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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.

Keywords: Sparse coding; Inhibitory neurotransmission; Glutamic acid decarboxylase (GAD); Brain-derived neurotrophic factor; Hippocampus

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, Finnkbeiner, 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

☆ Learning induces BDNF-highly positive neurons.
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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 ($30D \times 25W \times 30H$ cm^3) had a metal grid floor connected to a shock scrambler (SGS-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 naïve 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* upon 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) and injected at a dose of 0.3 mg/kg *i.p.*

2.2. Immunohistochemistry

Rats were anesthetized with sodium pentobarbital (50 mg/kg, *i.p.*) and perfused transcardially with chilled phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were post-fixed (4 °C, 2 h); immersed in 10%, 20%, and 30% sucrose in PB in series (4 °C, 24 h in total); frozen; and coronally sectioned at a thickness of 30 μm. Sections mounted on glass slides were incubated in 0.1% Triton X-100 for 10 min, in 5% goat serum for 1 h at 4 °C, in 0.25% goat serum with primary antibodies (rabbit anti-BDNF, Santa Cruz N-20, 0.2 μg/ml and mouse anti-GAD65, DSHB GAD6 (Chang & Gottlieb, 1988), 6 μg/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 + 2× 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