results not shown), consistent with previous studies (Danzon et al., 2004a,b). Somatic labeling of CA3 pyramidal cells was not observed to extend into proximal dendrites in the mossy fiber pathway, and none of the pyramidal cells with prominent somatic labeling was colabeled with GFP, so the relationship between somatic and thorny excrescence labeling of CA3 pyramidal cells is still unclear.

Astrocytes in the stratum lucidum contain BDNF

In the GFP-expressing mouse line used in the present study, a small number of astrocytes in the CA3 stratum lucidum expressed GFP. This provided the opportunity to determine whether BDNF is also localized to astrocytic processes. Eight GFP-expressing astrocytes with processes in the stratum lucidum of CA3a or CA3b were identified in the BDNF-immunostained sections isolated from three control animals. On superficial examination, these astrocytes do not appear to be BDNF-immunoreactive in that the cell bodies are not brightly immunoreactive, nor are the major processes outlined with BDNF immunoreactivity. Close examination of high-resolution confocal scans, however, revealed that seven of the eight astrocytes contained numerous submicrometer-sized BDNF-immunoreactive particles. These particles were found both in the soma (Fig. 7A) and in fibers (Fig. 7B,C), but were so sparse they did not reveal the outline of the astrocyte when BDNF immunoreactivity was viewed alone (Fig. 7, compare middle row with bottom row, which shows double-labeled regions in blue). Only when the structure of the astrocytes was revealed with GFP and astrocytic processes and BDNF immunoreactivity were followed through many focal planes was it clear that they contained BDNF immunoreactivity. Figure 7C, for example, shows an expansion of Figure 7B, boxed region, and demonstrates BDNF-immunoreactive particles within the astrocytic process. Although this process is clearly BDNF-immunoreactive, the sparse labeling contrasts to immunoreactivity seen in mossy fiber boutons, which frequently revealed the outline of the entire terminal. Significantly, the lack of labeling in one of the eight astrocytes examined strengthens the likelihood that labeling in the remaining seven is specific. Namely, this unlabeled astrocyte, which was located in an adjacent section on the same slide as two of the labeled astrocytes, followed a tortuous path, whereby dozens of its fibers neatly avoided all BDNF immunoreactivity in the section. If the positive astrocytic labeling were the result of random accumulation of nonspecific immunoreactive particles throughout the tissue (whereby all structures would accumulate some “hits”), then at least some of the many fibers of this astrocyte would be expected to be positive.

BDNF in astrocytes may be taken up from neurons (Biffo et al., 1995). If this is indeed the case, then this predicts that astrocytic processes will be found in contact with BDNF-immunoreactive neuronal processes. Assessment of the relationship between astrocytic processes and BDNF immunoreactivity revealed that astrocytes frequently enveloped BDNF-immunoreactive clusters (presumptive mossy fiber boutons). The degree of potential contact is striking, as shown in Figure 8. Note the BDNF-immunoreactive clusters (red, middle, bottom rows) and the extensive network of astrocytic processes (yellow, top, bottom rows) that envelop them. Processes from

Figure 7. BDNF immunoreactivity is localized to astrocytes in the mossy fiber pathway. Top row, GFP-expressing astrocytes located at the CA3b stratum lucidum. Middle row, BDNF-immunoreactivity in the same fields as shown in the top row. Bottom row, Merged GFP and BDNF images with double-labeled regions showing up as blue. *A*, Confocal scan from a single focal plane showing BDNF immunoreactivity in the cell body of an astrocyte. *B*, Stratum lucidum astrocyte containing numerous BDNF-immunoreactive particles in its processes. *C*, Magnified view of the boxed region shown in *B*. Images are a maximum projection of 20 confocal scans of focal planes separated by 0.2 μ m increments. The maximum projection shows the regions of highest intensity (the maximum) from the 20 scans. SR, Stratum radiatum; SL, stratum lucidum. Scale bars: *A*, 5 μ m; *B*, 10 μ m; *C*, 2 μ m.

single astrocytes were found adjacent to all sides, above and below a given cluster. Furthermore, the projections of these astrocytes are extensive, constituting hundreds of thin, continually branching fibers that envelope numerous BDNF-immunoreactive clusters within the projection field.

