

The Contribution of Low Affinity NGF Receptor (p75^{NGFR}) to Delayed Neuronal Death after Ischemia in the Gerbil Hippocampus

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The implication of low affinity nerve growth factor receptor (p75^{NGFR}), which is believed to play a pro-apoptotic role, in delayed neuronal death (DND) after ischemia in the gerbil hippocampus was investigated. Immunohistochemistry and Western blot analysis revealed that the presence of p75^{NGFR} immunoreactivity (IR) was negligible in the hippocampus of the sham control gerbil but appeared clearly in CA1 neurons 3 and 4 days after 5-min transient ischemia. Terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) positive nuclei appeared when the level of p75^{NGFR} IR increased. Furthermore, almost all TUNEL-positive CA1 neurons also co-stained for p75^{NGFR}. These results suggest that p75^{NGFR} contributes to DND after ischemia by an apoptotic mechanism.

Key words: p75^{NGFR}, apoptosis, delayed neuronal death, ischemia, gerbil

The biological functions of neurotrophins (NTs), including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4, promote cell survival and differentiation of central and peripheral neurons [1] through the activation of specific high-affinity tyrosine kinase (trk) receptors [2]. NGF binds to and activates trkA; BDNF and NT-4 activate trkB; and NT-3 activates trkC. All NTs also bind with equal affinity to another receptor, low affinity nerve growth factor receptor (p75^{NGFR}), which was originally characterized as an NGF receptor [3, 4]. Unlike the trks, p75^{NGFR} has a less clearly defined role but has been implicated in cell survival and apoptosis [5]. p75^{NGFR} cooperates with trk receptors to form a high-affinity binding site, and it modulates trk signaling [6]. On the other hand, p75^{NGFR} has also been found to have

a pro-apoptotic role [7]. In the presence of trkA receptors, p75^{NGFR} enhances NGF responsiveness which leads to the production of a survival signal; however, in the absence of trkA receptors, p75^{NGFR} generates an apoptotic signal [8]. The generation of the correct number of cells in the nervous system is a highly controlled and coordinated process that is the consequence of cell survival and cell death decisions made via p75^{NGFR}-induced apoptosis [5]. In addition, recent studies have demonstrated that apoptosis is involved in cell death in several pathological conditions, including ischemia [9, 10]. A 5-min occlusion of the bilateral common carotid artery induces neuronal death in the CA1 area 3 days after ischemia [11], and Nitatori *et al.* suggested that the delayed neuronal death (DND) that occurs in ischemia is related to apoptosis [9]. Expressions of p75^{NGFR} in the CA1 neurons have been observed after transient ischemia [12]. However, the relationship between p75^{NGFR} and apoptosis in DND is not yet clear. In this study, in order to examine the contribution of p75^{NGFR} to apoptosis in

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DND after cerebral ischemia, p75^{NGFR} expression and DNA fragmentation were investigated by means of immunohistochemistry, Western blot analysis, and the terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) method.

Materials and Methods

Animals and ischemia. Adult male Mongolian gerbils (wt. 60–70 g) were anesthetized with sodium pentobarbital (25 mg/kg), and transient forebrain ischemia was induced for 5 min by occlusion of the bilateral common carotid arteries. Rectal and tympanic temperatures were maintained at around 37 °C using a feedback-controlled heating pad (CMA, Stockholm, Sweden) and an overhead lamp during the operation. After restoration of blood flow, temperature was also maintained at 37 °C for 30 min. Sham-operated control animals were operated on in the same way except for the occlusion of carotid arteries.

