Antisense oligos to neuronal nitric oxide synthase aggravate motoneuron death induced by spinal root avulsion in adult rat

Lihua Zhou a,b, Wutian Wu a,*

a Department of Anatomy, Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong, China
b Department of Anatomy, Zhong Shan Medical College, Sun Yat-Sen University, Guangzhou, China

Received 12 April 2005; revised 2 August 2005; accepted 11 August 2005
Available online 24 October 2005

Abstract

The present study used nitric oxide synthase (nNOS) antisense oligos (nNOS AS-ODN) to assess the role of nNOS in motoneuron death induced by spinal root avulsion. A right seventh cervical (C7) spinal root avulsion was performed on adult male Sprague-Dawley rats. Two weeks later, FITC-labeled random oligos (FITC-R-ODN), nNOS AS-ODN, R-ODN or TE buffer was applied to the lesioned side of the C7 spinal segment and refreshed every 3 days. FITC-R-ODN was first detected inside the injured motoneurons at 10 h, accumulated to a maximum by 24 h and faded out from 72 h. Following avulsion, nNOS AS-ODN decreased the number of nNOS-positive motoneurons in the lesioned segment compared either with buffer (P < 0.001 at 15 days, 3 and 4 weeks post-injury) or with R-ODN control (P = 0.002 at 15 days, P < 0.001 at 3 and 4 weeks post-injury). Interestingly, nNOS AS-ODN also decreased the number of surviving motoneurons compared either with buffer (P = 0.005 at 15 days, P < 0.001 at 3 or 4 weeks) or with R-ODN control (P < 0.001 at 3 or 4 weeks). Meanwhile, there were no significant differences between R-ODN and buffer control either in the number of nNOS-positive motoneurons (P = 0.245 at 15 days, P = 0.089 at 3 weeks and P = 0.162 at 4 weeks) or in the number of surviving motoneurons (P = 0.426 at 15 days, P = 0.321 at 3 weeks or P = 0.344 at 4 weeks). These findings indicate that nNOS AS-ODN, applied from 2 weeks after avulsion, aggravates the motoneuron death due to root avulsion by specifically down-regulating nNOS gene expression and that the expression of nNOS in adult spinal motoneurons in response to root avulsion may play a beneficial role in the survival of injured neurons.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Antisense oligos; Neuronal nitric oxide synthase; Spinal motoneurons; Root avulsion; Adult rats

Introduction

In previous studies, we demonstrated that root avulsion of the brachial plexus induces dramatic spinal motoneuron death in both neonatal and adult rats (Li et al., 1993; Wu, 1993; Wu et al., 1994b). Other studies showed delayed motoneuron death when the C7 spinal nerve was transected about 10 mm distal to the DRG (Ma et al., 2001). Recently, lumbosacral ventral root avulsions were also proved to progressively deplete motor neurons and suggested apoptosis as a mechanism contributing to the neuronal death (Hoang et al., 2003). However, the mechanism of motoneuron death in this model is not fully understood. In primary cultures of rat embryonic motor neurons deprived of brain-derived neurotrophic factor (BDNF), induction of neuronal nitric oxide synthase (NOS) was correlated with subsequent apoptosis, in which peroxynitrite plays an important role (Estevez et al., 1998). We also found that the time course and density of avulsion-induced NOS expression are correlated with the severity of motoneuron death (Wu, 1993; Wu et al., 1995). In addition, implantation of a peripheral nerve graft or application of exogenous glial-cell-line-derived neurotrophic factor (GDNF) or BDNF blocks or down-regulates the existing NOS expression and protects the injured motoneurons from death (Wu et al., 1994a, 2003, 2004; Li et al., 1995; Chai et al., 1999, 2000; Yuan et al., 2000; Gu et al., 2004). These results seem to indicate that injury-induced NOS plays a neurotoxic role in motoneuron survival after root avulsion. However, these results cannot rule out the alternative hypothesis that NOS induction may represent an attempt of avulsed motoneurons to resist the directly lethal effects of an unknown molecule. NOS/NO might act as a replacement for neurotrophic factors in injured and deprived motoneurons.
neurons. This may explain why NOS is induced in motoneurons following root avulsion but not after distal axotomy (Wu, 1993; Wu et al., 1994c). Inhibition of NOS expression in regenerating motoneurons following PN graft implantation or ventral root reimplantation (Wu et al., 1994a, 2004; Chai et al., 2000) also seems to support this hypothesis since regenerating motoneurons may be able to obtain neurotrophic factors from the PN graft and NOS/NO may no longer be needed.

