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Synaptic and Extrasynaptic Localization of Brain-Derived Neurotrophic Factor and the Tyrosine Kinase B Receptor in Cultured Hippocampal Neurons

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Abstract

Brain-derived neurotrophic factor (BDNF) regulates synapses, but the distribution of BDNF and its receptor TrkB relative to the location of glutamatergic and γ -aminobutyric acidergic (GABAergic) synapses is presently unknown. Immunocytochemistry was performed in primary hippocampal neuron cultures to determine whether BDNF and TrkB are preferentially localized to excitatory or inhibitory markers at 7, 14, and 21 days in vitro (DIV). Glutamatergic sites were localized with vesicular glutamate transporter type 1 (VGLUT1) as presynaptic marker and the NR1 subunit of the NMDA receptor and the GluR1 subunit of the AMPA receptor as receptor markers. GABAergic sites were labeled with the 65-kDa isoform of glutamic acid decarboxylase (GAD-65) as presynaptic marker and the $\gamma 2$ subunit of the GABA_A receptor as receptor marker. During development, <30% of BDNF punctae and TrkB clusters were localized to glutamatergic and GABAergic markers. Because their rates of colocalization did not change from 7 to 21 DIV, this study details the distribution of BDNF and TrkB at 14 DIV. BDNF was preferentially colocalized with glutamatergic markers VGLUT1 and NR1 (~30% each). TrkB was also relatively highly colocalized with VGLUT1 and NR1 (~20% each) but was additionally highly colocalized with GABAergic markers GAD-65 (~20%) and $\gamma 2$ (~30%). NR1 clusters colocalized with BDNF puncta and TrkB clusters were mostly extrasynaptic, as were $\gamma 2$ clusters colocalized with TrkB clusters. These results show that, whereas most BDNF and TrkB protein is extrasynaptic, BDNF is preferentially associated with excitatory markers and that TrkB is associated equally with excitatory and inhibitory markers.

Indexing terms

neurotrophins; glutamate; GABA; synaptogenesis

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, modulates many aspects of synaptic structure and function. For instance, BDNF modulates axonal and dendritic branching. It increased branching and complexity of optic axon terminal arbors in *Xenopus* (Cohen-Cory and Fraser, 1995) and in chick (Inoue and Sanes, 1997) and increased the length and complexity of apical and basal dendrites in *Xenopus* retina (Lom and Cohen-Cory, 1999) and in mammalian visual cortex (McAllister et al., 1995). BDNF also modulates synaptic efficacy. It acutely potentiated excitatory transmission in the neuromuscular junction (Lohof et al., 1993) and hippocampus (Lessmann et al., 1994; Kang and Schuman, 1995; Levine et al., 1995; Messaoudi et al., 1998; Li et al., 1998), acutely suppressed inhibitory transmission

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in the hippocampus (Tanaka et al., 1997; Frerking et al., 1998; Brunig et al., 2001; Cheng and Yeh, 2003), and elicited action potentials in the hippocampus, cortex, and cerebellum by activating the tetrodotoxin-insensitive sodium channel Na_v1.9 (Kafitz et al., 1999; Blum et al., 2002). Furthermore, BDNF influences synaptic maturation. It increased the density of both excitatory and inhibitory synapses in the hippocampus (Vicario-Abejon et al., 1998; Martinez et al., 1998) and promoted activity-dependent inhibitory synaptogenesis (Seil and Drake-Baumann, 2000).

Despite its established role in synaptic development, the synaptic localization of BDNF is not fully characterized. BDNF is a ligand for the TrkB receptor, a high-affinity tyrosine kinase. Both BDNF and TrkB mRNA and protein are widely distributed throughout the brain, including the cerebral cortex, hippocampus, dentate gyrus, striatum, septal nuclei, substantia nigra, cerebellar Purkinje cells, brainstem, and spinal motor neurons (Zhou et al., 1993; Friedman et al., 1998). Particularly strong labeling is present in the hippocampus (Hofer et al., 1990; Klein et al., 1990a; Lamballe et al., 1991; Gall and Lauterborn, 1992; Fryer et al., 1996). Immunocytochemistry and in situ hybridization showed that BDNF and TrkB are present in hippocampal axons and dendrites (Conner et al., 1997; Tongiorgi et al., 1997; Yan et al., 1997), and electron microscopy demonstrated that BDNF and TrkB are present in hippocampal dendritic spines and at postsynaptic densities representative of both excitatory and inhibitory synapses (Drake et al., 1999; Aoki et al., 2000). However, these studies did not address the relative distribution of BDNF and TrkB to synapse type.