Status epilepticus alters the pattern of BDNF immunoreactivity in the mossy fiber pathway

Status epilepticus leads to a dramatic increase of BDNF immunoreactivity in the mossy fiber pathway (Wetmore et al., 1994; Yan et al., 1997; Rudge et al., 1998; Katoh-Semba et al., 1999). Whether BDNF is increased only in mossy fiber boutons already immunoreactive for BDNF or whether previously immunonegative boutons become immunopositive is not known. In the present study, status epilepticus was induced in six GFP-expressing mice with pilocarpine, whereas five littermate controls were treated with saline. Animals were anesthetized and perfused with paraformaldehyde 48 hr after the termination of seizure activity.

Consistent with previous studies, BDNF immunoreactivity was increased in the mossy fiber pathway in mice that underwent status epilepticus (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Also consistent with earlier studies (Shibley and Smith, 2002; Borges et al., 2003), the expected pattern of cell death was observed in these animals. Specifically, Fluoro-jade B staining (Histo-Chem Inc., Jefferson, AR), which labels dead and dying cells, revealed extensive loss of hilar neurons and also loss of a small number of pyramidal cells in CA3a (data not shown). Pyramidal cell loss varies depending on the

Figure 8. Confocal scans from single focal planes showing astrocytic processes enveloping BDNF-immunoreactive clusters. Top row, GFP-expressing astrocytic processes. Middle row, BDNF immunoreactivity in the same fields as shown in the top row. Bottom row, Merged GFP and BDNF images. Note the close apposition but negligible colocalization (shown as blue) between the processes of the astrocytes and the BDNF-immunoreactive clusters. Scale bar, 3 μ m.

severity of seizure activity induced (Borges et al., 2003), and, with our protocol, CA3 pyramidal cell loss was minimal (>75% survival). None of the GFP-labeled pyramidal cells examined in the present study exhibited noticeable pathologies (e.g., dendritic blebbing and retraction). In control mice, immunostaining in the mossy fiber pathway was organized into clusters, with a lower intensity of immunoreactivity between clusters (supplemental Fig. 1, left, available at www.jneurosci.org as supplemental material). These clusters almost certainly represent BDNF-immunoreactive mossy fiber boutons, as described previously (Fig. 3). In status epilepticus-induced mice, the number of immunostained clusters was increased (supplemental Fig. 1, right, available at www.jneurosci.org as supplemental material). In addition, close examination of the material revealed that staining in between obvious clusters was also increased. To ascertain the localization of BDNF immunoreactivity within the distinct terminal specializations of mossy fiber axons as well as alternative locales, BDNF immunoreactivity in GFP-labeled processes was examined.

Figure 9. Left graph, The percentage of BDNF-immunoreactive giant MFBs was significantly increased 48 hr after 3 hr of pilocarpine-induced status epilepticus (SE) relative to saline-treated controls (C). The numbers of BDNF-immunoreactive mossy fiber bouton filipodia and mossy fiber en passant terminals (EPT) were not significantly increased. $**p < 0.01$, rank-sum test. Right graph: The percentage of CA3 pyramidal cell thorny excrescence spine heads with BDNF immunoreactivity adjacent to the spine head was significantly increased after status epilepticus relative to control animals. $*p < 0.05$, rank-sum test.

Status epilepticus increases the number of BDNF-immunoreactive mossy fiber boutons

Examination of GFP-expressing mossy fiber boutons (MFBs) in control mice (87 MFB) and in mice after status epilepticus (75 MFB) revealed that the proportion of labeled boutons was significantly greater after status epilepticus (Fig. 9, left) (control, $47.3 \pm 7.7\%$; status epilepticus, $86.8 \pm 6.8\%$; $p < 0.01$), indicating that status epilepticus led previously BDNF-immunonegative mossy fiber boutons to begin to concentrate BDNF. Similar trends, although nonsignificant, were evident with both mossy fiber bouton filipodia and mossy fiber en passant terminals (Fig. 9, left).

