Amino Acids (1998) 14: 121–129

Amino
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Brain derived neurotrophic factor and insulin like growth factor-1 attenuate upregulation of nitric oxide synthase and cell injury following trauma to the spinal cord

An immunohistochemical study in the rat

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Accepted September 26, 1997

Summary. The possibility that brain derived neurotrophic factor (BDNF) and insulin like growth factor-1 (IGF) induced neuroprotection is influenced by mechanisms involving nitric oxide was examined in a rat model of focal spinal cord injury. BDNF or IGF-I (0.1 μg/10 μl in phosphate buffer saline) was applied topically 30 min before injury on the exposed spinal cord followed by repeated doses of growth factors immediately before and 30 min after injury. Thereafter application of BDNF or IGF was carried out at every 1 h interval until sacrifice. Five hours after injury, the tissue pieces from the T9 segment were processed for nNOS immunostaining, edema and cell injury. Untreated injured rats showed a profound upregulation of nNOS which was most pronounced in the nerve cells of the ipsilateral side. A marked increase in the blood-spinal cord barrier (BSCB) permeability to ^{125}I-albumin, water content and cell injury in these perifocal segments was also found. Pretreatment with BDNF and IGF significantly reduced the upregulation of nNOS in the spinal cord. This effect of the growth factors was most pronounced in the contralateral side. Rats treated with these neurotrophic factors showed much less signs of BSCB damage, edema and cell injury. These results suggest that BDNF and IGF pretreatment is neuroprotective in spinal cord injury and that these neurotrophic factors have the capacity to down regulate nNOS expression following trauma to the spinal cord. Our data provide new experimental evidences which suggest that BDNF and IGF may exert their potential neuroprotective effects probably via regulation of NOS activity.

Keywords: Brain derived neurotrophic factor – Insulin like growth factor-1 – Nitric oxide – Spinal cord injury – Edema – Cell injury – Blood-spinal cord barrier – Immunohistochemistry

Introduction

Nitric oxide (NO) is a free radical gaseous molecule which serve as a messenger molecule in the central nervous system (CNS) (Bredt et al., 1991; Dawson and Dawson, 1996). NO is synthesises by the enzyme nitric oxide synthase (NOS) which is found in various isoforms in different types of cells in the CNS or in the periphery (Kimura and Steinbusch, 1996). Nerve cells in the brain express a constitutive isoform of the enzyme which was originally isolated and cloned by Bredt, Snyder and their colleagues (Bredt et al., 1991). In adult spinal cord, NOS positive neurons are mainly located in the superficial dorsal horn and in lamina X (Dun et al., 1993).

Recent evidences suggest that NO is involved in the pathophysiological mechanisms of cell injury in the CNS following ischemia, stroke and trauma (Dawson and Dawson, 1996). Experiments carried out in our laboratory in the past suggest that a focal spinal cord injury has the capacity to induce an upregulation of NOS in spinal cord neurons which normally do not exhibit NOS activity (Sharma et al., 1996a; b). This upregulation of NOS is well correlated with edema and cell changes in the spinal cord 5 h after focal incision of the right dorsal horn (Sharma et al., 1996a). These observations point to a role of NO in the pathophysiology of spinal cord injury. However, the detail mechanisms of NOS induced cell injury are not known.

Neurotrophins constitute a family of five related polypeptides which are commonly recognised as neurotrophic factors or neuronal survival promoting factors (Davies, 1994; Hotzman et al., 1994). There are reports that neurotrophins can influence or induce neurotransmitter expression selectively. Thus nerve growth factor (NGF) and BDNF, have the capacity to induce somatostatin and neuropeptide Y synthesis in cultured cortical neurons without influencing the cell survival (Nawa et al., 1993). Intravenous administration of BDNF to the newborn rats induces synthesis of several neuropeptides in the CNS (Nawa et al., 1994) and NGF can induce selective expression of neuropeptides in the sensory neurons (Mulderry 1994). These observations suggest that neurotrophins can influence neural transmission and thus modify the consequences of cell injury following trauma. However the influence of neurotrophic factors on expression of NOS enzyme or NO production is so far not studied either in the developing or adult CNS.