Selective blockade of nNOS production offers a useful approach to understanding its precise role in motoneuron death. Pharmacologic inhibitors of NOS have been studied in peripheral nerve injury models. Following avulsion, nitroarginine significantly reduces the death of motoneurons due to spinal root avulsion (Wu and Li, 1993). \(N^\text{G}\)-nitro-L-arginine methyl ester (L-NAME) in a transected sciatic nerve is associated with enhanced regeneration of myelinated fibers in mice (Zochodne et al., 1997). L-NAME and 7-nitroindazole (7-NI) can suppress the degeneration of spinal motoneurons in wobbler mice (Ikeda et al., 1998). But, these substrate-based pharmacologic inhibitors lack high selectivity for the different isoforms of NOS and have produced conflicting results when applied to the central nervous system (Iadecola et al., 1994; Moore and Handy, 1997).

Targeted disruption of the nNOS gene has also been achieved in knockout mice (Huang et al., 1993) and studied in a sciatic nerve model comparing wild-type mice and mice lacking neuronal NOS after transection and microsurgical repair. In NOS knockout mice, delayed regeneration is preceded by a decelerated Wallerian degeneration, a substantial loss of small and medium-sized dorsal root ganglia neurons, spinal interneurons and, to a lesser extent, spinal motoneurons. On the other hand, a lack of endothelial or inducible NOS was well tolerated. Thus, NO supply turned out to be essential for cell survival and recovery with reference to the neuronal NOS isoform (Keilhoff et al., 2002a,b, 2003, 2004). But, in the root avulsion model, nNOS gene expression shows diversity among different rodent strains (Wu, 2000). It is difficult to assess the role of injury-induced nNOS in motoneuron death in the rat by using NOS knockout mice because although avulsion causes severe motoneuron death it does not induce nNOS expression in mice (Li et al., 1998).

The antisense gene knockdown strategy is thought to be a highly selective tool for arresting mRNA translation into the functional enzyme. Extensive studies have demonstrated that antisense oligos can enter cells and stop protein translation either by blocking the translocation of ribosomes (Boiziau et al., 1992) or by destroying the target mRNA through an RNAse H-mediated degradation (Sommer et al., 1998). Recently, it was demonstrated that the antisense inhibition of iNOS is more efficacious than currently available iNOS pharmacological agents in reducing several pathophysiological processes after spinal cord injury (Pearse et al., 2003). Studies have shown that antisense inhibition of nNOS can interfere with nNOS-related functions in the central nervous system in vivo (Maeda et al., 1999; Naassila et al., 2000; Cao et al., 2001; Chen and Ma, 2002, 2003; Li et al., 2003, 2004).

In the present study, nNOS AS-ODN was used to knockdown nNOS gene expression, and its effects on the expression of injury-induced nNOS and motoneuron survival were investigated after spinal root avulsion.

Materials and methods

Animal treatment

Male Sprague–Dawley rats weighing 280–350 g were housed under a 12 h light/dark cycle with free access to food and water. All procedures were approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong.

Spinal root avulsion

Spinal root avulsion surgery followed the procedures described in our previous publications (Wu, 1993, 1996; Li et al., 1995). Briefly, rats were anesthetized by intramuscular injection of ketamine (80 mg/kg) and xylazine (8 mg/kg). Under an Olympus surgical microscope, the right seventh spinal nerve (C7) of the brachial plexus was identified in the supine position. Extravertebral avulsion was carried out on the C7 ventral and dorsal roots by pulling the C7 spinal nerve out with a pair of microhemostatic forceps. The avulsed ventral and dorsal roots together with the dorsal root ganglia were cut away from the distal stem of the C7 spinal nerve and examined under the microscope to confirm the success of the surgery. The skin was then sutured, and the animals were allowed to recover for 1–2 h and returned to their cages.