This study investigated the synaptic and extrasynaptic localization of BDNF and TrkB in hippocampal pyramidal neurons during in vitro development. To determine the distribution of BDNF and TrkB to glutamatergic and γ -aminobutyric acidergic (GABAergic) sites, double-label and triple-label immunocytochemistry was performed on rat primary hippocampal neuron cultures, a method previously used to investigate synaptic development (Rao and Craig, 1997; Hering et al., 2003). First, the expression of BDNF and TrkB was characterized from 1 to 21 days in vitro (DIV). Then, the distribution of BDNF and TrkB to glutamatergic and GABAergic markers was analyzed at 7, 14, and 21 DIV. Glutamatergic sites were visualized by using the vesicular glutamate transporter type 1 (VGLUT1) as a presynaptic marker and the NR1 subunit of the NMDA receptor and the GluR1 subunit of the AMPA receptor as receptor markers. GABAergic sites were labeled by using the 65-kDa isoform of glutamic acid decarboxylase (GAD-65) as a presynaptic marker and the γ 2 subunit of the GABA_A receptor as a receptor marker. Because receptor markers label both synaptic and extra-synaptic receptors, triple labeling of BDNF or TrkB with both presynaptic and receptor markers was also performed.

MATERIALS AND METHODS

Cell culture

Neuronal hippocampal/glial cocultures were prepared from 18-day embryonic rats as described by Goslin et al. (1998). Glial cell cultures were prepared 10 days prior to coculturing with hippocampal neurons. In a laminar flow hood, neonatal Sprague-Dawley rat pups were decapitated after being placed on ice for 2–3 minutes. Hippocampal neuron cultures were prepared later when E18 Sprague-Dawley rat fetuses were removed from the pregnant mother after she had been anesthetized with halothane. Fetuses were then decapitated and brains removed. These methods were approved by the University of Virginia Animal Care and Use Committee and conform to NIH guidelines. Ten thousand cells were added to each of the dishes containing polylysine-coated coverslips and transferred to 35-mm dishes containing glial cell monolayers in 3 ml serum-free MEM with N2 supplement. N2 supplement (1 ml) was added every 10 days. The neurons were fixed 1–21 days after plating and used for immunocytochemistry.

Immunocytochemistry

Coverslips of neurons were fixed with 4% paraformaldehyde (PFA)/4% sucrose in phosphate-buffered saline (PBS; pH 7.1) for 20 minutes at room temperature, permeabilized with 0.10% Triton X-100 in PBS for 15 minutes at room temperature, blocked with 4% normal goat serum for 15 minutes at room temperature, and incubated with primary antibodies overnight at 4°C. Coverslips were washed with 1× PBS on the following day and then incubated overnight at 4°C with a second primary antibody. If a triple label was being performed, then, on the third day, the coverslips were again washed with 1× PBS and incubated overnight at 4°C. On the final day, the coverslips were washed with 1× PBS and then incubated with appropriate fluorochrome-conjugated secondary antibodies for 45 minutes on a shaker at room temperature in darkness. Coverslips were mounted in Gel/Mount (Biomedica Corp, Foster City, CA), the edge of each coverslip was sealed with clear nail polish, and slides were stored at −20°C.

To colabel with primary antibodies raised from the same species, neurons were incubated with the first primary antibody and labeled with secondary antibody the next day. Neurons were washed with PBS and blocked with an extremely high concentration of unlabeled secondary antibody (60 µg/ml) for 2 hours at room temperature, to block all sites previously labeled by that species. Neurons were then immediately incubated with the second primary antibody overnight at 4°C and labeled with a secondary antibody conjugated with a different fluorochrome on the next day.

Antibodies

Primary antibodies consisted of a sheep polyclonal antibody to BDNF (5 µg/ml; Chemicon International, Temecula, CA) and a mouse monoclonal antibody to TrkB (1 µg/ml; BD Transduction Laboratories, Lexington KY) used in combination with primary antibodies, including the presynaptic marker VGLUT1, the NR1 subunit of the NMDA receptor, the GluR1 subunit of the AMPA receptor, the presynaptic marker GAD-65, and the γ2 subunit of the GABA_A receptor. The antibody against BDNF has been characterized by Western blotting (Katoh-Semba et al., 1997) and immunohistochemistry (Zhou et al., 1994). The antibody against TrkB has been characterized by Western blotting (Jovanovic et al., 2000; Muller et al., 2000; Du et al., 2000). The guinea pig polyclonal antibody against VGLUT1 (1:5,000; Chemicon International) has been characterized by immunohistochemistry (Ni et al., 1994, 1995; Fremeau et al., 2001). The mouse monoclonal antibody against GAD-65 (1 µg/ml; Boehringer Mannheim, Germany) has been characterized by immunohistochemistry and immunoprecipitation (Gottlieb et al., 1986). The mouse monoclonal antibody to NR1 (2 µg/ml; BD PharMingen, San Diego, CA) has been characterized by immunocytochemistry (Brose et al., 1994; Siegel et al., 1994) and immunoprecipitation (Brose et al., 1994). The rabbit polyclonal antibody to GluR1 (1 µg/ml; Chemicon International) has been characterized by immunocytochemistry (Wenthold et al., 1992), immunoprecipitation (Hayashi et al., 1997), and Western blotting (Sutton et al., 2003). The rabbit antibody to γ2 raised against amino acids 319–366 of the subunit (2 µg/ml; gift from Werner Sieghart, Brain Research Institute, Vienna, Austria) has been characterized by immunocytochemistry (Sperk et al., 1997).