Status epilepticus increases BDNF immunoreactivity surrounding CA3 pyramidal cell thorny excrescences

To determine whether the number of thorny excrescence spine heads found adjacent to BDNF immunoreactivity was increased by seizure activity, 147 spine heads from five saline control mice and 116 spine heads from five mice that underwent status epilepticus were examined. (One animal that underwent status epilepticus was excluded because no GFP-labeled thorny excrescences were found.) The number of spine heads found adjacent to BDNF immunoreactivity was significantly increased after seizure activity (Fig. 9, right) (control, $56.4 \pm 13.8\%$; status epilepticus, $90.6 \pm 6.3\%$; $p < 0.05$). Interestingly, the number of spine heads projecting into BDNF-immunoreactive clusters increased from 4.4% in controls to 38% after status epilepticus. Although this precluded determining whether these enveloped spine heads contained or were merely completely surrounded by BDNF (in these cases the resolving power of the microscope was exceeded), the finding suggests that the exposure of these spines to BDNF may be dramatically increased.

BDNF immunoreactivity is similar in multiple terminals of single granule cells

The present study demonstrates that mossy fiber bouton BDNF content is heterogeneous. Some boutons contained high levels of BDNF, whereas others were devoid of immunoreactivity. To further characterize this heterogeneity in control animals, BDNF immunoreactivity was assessed in mossy fiber boutons belonging to the same axon. Specifically, GFP-expressing mossy fiber axons were examined to find segments of an axon containing two mossy fiber boutons. BDNF immunoreactivity in these mossy fiber bouton "pairs" was then imaged using confocal microscopy. Twenty mossy fiber bouton pairs from CA3a and CA3b were examined.

To facilitate comparing BDNF levels, the intensity of BDNF immunoreactivity in each mossy fiber bouton was quantified using Leica confocal software. Mean signal amplitude (pixel values, 0–255) for each bouton was determined, and subsequent analysis of these data revealed that BDNF immunoreactivity within mossy fiber bouton pairs was highly correlated (Pearson product moment correlation, $r = 0.678$; $p = 0.001$). That is, adjacent mossy fiber boutons along the same axon tended to have similar levels of BDNF immunoreactivity. In contrast, no correlation in BDNF levels was found between mossy fiber boutons in close proximity to each other (within 40 μm) but located along different axons (Pearson product moment correlation, $r = -0.172$; $p = 0.469$; $n = 20$ pairs).

Discussion

The organization of BDNF immunoreactivity in the mossy fiber pathway led us to hypothesize that BDNF was in dentate granule cell mossy fiber boutons. Confocal microscopy and BDNF immunohistochemical analyses of transgenic mice expressing GFP were used to localize BDNF within the mossy fiber pathway. Forty-seven percent of giant mossy fiber boutons exhibited BDNF immunoreactivity. To our surprise, however, 14% of dentate granule cell filopodial and en passant terminals were BDNF-immunoreactive as well, and BDNF immunoreactivity was also detected in CA3 pyramidal cell thorny excrescences and in astrocytes in the mossy fiber pathway. After status epilepticus, the number of BDNF-immunoreactive mossy fiber boutons was increased from 47 to 87%, demonstrating activity regulation of BDNF content within mossy fiber boutons. Similarity in BDNF immunoreactivity in pairs of giant boutons located on the same axon but not on neighboring boutons of distinct axons indicates that the axon to which a bouton belongs affects BDNF content to a greater extent than the local environment of the bouton. These studies demonstrate that mossy fiber BDNF is poised to regulate both direct excitatory and indirect feedforward inhibitory inputs to CA3 pyramidal cells and reveal that neuronal activity increases the pool of BDNF-expressing granule cell presynaptic terminals contacting CA3 pyramidal cells.

