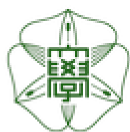

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Title page

Title:

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.

Keywords

focal ischemia; enriched environment; brain derived neurotrophic factor

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

Introduction

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 [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 [16], 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. 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.

Materials and Methods

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 to 96 hours 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 (610x460x460 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 (320x210x130 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°/sec, 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 strong 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.

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.

Results

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 (Figure 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 standard group (1.00±0.35, n=6). On the contralateral non-ischemic side, there was no significant difference in the BDNF/GAPDH ratio between the enriched group (1.45±0.63, n=8) and standard group (1.54±0.72, n=6).

Figure 2 summarizes the results of Western blotting for matBDNF and proBDNF. The antibodies for matBDNF and proBDNF were validated with Western blotting using recombinant matBDNF and C6 glioma cell lysate, respectively (Fig. 2A). There were no significant differences between the enriched group (n=8) and standard group (n=8) in either matBDNF on the ischemic side (Fig. 2B), matBDNF on the contralateral side (Fig. 2C), proBDNF on the ischemic side (Fig.

2D), or proBDNF on the contralateral side (Fig. 2E), although the level of matBDNF in ischemic side tended to be slightly lower in the enriched group than in the contralateral group.

Figure 3 shows the results of immunohistochemical staining of GFAP, SYP and BDNF. The areas stained with SYP and BDNF were quantified within the GFAP-stained area. The SYP-stained area on the ischemic side (n=14) was significantly increased in the enriched group (n=13) compared to the standard group (Fig. 3B, 2.57±0.28% vs 2.07±0.23%, $p<0.001$), although no significant difference was observed on the contralateral side (Fig. 3C, 1.56±0.29% vs 1.49±0.30%, $p=0.52$). On the other hand, the matBDNF-stained area on the ischemic side was slightly smaller in the enriched group (n=15) than in the standard group (n=12), although the difference did not reach the level of statistical significance (Fig. 3D). On the contralateral side, the enriched group showed an matBDNF-stained area comparable to that of the standard group without significant difference (Fig. 3E).

Discussion

This study showed that housing in an enriched environment for two weeks significantly enhanced the functional recovery of rats after ischemic stroke. In addition, the immunohistochemical findings of increased SYP staining indicated an increased density of synapses. On the other hand, no significant difference was observed in the volume of infarction, mRNA expression of BDNF, or protein expressions of BDNFs.

Our previous investigation using a 4-week period of housing demonstrated a decrease in

BDNF gene in the animals housed in an enriched environment based on microarray analysis and real-time RT-PCR as well as a continuous improvement of neurological functions until 4-week [16]. To further clarify the mechanisms of decreased BDNF expression, in the present study we measured the levels of the BDNF protein and gene after a shorter period of enriched environment, i.e., 2 weeks. The common finding between the 2-week and 4-week experiments was the lack of a clear increase in BDNF gene expression, despite the well-known beneficial effects of BDNF for neurons and neurological functions under various physiological and pathological conditions. These results, combined with our previous findings showing amelioration of neurological sign after 2 week, suggest that the functional recovery induced by an enriched environment might be brought about by mechanisms other than increased BDNF.

As an explanation for the relationship between BDNF and functional recovery, we hypothesized that proBDNF might play an important role. The primary product of the BDNF gene is the 32-kDa proBDNF protein, which is translated in neurons and released into the extracellular space. Proteolytic enzymes such as plasmin cleave proBDNF into 14-kDa matBDNF and another particle. While matBDNF binds to the Trk-B receptor on neurons and induces cell survival, differentiation, and long-term potentiation, proBDNF binds to the p75NTR receptor on neurons and induces apoptosis of neurons and long-term depression [9, 11, 17]. Thus, proBDNF and matBDNF, originating from the same gene, have opposite effects. Therefore, not only transcriptional regulation but also post-translational modification must be taken into consideration when we discuss the effects of BDNF. Via its pro-apoptotic effect, proBDNF might have an exacerbating effect on the ischemic damage of neurons and therefore neurological functions. If so, the decrease in proBDNF is

beneficial. However, to our knowledge, there has been no studies investigating proBDNF expression in relation to stroke and enriched environment, although Zhao et al. previously measured the total levels of matBDNF and proBDNF using ELISA with anti-matBDNF antibody [20]. In the present work, we first tried to measure proBDNF using commercially available antibodies. Theoretically, Western blotting using either a proBDNF-specific antibody or matBDNF antibody could be used to quantify proBDNF levels, since the matBDNF domain is common to proBDNF and matBDNF. However, we did not find an efficient antibody that clearly revealed the band of proBDNF. Therefore, in the present study, we produced a polyclonal antibody using a proBDNF-specific peptide. Contrary to our expectations, Western blotting revealed no significant difference in proBDNF expression between the enriched group and standard group. This finding clearly indicates that the functional improvement induced by an enriched environment cannot be ascribed to decreased proBDNF.