Secondary antibodies included Alexa 350, Alexa 488, and Alexa 594 fluorochromes conjugated with goat anti-sheep, goat anti-mouse, goat anti-rabbit, or goat anti-guinea pig IgG (4 µg/ml; Molecular Probes, Eugene, OR). All primary and secondary antibodies were diluted in 0.1 M PBS (pH 7.4) containing 2% normal goat serum.

Image acquisition and analysis

Image acquisition—Fluorescent images of cells were captured with a Roper Scientific Photometrics Cool-SNAPcf CCD camera mounted on a Nikon Eclipse TE200 fluorescent microscope (Japan) with either a 40 × 1.3 NA lens or 60 × 1.4 NA lens driven by Metamorph

imaging software (Universal Imaging Corporation, Downingtown, PA). For double labeling, high-resolution digital images were acquired of red and green fluorochromes with a $\times 60$ objective. For triple labeling, high-resolution digital images were acquired of red, green, and blue fluorochromes with a $\times 40$ objective.

Definition of punctae/clusters—In Metamorph, the brightness and contrast of fluorescent images were thresholded so that punctate fluorescence was two times higher than diffuse background labeling. The number of punctae was then measured. A puncta was defined as an aggregation of 2–1,000 pixels, corresponding to 0.08–41.67 μm diameter. Because of intense nonspecific binding in the cell body, only punctae on processes were quantitated for all neurons. Numbers of BDNF and TrkB punctae were quantified per field after a single neuron was centered in the field.

Analysis of colocalization—In Metamorph, a binary image was created from each thresholded image. Binary images were then added together to display overlapping punctae. Punctae were counted, and percentage colocalization was calculated as the number of colocalized punctae divided by the total number of BDNF or TrkB clusters. Data were analyzed in GraphPad Prism 4.0 (San Diego, CA). Significant differences between two groups were determined with an unpaired Student's *t*-test, and significant differences among multiple groups were determined with an ANOVA, followed by the Tukey's multiple-comparisons test. Differences were considered significant at $P < 0.05$. All values are reported as mean $\pm$ SEM, and *n* = number of neurons analyzed.

Photomicrograph production—As mentioned above, the brightness and contrast of fluorescent images were adjusted in Metamorph so that punctate fluorescence was two times higher than diffuse background labeling. Images were then saved as eight-bit TIFF files and opened in Adobe Photoshop 6.0 (San Jose, CA), in which overall brightness was increased for final production.

RESULTS

Development of BDNF and TrkB was studied in hippocampal pyramidal neurons by using primary hippocampal neuron cultures. Approximately 80–90% of neurons in these cultures were pyramidal and could be morphologically identified at 7–21 DIV (Benson et al., 1994). Neurons quantified at 1 DIV could only be assumed to be pyramidal based on morphology. Punctate labeling of BDNF and TrkB could be seen as early as 1 DIV. Approximately 80% of pyramidal neurons expressed BDNF punctae and TrkB clusters at all developmental time points.

Expression of BDNF in developing neurons

BDNF was distributed to the cell soma and neuronal processes from 1 to 21 DIV. BDNF in the soma appeared diffuse, whereas BDNF in neuronal processes appeared as punctate vesicular structures. BDNF punctae expressed in neuronal processes were examined from 1 to 21 DIV (Fig. 1). BDNF punctae were present on both axons and dendrites (data not shown). BDNF distribution in neuronal processes shifted from punctae interspersed with diffuse labeling at 1 DIV (Fig. 1A) to discrete punctae with little diffuse labeling at 7–21 DIV (Fig. 1B–D). Number of BDNF punctae in neuronal processes significantly increased during development, from 6.3 ± 0.9 per field at 1 DIV (*n* = 24 neurons) to 41.9 ± 15.4 per field at 7 DIV (*n* = 12 neurons), 50.1 ± 12.4 per field at 14 DIV (*n* = 23 neurons), and 117.5 ± 19.9 per field at 21 DIV (*n* = 6 neurons; $P < 0.01$; Fig. 1E). This increase in BDNF expression is due at least partially to neuron growth, insofar as it corresponded to increased development of processes. The number of neuronal processes significantly rose from 7.0 ± 0.5 per field at 1

DIV to 15.0 ± 1.0 per field at 7 DIV, 23.8 ± 1.1 per field at 14 DIV, and 31.3 ± 2.6 per field at 21 DIV ($P < 0.0001$; data not shown). However, size of BDNF punctae remained relatively constant, ranging from a diameter of $0.97 \pm 0.1 \mu\text{m}$ at 1 DIV to $1.5 \pm 0.2 \mu\text{m}$ at 7 DIV, $1.2 \pm 0.1 \mu\text{m}$ at 14 DIV, and $1.05 \pm 0.2 \mu\text{m}$ at 21 DIV (Fig. 1F).

