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Gene and protein analysis of brain derived neurotrophic factor expression in relation to neurological recovery induced by an enriched environment in a rat stroke model

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ABSTRACT

Although an enriched environment enhances functional recovery after ischemic stroke, the mechanism underlying this effect remains unclear. We previously reported that brain derived neurotrophic factor (BDNF) gene expression decreased in rats housed in an enriched environment for 4 weeks compared to those housed in a standard cage for the same period. To further clarify the relationship between the decrease in BDNF and functional recovery, we investigated the effects of differential 2-week housing conditions on the mRNA of BDNF and protein levels of proBDNF and mature BDNF (matBDNF). After transient occlusion of the right middle cerebral artery of male Sprague–Dawley rats, we divided the rats into two groups: (1) an enriched group housed multiply in large cages equipped with toys, and (2) a standard group housed alone in small cages without toys. Behavioral tests before and after 2-week differential housing showed better neurological recovery in the enriched group than in the standard group. Synaptophysin immunostaining demonstrated that the density of synapses in the peri-infarct area was increased in the enriched group compared to the standard group, while infarct volumes were not significantly different. Real-time reverse transcription polymerase chain reaction, Western blotting and immunostaining all revealed no significant difference between the groups. The present results suggest that functional recovery cannot be ascribed to an increase in matBDNF or a decrease in proBDNF but rather to other underlying mechanisms.

Functional impairment caused by stroke is a highly serious health problem throughout the world. Rehabilitation has been widely applied and has been shown to contribute greatly to neurological recovery. However, the mechanisms of the beneficial effects of rehabilitation remain unclear [3]. An enriched environment is a model of rehabilitation for rodents, in which multiple animals are housed together in a large cage equipped with toys. Enriched environments have been shown to enhance the recovery of neurological function impaired by experimental focal ischemia [13]. Brain-derived neurotrophic factor (BDNF), one of the neurotrophins, may be a key molecule in this effect, since it is central to many facets of the neural network, from differentiation and neuronal survival to synaptogenesis and activity-dependent forms of synaptic plasticity [9]. While an enriched environment increases BDNF expression in non-ischemic healthy animals [5], this is not the case with ischemic animals. The alteration of BDNF after ischemic stroke is not fully understood, although BDNF expression has been investigated in association with an enriched environment after experimental stroke. Zhao et al. demonstrated that BDNF mRNA

Abbreviations: BDNF, brain derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IPT, inclined plane test; MAP-2, microtubular-associated protein 2; matBDNF, mature BDNF; MCA, middle cerebral artery; NSS, neurological severity scores; ROI, region of interest; RT-PCR, reverse transcription polymerase chain reaction; SYP, synaptophysin; tMCAO, transient MCA occlusion.

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[19] and BDNF protein [20] were decreased after ischemic stroke and housing in an enriched environment. Nygren et al. reported that BDNF +/− mice, which express low levels of BDNF, showed better stroke recovery in an enriched environment than their wild-type counterparts [12]. On the other hand, Risedal et al. observed no significant change of BDNF mRNA between rats in an enriched environment and those in a standard environment in their experiments using a permanent occlusion model [15]. In our previous investigation, microarray analysis and real-time reverse transcription polymerase chain reaction (RT-PCR) revealed a significant decrease in expression of the BDNF gene in the contralateral cortex to ischemia in rats in 4-week enriched environment [16]. Generally, BDNF is a beneficial molecule for neurons and neurological functions, and there thus appears to be a discrepancy between the decreased BDNF and improved neurological functions in these studies. There are at least two possible explanations for this phenomenon. The first one is that BDNF may be down-regulated, since the functionally improved brain may no longer need elevated BDNF after 4-week enrichment. Another possibility is that BDNF itself is an exacerbating factor for neurological deficit, especially in the post-stroke state, and that an enriched environment may eliminate BDNF to avoid its potentially deleterious effects. To examine these hypotheses, it will be necessary to examine brain tissue at an earlier time point when functional recovery has not been completed. Furthermore, proBDNF, a precursor of the BDNF protein, must be investigated, since it negatively influences neurons [9]. Thus, the objective of the present study was to investigate the expression of BDNF in rats subjected to focal cerebral ischemia followed by housing for 2 weeks in an enriched environment by using RT-PCR, Western blotting, and immunohistochemical techniques.

Nine-week-old, male Sprague–Dawley rats were anesthetized by chloral hydrate and the right middle cerebral artery (MCA) was occluded intraluminally for 60 min with nylon monofilaments, as previously described [8]. At 72–96 h after transient MCA occlusion (tMCAO), the rats were randomly divided into two groups, an enriched group and a standard group. For the enriched group, 4–6 rats were housed together in a large cage (610 mm × 460 mm × 460 mm) containing toys including a running wheel, a tunnel, balls, logs and rings, rearranged twice a week. For the standard group, rats were housed alone in a standard-sized cage (320 mm × 210 mm × 130 mm) containing food and water.