On the other hand, an interesting difference between the 2-week and 4-week experiment is that a significant decrease in BDNF gene expression was observed after 4 weeks but not after 2 weeks of enrichment. Therefore, the difference in BDNF expression may be amplified between 2 weeks and 4 weeks. Considering that functional recovery and synaptogenesis were already observed at 2 weeks, there is a possibility that the decrease of BDNF expression at 4 weeks might result from secondary down-regulation due to the improved neurological functions. To prove the hypothesis, further investigation will be necessary to collect samples in earlier time points than 2 week especially before the significant improvement becomes evident in enriched group. Not only BDNF gene expression but also matBDNF protein showed a consistent trend of decrease by both Western

blotting and immunohistochemical staining, although the difference was not statistically significant. These results agree with the report by Zhao et al., which demonstrated a decrease in the total levels of BDNF protein in an enriched group [20]. Thus, we cannot completely rule out type-2 statistical error that small analyzing numbers concealed the possible decrease in BDNF genes or matBDNF in enriched group. Even if that is the case, however, there is no evidence of increase in BDNF genes or matBDNF.

Few genes are known to have a relationship to enriched housing environments. Dahlqvist et al. reported an alteration in the levels of nerve growth factor-induced gene A and glucocorticoid receptor following environmental enrichment in rats [4]. We previously found that animals in an enriched housing group showed a decrease in Early growth response-1 (Erg-1) mRNA [16], which is an inflammatory gene associated with exacerbation of neurological deficits after stroke [18]. To clarify the mechanisms of enriched environment-induced functional recovery, future investigations of neurotrophic factors other than BDNF (i.e., nerve growth factor, neurotrophin-3, and neurotrophin-4) and other inflammatory genes will be needed. Since the environmental stimulation involves a series of complicated processes through which many genes and proteins are expected to alter their expression, the combination of exhaustive analysis of the relevant genes and proteins will provide additional information.

We observed no decrease in infarction volume in the enriched group. This finding is consistent with previous reports [1, 13]. Functional recovery induced by an enriched environment is not due to a reduction of infarct volume but possibly due to functional compensation in the brain tissues that escaped from infarction. The increased density of synapses in peri-infarct cortex

measured using the SYP-stained area further supports this hypothesis.

One may argue that an enriched environment is a model enhancing voluntary exercise and thus the effect depends on individual activity, leading to a great individual variability in stroke recovery. However, as far as animal experiments are concerned, the effect of forced exercise such as treadmill running is controversial [7, 10, 14], while most studies on environmental enrichment agree on its positive effects. Therefore, we consider that enriched housing is a more feasible model to investigate the mechanisms of functional recovery after brain ischemia. Because it is established that environmental enrichment leads to an increase in BDNF in non-ischemic healthy animals [5], we compared only ischemic rats between enriched and standard environment according to the previous research designs [19, 20].

Conclusions

An improvement of neurological functions was induced by an enriched environment accompanied with an increased density of synapses but without a reduction of infarct volume. The 2-week environmental enrichment did not significantly alter BDNF expression, including BDNF mRNA, matBDNF protein, or proBDNF protein. These results suggest that the functional recovery might not be due to increased BDNF or decreased proBDNF but rather to other underlying mechanisms.

Acknowledgements

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Legends

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

Fig. 2. Western blotting. (A) Recombinant human matBDNF (Rec), C6 glioma cell lysate (C6), and rat brain tissue validated the use of the antibodies for matBDNF (14kDa) and proBDNF(32kDa). Rat brain tissue contained detectable levels of these molecules. The levels of (B) matBDNF on the ischemic side (p=0.27), (C) matBDNF in contralateral side (p=0.86), (D) proBDNF on the ischemic side (p=0.79), and (E) proBDNF on the contralateral side (p=0.25) were not significantly different between the enriched and standard groups. Abbr. E: enriched group; S: standard group.