Expression of TrkB in developing neurons

TrkB clusters were also distributed throughout the cell soma and neuronal processes from 1 to 21 DIV. TrkB expression in the soma appeared diffuse, but TrkB receptors in neuronal processes were clustered. TrkB clusters in neuronal processes were analyzed from 1 to 21 DIV (Fig. 2). Both axons and dendrites expressed TrkB clusters (data not shown). In contrast to the case for BDNF, TrkB clusters appeared as discrete punctae abundant throughout the processes as early as 1 DIV (Fig. 2A), and this distribution did not change from 7 to 21 DIV (Fig. 2B–D). Number of TrkB clusters in neuronal processes significantly increased from 37.7 ± 7.4 per field at 1 DIV ($n = 13$ neurons) to 137.7 ± 13.8 per field at 7 DIV ($n = 6$ neurons), 379.9 ± 62.3 per field at 14 DIV ($n = 9$ neurons; $P < 0.001$ vs. 1 and 7 DIV), and 482.8 ± 36.4 per field at 21 DIV ($n = 6$ neurons; $P < 0.001$ vs. 1, 7, and 14 DIV; Fig. 2E). This increase in TrkB cluster expression may also be due at least partially to a developmental increase in the number of processes, similarly to the case for BDNF. Size of TrkB clusters did not significantly change from 1 to 21 DIV, ranging from a diameter of $1.2 \pm 0.1 \mu\text{m}$ at 1 DIV to $0.89 \pm 0.1 \mu\text{m}$ at 7 DIV, $0.85 \pm 0.1 \mu\text{m}$ at 14 DIV, and $0.81 \pm 0.1 \mu\text{m}$ at 21 DIV (Fig. 2F).

Colocalization of BDNF with TrkB

It has been reported that BDNF moves rapidly throughout the cell (Kohara et al., 2001), suggesting that it is likely to be captured in transit rather than at its site of action. Therefore, before the synaptic distribution of BDNF and TrkB was analyzed, a reference level of colocalization of BDNF with its high-affinity receptor TrkB was measured (Fig. 3). At 14 DIV, the density of BDNF punctae per neuronal process appeared lower than the density of TrkB clusters (Fig. 3A,B). Moreover, when images of BDNF punctae and TrkB clusters were merged, few overlapping regions were evident (Fig. 3C). When quantified, $24.0\% \pm 6.4\%$ of BDNF punctae were colocalized with TrkB punctae ($n = 3$ neurons), and $6.1\% \pm 0.8\%$ of TrkB punctae were colocalized with BDNF punctae. This low rate shows that, despite the important role of BDNF at TrkB receptors, BDNF and TrkB are not often localized together.

Colocalization of BDNF and TrkB with glutamatergic and GABAergic markers

Synaptic and extrasynaptic distributions of BDNF and TrkB were examined during development of cultured hippocampal neurons. VGLUT1 was visualized as a presynaptic glutamatergic marker, whereas NR1, a common subunit of the NMDA receptor, and GluR1, a well-characterized subunit of the AMPA receptor, were used as glutamatergic receptor markers. Similarly, GAD-65, the synthetic enzyme for GABA, was visualized as a presynaptic GABAergic marker, and the $\gamma 2$ subunit of the GABA_A receptor, a subunit required for synaptic targeting of the GABA_A receptor (Essrich et al., 1998), was used as a GABAergic receptor marker. After conducting double-label or triple-label immunocytochemistry, we merged images and quantified the percentages of BDNF or TrkB punctae colocalized with glutamatergic and GABAergic markers. Because NMDA, AMPA, and GABA_A receptors may be located at extrasynaptic sites, the colocalization of BDNF to presynaptic markers was analyzed separately from receptor markers.

Colocalization of BDNF and TrkB to presynaptic and receptor markers was analyzed from 7 to 21 DIV. The rate of colocalization remained relatively constant during in vitro development (Tables 1, 2). Therefore, the discussion of results will focus on the synaptic localization of BDNF and TrkB at 14 DIV, a time at which the neurons are relatively mature.

BDNF—First, distribution of BDNF to presynaptic and receptor markers was analyzed (Fig. 4). BDNF punctae appeared as previously described (Fig. 4A,D,G,J,M). The excitatory presynaptic marker VGLUT1 appeared as discrete, large punctae (Fig. 4B), whereas the NMDA receptor marker NR1 labeled large amounts of discrete, smaller clusters (Fig. 4E), and the AMPA receptor marker GluR1 appeared as varying sizes of discrete clusters, most of which were fairly large (Fig. 4H). The inhibitory presynaptic marker GAD-65 also appeared as discrete, large punctae (Fig. 4K), and the GABA_A receptor marker $\gamma 2$ labeled discrete, round clusters of varying sizes, mostly large (Fig. 4N). Overall, presynaptic markers appeared as smooth-lined punctae relatively constant in size, whereas receptor markers labeled clusters of various sizes that appeared rounder and rougher in outline than presynaptic markers. Receptor markers also seemed to be present at higher density than presynaptic markers. This is most likely because receptor markers label both synaptic and extrasynaptic receptors.

