Contextual learning induces an increase in the number of hippocampal CA1 neurons expressing high levels of BDNF

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Abstract

We examined behaviorally induced expression of brain-derived neurotrophic factor (BDNF) in area CA1 of the hippocampus. Sprague–Dawley rats were trained in a contextual fear conditioning (CFC) task, sacrificed 4 h later, and their brains were processed for immunohistochemistry. We found distinctively high levels of BDNF immunoreactivity in a small number (~1%) of CA1 neurons in untrained animals. The number of these exceptional neurons, which are identified as BDNF(++) in this study, was increased by up to ~3% after CFC. This increase was blocked in the presence of a memory-impairing dose of a NMDA receptor antagonist (MK801 0.3 mg/kg, i.p.) given 30 min prior to training. The BDNF signal intensity in BDNF(++) neurons correlated with that of surrounding glutamic acid decarboxylase (GAD) 65. This correlation between GAD65 and BDNF signal intensities suggests that BDNF upregulation was associated with increased signaling via inhibitory GABAergic synapses that would lessen further intervening neuronal activity. Our observation that neurons which upregulate BDNF expression following a learning experience are rich in GAD65-enriched afferent synapses suggests that these neurons may have distinct roles in memory consolidation.

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1. Introduction

Brain-derived neurotrophic factor (BDNF), a secretory protein expressed mainly in excitatory neurons, is a major candidate molecule for participation in contextual memory formation (Liu, Lyons, Mamounas, & Thompson, 2004; Monteggia et al., 2004). Intra-hippocampal infusion of anti-BDNF antibody impaired long term memory when given 15 min before or 1 and 4 h after training, but not when given 0 or 6 h post-training, indicating that there are two hippocampal BDNF-sensitive time windows (Alonso et al., 2002). These findings suggest that BDNF protein expression is critically involved in the consolidation phase of long-term memory. As would be predicted for a molecule critically involved in memory consolidation processes, neuronal activity has been shown to induce BDNF expression (Tao, Finnkeiner, Arnold, Shaywitz, & Greenberg, 1998; Zafra, Castren, Thoenen, & Lindholm, 1991). Furthermore, BDNF expression is induced in the hippocampus following behavioral training in a contextual fear conditioning (CFC) task (Hall, Thomas, & Everitt, 2000).

Several studies have assessed the number of Fos-positive neurons as a marker of neuronal activity following training in various learning paradigms (Aggleton & Brown, 2005; Jenkins, Amin, Harold, Pearce, & Aggleton, 2003; Kee, Teixeira, Wang, & Frankland, 2007; Touzani, Marighetto, & Jaffard, 2003; Vann, Brown, Erichsen, & Aggleton, 2000). For example, Fos was upregulated and expressed in approximately 2% of mature dentate neurons following water maze testing (Kee et al., 2007). Riboprobe in situ hybridization and immunohistochemistry experiments examining the spatial distribution pattern of BDNF expressing neurons in the CA1 subfield of naïve rats...
revealed a few scattered densely labeled cells (Conner, Lauterborn, Yan, Gall, & Varon, 1997). These findings of sparsely distributed activated cells are consistent with the experimental suggestion that only 6–10% of hippocampal CA1 pyramidal cells should be recorded as units (Henze et al., 2000), and that 30–50% of the recorded units behave as place cells (Wilson & McNaughton, 1993). Moreover, such findings support the view that spatial information is sparsely encoded (Jung & McNaughton, 1993).

Although there has been great interest in BDNF as a putative molecular player in memory consolidation, a detailed examination of the distribution of behaviorally induced BDNF protein has not yet been reported. Therefore, in the present study, we examined BDNF protein expression in hippocampal CA1 neurons induced by CFC.