Immunohistochemistry and TUNEL staining. 1, 2, 3, 4, and 6 days after ischemia (n = 3, respectively), the gerbils were anesthetized with sodium pentobarbital (50 mg/kg) and perfused transcardially with 1% NaNO₃ in 0.01 M phosphate buffer (PB, pH 7.4) followed by a fixative consisting of 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4) for 30 min. Sham control gerbils were also fixed in the same way (n = 3). The brains were removed, postfixed in the same fixative for 2.5 h, cryoprotected in 30% sucrose at 4 °C, and stored at –70 °C until further processing. For immunohistochemistry, 25 µm brain sections at the dorsal hippocampus level were prepared by a freezing microtome, blocked with 10% normal horse serum for 30 min, and then incubated overnight with purified MC192 (1 µg/ml at 4 °C, Boehringer Mannheim Biochemica, Indianapolis, IN, USA), a monoclonal antibody that recognizes p75^{NGFR} [13]. After being washed with PBS, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:40 dilution, Vector Laboratories Inc., Burlingame, CA, USA) for 1 h. Immunostaining of p75^{NGFR} was observed with a confocal microscope (LSM-GB200, Olympus, Tokyo, Japan) using excitation at 488 nm. Other sections were also stained with hematoxylin and eosin (HE). To detect DNA fragmentation, TUNEL was performed using an *in situ* cell death detection kit as per the manufacturer's instructions (Intergen Company, NY,

USA). TUNEL-positive nuclei were visualized using DAB and H₂O₂ with peroxidase-conjugated anti-digoxigenin. p75^{NGFR} and TUNEL co-staining were also performed 4 days after ischemia. Sections were blocked with 10% normal horse serum for 30 min after the TUNEL reaction, and this was followed by overnight incubation with MC192 at 4 °C. After being washed with PBS, the sections were incubated with a biotinylated second antibody for 30 min, followed by incubation with an avidin-biotin-alkaline phosphatase complex (ABC) for 1 h according to the supplier's recommendations (Vectastain elite ABC kit; Vector Laboratories, Burlingame, CA, USA). The sections were finally reacted with an alkaline phosphatase substrate kit (BCIP/NBT, Vector Laboratories).

Western blot analysis. The gerbils were anesthetized with ether and decapitated 4 days after ischemia or the sham operation (n = 5, respectively). Hippocampal slices (500 µm, 10–12 slices per gerbil) were made with a MacIlwain chopper and stored in ice-cold artificial cerebrospinal fluid as previously reported [14]. Slices were dissected into CA1 and other regions with a microsurgical knife under a microscope, immediately frozen with powdered dry ice, and stored at –70 °C until analysis. Western blot was performed along the lines of a previously reported method [15]. Briefly, samples were homogenized using a polytron homogenizer in a homogenizing buffer containing 10.8% sucrose and then centrifuged at 3,000 × g for 5 min. The precipitate was then dissolved in 0.1 M phosphate buffered saline with 0.5% Nonidet p-40 (Sigma, St. Louis, MO, USA) and a 10% protease inhibitor cocktail (P2714, Sigma) and then incubated on ice for 10 min. Protein concentration was determined using the Bradford method [16]. About 15 µg of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-page), and then the proteins were transferred to nitrocellulose membranes. The membranes were incubated for 3 h with MC192 (1:50 dilution) in Tris-buffered saline (TTBS) and 1% bovine albumin, and they were then incubated with peroxidase-conjugated anti-mouse IgG for 1 h. After the membranes were washed with TTBS, the ECL-WB detection system (Amersham, Tokyo, Japan) was employed as described elsewhere [14]. Immunoblot bands were quantified by a densitometric scanner (Shimadzu model CS-90000, Tokyo, Japan). Data was analyzed by the Mann-Whitney *U* test. A probability level of < 0.05 was considered statistically significant.

Results

Immunohistochemistry and TUNEL staining. Ischemic changes were observed in CA1 neurons 3 days after transient ischemia as previously reported [11]. Six days after ischemia, almost all CA1 neurons were destroyed. The presence of p75^{NGFR} immunoreactivity (IR) was negligible in the sham operation group (Fig. 1). However, 2 days after ischemia, the p75^{NGFR} IR was slightly expressed in the CA1 neurons and clearly increased 3 and 4 days after ischemia. In other areas, such as the CA3 and hilus, some p75^{NGFR} positive cells were also observed at this time. p75^{NGFR} IR decreased but still existed in the remaining cells 6 days after ischemia. TUNEL-positive nuclei were detected 3 days and increased 4 days after ischemia as previously reported [9]. Co-staining with p75^{NGFR} and TUNEL revealed that almost all TUNEL-positive neurons also exhibited p75^{NGFR} IR (Fig. 2B).