Mossy fiber bouton $\rightarrow$ CA3 pyramidal cell synapses

The powerful effects of BDNF on synaptic efficacy together with the high concentration of BDNF in mossy fiber boutons suggest that BDNF may regulate the efficacy of mossy fiber $\rightarrow$ CA3 pyramidal cell synapses. Importantly, BDNF has been implicated in several forms of LTP of the Shaffer collateral $\rightarrow$ CA1 pyramidal cell synapse (Korte et al., 1995; Patterson et al., 1996; Korte et al., 1998); one form, theta burst LTP, putatively involves a presynaptic component of expression and requires BDNF in the CA3 afferents (Zakharenko et al., 2003). LTP of the mossy fiber $\rightarrow$ CA3 synapse is expressed presynaptically (Xiang et al., 1994; Weisskopf et al., 1994; Weisskopf and Nicoll, 1995; Langdon et al., 1995; Maccaferri et al., 1998). The present finding that BDNF is localized to the giant mossy fiber bouton places it in a site poised to regulate the efficacy of mossy fiber synapses onto CA3 pyramidal cells. Whether increased BDNF content of mossy fiber boutons contributes to pyramidal cell hyperexcitability during epileptogenesis (King et al., 1985; Scharfman et al., 2000, 2001) is an open, albeit intriguing, question.

The importance of a signal that could regulate synaptic strength at the granule cell $\rightarrow$ CA3 pyramidal cell synapse is heightened by the unique anatomy and physiology of this region. In particular, input from a single granule cell is capable of firing its target pyramidal cell (Henze et al., 2002). Furthermore, an individual granule cell typically contacts a given CA3 pyramidal

cell with only one mossy fiber bouton (although individual pyramidal cells are innervated by boutons from many granule cells). Changes in the strength of a single granule cell–CA3 pyramidal cell contact, therefore, may profoundly affect the activity of that pyramidal cell. The present data suggest, therefore, that BDNF may be a critical factor mediating synaptic plasticity in a circuit in which changes in the strength of a single bouton can regulate the firing of the target neuron.

Mossy fiber en passant terminal $\rightarrow$ interneuron synapses

Unlike mossy fiber $\rightarrow$ CA3 pyramidal cell synapses, mossy fiber $\rightarrow$ interneuron synapses do not undergo LTP (Maccaferri et al., 1998). What role, then, does BDNF play at filopodial and en passant terminals containing BDNF? One possibility is that BDNF regulates peptide expression by interneurons. Specifically, in BDNF homozygous knock-out mice, expression of neuropeptide Y is decreased (Jones et al., 1994), whereas BDNF infusion increases neuropeptide Y expression in interneurons (Nawa et al., 1994; Reibel et al., 2000; Scharfman et al., 2002). Innervation of hippocampal interneurons (Frotscher, 1985; Frotscher, 1989; Acsády et al., 1998; Scharfman, 1999) by BDNF-containing granule cell terminals provides a mechanism whereby neuropeptide Y expression could be regulated by granule cell-derived BDNF. Consistent with this idea, hippocampal interneurons express mRNA for the BDNF receptor TrkB (Altar et al., 1994; Marty et al., 1996) but appear not to express BDNF (Ernfors et al., 1990; Pascual et al., 1999). Finally, the trend toward increased filopodial and en passant terminal BDNF content after seizures coincides with increased neuropeptide Y expression after seizures (Gall et al., 1990; Vezzani et al., 1996). Neuropeptide Y possesses antiseizure properties (Baraban et al., 1997), so its increased expression after seizures likely reflects a compensatory response.

BDNF protein content in mossy fiber boutons may reflect granule cell activity

Mossy fiber boutons along the same axon tend to have similar levels of BDNF immunoreactivity, whereas no correlation was found between boutons in close proximity to each other but on different axons. The results suggest that the parent axon has a greater impact on the BDNF content of a given bouton than that of the local environment, at least for the time and conditions examined. Although the mechanisms underlying this distribution are unclear, we favor the idea that BDNF content among boutons along the same axon is coordinated by the parent dentate granule cell. Increased activity produces increased mRNA content of granule cell somata, presumably because of increased transcription of the BDNF gene (Zafra et al., 1990, 1992; Wetmore et al., 1994); it seems plausible that high levels of neuronal activity in some granule cells but not others may account for the heterogeneous distribution of BDNF among granule cells in the absence of seizures. Consistent with the idea, studies examining immediate early gene expression, an indicator of neuronal activity levels, reveal heterogeneous expression among granule cells under physiological conditions (Guzowski et al., 1999).