Experimental evidence suggests that local application of neurotrophins in the region of ischemia attenuates cell death and promote cell survival (Huber et al., 1995; Yan et al., 1992). Thus, brain-derived neurotrophic factor (BDNF) and insulin like growth factor (IGF-I) are found to be neuroprotective in various other models of ischemic brain injury (Davies, 1994; Yan et al., 1992; Yu, 1994). This indicates that neurotrophins are neuroprotective agents in traumatic or ischemic insults to the adult CNS as well. The probable mechanisms of neurotrophins induced neuroprotection are not known in all details.

This investigation was undertaken to examine whether pretreatment with BDNF and IGF is neuroprotective in our model of rat spinal cord injury and if so whether this neuroprotection offered by these neurotrophic factors is somehow associated with NOS upregulation.

Materials and methods

Animals

Experiments were carried out on 30 male Sprague Dawley rats (body weight 250–300 g) under equithesin (3 ml/kg, i.p.) anaesthesia. The animals were housed at controlled room temperature (21 ± 1°C) with 12 h light and 12 h dark schedule. The rat food and tap water were supplied *ad libitum* before the experiment.

Spinal cord injury

One segment laminectomy was done on the T10-11 segment and the spinal cord was exposed by carefully removing the dura mater. The exposed spinal cord was covered with surgical cotton soaked in physiological saline at room temperature. About 30 min after stabilisation period, spinal cord injury was made using a sterile scalpel blade. A focal injury was made by hand on the exposed spinal cord by making an incision of the right dorsal horn of the T10-11 segments. The deepest part of the lesion was very close to the lamina VII-VIII (Sharma et al., 1996a). This experimental condition was approved by the Ethical Committee of Banaras Hindu University, Varanasi, India and Uppsala University, Uppsala, Sweden.

Treatment with neurotrophins

In separate group of animals BDNF or IGF-I (0.1 μg/10 μl in phosphate buffer saline) was applied topically 30 min before injury on the exposed spinal cord followed by repeated doses of growth factors immediately before and 30 min after injury. Thereafter application of BDNF or IGF was carried out at every 1 h interval until sacrifice. Five hours after injury, the tissue pieces from the T9 and T12 segments were processed for neuronal NOS immunostaining.

Control group

Normal animals served as intact controls. For comparison with the drug treatment group, spinal cord traumatised rats subjected to saline application were used as experimental controls.

Parameters measured

The following parameters were examined in control, spinal cord injured and neurotrophins treated injured animals.

NOS immunohistochemistry. Five h after spinal cord injury, the animals were perfused with about 200 ml of 4% paraformaldehyde fixative in 0.1 M phosphate buffer pH 7.4 at 4°C preceded with a brief saline rinse. After perfusion, the spinal cord was dissected out and the T9, T10-11 and T12 segments were dissected out. About 60 μm thick vibratome multiple sections were cut from the T9 and T12 segments and processed for NOS immunoreactivity using monoclonal antiserum to constitutive isoform of neuronal NOS as described earlier (Sharma et al., 1996a; 1997a; 1998).

Microvascular permeability and edema. In separate group of rats fresh spinal cord tissues from the T9 and T12 segments were used for determination of spinal cord water content and microvascular permeability to $^{[131]}$I-sodium (Sharma et al., 1997b). For this purpose radioactive iodine (about 0.5×10^6 cpm) in 0.9% saline was injected into the right femoral vein 5 min before termination of the experiment. The intravascular tracer was washed out by a brief saline rinse through heart (Sharma et al., 1990). Tissue pieces from T9, T10-11 and T12 were dissected out, weighed immediately and counted in a 3-in well type gamma counter (energy window 500–800 KeV). After counting the radioactivity the tissue samples were dried in an incubator maintained at 90°C for 72 h (Sharma et al., 1997b).

Cell changes. Some tissue pieces were embedded in epon for routine light and electron microscopy for morphological examination of cell changes using standard procedures (Sharma et al., 1997a, b).

Statistical analysis

The quantitative data obtained was analysed for statistical significance using unpaired Student's t-test. A p value less than 0.05 was considered to be significant.

Results

Effect of neurotrophins on visual swelling

Spinal cord traumatised rats showed signs of haemorrhage and visual swelling immediately after lesion. This swelling continued to expand over a 5 h period. Pretreatment with neurotrophins either BDNF or IGF-1 markedly attenuated the occurrence of gross visual swelling after injury without influencing the occurrence of microhaemorrhages immediately following lesion. The visual swelling seen after 5 h injury in neurotrophins treated rats is markedly less compared to untreated injured rats.

