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Abstract

Background

The immature brain is more susceptible to seizures than mature brains but less vulnerable to seizure-induced neuronal loss. We studied age-related susceptibility and vulnerability to kainic acid-induced status epilepticus (KASE) in rats in terms of hippocampal expression of brain-derived neurotrophic factor (BDNF) and tyrosine kinase B receptor (TrkB).

Methods

Immunohistochemical and Western analysis were performed after kainic acid (KA)-induced status epilepticus (SE).

Results

KA doses required to induce SE increased from 1.5 mg/kg in 1-week-old rats to 10 mg/kg at 4 weeks of older. After SE the older rats showed spontaneous seizures and hippocampal pyramidal neuronal loss-unlike rats under 4 weeks old. Hippocampal BDNF protein expression had increased fivefold in 1-week-old rats and threefold in 8-week-old rats 1 day after SE, returning to baseline 2 days after SE. TrkB expression showed little effect from KASE at either age.

Conclusions

These results indicated that the critical period as for vulnerability to SE was the age of 4-week-old and older in the rat. Since the response patterns of BDNF and TrkB to SE were similar between neonatal and the adult rats, our study revealed that the observed transient up-regulation of BDNF did not contribute to cause epilepsy in neonatal rats.

Key Words: Brain-derived neurotrophic factor (BDNF); Epileptogenesis; Hippocampus; Status epilepticus; Tyrosine kinase B receptor (TrkB)

Introduction

In comparison with adult brains, the immature brain is more susceptible to seizures but more resistant to neuronal loss induced by these seizures[1,2]. Thus, maturation of the brain has

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opposing effects on seizure thresholds and vulnerability to potentially epileptogenic brain injury from an initial seizure episode. The mechanisms underlying these differences, the manner in which development affects seizure susceptibility, and the critical period for vulnerability to brain injury all require elucidation, representing the rationale for this experimental study.

Brain-derived neurotrophic factor (BDNF) is the most abundant and widely distributed neurotrophin in the brain, and is especially, in the hippocampus[3-5]. The high-affinity receptor for BDNF, tyrosine kinase B receptor (TrkB), also is expressed most prominently in the hippocampus[4-6]. BDNF acts importantly to promote survival, differentiation, and maintenance of neurons during development, and also to mediate synaptic plasticity and excitability[7-11]. Several reports have indicated that status epilepticus (SE) increased the expression of hippocampal BDNF, which induced proliferation of ectopic granule cells in the dentate gyrus in adult rats, contributing to subsequent epilepsy after SE[12-16]. However, BDNF and TrkB expression have been studied considerably less in neonatal rats with SE[17,18]. If BDNF were pivotal in epileptogenesis after SE, expression of BDNF and TrkB after SE might be expected to differ between neonatal and adult rats. We examined hippocampal BDNF and TrkB protein expression by immunohistochemical and Western analysis in neonatal and adult rats after kainic acid (KA)-induced SE (KASE).

Methods

Animal treatment paradigms

All animal experiments were carried out in accordance with the Guidelines for Use and Care of Experimental Animals approved by the Animal Committee of Osaka City University Graduate School of Medicine. Efforts were made to minimize numbers of animals used as well as their suffering. Male Wistar rats were studied at weekly intervals from 1 to 8 weeks of age with the respective extremes of the range designated “neonatal” and “adult”. To induce SE, 1-, 2-, and 3-week-old rats were injected intraperitoneally with 1.5 mg/kg, 3 mg/kg, and 6 mg/kg of kainic acid respectively, since at each age the rats died when doses exceeded those specified. Rats 4 to 8 weeks old were injected with 10 mg/kg of kainic acid, uniformly resulting in SE. Rats were observed for up to 60 days after KASE. Age-matched control rats were injected with saline in lieu of KA.

Histologic examination

To examine hippocampal damage, groups of 12 rats representing ages from 1 to 8 weeks at time of KASE were anesthetized by intraperitoneal injection of pentobarbital and killed by intracardiac perfusion with cold 2% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at 1, 7, 21, and 28 days later. Brains were removed rapidly, fixed in Bouin’s solution, and dehydrated stepwise using graded ethanol solutions prior to embedding in paraffin. Embedded brains were sectioned coronally through the hippocampus to be stained with hematoxylin and eosin (H and E) for routine light microscopy.