An interesting implication of these data are that they suggest that all of the mossy fiber bouton–CA3 pyramidal cell contacts of an individual granule cell are regulated coordinately by BDNF. Individual granule cells contact 10–15 CA3 pyramidal cells via 10–15 distinct mossy fiber boutons. Our data suggest that each of the boutons of an individual granule cell contains similar levels of BDNF, and these levels are different among neighboring granule cells. Because BDNF almost certainly regulates the efficacy of the synapses formed by these boutons, our data predict that all of the

CA3 pyramidal cell targets of a granule cell are coordinately regulated by BDNF. If true, this provides a mechanism whereby the recent experience or activity of a granule cell determines the expression of BDNF in all of its terminals and, in turn, increases the likelihood that this granule cell and all of its pyramidal cell targets act as a functional unit distinct from neighboring functional units. Whether this is indeed the case will require additional studies.

Significance of BDNF immunoreactivity in astrocytes

Hippocampal astrocytes can express BDNF mRNA *in vitro* (Rudge et al., 1992; Zafra et al., 1992); however, evidence for mRNA expression *in vivo* is lacking (Ernfors et al., 1990; Conner et al., 1997), raising the possibility that BDNF protein detected in astrocytes may be taken up from neuronal processes, perhaps via truncated TrkB receptors (Frisén et al., 1992; Beck et al., 1993; Condorelli et al., 1994; Biffo et al., 1995; Alderson et al., 2000). Consistent with this hypothesis, electron microscopy studies reveal that mossy fiber boutons, shown to contain BDNF in the present study, make contact with astrocytic processes (Blackstad and Kjaerheim, 1961). Furthermore, the present study demonstrates that astrocytes envelop BDNF-immunoreactive clusters (structures likely representing mossy fiber boutons). Neuronal packing density in the hippocampus and, correspondingly, the density of granule cell→CA3 pyramidal cell synapses are extremely high. If BDNF is released from mossy fiber terminals, restricting BDNF availability to the targeted postsynaptic cells is likely to pose a significant problem. The present findings suggest that astrocytes may play an important role in solving this problem.

In summary, the history of dentate granule cell activity may well dictate BDNF protein content in mossy fiber terminals. Under basal conditions, a subset of mossy fiber boutons express BDNF, and after intense granule cell stimulation, most express BDNF. BDNF in mossy fiber terminals, in turn, is positioned to regulate the efficacy of both excitatory and inhibitory synaptic inputs to CA3 pyramidal cells. Once released, access of BDNF to its various postsynaptic targets is likely further regulated by astrocytes. The complex localization provides information critical to optimal design and interpretation of electrophysiological studies aimed at elucidating how BDNF regulates granule cell synaptic control of CA3 pyramidal cells.

In retrospect, that BDNF would regulate excitation and feedforward inhibition of CA3 pyramidal cells fits perfectly with our current understanding of the mossy fiber pathway. Unlike almost all other populations of principle neurons, inhibitory interneurons, rather than excitatory neurons, are the primary targets of dentate granule cells (Acsády et al., 1998). Clearly this feedforward inhibitory circuit is critical to hippocampal function, and, indeed, compromising this circuit may underlie spontaneous seizures (Behr et al., 1998; Lawrence and McBain, 2003), an undeniably catastrophic condition for any circuit designed to perform a useful function. Seizure-induced increases in pyramidal cell excitability, therefore, may be kept partly in check by increased expression of neuropeptide Y by interneurons, and both effects may be mediated by granule cell-derived BDNF.

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