Effect of neurotrophins on NOS upregulation

A representative example of NOS upregulation following 5 h spinal cord injury in the ventral gray matter of the ipsilateral side of the cord and its modification with BDNF treatment is shown in Fig. 1. As apparent from the figure, pretreatment of rats with BDNF significantly attenuated NOS upregulation in the motoneuron compared to the untreated injured rat.

Similar results were also obtained with IGF-1 treatment (Fig 2). Semiquantitative analysis showed that untreated injured rats showed marked upregulation of the constitutive isoform of neuronal NOS. This effect was most pronounced in the motoneurons of the ipsilateral side (Fig. 2). Pretreatment with BDNF and IGF-1 markedly reduced the upregulation of neuronal NOS in the spinal cord. This effect of the growth factors was most pronounced in the contralateral side (Fig. 2).

Effect of neurotrophins on edema and microvascular permeability

Spinal cord injured rats exhibited marked increase in the blood-spinal cord barrier (BSCB) permeability to ^{125}I-albumin and water content in T9 and T12 perifocal segments (Sharma et al., 1996). Pretreatment with neurotrophic factors significantly attenuated the extravasation of iodine across the BSCB (Sharma et al., unpublished observations) and reduced the edema formation as seen by changes in the spinal cord water content (Fig. 2).

Effect of neurotrophins on cell changes

The cell changes following spinal cord injury were considerable reduced by application of neurotrophins (results not shown). Thus, spinal cord samples of

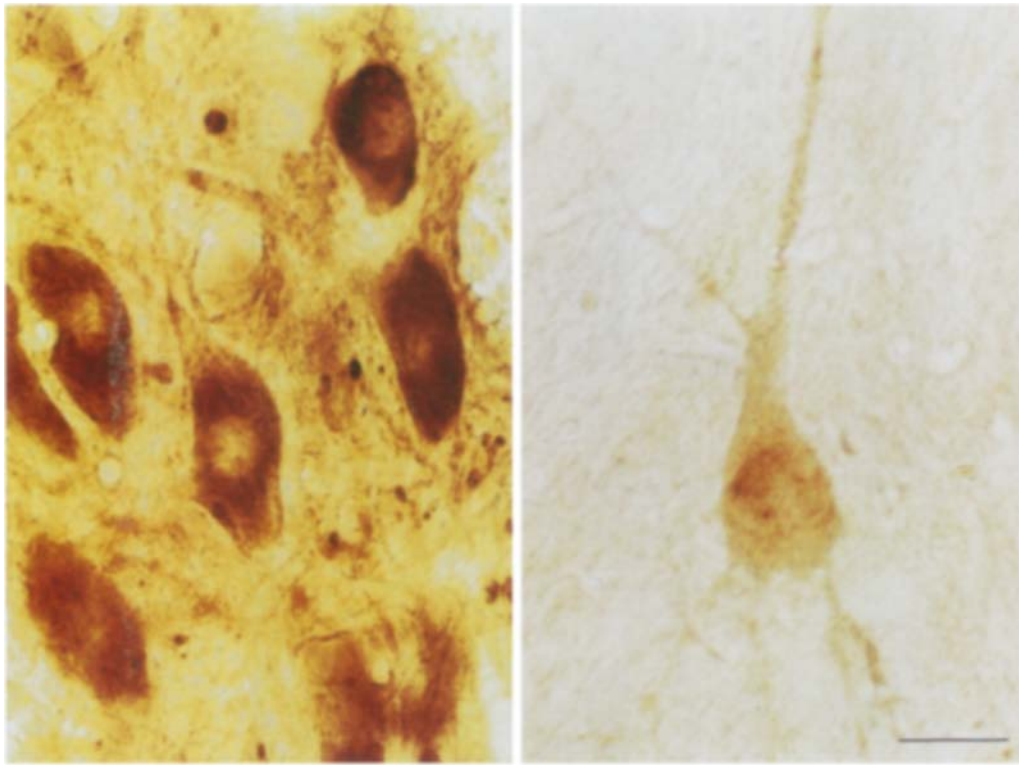

Fig. 1. A representative example of NOS immunoreactivity in the ipsilateral ventral horn of T9 segment after 5 h spinal cord injury (left) and its modification with BDNF treatment (right). Pretreatment with BDNF significantly thwarted the NOS immunostaining in the traumatised cord. Thus only a few NOS positive cells are visible in the treated rat compared to untreated injured rat (bar = 20 μm)

T9 and T12 segments from untreated rats showed profound cell injury and damage to the nerve cells, glial cells and myelin. Application of BDNF or IGF significantly attenuated the cell injury following trauma (results not shown).