Fig. 3. Immunohistochemical analysis of GFAP, SYP, and matBDNF levels on the ischemic side and contralateral side. (A) Representative whole brain images (left 2 columns) and enlarged images (right 4 columns). The ischemic side was the left in the picture (right hemisphere of the rats). Arrows indicate the enlarged sites. The bar represents 100 μm. (B-D) The SYP- and BDNF-stained areas were quantified in the GFAP-stained area within the GFAP-stained area. The ischemic side in the enriched group showed significantly stronger staining than the ischemic side in the standard

group (B) ($p<0.001$), while the contralateral side in the enriched group and contralateral side in the standard group did not show a significant difference (C) ($p=0.52$). On the other hand, the matBDNF-stained area on the ischemic side (D) ($p=0.24$) and contralateral side (E) ($p=0.85$) showed no significant difference.

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Table 1. Behavioral test results

	Pre-MCAO		Day 0		Day 14	
	Enriched n=24	Standard n=22	Enriched	Standard	Enriched	Standard
NSS	0.00 ± 0.00	0.00 ± 0.00	8.00 ± 1.02	7.55 ± 0.74	4.92 ± 1.32 *	5.73 ± 1.03
NSS recovery rate					37.83 ± 17.95 *	23.67 ± 14.08
IPT Up-headed	51.72 ± 2.00	50.80 ± 1.74	42.26 ± 2.61	43.52 ± 2.34	47.54 ± 1.63 *	45.37 ± 2.30
IPT Right-headed	50.60 ± 2.86	50.79 ± 2.62	41.63 ± 2.62	42.80 ± 2.94	47.08 ± 2.11 *	45.61 ± 2.11
IPT Mean	51.16 ± 2.16	50.79 ± 1.90	41.94 ± 2.39	43.16 ± 2.37	47.31 ± 1.70 *	45.49 ± 2.15
IPT improvement index					59.17 ± 29.51 *	27.75 ± 40.25

* $p<0.05$ compared to the standard group. Data are presented as the means±SD.