Western blot analysis. Fig. 3A shows the immunoreactive bands of p75^{NGFR}, which are similar to previously reported molecular weights [13, 15]. Immunoreactivity was quantified by densitometric scanning (Fig. 3B). In the sham operation group, the p75^{NGFR} IR of both CA1 and other areas was faint, whereas the immunoreactivity in the CA1 subfield increased significantly 4 days after ischemia ($P < 0.05$ vs. sham).

Discussion

p75^{NGFR} IR in the hippocampus was only slightly present in sham control gerbils as previously reported [17]. However, CA1 neurons with p75^{NGFR} staining began to appear on postischemic day 2, and they increased on postischemic days 3 and 4. Western blot analysis also showed a significant increase of p75^{NGFR} IR in the CA1 subfield 4 days after ischemia. On the other hand, TUNEL-positive CA1 neurons, which indicated that cell death with DNA fragmentation had occurred [18], began to appear on postischemic day 3 which was the same time the p75^{NGFR} IR increased. Furthermore, almost all CA1 neurons after ischemia showed co-staining with TUNEL and p75^{NGFR}. The fact that p75^{NGFR} IR appeared earlier than TUNEL staining suggests that p75^{NGFR} plays a pro-apoptotic role although the simple co-localization of p75^{NGFR} and TUNEL positive nuclei does not necessarily mean there is a direct relation between them.

Lee *et al.* reported that p75^{NGFR} IR was induced at 1 h, increased 1 day after recirculation in the CA1 neurons, and was widely expressed in the surviving neurons at 7 days after ischemia [12]. On the other hand, in the present study, p75^{NGFR} IR was induced 2 days after recirculation with the peak occurring at 3 or 4 days and decreasing 6 days after ischemia. This discrepancy may have arisen from a difference in the duration time of ischemia in these experiments. In the present study, ischemia was induced for 5 min by occlusion of the bilateral common carotid arteries, while occlusion was induced for 3.5 min in Lee's study [12]. The expression of p75^{NGFR} might be influenced by the extent of the ischemia.

NGF in the rat CA1 subfield was found to decrease after 2 days in a previous study, but it recovered to the control level 2–7 days after transient ischemia [19]. On the other hand, trkA-expression was downregulated in the hippocampal formation by kainic acid injection [20]. p75^{NGFR} expression after ischemia might cause ischemic cell death because the binding of NGF to p75^{NGFR} could promote cell death in the absence of a trkA-initiated signaling cascade [8]. Oh *et al.* also demonstrated that overexpression of p75^{NGFR} in cholinergic neurons was induced by administration of kainic acid into the rat basal forebrain and contributed to excitotoxin-induced cell death by an apoptotic-like mechanism [21]. Results from such studies, taken together with the present results, suggest that the activation of p75^{NGFR} is caused by ischemia and that the resulting substantial increase in p75^{NGFR} expression contributes to DND by an apoptotic mechanism. In addition, the roles of p75^{NGFR} in cooperating with trk and in mediating apoptosis are complex, and the p75^{NGFR} signaling system is not fully understood. There is still a possibility that the marked expression of p75^{NGFR} is due to upregulation for the prevention of neuronal cell death [12]. Further investigation is needed to elucidate the mechanism of p75^{NGFR} induced apoptosis. The transient ischemic model may be useful in investigating the role of p75^{NGFR} in cell survival and death because of the concurrence of p75^{NGFR} expression and apoptosis in this model.

In conclusion, p75^{NGFR}, which is suspected to be a pro-apoptotic receptor, was found to be clearly expressed in CA1 neurons after ischemia, and these neurons were also found to contain TUNEL-positive nuclei. These results support the suggestion that p75^{NGFR} plays a pro-apoptotic role and suggest that p75^{NGFR} induces apoptosis in DND after ischemia.