Antibodies

Anti-BDNF (sc-546) antiserum purchased from Santa Cruz Biotechnology (Santa Cruz, CA) reacted with the N-terminal region of 14-kDa mature BDNF. Anti-TrkB (sc-12) antibody purchased from the same supplier reacted with the C-terminal region of 145-kDa full-length

TrkB.

Competition experiments were performed using the immunoreactive peptides for BDNF (sc-546P) and TrkB (sc-12P) to ensure specificity of immunoreactivity.

Immunohistochemistry

Thirty neonatal (1-week-old) and thirty adult (8-week-old) rats were used for immunohistochemical studies. Hippocampal sections were examined immunohistochemically without KASE and at days 1 (24 hr), 2, 3, 4, and 5 after KASE using anti-BDNF and -TrkB antibodies.

Rats were anesthetized by intraperitoneal injection of pentobarbital and killed by intracardiac perfusion with PBS. Brains were removed and immersed in the same fixative for 2 hr, and then immersed in 0.1 M phosphate buffer containing 30% sucrose at 4℃ for 2 days. The brains were sectioned at a 20 μm thickness using a cryostat (Leica Instruments, Nußloch, Germany) in a coronal plane, and sections were affixed to glass slides. Slides were kept in 0.1 M PBS with 0.3% Triton-X (PBST) for 3 days. Slides then were incubated for 72 hr at 4℃ with primary antibody against BDNF (1:1000) or TrkB (1:1000) in 0.1 M PBST. Sections were washed at room temperature in PBST and then incubated for 1 hr with biotinylated goat anti-rabbit IgG (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) followed by washing in PBST and incubation with avidin-biotin-horseradish peroxidase complex (Vectastain ABC Kit, Vector Laboratories) for 1 hr. After three more washes at room temperature in PBST, reaction product was visualized with Tris-buffered saline solution containing nickel-intensified diaminobenzidine (DAB. Vector Laboratories) and hydrogen peroxide (0.01%) for 5 min. Nickel-intensified DAB was prepared by adding 0.3 mg/mL of nickel ammonium sulfate to the DAB solution, staining the reaction product dark blue.

Western analysis

Thirty neonatal (1-week-old) and thirty adult (8-week-old) rats also were used for Western analysis of BDNF and TrkB. Rats were anesthetized by intraperitoneal injection of pentobarbital and killed by intracardiac perfusion with PBS with no KASE or at day 1 (24 hr), 2, 3, 4, or 5 after KASE. The hippocampus was dissected from the rest of the brain and frozen at −80℃. Hippocampi were homogenized individually in 200 μL of ice-cold buffer containing 50 mM Tris-HCl (pH 7.2), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 μg/mL antipain, and 1 μg/mL aprotinin. Homogenates then were centrifuged at 12000×g for 20 min at 4℃ in an ultracentrifuge (model L765, Beckman-Coulter Electronics, Fullerton, CA), and supernatants were collected. Protein concentrations were determined using a DC Protein Assay (Bio-Rad, Hercules, CA). Proteins were mixed 1:1 with sample buffer containing 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% ß-mercaptoethanol, and 0.05% bromophenol blue, and boiled for 5 min. Each well was loaded with 20 μg of protein. Samples were separated on 8% (TrkB) or 12% (BDNF) SDS-polyacrylamide gels. After electrophoresis, proteins were transferred electrically to polyvinylidene fluoride (PVDF) membranes (WBK-204; TOYOBO, Osaka, Japan) 6 V for 1 hr. After transfer, blots were incubated in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% nonfat milk. Subsequently, the blots were incubated overnight with either BDNF (1:500) or TrkB (1:1000) antiserum at 4℃ in TBST. Control blots were processed without primary antibodies or with antisera preabsorbed with immunoreactive peptide. After primary

antibody incubation, blots were incubated for 15 min in TBST containing 0.5% nonfat milk, and washed for 15 min in TBST. Blots then were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies (1:1500; Vector Laboratories) for 1 hr. Immunoreactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Arlington Heights, IL) according to instructions provided by the manufacturer.

To ensure that each lane was loaded with an equivalent amount of protein, blots were submerged in stripping buffer (100 mM ß-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl; pH 6.7) for incubation at 50℃ for 30 min followed by reincubated with anti-ß actin serum (1:3000, Sigma Chemical, St. Louis, MO) as described above.