2. Materials and methods

2.1. Contextual fear conditioning

Male Sprague-Dawley rats (SLC Japan, Shizuoka, Japan), 4 weeks old (76–104 g), were housed with free access to food and water on a 12 h light/dark cycle. Rats were handled for 7 d, and on the day prior to training, they were placed in a non-electrified experimental chamber with a red acrylic flat floor and the same dimensions as the training chamber for 300 s, in order to familiarize them with the experience of being placed in a different chamber by the experimenter. The training chamber (30\( \times \)25\( \times \)30 cm\(^3\)) had a metal grid floor connected to a shock scrambler (SFS-003DX; Muromachi, Tokyo, Japan) and was equipped with a CCD camera. On the training day, rats were subjected to a conditioning session, which consisted of being placed in the training chamber and receiving footshocks (1.5 mA, 2 s) 120 and 240 s later. CFC-trained rats were returned to their homecages 60 s after the last footshock (CFC group). Three control groups were included: (1) rats in the naive group were handled for 7 d, but never placed in the training chamber; (2) rats in the no shock “control” group were exposed to the chamber for 300 s without receiving any footshocks; and (3) rats in the unpaired “footshock-context” (F-C) group were exposed to one footshock (1.5 mA, 4 s) immediately after being placed in the chamber and remained in the chamber for 300 s. This F-C paradigm has previously been reported to not support footshock-context learning (Fanselow, DeCola, & Young, 1993; Milanovic et al., 1998; Radulovic, Kammermeier, & Spiess, 1998; Sananbenesi, Fischer, Schrick, Spiess, & Radulovic, 2002).

The 24 h retention test session lasted for 10 min and was conducted in the training chamber. During testing, images were recorded at 2 frames/s, and the period within which the difference in the successive images (reflecting rat movement) did not exceed a pre-determined threshold (i.e., 173 pixels, about one-third of the average body area) was automatically scored using a custom-made macro of ImageJ software (National Institutes of Health, Bethesda, MD). MK-801 (Tocris, Ellisville, MO) was dissolved in saline (0.3 mg/10 ml) at 4 °C for 18 h, and then in 1% goat serum with Alexa 488-anti-rabbit IgG, Alexa 594-anti-mouse IgG (1:500–1000, Invitrogen, Eugene, OR) and NeuroTrace 435/455 blue fluorescent Nissl stain (Invitrogen) for 1 h at room temperature. No signals exceeded the employed threshold when the BDNF antibody was pre-incubated with antigen peptide (0.27 μg/ml, Santa Cruz) before immunohistochemistry of sections from wild-type mouse brains or when sections prepared from 14-day-old BDNF-deficient mouse brains were processed for BDNF immunohistochemistry (Supplementary Fig. 1). The dorsal hippocampus was selected for analysis because it has been strongly implicated in CFC memory (Bast, Zhang, & Feldon, 2003; Moser, Moser, Forrest, Andersen, & Morris, 1995). We arrayed sections from all comparing groups on each slide and processed them in the same pool of solutions. Three non-overlapping images nearly spanning area CA1 were obtained from each section and six sections per animal were used.

Double immunolabeling experiments with anti-BDNF plus anti-CaMKII antibodies and with anti-BDNF plus anti-GAD67 antibodies were conducted to determine whether the BDNF immunoreactive cells were excitatory or inhibitory. Sections were incubated in 0.5% Triton X-100 for 30 min, 2% goat serum for 60 min, Avidin solution (Vector Laboratories, 1:1) for 15 min, Biotin solution (Vector Laboratories, 1:1) for 15 min, primary antibodies (anti-BDNF, generous gift from Amgen, 0.5 μg/ml, mouse anti-CaMKII, Santa Cruz, 1:200 or mouse anti-GAD67, Chemicon, 1:1000) for 18 h at 4 °C, conjugated secondary antibodies (biotinylated anti-rabbit IgG, Vector Laboratories, 1:1000, Alexa-488 conjugated anti-mouse IgG, Invitrogen, 1:400) together with a Nissl dye (NeuroTrace fluorescent Nissl, Invitrogen, 1:50) for 3 h, ABC complex (Vector Laboratories, 1:100) for 90 min, and Cy3-Tyramide Working solution (1:4, Perkin Elmer) 60 min.

2.3. Imaging

Focus points and image frames were decided based on observations of Nissl staining. Images were captured with a cooled CCD camera, Orca-II (Hamamatsu photonics, Shizuoka, Japan) and with an MRC-1000 confocal system (Bio-Rad) on Nikon TE300 microscopes (10× objective lenses). The 8-bit images were analyzed with ImageJ and MetaMorph (Universal Imaging Corp., West Chester, PA). The threshold values were set in each image as the average ± 2x the standard deviation of intensity of each CA1 cell layer. The threshold values did not show any group-dependent tendencies and did not correlate with the number of BDNF(++) cells. At least 2/3 of the samples were processed in a blind manner; the same results were obtained under both conditions.

