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Temporal changes in the level of neurotrophins in the spinal cord and associated precentral gyrus following spinal hemisection in adult Rhesus monkeys

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ABSTRACT

Neurotrophins (NTs) appear to be crucial for the survival and potential regeneration of injured neurons. However, their temporal changes and remote regulations following spinal cord injury (SCI) have been only partially determined, especially in primates. In this study, ELISA was performed on the extracts of injured spinal cord and the associated precentral gyrus contralateral to the site of spinal cord hemisection to investigate the temporal changes in the levels of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) in adult rhesus monkeys subjected to T8 spinal hemisection. Animals were allowed to survive 3, 7, 14, 30 and 90 days post-operation (dpo). In the spinal cord, the levels of NGF, BDNF and NT-3 sharply decreased between 3 and 7 dpo. Thereafter, the levels of NGF and BDNF were transiently elevated while NT-3 level continuously increased and recovered to normal level at 30 dpo. In the contralateral precentral gyrus (cPG), only the NT-3 level was altered and in fact elevated above the normal value. No obvious changes were observed in NT-4 level in any of the regions studied. Taken together, the present findings indicated that intrinsic NGF, BDNF and NT-3 may play a local role in the responses to the SCI in primates. Especially, the increase of NT-3 level occurred continuously in both the cPG and the spinal cord pointed to a possible transportation of NT-3 to the cord following SCI.

1. Introduction

Spinal cord injury (SCI) often results in significant neurological dysfunction and disability, including muscle atrophy, faster contractile properties, and increased fatigability. Currently, there are still no effective measures for repair of the damaged spinal cord (SC). The limited regenerative ability of the mammalian central nervous system (CNS) may be attributed to the lack of trophic support, activation of immune response and inflammatory processes, the presence of glial scars, which are inhibitory to structural repairs (Widenfalk et al., 2001; Koda et al., 2001), and other factors.

It has long been held that endogenous neurotrophins (NTs) including nerve growth factor (NGF) (Levi-Montalcini and Hamburger, 1953), brain-derived neurotrophic factor (BDNF) (Barde et al., 1982), neurotrophin-3 (NT-3) (Hohn et al., 1990) and neurotrophin-4/5 (NT-4/5) (Berkemeier et al., 1991) can lessen the loss of neurons and stimulate the elongation of axons. NTs have been reported to exert beneficial effects when delivered through different carriers (Grill et al., 1997; Kobayash et al., 1997; Ye and Houle, 1997; Koda et al., 2001; Kamada et al., 2004) or combined with fetal spinal cord transplantation (Bregman et al., 1997, 1998) after SCI. However, the expression levels of endogenous NTs following SCI has only been partially investigated (Jakeman et al., 1998; Dreyfus et al., 1999; Johnson et al., 2000; Nakamura and Bregman, 2001; Widenfalk et al., 2001; Murakami et al., 2002; Brown et al., 2004; Gulino et al., 2004; Qin et al., 2006). Furthermore, most of these studies used rats as the animal model. Little was known in the changes of NTs in the SC of primates. As the anatomical structures and physiological functions of primates are closer to those of human in comparison with rodents, it would be an advantage to use primates as the animal model to gain a better understanding of SCI in the human. Our recent study showed that the numbers of NTs immunopositive neurons and their optical densities all increased in the hemisected spinal cord in the rat (Qin et al., 2006). Would NTs be also conserved across different

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mammalian genera? Would NTs be also involved in neuroplasticity in primates subjected to SCI?

In addition, NTs are generally known to be target-derived (Mufson et al., 1996) and axonal transportation of NTs between brain and SC has been demonstrated (Altar et al., 1997; Kohara et al., 2001). Should this axonal transportation be interrupted by SCI, for example, would there be changes in the NTs expression in the precentral gyrus following SCI?

To answer the above questions, this study performed spinal cord hemisection in adult Rhesus monkeys and investigated possible changes in the levels of selective NTs in the spinal cord and the precentral gyrus contralateral to the site of injury at different time points by using ELISA. Various changes were observed in the levels of NGF, BDNF and NT-3 in the SC. However, only changes in the NT-3 level were detected in both the SC and contralateral precentral gyrus (cPG). The increase of NT-3 in the spinal cord could be attributed to its transport from the cPG. The study opens the possibility of exogenous administration of NT-3 into the precentral gyrus to aid the said phenomenon.

2. Materials and methods

2.1. Animals and treatment

Twenty-two adult male Rhesus monkeys weighing about 5–7 kg were obtained from the Animal Experimental Center of Sichuan University. They were individually housed in a 12/12 h light/dark, quiet and without strong-light vivarium with free access to water and food. The animals were cared for in accordance with the Guidelines for the care and use of Laboratory Animal published by NIH (1996). Every effort was made to minimize both the suffering and wastage of animals. Two normal animals were used to determine the immunohistochemical distributions of NGF, BDNF, NT-3 and NT-3. Another two animals were used to determine the characteristics of antibodies and to detect the normal levels of the above mentioned NTs in the SC and cPG. The remaining animals were divided into the following 6 groups: 3 of them were sham-operated (Group A); the remaining ones ($n = 3$ in each group) were subjected to spinal cord hemisection and killed at 3 days (Group B), 7 days (Group C), 14 days (Group D), 30 days (Group E), and 90 days (Group F) after spinal cord hemisection.