Design of antisense oligos to nNOS

All oligos used in this study were purchased from Biognostik® (GmbH, Goettingen, Germany). The phosphorothioate forms of the antisense oligos targeting rat nNOS had been used previously (Chen and Ma, 2002, 2003). The sequence of nNOS AS-ODN corresponded to the translation start regions of rat nNOS mRNA (GGA GAC GCA CGA AG). R-ODN (ACC GAC CGA CGT GT), which had a random sequence but the same sequence length, G/C contents and phosphorothioate modifications as those of nNOS AS-ODN, was used as ODN control. Fluorescein isothiocyanate-labeled R-ODN (FITC-R-ODN) was used to check the cellular uptake of oligos. All oligos used in this study were subjected to a BLAST check. No cross-homologies were found in the GenBank database. nNOS AS-ODN had positive matches only for the targeted nNOS mRNA sequence. No positive matches were produced for R-ODN. All oligos were reconstituted in TE buffer, pH 7.2 (Biognostik, GmbH, Goettingen, Germany) at a final concentration of 4 nmol/μl. TE buffer served as buffer control.
Intrathecal application of oligos to nNOS

Two weeks after avulsion, the animals were anesthetized as above. Under a surgical microscope, all animals received a laminectomy at the C7 segment. The dura mater was cut and the subarachnoid space opened. The site of the injured C7 ventral root was excised. A small piece of gelfoam (Pharmacia and Upjohn, Peapack, NJ) soaked in 4 μl of nNOS AS-ODN (16 nM), R-ODN (16 nM), FITC-R-ODN (16 nM) or TE buffer was then placed in contact with the site of the injured C7 ventral root (Wu et al., 2003).

The first group of animals was treated with 16 nM FITC-R-ODN in order to study the cellular uptake of oligos. These animals were allowed to survive for 10, 24, 48 or 72 h or 1 week after treatment (n = 3 at each survival time).

The second group of animals was used to study the effect of nNOS AS-ODN on avulsion-induced nNOS expression. These animals were further divided into three subgroups, in which each animal received 16 nM nNOS AS-ODN, 16 nM R-ODN or TE buffer (n = 6 in each group). They were allowed to survive for 15 days following avulsion (i.e. 24 h after antisense treatment).

The third group of animals received multiple repeated antisense treatments to study the effect of nNOS AS-ODN on motoneuron survival after root avulsion. The repeated treatments were carried out at 14, 18, 22 and 26 days following avulsion. Under a surgical microscope, the previous skin and muscle sutures were re-opened, and the remnants of gelfoam were removed. Fresh gelfoam presoaked in a 4 μl solution of 16 nM nNOS AS-ODN, 16 nM R-ODN or TE buffer was then placed in the lesioned side of the C7 spinal segment. The muscle and skin were sutured again, and the rats were returned to their cages. After survival periods of 10, 24, 48 or 72 h, the animals were sacrificed with a lethal dose of ketamine and xylazine, and the C7 segments were removed. The C7 spinal segment was defined from the dorsal vertebral column was dissected, and the spinal cord was immersion-fixed in cold-buffered 4% paraformaldehyde. The following day, all segments were stored in cold-buffered 30% (v/v) sucrose until ready for sectioning. All C7 segments were sectioned serially at 40 μm in the transverse plane with a freezing microtome, and sections were collected in 0.1 M PBS at pH 7.4.

Detection of cellular uptake of oligos

At the end of 10, 24, 48 or 72 h or 1 week after FITC-R-ODN labeling, every 3rd FITC-R-ODN-labeled section was mounted and allowed to dry for 1 h in the dark. By coverslipping using DAKO fluorescent mounting, all sections were examined under the Leica fluorescent microscope, and the results were recorded with a digital camera. Injured motoneurons with cytoplasmic labeling of the soma by FITC-R-ODN were considered able to take up the oligos (Sommer et al., 1998).

Immunocytochemical staining for nNOS

One third of the sections were subjected to immunocytochemical (ICC) staining. The procedures for nNOS ICC were similar to those used in a previous study (Wu, 1996). Briefly, sections were incubated in primary antibody, rabbit anti-nNOS (Santa Cruz Biotechnology, Inc.), for 48 h at 4°C. The antibody was applied in 1:1500 dilutions in 0.1 M PBS containing 2% normal serum and 0.2% Triton X 100. Then, sections were washed three times in PBS for 10 min each and incubated in biotinylated secondary antiserum (Vectastain ABC kit, Vector Lab, Burlingame, CA) for 40 min at room temperature. Sections were rinsed and incubated in ABC reagents (Vectastain ABC kit) for 60 min then washed thoroughly and incubated in 0.05% DAB and 0.03% H2O2 for 3–5 min until a brown reaction product was observed. The images were captured using a camera Lucida attached to the Olympus BX50 microscope.

NADPH-diaphorase histochemistry

The remaining sections were stained with NADPH-diaphorase (NADPH-d). Sections were incubated at 37°C for 1 h in 10 ml of 0.1 M Tris–HCL (pH 8.0) containing 10 mg of NADPH (Sigma) and 2.5 mg of nitro blue tetrazolium and then counterstained with neutral red. These sections were used to count the numbers of NOS-positive and surviving motoneurons. The NADPH-d technique was reliable for the expression of nNOS since we had previously demonstrated that NADPH-d stains exactly the same population of injured motoneurons visualized by nNOS ICC and nNOS in situ hybridization (Wu et al., 1994c).