Discussion

Our results strongly indicate that neurotrophins can induce significant neuroprotection in the injured spinal cord of the adult rat. This is evident from the fact that pretreatment with neurotrophins significantly reduced the occurrence of visual swelling in the traumatised spinal cord seen at 5 h after injury. This observation is in line with previous data in ischemia in which application of neurotrophins has reduced the ischemic cell damage (Yan et al., 1992). Our results further suggest that neurotrophins can reduce microvascular permeability disturbances and edema formation following spinal cord injury. This suggests that neurotrophins also influence the secondary injury process and alterations in the microfluid environment.

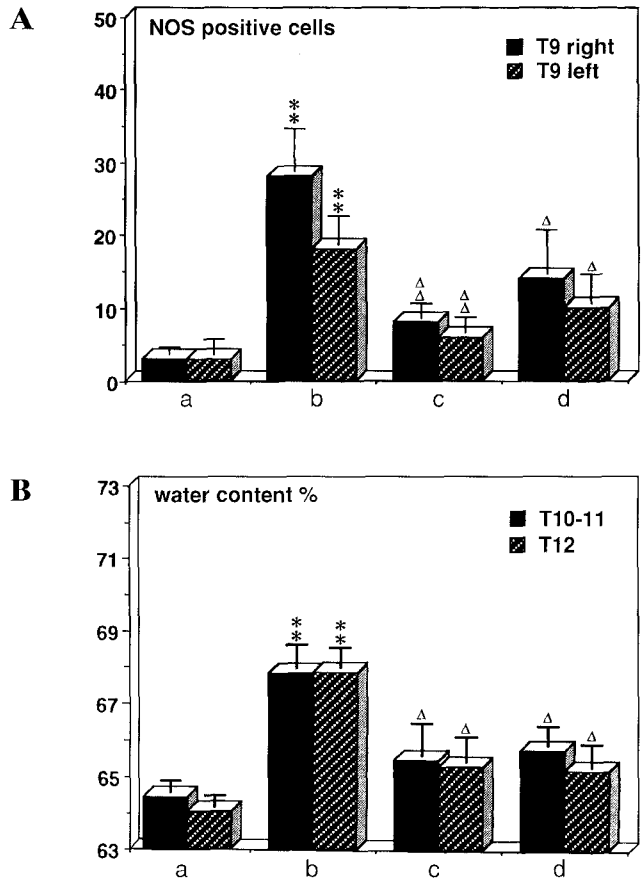

Fig. 2. A Semiquantitative analysis of NOS upregulation in the spinal cord following trauma and its modification by BDNF or IGF-1 treatment. **B** Water content following spinal cord injury and its modification with BDNF and IGF-1 treatment (**$p < 0.01$, Student's unpaired t-test compared from control group; $\Delta\Delta p < 0.01$, compared from spinal cord injury group). *a* control; *b* SCI; *c* BDNF + SCI; *d* IGF + SCI; *SCI* spinal cord injury

The mechanisms by which neurotrophins exert neuroprotective effects are still unclear. There are recent data suggesting that motoneurons and other neuronal populations in the spinal cord are important targets of neurotrophins. BDNF mRNA is produced in dorsal root ganglion which is anterogradely transported to the neurons in the dorsal horn of the spinal cord (Huber et al., 1995). Most of the neuronal population in the spinal cord express receptors for neurotrophins such as trk-B, trk-C and low-affinity neurotrophin receptor p75 (Schwab and Bartholdi, 1996).