Ischemic animals were subjected to two behavioral tests, the neurological severity score test (NSS) [2] and the inclined plane test (IPT) [6]. These tests were performed 3 times, once before tMCAO, once at 3 or 4 days after tMCAO (defined as day 0), and once at 14 days after initiation of differential housing (defined as day 14). The NSS is a composite of motor, sensory, reflex, and balance tests. The score ranges from 0 to 18, with the higher score indicating severe neurological impairment. In this study, we analyzed rats that scored between 7 and 12 in the second test (day 0). The recovery rate was defined as $(NSS_{2nd} - NSS_{3rd})/NSS_{2nd}$. The IPT was performed to evaluate motor deficits. Each rat was placed up-headed or right-headed on a stainless steel plane steepening at a rate of $2^{\circ}\,s^{-1}$, and we recorded the angle when the rat slipped on the plate. The improvement index was calculated as $(IPT_{3rd} - IPT_{2nd})/(IPT_{1st} - IPT_{2nd})$. After the behavioral tests on day 14, rats were sacrificed and the brains were cut into 3 coronal sections with a thickness of 3 mm from the frontal pole.

The second blocks from the frontal pole were embedded in paraffin for the histological study. Microtubular-associated protein 2 (MAP-2), glial fibrillary acidic protein (GFAP), synaptophysin (SYP), and matBDNF were immunohistochemically stained. The infarct volume was calculated as $(C - I)/C$, where C represents MAP-2-stained volume in contralateral side, and I represents MAP-2-stained volume in ischemic side. To set regions of interest in peri-infarct area, we assessed both neuronal viability using MAP-2 staining and glial activity using GFAP staining. MAP-2 mainly distinguishes infarct area from non-infarct area, and GFAP mainly distinguishes peri-infarct area and distant intact area. The area with both preserved MAP-2 staining and intense GFAP staining was defined as peri-infarct area. SYP and BDNF immunoreactivity was quantified in peri-infarct area and its contralateral cortex. The rate of the positively stained area was compared between the two groups.

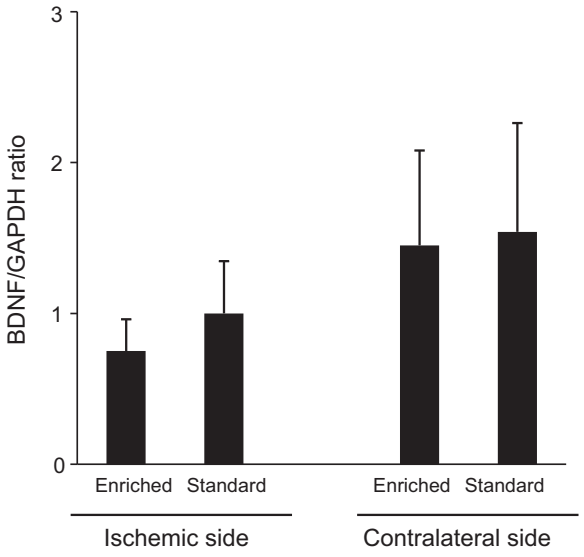

Fig. 1. BDNF gene expression showed no significant differences between the enriched and standard groups on the ischemic side ($p = 0.16$) or contralateral side ($p = 0.81$). Data were normalized to the ischemic side of the standard group.

The peri-infarct cortex and its contralateral cortex of the third blocks were subjected to real-time RT-PCR or Western blotting. Total RNA from the peri-infarct cortex and contralateral cortex was isolated and analyzed for gene expression by real-time quantitative RT-PCR. Expression levels of BDNF mRNA were normalized to those of GAPDH mRNA. For Western blotting, primary antibodies were HRP-conjugated anti-β actin antibody, anti-BDNF antibody, and anti-proBDNF antibody. The anti-proBDNF antibody was produced in a rabbit by intravenous injection of proBDNF-specific peptide. The secondary antibody was HRP-conjugated anti-rabbit goat IgG. The chemiluminescence agents were ECL or ECL+Plus (GE Healthcare). An LAS-4000miniEPUV (FUJIFILM) CCD camera was used to quantify the band intensity. As positive controls, recombinant human BDNF and C6 glioma cell lysate was applied for Western blotting.

Data are expressed as the means ± SD, and a p-value less than 0.05 was considered statistically significant. See the supplementary document for more information about the methods.

The neurological and motor functions of rats in both groups were impaired after t-MCAO and improved on day 14 (Table 1). A significant difference was observed in both NSS and IPT on day 14, but not on day 0, between the enriched ($n = 24$) and standard ($n = 22$) groups. The recovery rate for NSS and improvement index for IPT both indicated a significant improvement in function in the enriched group compared to the standard group.

The infarction area evaluated by immunoreactivity to MAP-2 in the enriched group (56.82 ± 7.31%, $n = 14$) was not significantly different from that in the standard group (55.44 ± 11.50%, $n = 13$, $p = 0.72$).

We performed real-time RT-PCR to examine the changes in BDNF levels (Fig. 1). The data presented were normalized to the ischemic side of the standard group. On the ischemic side, the BDNF/GAPDH ratio was 0.75 ± 0.21 ($n = 7$) in the enriched group, which was slightly but not significantly lower than that in the stan-