Images of BDNF with VGLUT1 (Fig. 4C), NR1 (Fig. 4F), GluR1 (Fig. 4I), GAD-65 (Fig. 4L), or $\gamma 2$ (Fig. 4O) were merged to quantify colocalization. When presynaptic markers were analyzed, $29.9\% \pm 4.6\%$ of BDNF punctae were colocalized with VGLUT1 punctae ($n = 8$ neurons), and $7.6\% \pm 2.4\%$ of BDNF punctae were colocalized with GAD-65 punctae ($n = 9$ neurons; $P < 0.001$; Fig. 4P). This indicates that presynaptically BDNF is more frequently colocalized with VGLUT1. When receptor markers NR1, GluR1, and $\gamma 2$ were examined, $27.2\% \pm 3.1\%$ of BDNF punctae were colocalized with NR1 clusters ($n = 6$ neurons; $P < 0.05$ vs. GluR1 and $\gamma 2$), $14.4\% \pm 3.2\%$ with GluR1 clusters ($n = 6$ neurons); and $18.2\% \pm 2.4\%$ with $\gamma 2$ clusters ($n = 6$ neurons; Fig. 4Q). This shows that postsynaptically BDNF is most often colocalized with NR1. Overall, these results suggest that, although BDNF is colocalized with all marker types, it is preferentially colocalized with glutamatergic presynaptic and receptor markers. Moreover, BDNF was more likely to be colocalized with NMDA receptors than with AMPA receptors. To clarify the rate of colocalization of NMDA and AMPA receptors, double labeling of NR1 and GluR1 was performed. At 14 DIV, $11.2\% \pm 2.5\%$ of NR1 clusters were colocalized with GluR1 clusters, and $16.5\% \pm 3.4\%$ of GluR1 clusters were colocalized with NR1 clusters ($n = 5$ neurons; data not shown). Similar low colocalization levels of NMDA and AMPA receptor clusters have also been found in cultured hippocampal neurons in studies from other laboratories (Rao et al., 1998).

NR1 labels both synaptic and extrasynaptic NMDA receptors. To determine what fraction of NMDA receptor clusters colocalized with BDNF punctae was synaptic, triple labeling of BDNF, NR1, and VGLUT1 was performed (Fig. 5). VGLUT1 (Fig. 5A), NR1 (Fig. 5B), and BDNF (Fig. 5C) appeared as previously described. BDNF punctae were much less densely dispersed than VGLUT1 or NR1. All three images were then merged (Fig. 5D) to analyze colocalization. At 14 DIV, $8.2\% \pm 5.6\%$ of BDNF punctae were colocalized with both NR1 clusters and VGLUT1 punctae ($n = 5$ neurons). This percentage is approximately 30% of the amount of colocalization of BDNF punctae with VGLUT1 punctae ($29.9\% \pm 4.6\%$) and NR1 clusters ($27.2\% \pm 3.1\%$) individually. This suggests that, at 14 DIV, most NMDA receptor clusters colocalized with BDNF punctae are not also colocalized with VGLUT1 punctae and are therefore extrasynaptic.

TrkB—The distribution of TrkB to presynaptic and receptor markers was examined next (Fig. 6). TrkB clusters appeared as previously described (Fig. 6A,D,G,J,M). Excitatory markers VGLUT1 (Fig. 6B), NR1 (Fig. 6E), and GluR1 (Fig. 6H) and inhibitory markers GAD-65 (Fig. 6K) and $\gamma 2$ (Fig. 6N) also appeared as previously described. Images of TrkB with VGLUT1 (Fig. 6C), NR1 (Fig. 6F), GluR1 (Fig. 6I), GAD-65 (Fig. 6L), or $\gamma 2$ (Fig. 6O) were merged to quantify colocalization. Presynaptically there was no significant difference in the rate of colocalization of TrkB to glutamatergic and GABAergic markers, in that $23.2\% \pm 5.2\%$ of TrkB clusters were colocalized with VGLUT1 punctae ($n = 8$ neurons) and $20.4\% \pm 3.2\%$ of TrkB clusters were colocalized with GAD-65 punctae ($n = 9$ neurons; Fig. 6P). However, when

receptor markers were analyzed, $23.2\% \pm 1.8\%$ of TrkB clusters were colocalized with NR1 clusters ($n = 5$ neurons; $P < 0.05$ vs. GluR1), $3.8\% \pm 0.8\%$ with GluR1 clusters ($n = 4$ neurons), and $34.5\% \pm 6.6\%$ with $\gamma 2$ ($n = 9$ neurons; $P < 0.01$ vs. GluR1; Fig. 6Q). Therefore, postsynaptically TrkB clusters are preferentially colocalized with NR1 and $\gamma 2$ subunit clusters. These results suggest that, as with BDNF, TrkB is relatively highly colocalized with presynaptic glutamatergic markers and NMDA receptors. However, unlike BDNF, TrkB is also relatively highly colocalized with presynaptic GABAergic markers and GABA_A receptors.

Receptor markers NR1 and $\gamma 2$ label both synaptic and extrasynaptic NMDA receptors and GABA_A receptors. To determine the synaptic fraction of NMDA receptors colocalized with TrkB clusters, triple labeling of TrkB, NR1, and VGLUT1 was performed (Fig. 7). VGLUT1 (Fig. 7A), TrkB (Fig. 7B), and NR1 (Fig. 7C) appeared as previously described. All three images were then merged (Fig. 7D) to analyze colocalization. At 14 DIV, $7.2\% \pm 2.5\%$ of TrkB clusters were colocalized with both NR1 clusters and VGLUT1 punctae ($n = 5$ neurons). This percentage is approximately 50% of the amount of colocalization of TrkB clusters to VGLUT1 punctae ($13.8\% \pm 4.2\%$) and approximately 30% of the amount of colocalization to NR1 clusters ($23.2\% \pm 1.8\%$). This suggests that, at 14 DIV, most NMDA receptor clusters colocalized with TrkB punctae are not also colocalized with VGLUT1 punctae and are therefore extrasynaptic.