Fig. 1

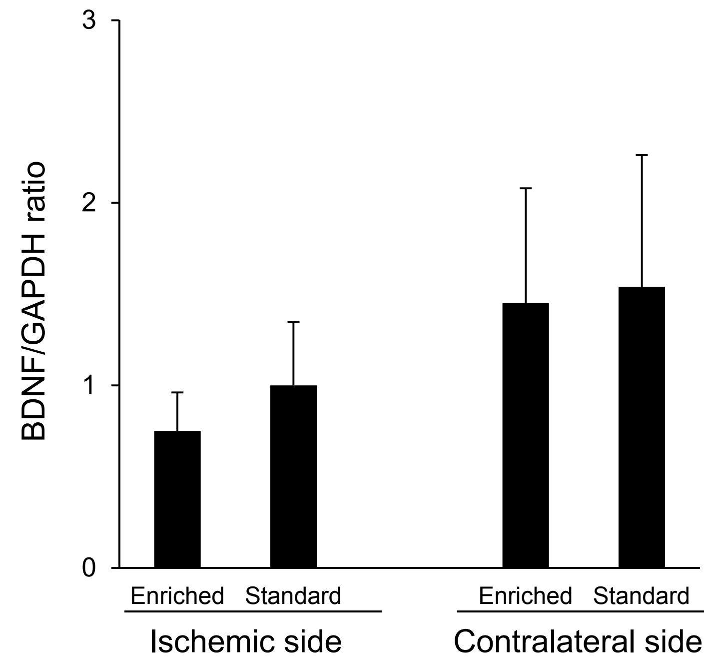


Fig. 2

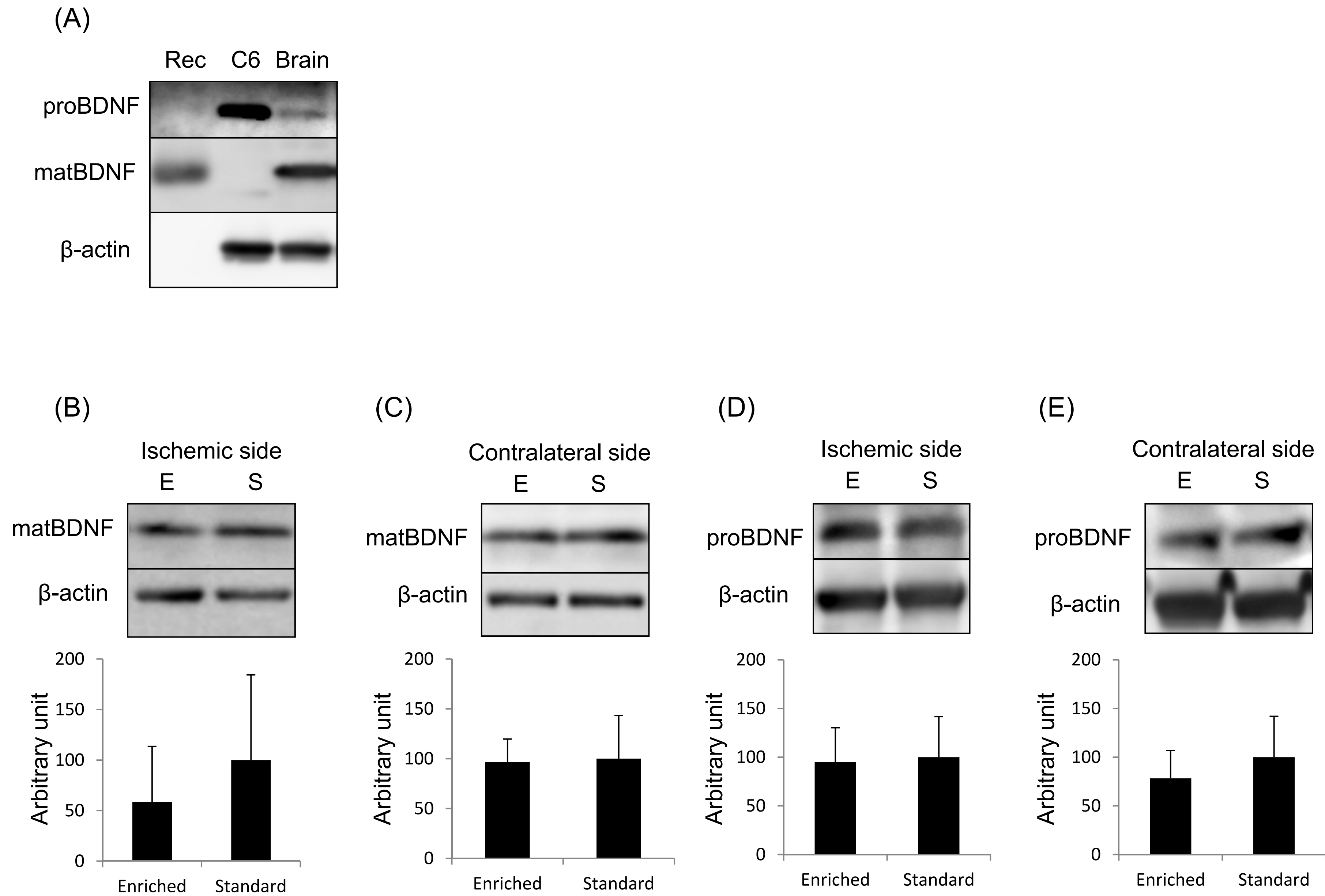


Fig. 3

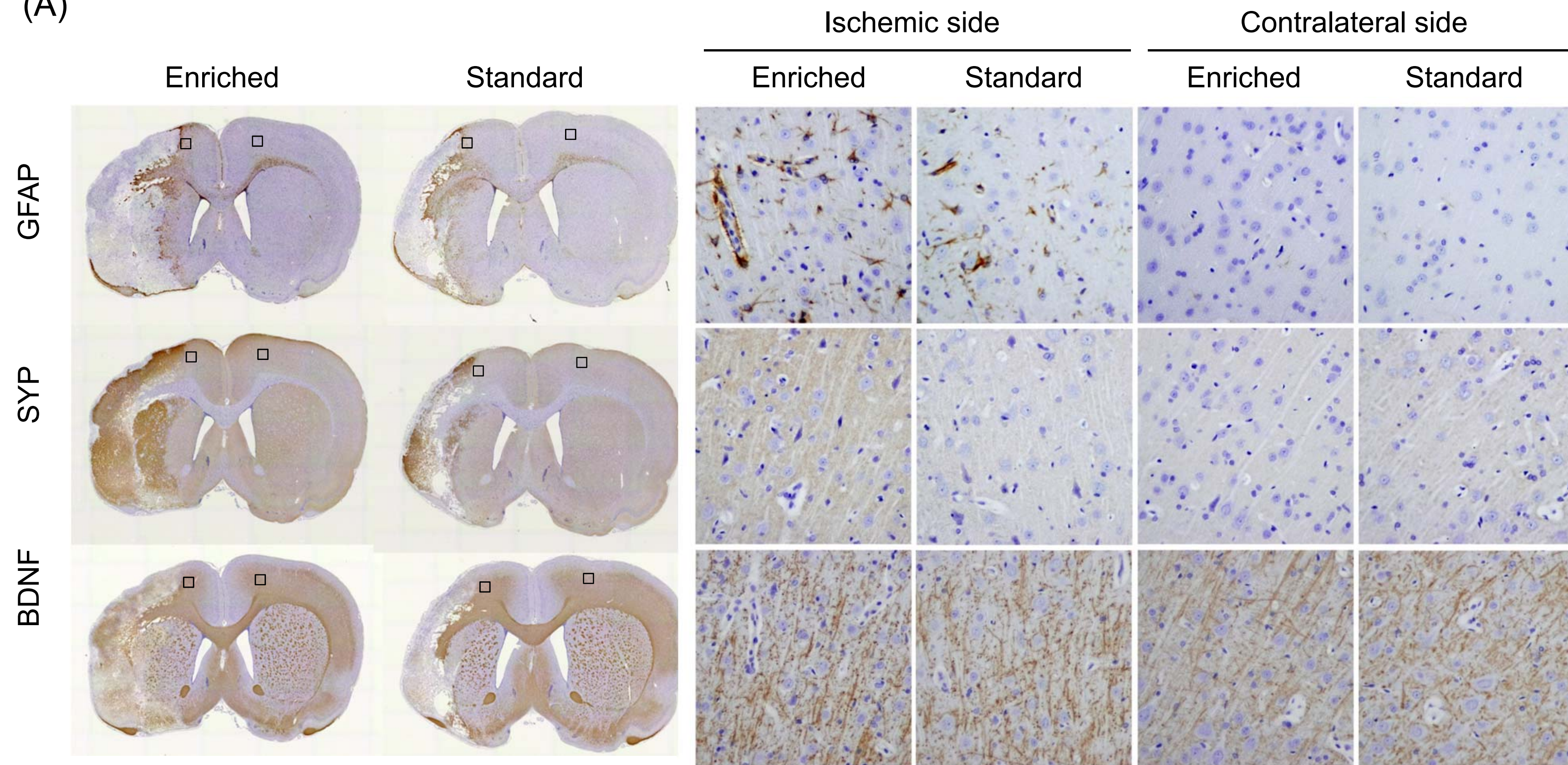
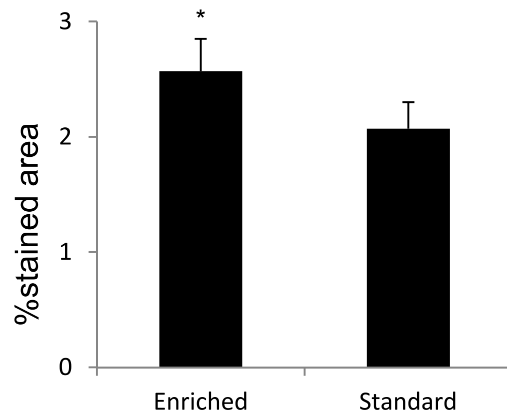