After immunoblotting, digitized images of immunoreactive bands for target (BDNF and TrkB) and control (ß-actin) products were imported into NIH Image software (version 1.62), with which the average optical density of each band was measured.

Statistical analysis

All data are expressed as the mean±SEM. Differences between groups were examined for statistical significance using repeated measures analysis of variance (ANOVA; StatView; SAS Institute, Cary, NC). Apparently significant values then were examined by Tukey-Kramer post hoc analysis. A p value less than 0.05 was considered to indicate a statistically significant difference.

Results

Behavioral observation and histology

Rats at the time of KASE 4 to 8 weeks old began to have spontaneous seizures at day 7 after KASE, while 1 to 3 weeks old rats did not develop epilepsy after KASE. Histologic examination of rats 4 to 8 weeks old at the time of KASE showed scattered pyramidal cells with pyknosis at CA1 on day 1, while pyramidal cell numbers in CA1 decreased gradually from days 7 to 21 after KASE. Pyramidal cell loss was noted to a lesser extent in CA3 and CA4 regions as well (Fig. 1A); pyramidal cells at CA2 were not affected by KASE. Following pyramidal cell loss at CA1, astrocytes in that area increased in number to result in gliosis by day 28. In the hippocampus of rats with KASE at 1 to 3 weeks of age, no cell loss was observed in the pyramidal cell layer through day 28 (Fig. 1B).

Immunohistochemistry

In the hippocampus of adult rats without KASE, granule cells in the dentate gyrus as well as pyramidal cells were stained intensely by anti-BDNF antibody (Fig. 1C). Even at day 1 after KASE, staining intensities for BDNF in both cell types were similar to those before SE (Fig. 1D). Surviving pyramidal cells as well as granule cells continued to show strong immunoreactivity for BDNF through day 5 after KASE.

In the hippocampus of neonatal rats without SE, granule cells and pyramidal cells also were stained with anti-BDNF antibody (Fig. 1E), with no apparent change in staining intensity at 1 to 5 days after SE.

TrkB immunoreactivity also was detected in pyramidal cells and in the granule cells of the dentate gyrus. Staining intensities without KASE were similar to those seen up to day 5 after KASE in both neonatal and adult rats.