Neurotrophins promote survival of the embryonic motoneurons *w* and prevent loss of motoneurons following axotomy *in vivo* (Yan et al., 1992). There are also reports that peripheral axotomy induces NOS upregulation in the spinal cord motoneurons (Yu et al., 1994). This indicates that NOS upregulation is somehow involved in the mechanisms of damage to motoneurons. In experimental model, Novikov et al. (1995) showed that BDNF

pretreatment significantly reduced the upregulation of NOS following axotomy. This indicates that probably neurotrophins influence NOS upregulation.

Neurotrophins apart from their role as trophic support, are capable of modifying intracellular signal transduction mechanisms by influencing various neurotransmitter regulation. Thus NGF is known to induce selective neuropeptide upregulation in sensory neurons (Lindsay and Harmer, 1991). In one study, NGF was shown to induce NOS in forebrain septal cholinergic neurons (Hotzman et al., 1994). These observations suggest that neurotrophins significantly influences the microenvironment of the CNS by influencing various transmitter and their receptor regulation.

Recent evidences that neurotrophins prevent naturally occurring cell death commonly known as apoptosis. Neurotrophins suppress the mechanisms involved in apoptosis thus enhancing cell survival. Since apoptosis is a common feature following trauma, ischemia or stroke it seems quite likely that neurotrophins exert their influence by mechanisms responsible for suppression of apoptosis (Schwab and Bartholdi, 1996). To further confirm this, further studies using particular markers of apoptosis are needed to find out such a relationship between neurotrophin induced neuroprotection and apoptosis in various models of cell injury or neurodegeneration, a feature which require additional investigation.

The other possibility may include a high affinity binding of neurotrophins to their receptors following injury. Adult motoneurons express high-affinity trk-B receptor which is also found to be increased following axotomy or trauma (Schwab and Bartholdi, 1996). An upregulation of these receptors may provide an increased capacity of neurotrophins to bind with these receptors in order to promote cell survival.

Our observation that pretreatment with neurotrophins prevents the NOS regulation following trauma suggests that neurotrophins can induce neuroprotection probably by inhibiting NOS upregulation in the neurons following trauma. This is evident from the fact that NOS labelled neurons following trauma which normally do not exhibit NOS activity are going to die (Sharma et al., 1996a, 1997a, 1998). NOS generates free radical formation and induces production of NO (Dawson and Dawson, 1996). Production of NO exerts its neurotoxic effect on cells probably via activation of NMDA receptor and Ca^{2+} influx. Prevention of NMDA receptor activation by MK-801 or inhibition of NOS are neuroprotective (Dawson and Dawson, 1996).

The mechanisms by which neurotrophins attenuate NOS upregulation is not clear from this study. However it appears that neurotrophins can attenuate oxidative stress. Thus, it would be possible that neurotrophins are able to reduce oxidative stress or cellular stress following spinal trauma (Hökfelt et al., 1994; Huber et al., 1995). This could be one of the most important mechanisms for reducing NOS upregulation and consequently cell injury.

A significant attenuation of BSCB permeability by neurotrophins suggests that secondary injury mechanisms following trauma are thwarted by neurotrophins. A reduction in BSCB permeability will attenuate leakage of plasma proteins and thereby reducing vasogenic edema which is clearly evident from our findings of water content determination in this model. Obviously a less

vascular or cellular reaction in neurotrophin treated injured spinal cord is primarily responsible for less adverse cell changes.

In conclusion, our results suggest that BDNF and IGF-1 pretreatment is neuroprotective in spinal cord injury and these neurotrophic factors have the capacity to downregulate nNOS expression following trauma to the spinal cord. Taken together, our results provide new experimental evidences suggesting that BDNF and IGF may exert their potential neuroprotective effect via NOS regulation.

Acknowledgements

This investigation is supported by grants from Swedish Medical Research Council nr 2710, 9459, 11205 and Swedish Cancer Research Foundation 3857-B97-OIVAA, The University Grants Commission, New Delhi, India; Alexander von Humboldt Foundation, Bonn, Germany. The skilful technical assistance of Kärstin Flink and Ingmarie Olsson; and secretarial assistance of Gun-Britt Lind and Aruna Misra are greatly appreciated.