Similarly, to determine what percentage of GABA_A receptor clusters colocalized with TrkB punctae was synaptic, triple labeling of TrkB, $\gamma 2$, and GAD-65 was performed (Fig. 8). GAD-65 (Fig. 8A), TrkB (Fig. 8B), and $\gamma 2$ (Fig. 8C) appeared as previously described. All three images were then merged (Fig. 8D) to analyze colocalization. At 14 DIV, $4.3\% \pm 1.3\%$ of colocalized TrkB punctae were colocalized with both $\gamma 2$ clusters and GAD-65 punctae ($n = 5$ neurons). This percentage is approximately 20% of the colocalization of TrkB to GAD-65 ($20.4\% \pm 3.2\%$) and approximately 12% of the colocalization of TrkB to $\gamma 2$ ($34.5\% \pm 6.6\%$). This suggests that, at 14 DIV, most GABA_A receptor clusters colocalized with TrkB punctae are not also colocalized with GAD-65 punctae and, therefore, like NMDA receptor clusters, are extrasynaptic.

DISCUSSION

This study characterizes the synaptic and extrasynaptic localization of BDNF and TrkB in hippocampal pyramidal neurons. The primary conclusions are that, during in vitro development of pyramidal neurons, 1) presynaptically, BDNF is preferentially localized to glutamatergic terminals, whereas TrkB is equally localized with both glutamatergic and GABAergic terminals; 2) BDNF is more often colocalized with NMDA receptors than AMPA or GABA_A receptors, and most of these NMDA receptors are extrasynaptic; 3) TrkB is more often localized with NMDA and GABA_A receptors than AMPA receptors, and most of these NMDA and GABA_A receptors are extrasynaptic.

Previous studies have established that BDNF and TrkB can be present at both excitatory and inhibitory synapses, but this study extends analysis to measure the relative distribution of BDNF and TrkB to these synapses as well as extrasynaptic excitatory and inhibitory sites. Electron microscopy has been used to show that BDNF and TrkB are present at postsynaptic densities representative of both excitatory and inhibitory synapses (Wu et al., 1996; Drake et al., 1999; Aoki et al., 2000), but this technique could be performed on only a limited number of synapses. Immunocytochemistry, as used in this study, provided the advantage of quantifying a vast number of synapses ($>10,000$).

Even though BDNF and TrkB have been shown to modulate synapses, the percentage of immunoreactive BDNF punctae and TrkB clusters colocalized with excitatory and inhibitory synapses was low ($\leq 30\%$). However, Kohara et al. (2001) used real-time visualization of green fluorescent protein (GFP)-tagged BDNF to demonstrate that BDNF moves rapidly throughout the neuron, suggesting that most immunoreactive BDNF punctae represent molecules in transit. In confirmation, the present study shows that even the percentage of BDNF colocalized with its high-affinity receptor TrkB was low ($\sim 25\%$), suggesting that immunocytochemistry is unlikely to capture BDNF at its site of action. Perhaps immunoreactive punctae of BDNF and TrkB represent internalized proteins in the process of anterograde or retrograde transport. In addition, the low level of colocalization of TrkB with synapses may be due to its signaling mechanisms. The TrkB receptor activates numerous second messenger signaling cascades, such as protein kinase C (PKC), that can act as diffusible messengers to the synapse. Therefore, despite the reported TrkB modulation of synapses, TrkB does not necessarily have to be colocalized with glutamatergic or GABAergic synapses.

The relatively high localization of BDNF and TrkB to glutamatergic markers was expected, given that BDNF modulates glutamatergic synapses via the TrkB receptor (for review see Poo, 2001). BDNF acutely enhanced glutamatergic synaptic transmission in the hippocampus via the TrkB receptor (Lessmann et al., 1994; Kang and Schuman, 1995; Levine et al., 1995; Messaoudi et al., 1998). Also, BDNF is required for long-term potentiation (LTP) in hippocampal neurons by acting on the TrkB receptor, in that chronic BDNF results in long-term enhancement of central synaptic transmission (Sherwood and Lo, 1999), and virus-mediated transfer of the BDNF gene restored LTP in the hippocampus of BDNF mutant mice (Korte et al., 1996).

BDNF has also been reported to modulate GABAergic synapses via the TrkB receptor (Tanaka et al., 1997; Frerking et al., 1998; Brunig et al., 2001; Cheng and Yeh, 2003), making the preferential association of TrkB but not BDNF to GABAergic markers unexpected. Some BDNF punctae are still present at GABAergic markers to activate TrkB at these sites. The most likely explanation is that the transmembrane protein TrkB remains stationary at sites of action, whereas, as mentioned previously, most BDNF is moving rapidly throughout the cell.