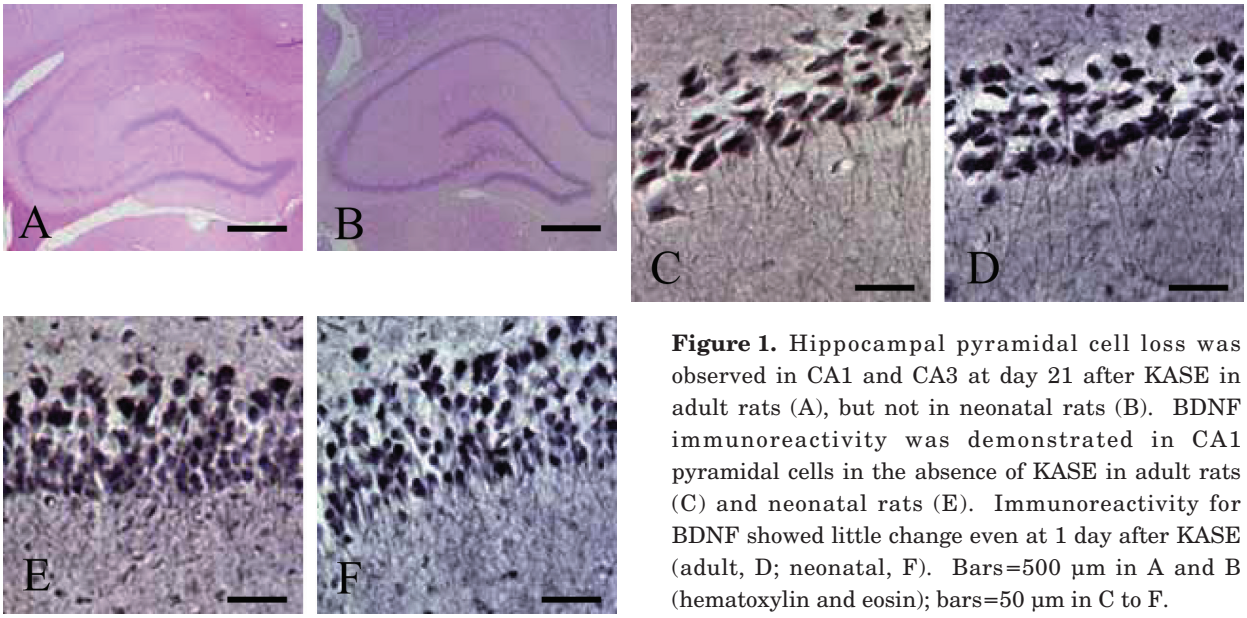

Figure 1. Hippocampal pyramidal cell loss was observed in CA1 and CA3 at day 21 after KASE in adult rats (A), but not in neonatal rats (B). BDNF immunoreactivity was demonstrated in CA1 pyramidal cells in the absence of KASE in adult rats (C) and neonatal rats (E). Immunoreactivity for BDNF showed little change even at 1 day after KASE (adult, D; neonatal, F). Bars=500 μm in A and B (hematoxylin and eosin); bars=50 μm in C to F.

Western analysis

In Western analysis of BDNF and TrkB, immunoreactive BDNF was seen as a single 14-kDa band that showed clearly increased density only at day 1 after SE, in both neonatal and adult rats (Figs. 2A and 2B). In contrast, the single 145-kDa TrkB band appeared unchanged in neonatal and adult rats through day 5 after KASE (Figs. 2C and 2D).

Densitometric analysis in rats not subjected to KASE indicated that mean BDNF expression in the hippocampus of adult rats was 492±22% of expression in neonatal rats (Fig. 3A).

In the hippocampus of neonatal rats, BDNF expression at 1, 2, 3, 4, and 5 days after SE respectively was 515±112%, 142±19%, 110±27%, 138±29%, and 158±23% of expression in neonatal rats with no SE (Fig. 3B). In summary, BDNF expression increased about five-fold at day 1 and returned to near-baseline expression by day 2 after SE. In the hippocampus of adult SE rats, BDNF expression at 1, 2, 3, 4, and 5 days after SE respectively was 314±19%, 153±21%, 112±23%, 112±13%, and 101±13% of expression in adult rats with no SE (Fig. 3). Thus, in adult rats, BDNF expression had increased about three times at day 1 after SE, returning to baseline at day 2, resembling the chronologic pattern in neonatal SE rats.

Densitometric analysis of hippocampal TrkB expression in adult rats not subjected to KASE showed mean expression to be 197±23% of expression in neonatal rat without SE (Fig. 4A).

At days 1, 2, 3, 4, and 5 after SE, hippocampal TrkB expression in neonatal rats respectively was 107±19%, 133±20%, 136±25%, 122±23%, and 115±33% of expression in neonatal rats without SE (Fig. 4B). TrkB expression in adult rats at days 1, 2, 3, 4, and 5 after SE respectively was 67±11%, 91±11%,77±17%, 57±9%, and 67±20% of expression in adult rats without SE (Fig. 4). Thus, SE slightly reduced TrkB expression in adult rats, while having essentially no effect on expression in neonatal rats.

Discussion

In our study, doses of KA required to induce SE increased from 1.5 mg/kg at 1 week of age to

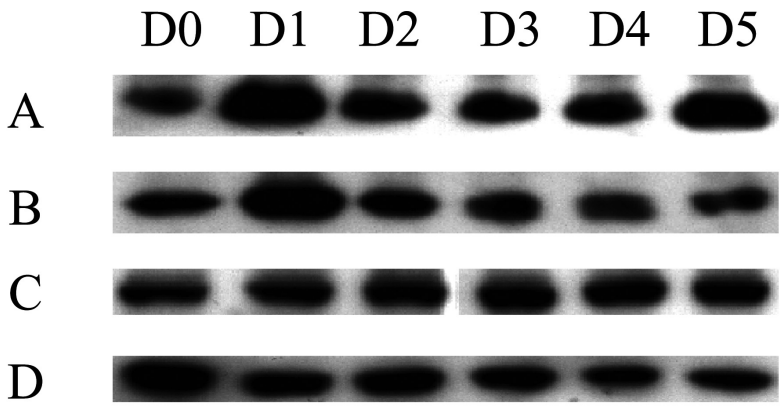

Figure 2. Western analysis of BDNF and TrkB proteins in hippocampus from neonatal and adult rats following KASE. A distinct increase in BDNF can be seen at day 1 after KASE in both neonates (A) and adults (B). TrkB expression showed little apparent difference between pre-KASE and post-KASE blots in both neonates (C) and adults (D). D0: baseline, before KASE; D1 to D5, days 1 to 5 after KASE.