Motoneuron counts and statistics

Motoneurons were counted on both sides of each C7 spinal segment in neutral red stained sections, as described previously (Wu, 1993; Wu and Li, 1993). The number of motoneurons on the contralateral side served as normal control and was expressed as 100%. The number of NADPH-positive motoneurons on the ipsilateral side was expressed as a percentage of the surviving motoneurons on the same side. The number of surviving motoneurons on the ipsilateral side was expressed as a percentage of the motoneurons on the contralateral side. Statistical evaluation of counts and comparison among groups were performed with the Statistical Package for Scientific Software. A one-way ANOVA was applied to detect differences among groups followed by Tukey–Kramer multiple comparison tests. A P
value of <0.05 was considered statistically significant, and all data were presented as mean ± SEM.

Results

Uptake of FITC-R-ODN by injured motoneurons

FITC-R-ODN was taken up and accumulated within 10 h by large numbers of injured motoneurons on the lesioned side of the spinal cord. The labeling did not diffuse to the contralateral side (Fig. 1A). Within 24 h, the FITC-R-ODN distribution displayed a punctate cytoplasmic pattern in injured motoneurons (Fig. 1B). According to morphologic characteristics following Nissl staining, we found that 83 ± 12% of the motoneurons on the ipsilateral side were labeled at 24 h (data not shown). The injured motoneurons showed strong cytoplasmic labeling of cell bodies and dendrites (Fig. 1C). The intracellular labeling with FITC-R-ODN attenuated gradually from 72 h and could not be detected after 1 week (data not shown). From this result, we decided to repeat the antisense treatment every 3 days.

Expression of nNOS in injured motoneurons after antisense treatment

In rats treated with buffer control, nNOS ICC was widely distributed in almost every injured motoneuron and was evident in the somatic cytoplasm and dendrites (Fig. 1D). In nNOS AS-ODN-treated rats, expression of nNOS was significantly down-regulated. Only a few nNOS ICC-positive neurons with weak staining in the soma were observed on the lesioned side (Fig. 1E). The pattern and density of nNOS expression in R-ODN control (Fig. 1F) were similar to those in buffer control.

Quantitative analysis using NADPH-d-stained slides showed that the numbers of nNOS-positive motoneurons in buffer control were 51 ± 2.8% at 15 days, 65 ± 3.4% at 3 weeks and 54 ± 6.2% at 4 weeks after root avulsion (Fig. 2). In the nNOS AS-ODN-treated group, these numbers were only 32 ± 6.2% at 15 days, 30 ± 5.8% at 3 weeks and 28 ± 5.1% at 4 weeks (Fig. 2). In R-ODN control, these numbers were 48 ± 6.8% at 15 days, 61 ± 3.9% at 3 weeks and 49 ± 5.5% at 4 weeks (Fig. 2). Statistical analysis showed that the differences among groups were significant (P < 0.001). The differences...
between nNOS AS-ODN and buffer control were significant ($P < 0.001$) at 15 days, 3 weeks and 4 weeks. The differences between nNOS AS-ODN and R-ODN control were also significant at 15 days ($P < 0.002$) and at 3 and 4 weeks ($P < 0.001$). The differences between R-ODN and buffer control were not significant at 15 days, 3 weeks and 4 weeks ($P > 0.05$, Fig. 2). Morphologically, many surviving motoneurons were NADPH-d-positive in the R-ODN-treated group by 3 weeks post-injury (Fig. 3A), which was similar to the buffer control group (Fig. 3B). However, very few NADPH-d-positive motoneurons were observed at the same time post-injury in the nNOS AS-ODN-treated group, and most remaining motoneurons were NADPH-d-negative (Fig. 3C).