A higher amount of BDNF may be found at excitatory presynaptic terminals and NMDA receptors because it is synthesized there. NMDA receptor-mediated calcium influx is tightly linked to the induction of BDNF, insofar as the BDNF promoter is regulated by two calcium response elements via two transcription factors: CREB and CaRF (Shieh and Ghosh, 1999; Tao et al., 2002). NMDA-induced synthesis could be local or could occur in the cell body, whereupon newly synthesized BDNF would be targeted to NMDA receptors upon activation (Tongiorgi et al., 1997). It should be noted that the majority of NMDA receptor clusters colocalized with BDNF punctae were extrasynaptic. However, extrasynaptic receptors still retain spontaneous activity, which might be responsible for the induction of BDNF expression.

It is interesting that BDNF is preferentially localized to NMDA receptors and not AMPA receptors. NMDA receptors require depolarization to remove their Mg^{2+} block and allow them to function, and AMPA receptors are often the source of this depolarization. Analysis of glutamatergic receptor markers in these neurons confirmed that a low percentage of NMDA receptor clusters was colocalized with AMPA receptor clusters. This suggests that NMDA receptors in these neurons are inactive. However, electrophysiological recordings previously performed on these cultured neurons showed that NMDA receptors are functional at 14 DIV (Mangan and Kapur, 2004). Therefore, low amounts of AMPA receptor protein, which label diffusely instead of as clusters, might be present with NMDA receptors. Alternatively, AMPA receptor trafficking is very dynamic (for review see Brecht and Nicoll, 2003), so therefore AMPA receptors may rapidly translocate to NMDA receptors upon receiving some cellular

signal, such as depolarization. However, other explanations exist for the high level of colocalization of BDNF to presynaptic glutamatergic terminals and NMDA receptors. BDNF might be docked at presynaptic sites, ready for release into the synaptic cleft. Alternatively, BDNF might have been recently endocytosed, presynaptically or at NMDA receptors, to affect the growth and survival of the neuron.

TrkB was found at NMDA, AMPA, and GABA_A receptors. However, TrkB was more often localized with NMDA and GABA_A receptors, suggesting additional important biological activity at these receptors. Indeed, TrkB receptor signaling has been shown to modulate NMDA and GABA_A receptors in various ways. As previously discussed, TrkB signaling enhances glutamatergic synaptic transmission (Lessmann et al., 1994; Kang and Schuman, 1995; Levine et al., 1995; Messaoudi et al., 1998) and is even required for LTP. TrkB signaling also affects GABA_A receptors in many ways, such as interneuron migration in the developing cerebral cortex (Polleux et al., 2002), formation of GABAergic synapses in the cerebellum (Seil and Drake-Baumann, 2000; Rico et al., 2002), and synaptic protein distribution in the hippocampus (Pozzo-Miller et al., 1999). However, it is possible that TrkB has additional functions at NMDA and GABA_A receptors. Elmariah and colleagues (2004) showed that TrkB receptor signaling can modulate clustering of NMDA and GABA_A receptors. Perhaps a higher percentage of TrkB is colocalized with NMDA and GABA_A receptors because it regulates assembly of those receptors. Future experiments should explore the role of TrkB at NMDA and GABA_A receptors.

One unexplored question is the synaptic localization of truncated TrkB receptors. Alternative splicing of TrkB mRNA produces at least three different TrkB receptor isoforms with different signaling capabilities, the full-length receptor and two truncated forms, TrkBT1 and TrkBT2 (Klein et al., 1990b). The truncated isoforms contain normal extracellular ligand-binding domains but lack the catalytic tyrosine kinase domain. Our antibody did not distinguish between full-length and truncated TrkB isoforms, so we do not know the type of TrkB receptors that was highly colocalized to GABAergic synapses. Future studies should focus on distinguishing the synaptic localization of truncated TrkB receptors vs. full-length TrkB receptors.

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Fig. 1. Development of BDNF expression from 1 to 21 DIV. BDNF punctae at 1 DIV (A), 7 DIV (B), 14 DIV (C), and 21 DIV (D) and quantitation of BDNF cluster number per field from 1 to 21 DIV (E) and quantitation of BDNF cluster size from 1 to 21 DIV (F). Number of BDNF punctae per field significantly increased (* $P < 0.05$), but size of BDNF punctae remained relatively constant. Scale bars = 10 μm .

Fig. 2. Development of TrkB expression from 1 to 21 DIV. TrkB clusters at 1 DIV (**A**), 7 DIV (**B**), 14 DIV (**C**), and 21 DIV (**D**) and quantitation of TrkB cluster number per field from 1 to 21 DIV (**E**) and quantitation of TrkB cluster size from 1 to 21 DIV (**F**). Number of TrkB clusters per field significantly increased (* $P < 0.05$), but size of TrkB clusters remained relatively constant. Scale bars = 10 μm .

Fig. 3. Colocalization of BDNF and TrkB. BDNF (**A**), TrkB (**B**), and merged image of BDNF (green) and TrkB (red; **C**), BDNF and TrkB have relatively low levels of colocalization. Arrows mark examples of colocalization. Arrowheads mark examples of noncolocalization. Scale = 1 μ m.

Fig. 4.