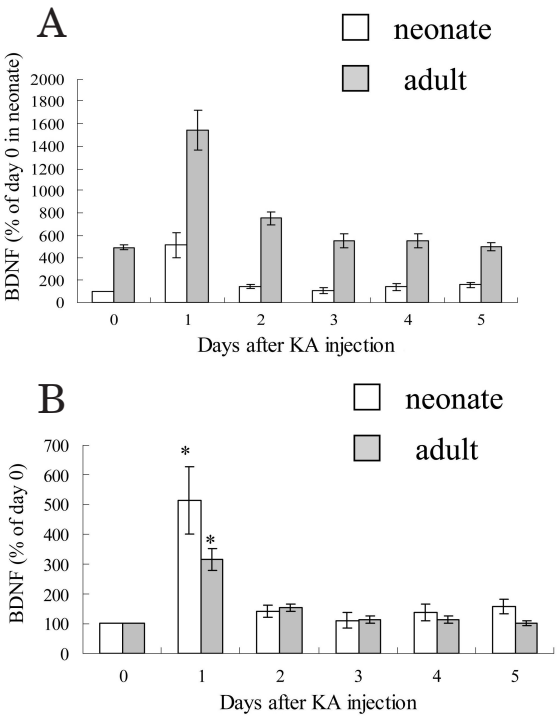

Figure 3. A, Densitometric analysis of BDNF expression after KASE according to Western blotting, relative to BDNF expression in neonatal rats with no SE (day 0). BDNF expression in the hippocampus of adult rats not subjected to KASE was 492±22% of expression in neonatal rats without SE. Data are presented as the mean±SEM for five rats. B, Densitometric analysis of BDNF expression after KASE according to Western blotting, relative to BDNF expression in neonatal or adult rats with no SE (day 0). BDNF expression in neonatal and adult rats increased about five- and threefold respectively at day 1 after KASE, returning to baseline at day 2. Data are presented as the mean±SEM for five rats. *p<0.005 vs BDNF expression in neonatal or adult rats with no SE (day 0).

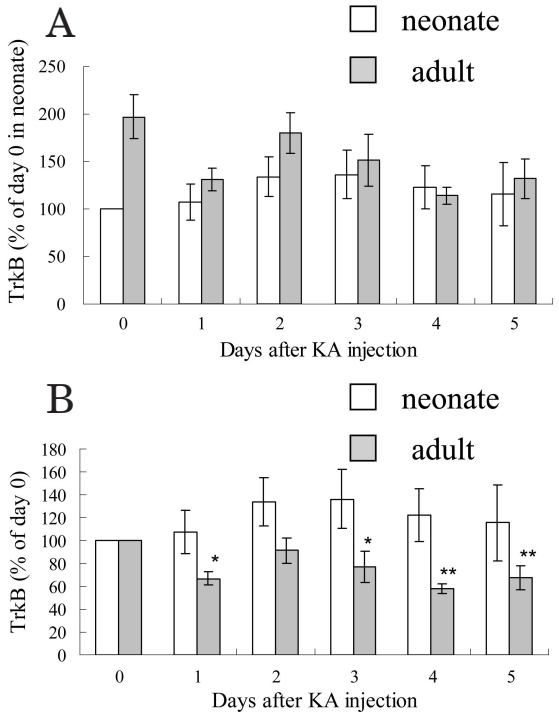

Figure 4. A, Densitometric analysis of TrkB expression after KASE according to Western blotting, relative to BDNF expression in neonatal rats with no SE (day 0). TrkB expression in the hippocampus of adult rats not subjected to KASE was 197±23% of expression in neonatal rats without SE. Data are presented as the mean±SEM for five rats. B, Densitometric analysis of TrkB expression after KASE according to Western blotting, relative to TrkB expression in neonatal or adult rats with no SE (day 0). KASE reduced TrkB slightly at days 1 and 3 to 5 after SE in adult rats, and had essentially no effect on TrkB in neonatal rats. Data are presented as the mean±SEM for five rats. *p<0.005, **p<0.05 vs TrkB expression in neonatal or adult rats without SE (day 0).