References

Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH (1991) Cloned and expressed nitric oxide synthase structurally resembles P-450 reductase. Nature 351: 714–718

Davies AM (1994) The role of neurotrophins in the developing nervous system. J Neurobiol 25: 1334–1348

Dawson VL, Dawson TM (1996) Nitric oxide neurotoxicity. J Chem Neuroanat 10: 179–190

Dun NJ, Dun SL, Wu SY, Förstermann U, Schmidt HHW, Tseng LF (1993) Nitric oxide synthase-immunoreactivity in the rat, mouse, cat and squirrel monkey spinal cord. Neuroscience 54: 845–857

Hökfelt T, Ceccatelli S, Gustafsson L, Hulting A-L, Verge V, Villar M, Xu X-J, Xu Z-Q, Wiessenfeld-Hallin Z (1994) Plasticity of NO synthase expression in the nervous and endocrine systems. Neuropharmacology 33: 1221–1227

Holtzman DM, Kilbridge J, Bredt DS, Black S M, Li Y, Clary DO, Reichardt LF, Mobley WC (1994) NOS induction by NGF in basal forebrain cholinergic neurons: evidence for regulation of brain NOS by a neurotrophin. Neurobiol Dis 1: 51–60

Huber KA, Krieglstein K, Unsicker K (1995) The neurotrophins BDNF, NT-3 and NT-4 but not NGF, TGF-β1 and GDNF, increases the number of NADPH-diaphorase-reactive neurons in rat spinal cord cultures. Neuroscience 69: 771–779

Kimura H, Steinbusch HWM (1996) The role of nitric oxide in the central nervous system. From basic to therapeutic aspect. J Chem Neuroanat 10: 179–322

Lindsay RM, Harmar AJ (1989) Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 337: 362–364

Mulderry PK (1994) Neuropeptide expression by newborn and adult rat sensory neurons in culture. effects of nerve grwith factor and other neurotrophic factor. Neuroscience 59: 673–688

Nawa H, Bassho Y, Carnahan J, Nakanishi S, Mizuno K (1993) Regulation of neuropeptide expression in cultured cerebral cortical neurons by brain-derived neurotrophic factor. J Neurochem 60: 772–775

Nawa H, Peleymounter MA, Carnahan J (1994) Intraventricular administration of BDNF increases neuropeptide expression in newborn rat brain. J Neurosci 14: 3751–3765

Novikov L, Novikova L, Kellerth J-O (1995) Brain-derived neurotrophic factor promotes survival and blocks nitric oxide synthase expression in adult rat spinal motoneurons after ventral root avulsion. Neurosci Lett 200: 45–48

Schwab ME, Bartholdi D (1996) Degeneration and regeneration of axons in the lesioned spinal cord. Physiol Rev 76: 319–370

Sharma HS, Westman J, Olsson Y, Alm P (1996a) Involvement of nitric oxide in acute spinal cord injury. An immunohistochemical study using light and electron microscopy in the rat. Neurosci Res 24: 373–384

Sharma HS, Lindholm D, Alm P, Gordh T, Olsson Y, Westman J (1996b) Topical application of brain-derived neurotrophic factor reduces upregulation of neuronal nitric oxide synthase following focal trauma to the rat spinal cord. Swiss International Winter Meeting, St. Mauritz, March 20–23, 1996, pp 16–17

Sharma HS, Westman J, Alm P, Sjöquist P-O, Cervós-Navarro J, Nyberg F (1997a) Involvement of nitric oxide in the pathophysiology of acute heat stress in the rat. Influence of a new antioxidant compound H-290/51. Ann NY Acad Sci 813: 581–590

Sharma HS, Westman J, Cervós-Navarro J, Dey P K, Nyberg F (1997b) Opioid receptor antagonists attenuate heat stress-induced reduction in cerebral blood flow, increased blood-brain barrier permeability, vasogenic edema and cell changes in the rat. Ann NY Acad Sci 813: 559–571

Sharma HS, Alm P, Westman J (1998) Nitric oxide and carbon monoxide in the brain pathology of heat stress. In: Sharma HS, Westman J (eds) Brain function in hot environment. Basic and clinical perspectives. Prog Brain Res vol 115. Elsevier Science Publishers, Amsterdam, pp 297–333

Yan Q, Elliott J, Snider WD (1992) Brain-derived neurotrophic factor rescues spinal cord motor neurons from axotomy induced cell death. Nature 360: 753–755

Yu W-HA (1994) Nitric oxide synthase in motor neurons after axotomy. J Histochem Cytochem 42: 451–457

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Received August 25, 1997