3. Results

3.1. CFC training increased the number of neurons expressing high levels of BDNF in the CA1 pyramidal cell layer

Immunohistochemical analysis of BDNF expression in a preliminary study revealed a small number of neuronal somata that were highly positive for BDNF-immunoreactivity, BDNF(++) in both trained and non-trained rats. As shown in Fig. 1A and B, subsequent double immunolabeling experiments revealed that all BDNF(++) neurons were also CaMKII-positive (n = 26), while no BDNF(++) neurons were GAD67-positive (n = 45).

Among brains obtained 2.5, 4, 6, and 24 h after CFC training in that preliminary study, the difference relative to non-trained controls was most apparent in the rats sacrificed 4 h after training. Therefore we conducted the present study in rats sacrificed 4 h after training. The average
intensities of BDNF immunolabeling in the whole CA1 pyramidal cell layer, stratum radiatum and stratum oriens did not differ between control and trained rats (trained level in the pyramidal layer was 98.3 ± 2.2% of the control level, *P* = .49, Student's *t* test)—presumably because the number of cells that upregulate BDNF expression in response to CFC training is small relative to the entire population of cells. Due to the similarity of BDNF immunoreactivity in trained and control groups, the effects of training on BDNF expression could not be detected by measuring immunolabeling intensity as a whole. Therefore we subsequently applied a semi-automated procedure to define the neurons expressing high levels of BDNF as BDNF(++) neurons (Fig. 1C), and proceeded to count the number of BDNF(++) neurons in brains from the context-exposed no-shock “control” group, the F-C group exposed to the footshock immediately upon being placed in the context, the CFC-trained group, and the naïve caged control group of rats.

The F-C group was included to exclude the possibility that the increase in the number of BDNF(++) neurons could be independent of the learning but instead due to footshock stimulation and/or exposure to a novel environment. In a separate experiment we confirmed that the amount of time spent freezing by CFC rats in a 24 h retention test was significantly greater than that of F-C and non-trained control rats (Fig. 1D). Rats in the CFC group spent more time freezing during the 24 h retention test, presumably due to the associative memory of the footshock in the behavioral chamber. F-C rats in our hands spent more time freezing than non-shocked control rats (*P* < .05). This freezing was most likely a result of generalized anxiety elicited by being subjected to a similar experience (transport and transfer) which 24 h earlier resulted in a non-cued footshock. Indeed freezing is an innate and broad-spectrum fear behavior in rats which has been adopted as an indirect index of fear memory retrieval in the laboratory (Vazdarjanova, Cahill, & McGaugh, 2001). Demonstrations in other laboratories that F-C rats did not acquire CFC under similar conditions as those employed here are consistent with this interpretation (Fanselow et al., 1993; Milanovic et al., 1998; Radulovic et al., 1998; Sananbenesi et al., 2002). While it is difficult to know precisely why others’ methods may have induced...
less freezing than ours, there are many plausible factors, such as differences in handling procedures, presence of reminder cues during testing, etc.

As summarized in Fig. 1E, an approximately 2-fold increase in the number of BDNF(++) CA1 neurons was observed in the CFC group (2.6 ± 0.07 × 10^2/mm^2) relative to the non-trained control group (1.2 ± 0.16 × 10^2/mm^2, P < .01). The number of BDNF(++) CA1 neurons in the F-C group (1.3 ± 0.05 × 10^2/mm^2) did not differ from that in the non-trained control group (P > .05). These BDNF(++) neurons represent 1.4%, 1.5%, and 3.2% of total neurons in control, F-C, and CFC groups, respectively. In area CA1 of naïve caged rats there were 0.82 ± 0.06 × 10^2/mm^2 BDNF(++) cells, accounting for 1.0% of the total CA1 cells. Note that the footshock experience not paired with the context in F-C rats did not induce an increase in the number of BDNF(++) neurons. This dissociation is consistent with the interpretation that the observed upregulation of BDNF in CFC rats is related to associative learning and is not a non-specific effect of the stress associated with being exposed to an aversive stimulus.