10 mg/kg at 4 or more weeks of age. KASE induced at 8 weeks of age was followed by hippocampal pyramidal cell loss and epilepsy, which did not occur in rats under the age of 4 weeks at the time of SE. Since only about one-seventh of the dose of KA required to induce SE in the adult rats was sufficient to cause SE in 1-week-old rats, the immature brain was extremely susceptible to seizures. Additionally, the results suggested that the critical period for vulnerability to epileptogenic hippocampal neuronal injury from SE began after 3 weeks of age in the rat. In human clinical observations, SE in young children induced hippocampal damage resulting in epilepsy[16,19], but SE has not been found to have this effect in the neonatal period. More observations will be important in further delineation of this critical period in humans.

Differences in seizure susceptibility and vulnerability to damage between immature and adult brains are speculated to arise from maturation, but details have remained unclear. We explored possible mechanisms underlying this difference by determining changes in expression of BDNF and TrkB in the hippocampus of neonatal and adult rats after SE.

BDNF, the most abundant neurotrophin in the brain is distributed widely; expression is particularly high in the hippocampus[3-5]. TrkB similarly is expressed mainly in the hippocampus of adult rats[4,6]. BDNF plays a critical role in survival, differentiation, and maintenance of neuronal populations during development, and also mediates synaptic plasticity and excitability[7-11]. BDNF also has been reported to be neuroprotective against excitotoxic insults in the mature brains[5,20-23], and also to protect the brain against hypoxic-ischemic injury in neonatal rats[24]. Thus, one might hypothesize that BDNF expression might be increased in the hippocampus as a protective mechanism after an insult such as SE. Several reports have concluded that SE increased hippocampal BDNF expression in adult rats and in humans[12-15]. Much less information has been available about induction of BDNF and TrkB in the neonatal rat by SE.

BDNF was demonstrated immunohistochemically in pyramidal and granule cells of the hippocampus in our neonatal and adult rats. Western analysis showed that BDNF in adult rats to be about five times higher than in neonatal rats, agreeing with prior observations that BDNF increased gradually in parallel with neuronal maturation[3,25]. SE caused threefold elevation of BDNF expression in adult rats and fivefold elevation in neonatal rats on day 1, but expression returned to baseline at day 2 after SE at both ages. Since BDNF has a neuroprotective function, this transient, rapid elevation could be interpreted as a neuroprotective mechanism. However, the response pattern of BDNF following SE in the neonatal rat was quite similar to that in the adult rat, despite a slightly somewhat greater relative elevation in neonates. This similarity of time course suggests the possibility that the age-related difference in vulnerability to injury from SE might depend on factors other than the BDNF response[26,27].

Other reports have included observations that increased expression of BDNF following SE was associated with axonal sprouting and neurogenesis of granule cells in the dentate gyrus, contributing to hyperexcitability and epileptiform activity in the adult rat[12-15,28]. Our adult rats developed spontaneous seizures after SE, but the neonatal rats did not develop epilepsy after SE despite a similar expression pattern for BDNF over time. Enhancement of BDNF expression by SE thus did not induce epilepsy in the neonatal rat brain, with effects of this enhancement being uncertain in the adult rat brain.

TrkB also was expressed on pyramidal cells and granule cells in the hippocampus. In the

absence of SE, Western analysis showed expression of TrkB in adult rats to be double that in neonatal rats. Previous results concerning expression of TrkB after SE have varied[23,29-31]. In our adult rats, TrkB expression fell slightly on days 1 and 3 to 5 after KASE. Reduction of TrkB at days 3 to 5 may have reflected pyramidal cell loss induced by KASE. A reduction in TrkB was not observed in our neonatal after KASE. Continuous administration of BDNF has been reported to lead to down-regulation of TrkB in adult rats, thus decreasing neuronal responsiveness to BDNF[13]. Considering the transient induction of BDNF in both neonatal and adult rats, SE probably had little direct effect on expression of TrkB.

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