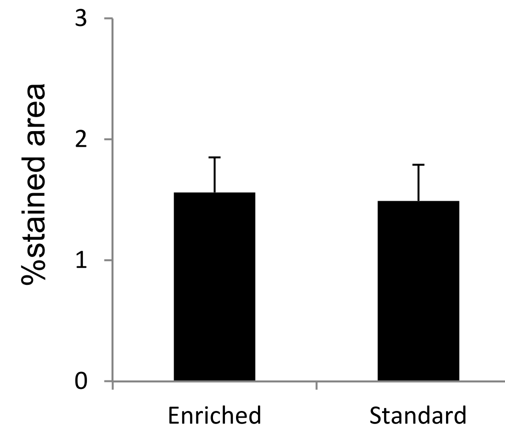


Fig. 3

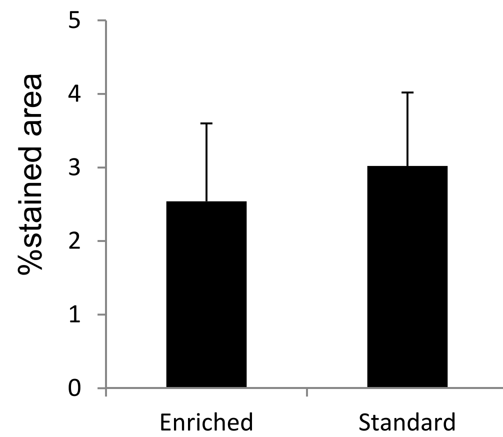
(B) SYP in ischemic side



(C) SYP in Contralateral side



(D) BDNF in ischemic side



(E) BDNF in contralateral side