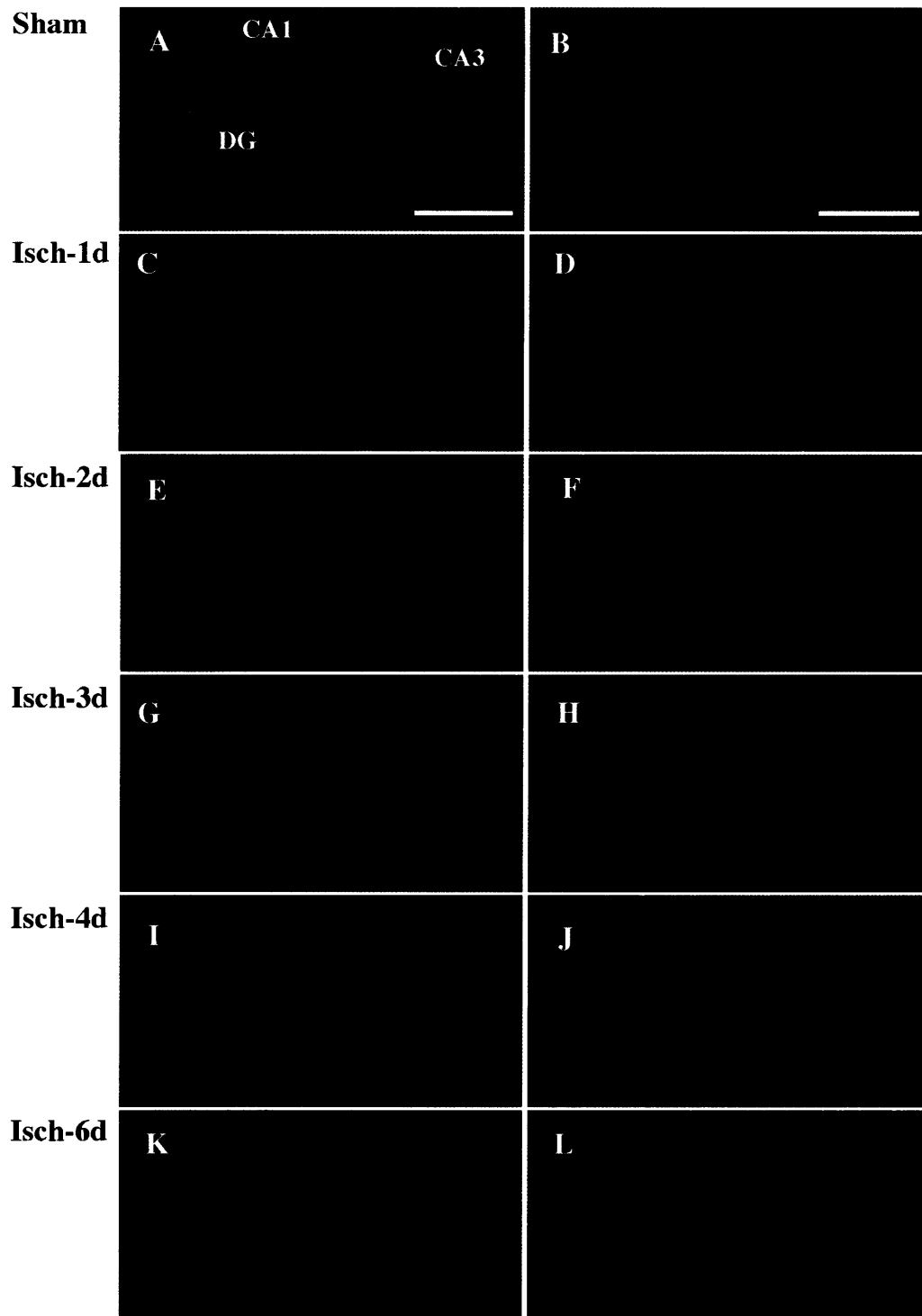


Fig. 1 Representative immunohistochemistry of p75^{NGFR} from the hippocampus (A, C, E, G, I, K) and high magnification of the CA1 pyramidal layer (B, D, F, H, J, L) following transient forebrain ischemia. Sham control (A, B) and postischemic days 1 (C, D), 2 (E, F), 3 (G, H), 4 (I, J), and 6 (K, L), respectively. p75^{NGFR} immunoreactivity (IR) was slightly present in the hippocampus (A, B); however, it was clearly expressed in CA1 neurons on postischemic days 3 and 4 (G, H, I, J). p75^{NGFR} IR was still observed in surviving CA1 cells on postischemic day 6 (K, L). Bars = 500 μ m in low magnification and 50 μ m in high magnification.