Colocalization of BDNF with synaptic markers at 14 DIV. BDNF (A), VGLUT1 (B), merged image of BDNF (green) and VGLUT1 (red; C); BDNF (D), NR1 (E), merged image of BDNF (green) and NR1 (red; F); BDNF (G), GluR1 (H), merged image of BDNF (green) and GluR1 (red; I); BDNF (J), GAD-65 (K), merged image of BDNF (green) and GAD-65 (red; L); BDNF (M), $\gamma 2$ (N), merged image of BDNF (green) and $\gamma 2$ (red; O); percentage of BDNF colocalized with presynaptic markers (P); and percentage of BDNF colocalized with postsynaptic markers (Q). BDNF was significantly more colocalized with VGLUT1 than GAD-65 and with NR1 than GluR1 or $\gamma 2$ (* $P < 0.05$). Arrows mark examples of colocalization. Arrowheads mark examples of noncolocalization. Scale bars = 10 μ m.

Fig. 5. Triple labeling of BDNF, NR1, and VGLUT1 at 14 DIV. VGLUT1 (**A**), NR1 (**B**), BDNF (**C**), and merged image of BDNF (blue) + NR1 (green) + VGLUT1 (red; **D**), BDNF was rarely colocalized with both NR1 and VGLUT1. Arrows mark examples of colocalized BDNF puncta and NR1 cluster. Arrowheads mark examples of VGLUT puncta not colocalized with BDNF puncta and NR1 clusters. Scale bar = 1 μ m.

Fig. 6. Colocalization of TrkB with synaptic markers at 14 DIV. TrkB (A), VGLUT1 (B), merged image of TrkB (green) and VGLUT1 (red; C); TrkB (D), NR1 (E), merged image of TrkB (green) and NR1 (red; F); TrkB (G), GluR1 (H), merged image of TrkB (green) and GluR1 (red; I); TrkB (J), GAD-65 (K), merged image of TrkB (green) and GAD-65 (red; L); TrkB (M), $\gamma 2$ (N), merged image of TrkB (green) and $\gamma 2$ (red; O); percentage of TrkB colocalized with presynaptic markers (P); and percentage of TrkB colocalized with postsynaptic markers (Q). TrkB was approximately equally colocalized with VGLUT1 and GAD-65 but was significantly more colocalized with $\gamma 2$ than with NR1 or GluR1 ($*P < 0.05$). Arrows mark examples of colocalization. Arrowheads mark examples of noncolocalization. Scale bars = 10 μm .

Fig. 7. Triple labeling of TrkB, NR1, and VGLUT1. VGLUT1 (**A**); TrkB (**B**); NR1 (**C**) and merged image of VGLUT1 (red), TrkB (green), and NR1 (blue; **D**); TrkB was rarely colocalized with both VGLUT1 and NR1. Arrows mark an example of colocalized TrkB and NR1 clusters. Arrowheads mark an example of VGLUT1 puncta not colocalized with TrkB and NR1 clusters. Scale bar = 1 μ m.

Fig. 8. Triple labeling of TrkB, $\gamma 2$, and GAD-65. GAD-65 (**A**); TrkB (**B**); $\gamma 2$ (**C**) and merged image of GAD-65 (red), TrkB (green), and $\gamma 2$ (blue; **D**). TrkB was rarely colocalized with both GAD-65 and $\gamma 2$. Arrowheads mark examples of noncolocalization. Scale bar = 1 μ m.

TABLE 1

Percentage of BDNF Colocalized With Synaptic Markers During Development

	7 DIV	14 DIV	21 DIV
VGLUT1	31.00 ± 4.3 (n = 14)	29.94 ± 4.6 (n = 8)	24.58 ± 4.1 (n = 8)
GAD-65	9.06 ± 3.1 (n = 9)	7.64 ± 2.4 (n = 9)	4.29 ± 2.3 (n = 3)
NR1	22.51 ± 2.1 (n = 6)	27.21 ± 3.1 (n = 6)	36.32 ± 4.5 (n = 6)
GluR1	22.48 ± 3.5 (n = 4)	14.37 ± 3.2 (n = 6)	20.50 ± 3.6 (n = 7)
γ2	20.85 ± 5.9 (n = 3)	18.25 ± 2.4 (n = 6)	15.58 ± 2.3 (n = 6)

TABLE 2

Percentage of TrkB Colocalized With Synaptic Markers During Development

	7 DIV	14 DIV	21 DIV
VGLUT1	13.44 ± 4.8 (n = 6)	13.78 ± 4.2 (n = 8)	32.70 ± 5.1 (n = 3)
GAD-65	36.09 ± 7.2 (n = 6)	20.42 ± 3.2 (n = 9)	31.03 ± 4.1 (n = 6)
NR1	33.74 ± 3.3 (n = 5)	23.18 ± 1.8 (n = 5)	16.76 ± 2.6 (n = 5)
GluR1	7.39 ± 1.8 (n = 3)	3.76 ± 0.8 (n = 4)	8.26 ± 2.2 (n = 3)
γ2	19.33 ± 0.8 (n = 6)	34.49 ± 6.6 (n = 9)	25.20 ± 4.1 (n = 6)