**Survival of injured motoneurons after antisense treatment**

Following avulsion, motoneuron loss in the C7 spinal segments was dramatic at the end of 4 weeks in the groups treated with buffer or R-ODN. Antisense treatment shortened the latency of death, with remarkable motoneuron loss at the end of 3 weeks. Quantitative analysis showed that the numbers of surviving motoneurons in the buffer-treated group were $88 \pm 1.7\%$ at 15 days, $83 \pm 2.1\%$ at 3 weeks and $69 \pm 14.6\%$ at 4 weeks (Fig. 4). In the nNOS AS-ODN-treated group, these numbers were $83 \pm 3.7\%$ at 15 days, $55 \pm 4.4\%$ at 3 weeks and $35 \pm 5.4\%$ at 4 weeks (Fig. 4). In the R-ODN control, the numbers were $87 \pm 5.1\%$ at 15 days, $80 \pm 7.9\%$ at 3 weeks and $62 \pm 8.8\%$ at 4 weeks (Fig. 4). Statistical analysis showed that the differences among groups were significant ($P < 0.001$). The differences between nNOS AS-ODN and buffer control were significant at 15 days ($P = 0.005$) and at 3 and 4 weeks ($P < 0.001$). The differences between nNOS AS-ODN and R-ODN control were also significant with $P < 0.001$ at 3 and 4 weeks but were not significant at 15 days ($P = 0.15$). The differences between R-ODN and buffer control were not significant at 15 days, 3 weeks and 4 weeks ($P > 0.05$, Fig. 4). Morphologically, many remaining motoneurons were still NADPH-d-positive in the R-ODN-treated group by 4 weeks post-injury (Fig. 3D), which was similar to the buffer control group (Fig. 3E). However, few motoneurons remained by this time in the nNOS AS-ODN-treated group (Fig. 3F).

**Discussion**

**Cellular uptake and intracellular localization of intrathecally administered oligos**

In the present study, the intrathecally administered oligos diffused and spread only in the ipsilateral spinal segment. The
majority of motoneurons and some glial cells in this segment took up the oligos. This result was similar to studies in which oligos were injected by the intracerebroventricular, intracerebral or intrathecal routes, where oligos only diffused and spread in nearby brain tissue, with the majority of neurons around the injection site taking them up and small numbers of glial cells being labeled (Sommer et al., 1998). Most intrathecal injections have been carried out with intrathecal tubing (Paul et al., 2001; Liu et al., 2004). Alternatively, we placed oligos not only intrathecally but also in contact with the injured axons of the C7 motoneurons. We could not compare the extent of cellular uptake to other studies because few show the quantitative data on cellular uptake of FITC-labeled ODNs. Furthermore, the degree of cellular uptake may not necessarily be the same in different brain regions, probably due to the different cell types (Sommer et al., 1998). The punctuate pattern of FITC-R-ODN in our study was often observed in in vivo studies with phosphorothioate-modified oligos, which represent transport of oligos into intracellular organelles other than lysosomes or endosomes (Iversen et al., 1992; Copper et al., 1995). It is suggested that our antisense oligos may directly react with their target nNOS mRNA inside the cell.

**Antisense knockdown of avulsion-induced nNOS expression**

Our results showed that nNOS AS-ODN down-regulated de novo expression of nNOS in spinal motoneurons following root avulsion. This effect is specific since the R-ODN control did not have any significant biological effects and did not inhibit nNOS expression as compared to buffer control. A number of recent studies, using the same oligos designed by Biognostik®, also demonstrated that injection of nNOS AS-ODN into rat gracile nuclei down-regulated nNOS expression and specifically attenuated nNOS-induced cardiovascular responses to electroacupuncture at ST36 (Chen and Ma, 2002, 2003). Therefore, in the present study, knockdown of the nNOS gene expression in injured motoneurons was due to the sequence-dependent properties of nNOS AS-ODN.

The response to antisense oligos depends on the transfection, the method of administration, and the half-life of the target proteins (Koller et al., 2000). But, the dosages of nNOS AS-ODN used in vivo vary widely as to the degree of inhibition of nNOS gene expression. In mice, intracerebroventricular (i.c.v.) injection of 20 µg nNOS AS-ODN reduced the level of nNOS mRNA and activity in periaqueductal gray matter within 24 h (Kolesnikov et al., 1997), while 3 × 25 µg nNOS AS-ODN i.c.v. was required to reduce the levels of nNOS activity in cerebellum and hippocampus within 6 days (Li et al., 2003, 2004). In rat, 8 × 25 µg nNOS AS-ODN i.c.v. was reported to decrease both NOS activity and nNOS mRNA levels in hippocampus within 5 days (Naassila et al., 2000). In the present study, we tried several dosages of single treatment and different intervals of repeated treatments (data not shown). We found that 16 nM nNOS AS-ODN (intrathecal application) significantly reduced the number of nNOS-positive motoneurons within 24 h, whereas the 16 nM R-ODN controls did not influence the expression of nNOS or survival in injured motoneurons as compared with buffer controls. Therefore, 16 nM was used as the effective dose in the present study. We also found that the effect of a single dose of nNOS AS-ODN on inhibition of nNOS expression was attenuated after 72 h, as assessed by FITC-R-ODN labeling, nNOS ICC and NADPH-d staining. We therefore used 3 days as the interval for repeated antisense treatments. Our result showed that 16 nM, 2 × 16 nM or 4 × 16 nM nNOS AS-ODN effectively inhibited avulsion-induced nNOS activity in rat spinal motoneurons for 24 h, 7 days or 14 days respectively, which parallel the studies mentioned above.