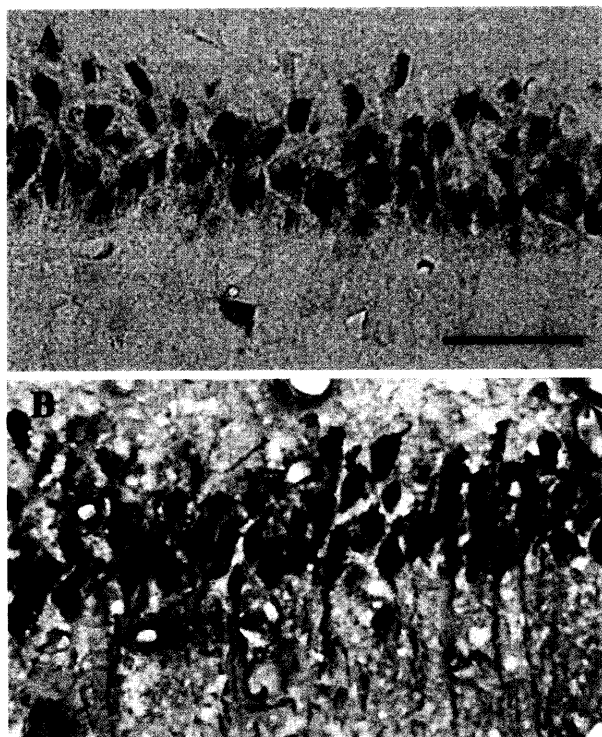


Fig. 2 Terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) (A) and co-labeling with p75^{NGFR} and TUNEL (B) in CA1 hippocampal neurons 4 days after transient ischemia. p75^{NGFR} immunoreactivity is visualized in blue and TUNEL in brown in panel B. There is almost an overlap between p75^{NGFR} expression and the presence of TUNEL-positive nuclei in CA1 neurons. Bar = 50 μm.

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References

- Lewin GR and Barde YA: Physiology of the neurotrophins. *Annu Rev Neurosci* (1996) **19**, 289–317.
- Bothwell M: Functional interactions of neurotrophins and neurotrophin receptors. *Annu Rev Neurosci* (1995) **18**, 223–253.
- Gotz R, Kolbeck R, Lottspeich F and Barde YA: Production and characterization of recombinant mouse neurotrophin-3. *Eur J Biochem* (1992) **204**, 745–749.
- Rodriguez-Tebar A, Dechant G and Barde YA: Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron* (1990) **4**, 487–492.
- Casaccia-Bonnel P, Gu C, Khursigara G and Chao MV: p75 neurotrophin receptor as a modulator of survival and death decisions. *Microsc Res Tech* (1999) **45**, 217–224.
- Chao MV and Hempstead BL: p75 and Trk: A two-receptor system. *Trends Neurosci* (1995) **18**, 321–326.
- Bono F, Lamarche I, Bornia J, Savi P, Della Valle G and Herbert JM: Nerve growth factor (NGF) exerts its pro-apoptotic effect via the p75NTR receptor in a cell cycle-dependent manner. *FEBS Lett* (1999) **457**, 93–97.
- Yoon SO, Casaccia-Bonnel P, Carter B and Chao MV: Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. *J Neurosci* (1998) **18**, 3273–3281.
- Nitatori T, Sato N, Waguri S, Karasawa Y, Araki H, Shibani K, Kominami E and Uchiyama Y: Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. *J Neurosci* (1995) **15**, 1001–1011.
- Hara A, Niwa M, Iwai T, Nakashima M, Yano H, Uematsu T, Yoshimi N and Mori H: Transport of fragmented DNA in apical dendrites of gerbil CA1 pyramidal neurons following transient forebrain ischemia. *Brain Res* (1998) **806**, 274–277.
- Kirino T: Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res* (1982) **239**, 57–69.
- Lee TH, Abe K, Kogure K and Itoyama Y: Expressions of nerve growth factor and p75 low affinity receptor after transient forebrain ischemia in gerbil hippocampal CA1 neurons. *J Neurosci Res* (1995) **41**, 684–695.
- Chandler CE, Parsons LM, Hosang M and Shooter EM: A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. *J Biol Chem* (1984) **259**, 6882–6889.
- Tokuda M, Ahmed BY, Lu YF, Matsui H, Miyamoto O, Yamaguchi F, Konishi R and Hatase O: Involvement of calmodulin-dependent protein kinases-I and -IV in long-term potentiation. *Brain Res* (1997) **755**,