2.2. Surgery

The animals were deeply anesthetized by intramuscular injection of chloramines (0.25 ml/kg, Sigma Chemical Co., St. Louis, MO). The T11 vertebral lamina was removed and the dura mater was cut with a microsurgical knife with the aid of an operating microscope. About 1 mm segment of the left half of the spinal cord (corresponding to T8 spinal segment) was removed. The hemisection site was then filled with sterile gelfoam, and the superficial back muscles and skin were sutured along the midline. For sham-operation, the above procedure was carried out short of spinal cord hemisection. Post-operative care included evacuating the urinary bladder three times daily by manual abdominal compression until urinary reflex was once again established. All the animals survived after operation and were housed in individual cages without the administration of any drugs.

2.3. Tissue preparation

For immunohistochemical study, the monkeys were perfused through the ascending aorta with 500 ml of normal saline, followed by 1 l of Zamboni's fixative (2% paraformaldehyde + 15% saturated picric acid). The SC and the brain tissues were quickly removed and stored at −80 until use. For Western blot and ELISA analysis, the cerebral cortex, hippocampus and T8–T9 spinal segments were removed and dissected (~0.5 cm^2) on ice, followed by careful removal of the meninges, vessels, spinal roots and dorsal root ganglia. All the tissue samples obtained were stored at −80 until use.

2.4. ELISA

ELISA methods were based on the procedure described by Takumi et al. (2005). We used each of four kinds of antibodies as the coating antibody: anti-NGF mouse monoclonal IgG (Promega), anti-BDNF mouse monoclonal IgG (Promega), anti-NT-3 mouse monoclonal IgG (Promega) and anti-NT-4 mouse monoclonal IgG (Promega). For the primary antibody, we used the following: anti-NGF chick polyclonal IgY (Promega), anti-BDNF chick polyclonal IgY (Promega), anti-NT-4 chick polyclonal IgY (Promega), anti-NT-3 chick polyclonal IgY (Promega) and normal chick IgY (Promega). Horseradish peroxidase (HRP)-conjugated anti-chick IgY antibody (Promega) was used as secondary antibody. Standard curves of ELISAs of NGF, BDNF, NT-3 and NT-4 were used to determine of concentrations.

2.5. Western blot and immunohistochemical procedure

Western blot and immunohistochemical procedure have been described in our previous study (Qin et al., 2006; Zhang et al., 2007). The primary antibodies used were as followed: affinity-purified rabbit polyclonal antibody for NGF (Chemicon, Temecula; No. AB1526P, 1:200 dilution), BDNF (Chemicon, Temecula; No. AB1779, 1:200 dilution), NT-3 (a gift from Prof. Xin-Fu Zhou (Centre for neuroscience, Department of Physiology, Flinders University of South Australia), 1:200 dilution) and NT-4 (Chemicon, Temecula; No. AB1781, 1:200 dilution). Biotinylated anti-rabbit IgG was used as secondary antibody (Vector Labs; 1:400 dilution). Negative control for immunostaining specificity was performed by omitting the primary antibody or by the preabsorption of antibodies with the appropriate NTs. These controls did not exhibit any specific immunostaining. A positive control labeling with each of these antibodies in the hippocampus was performed at the same time. In addition, Western blot analysis gives further support for the specificities of the antibodies used for monkeys.

2.6. Statistical analysis

The values of NTs were expressed as picograms per gram of tissue wet weight. All values were expressed as means ± S.E.M. The statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison tests. $P < 0.05$ or $P < 0.01$ was considered significant. Each experiment consisted of at least three replicates per condition. Statistical analyses were performed using SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Specificities of ELISA system and antibodies for immunostaining

The two-site ELISA system reported in this study was highly specific for NGF, BDNF, NT-3 and NT-4 (Fig. 1A). The sensitivity of our ELISA system for NTs were as low as 1 pg/ml and we could detect low levels of NTs without any cross-reactivity with the other NTs.

The specificity of the antisera for NTs was initially tested by Western blot analysis. Western blot analysis showed that each antibody specifically recognized appropriate bands at a molecular weight of approximately 14.7, 14.2, 13.6 and 14.5 kDa, for NGF, BDNF, NT-3 and NT-4, respectively (Fig. 1B). These positions coincided with the molecular weights of the NTs studied. Immunohistochemical control was also used to detect the specificity of the antisera. Intense to moderate labellings of NGF, BDNF, NT-3 and NT-4 were observed in the hippocampus (Fig. 1C). This pattern of immunostaining could be eliminated by incubation with the pre-absorbed primary antibodies (Fig. 1C).

3.2. Distribution and levels of NTs in normal monkey SC and cPG

3.2.1. Spinal cord

The immunoreactive products for all the NTs in the spinal ventral horn were present in neurons that appeared to be motoneurons and interneurons. NGF- and NT-4 immunoreactive products appeared lightly stained in the cytoplasm of the ventral motoneurons. Most of the nuclei and fibers appeared unstained. BDNF immunoreactive products were localized mainly in the cytoplasm of neurons, but some nuclei were also stained. A large dense body showing NT-3 IR is evident around the nuclei; some dendritic arborizations and axons were intensely stained (Fig. 2A).

In the normal monkey SC, we detected 400 pg of NGF/g wet tissue, 580–590 pg of BDNF/g wet tissue, 150–160 pg of NT-3/g wet tissue, and 70–80 pg of NT-4/g wet tissue (Fig. 2A).

3.2.2. cPG

The NGF-, BDNF-, NT-3- and NT-4-immunopositive neurons were observed throughout all layers (except layer I) of the motor cortex in the precentral gyrus (areas 4 and 6). Intensely labeled neurons were scattered in layers II/III (external pyramidal layer) and V (internal pyramidal layer). Based upon their somatodendritic