Analysis of the distribution patterns (Fukuda, Kosaka, Singer, & Galuske, 2006) of BDNF(++) neuronal somata revealed that the BDNF(++) cells were regularly spaced, rather than clustered or randomly distributed. The most common spatial interval between BDNF(++) cells was ∼100 µm. The coefficient of variation of the intervals was significantly smaller than that estimated by Monte Carlo simulations (P < .05 by Welch’s test, detailed data not shown).

3.2. Training-induced increase in the number of BDNF(++) neurons requires N-methyl-D-aspartate (NMDA) receptor activity

To further investigate whether the change in BDNF expression was related to associative learning, we analyzed the effect of a pharmacological blockade of memory acquisition. As shown previously (Bast et al., 2003; Bordi, Marcon, Chiamulera, & Reggiani, 1996), CFC long-term memory in rats was attenuated when the NMDA antagonist MK-801 was injected systemically 30 min prior to training (Fig. 2a). The training-induced increase in the number of BDNF(++) neurons was also blocked in rats given a systemic injection of MK-801 30 min prior to training (Fig. 2; P < .01 vs. saline-injected trained group and P > .05 vs. non-trained control groups). Meanwhile injection of MK-801 in the absence of CFC training did not affect the number of BDNF(++) neurons (Fig. 2b), demonstrating that the effects of MK-801 on BDNF expression in CFC rats were dependent upon the presence of learning.

3.3. Positive correlation between BDNF expression in cells and the intensity of the surrounding GAD65 signal

In a previous study, we observed that introduction of BDNF cDNA into approximately 1% of the neurons in a dissociated neuronal culture caused a selective increase in the level of GAD65 immunoreactivity around BDNF-expressing neurons (Ohba et al., 2005). Given that GAD65 is a GABA synthesizing enzyme that is primarily present in the presynaptic terminals of inhibitory neurons, we posited that BDNF(++) neurons in vivo may selectively receive augmented inhibition (Henneberger, Kirischuk, & Grantyn, 2005; Swanwick, Murthy, & Kapur, 2006). Here, we noticed that the GAD65 signal around the BDNF(++) cells was augmented relative to GAD65 labeling around other neighboring cells and examined the GAD65 signal around the BDNF(++) neurons (Fig. 3A). None of the BDNF(++) neurons examined (n = 193) expressed GAD65 signals homogeneously within the somata as would be expected for inhibitory GABAergic neurons themselves, but rather they had punctate signals surrounding the somata. This punctate external labeling was consistent with that which is expected for presynaptic terminals of inhibitory GABAergic neurons. Quantitative analyses of the BDNF and GAD65 signals revealed a significant positive correlation (R^2 = .30, P < .01) between the intensity of BDNF labeling in the somata and that for GAD65 surrounding the somata (Fig. 3B). The data in Fig. 3B are from animals sacrificed 4 h after CFC training; the correlation between BDNF and surrounding GAD65 signals was found in both trained and control animals.
Quantitative analysis of average staining intensity (GAD65 as well as BDNF) was not reliable given the variability of staining intensity among the slides. Therefore intensities were normalized to the average intensity observed on each slice (Fig. 3). Determining whether BDNF induction is really causes the GAD increase will involve future detailed time course experiments of the changes in expression.

4. Discussion

4.1. BDNF as a potential marker of cells involved in associative learning

These results provide the first evidence that the number of neurons expressing high levels of BDNF in the hippocampal CA1 pyramidal cell layer increases in parallel with successful learning. Furthermore, our experiments reveal the existence of a specific population of neurons that express greater amounts of BDNF than neighboring neurons in response to learning.

The immediate early gene Arc has been used as a marker of neuronal activity (Guzowski, McNaughton, Barnes, & Worley, 1999; Ramirez-Amaya et al., 2005). It was observed that the ratio of Arc-expressing neurons increases by as much as 30% in CA1 following a simple spatial exploration task. This robust increase in Arc expression is considerably greater than the increase we observed in the number of BDNF(++) neurons following CFC training. It is likely that the difference in induction of Arc and BDNF reflects a difference in the thresholds of neuronal activation which may be relevant to differences in the functions of these proteins during memory consolidation processes (Shepherd et al., 2006).