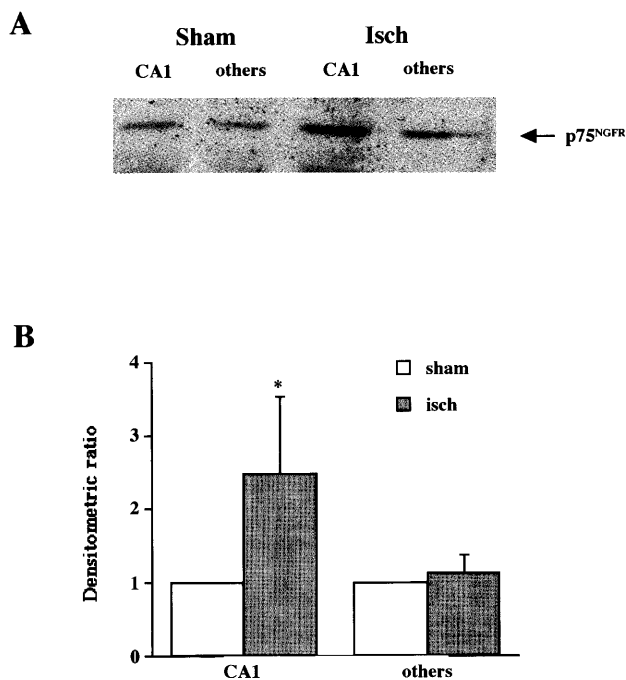


Fig. 3 Western blot analysis of p75^{NGFR}. A, Immunoreactive band of p75^{NGFR} from the CA1 region (CA1) and other regions (others) of the hippocampus in the sham control group (Sham) and in the ischemic group 4 days after ischemia (Isch). B, Densitometric intensities of the immunodetected bands shown in A. A significant increase of p75^{NGFR} IR was observed in the dissected tissues from the CA1 region 4 days after ischemia. The values are given as the mean ± SD *P < 0.05 compared with sham control.

- 162-166.
15. Allendoerfer KL, Shelton DL, Shooter EM and Shatz CJ: Nerve growth factor receptor immunoreactivity is transiently associated with the subplate neurons of the mammalian cerebral cortex. *Proc Natl Acad Sci USA* (1990) **87**, 187-190.
 16. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* (1976) **72**, 248-254.
 17. Pioro EP and Cuello AC: Distribution of nerve growth factor receptor-like immunoreactivity in the adult rat central nervous system. Effect of colchicine and correlation with the cholinergic system-I. *Forebrain. Neuroscience* (1990) **34**, 57-87.
 18. Gavrieli Y, Sherman Y and Ben-Sasson SA: Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J Cell Biol* (1992) **119**, 493-501.
 19. Shozuhara H, Onodera H, Katoh-Semba R, Kato K, Yamasaki Y and Kogure K: Temporal profiles of nerve growth factor beta-subunit level in rat brain regions after transient ischemia. *J Neurochem* (1992) **59**, 175-180.
 20. Venero JL and Hefti F: Regionally specific induction of BDNF and truncated trkB.T1 receptors in the hippocampal formation after intraseptal injection of kainic acid. *Brain Res* (1998) **790**, 270-277.
 21. Oh JD, Chartisathian K, Chase TN and Butcher LL: Overexpression of neurotrophin receptor p75 contributes to the excitotoxin-induced cholinergic neuronal death in rat basal forebrain. *Brain Res* (2000) **853**, 174-185.