The immediate early gene c-fos has also been used extensively as a neuronal activity marker. The number of Fos-positive neurons in CA1 has been reported to be increased following various learning paradigms (Aggleton & Brown, 2005; Jenkins et al., 2003; Touzani et al., 2003; Vann et al., 2000). Similar to the present observations with BDNF, this increase was reflected in a sparse distribution of Fos-expressing neurons. The number of nuclear Fos-positive neurons observed 1.5 h after an eight-arm maze test trial is similar to that of BDNF(++) neurons reported here; 10 Fos-positive neurons in a 0.43 mm² image (Vann et al., 2000) is representative of ~3 × 10² neurons/mm² of the CA1 pyramidal cell layer. However, in the case of CFC learning, two studies have shown that the number of Fos-positive neurons in CFC-trained rats relative to that in non-trained rats exposed to context without shock differs in the amygdala, but not the hippocampus (Milanovic et al., 1998; Radulovic et al., 1998). The high level of selectivity in the number of BDNF(++) neurons following behavioral training and the NMDA receptor-dependence of the learning-induced increase in BDNF(++) neurons together suggest that BDNF(++) might be a more tightly associated marker for learning than Arc or Fos.

Recently, it was reported that training for contextual memory caused a 5- to 6-fold increase in MAPK activation in approximately 10% of CA1 pyramidal neurons within 30 min of training, and that those neurons that had the highest degree of nuclear MAPK or MSK1 phosphorylation after training showed significantly increased levels of phosphorylated CREB relative to that expressing lower level of these molecules (Sindreu, Scheiner, & Storm, 2007). Since BDNF has been shown to be induced downstream of CREB activation, these signaling molecules could act to increase BDNF (Tao et al., 1998).

4.2. Relationship of BDNF and GAD65

We found a positive correlation between BDNF expression in cells and the intensity of GAD65 immunoreactive

Fig. 3. Positive correlation between BDNF expression in cells and the level of surrounding GAD65 signal. (A) Double immunolabeling for BDNF (green) and GAD65 (purple) suggested that the GAD65 signal surrounding the BDNF(++) neurons (arrows) was selectively augmented. Note that the GAD signal was not superimposed upon the somatically localized BDNF signals, but rather surrounded them. Scale bar, 50 μm. The method employed for intensity analysis using MetaMorph software is summarized on the right. Each BDNF(++) cell was enclosed in a fitted ellipse (upper), and the ellipse was transferred to the GAD65 image (middle). Then another concentric outer ellipse with 5 μm difference between the radii of the ellipses was drawn (lower). The averaged green signal intensity inside the inner ellipse was defined as the BDNF intensity, and the averaged intensity between the ellipses was taken as the surrounding GAD65 intensity. (B) Average BDNF intensity correlated with that of the surrounding GAD65 signal. The data were normalized to the mean intensity. The regression line is Y = 0.50X + 51 with R² = .30; P < .01, Spearman’s correlation test, n = 193 BDNF(++) neurons.
puncta surrounding those cells. This observation reveals for the first time the existence of a distinct subpopulation of hippocampal neurons with functionally linked expression of postsynaptic BDNF and presynaptic GAD65; the causal relationship between induced BDNF expression and enhanced GAD65 expression however remains unclear. Our prior observations that introduction of BDNF cDNA in a dissociated neuronal culture caused the level of GAD65 immunoreactivity around the BDNF-expressing neurons to be selectively enhanced (Ohba et al., 2005) are consistent with the possibility that BDNF expression may be causing the localized increase in GAD65 levels.

There have been reports that have suggested that BDNF-induced increases in GAD expression are accompanied by enhancement of GABA release (Henneberger et al., 2005; Swanwick et al., 2006). Given that perisomatic GABAergic synapses govern the firing of pyramidal cells (Klausberger et al., 2003), the possible selective enhancement of inhibitory potency suggested by the present study may reflect more efficient restriction of excitatory transmission via the BDNF-expressing neurons. This limitation on intervening neuronal activity might play a role in consolidation within neuronal circuits if the BDNF(++) neurons encode the information that has been learned. Thus the co-selectivity of BDNF(++) neurons and GAD65-augmented synapses raises the intriguing hypothesis that BDNF expression may serve as a functional marker of the specific neuronal population that mainly carries information about particular associative memories.

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