**Role of injury-induced nNOS in motoneuron survival after spinal root avulsion**

The functional significance of nNOS expression in adult motoneurons following peripheral nerve injury remains unclear, and there are many controversial reports regarding its roles in neuronal injury. It seems that nNOS plays different roles in different populations of neurons, different types of injuries, different ages and in different strains of animals (for review, see Wu, 2000).

Deprivation of trophic factors induces expression of nNOS and NO production in cultured motoneurons. Cultured motoneuron apoptosis requires the simultaneous production of NO and superoxide. On the other hand, nitric oxide also stimulates cGMP synthesis, which enhances the survival of motoneurons treated with BDNF (Estevez et al., 2002).

In cranial nerve injury, axotomy of the facial nerve in newborn or early postnatal rats does not induce nNOS expression, while extensive motoneuron death is detectable in the facial nucleus; in contrast, the same injury causes nNOS expression, while facial motoneuron loss is less severe in adult rats (Mariotti et al., 1997; Yu, 2002). After unilateral compression of the facial nerve, increased nNOS was thought to be a contributing factor to the restoration of facial innervation (Wong et al., 2000). Axotomy of the hypoglossal nerve induces nNOS expression, but there is no significant neuron loss in the hypoglossal motor nuclei (Yu, 1994, 1997). This nNOS expression is not related to the death of injured neurons but is correlated with survival or axonal regeneration (Yu, 2002). However, massive production of NO has also been implicated in the neuropathogenesis following hypoglossal nerve transection (Chang et al., 2000).

In rats, expression of nNOS coincides with the death of immature motoneurons following both sciatic and brachial nerve axotomy, implying that nNOS may play a role in the target-deprived degeneration of motoneurons in early development (Clowry, 1993; Li et al., 1993). But, in mice, expression of nNOS was thought not to be responsible for cell death but reflected a failed attempt at recovery of immature motoneurons following sciatic nerve axotomy (He et al., 1996).

Sciatic or pelvic nerve transection induces NOS expression in lumbosacral dorsal root ganglion (DRG) cells and was either thought to play a role in capsaicin-induced neurotoxicity (Vizzard et al., 1995) or to exert a neuroprotective effect through a cGMP pathway (Shi et al., 1998;
Thippeswamy and Morris, 2001; Thippeswamy et al., 2001). In view of the lack of atrophic changes in most motoneurons, the increased levels of NO production after blast injury were considered to be involved in a neuroprotective function (Kaur et al., 1999).

In root avulsion of the brachial plexus, nNOS expression coincides with severe motoneuron loss in both developing and adult rats (Wu, 1993; Wu et al., 1995). That injury-induced nNOS expression is inhibited and survival of motoneurons is enhanced by PN graft implantation (Wu et al., 1994a,b,c; Wu, 1996; Wu et al., 2004) or treatment with exogenous neurotrophic factors such as GDNF or BDNF (Li et al., 1995; Sakamoto et al., 2000; Wu et al., 2003) also suggests a role of injury-induced nNOS in motoneuron degeneration.

In ventral root avulsion of the L5 nerve, only 40% of the medial gastrocnemius (MG) motoneuron pool remain and about 50% of the remaining L5 motoneurons express strong NOS activity. BDNF completely blocks the NOS expression and resulted in 87% survival of the MG cells.CNTF could not block NOS activity in 46% of MG motoneurons and resulted in 73% motoneuron loss. It seems that, in adult spinal motoneurons, retrograde cell death is induced by ventral root avulsion. A lethal effect of NO has been implicated since NOS is expressed in the motoneurons destined to die (Novikov et al., 1995). In another lumbar ventral root avulsion study, only 10% of the avulsed motoneurons remained at 12 weeks postoperatively, 20–40% of which displayed NOS activity. Treatment with BDNF during the initial four postoperative weeks resulted in 45% motoneuron survival and a complete blockage of NOS expression at 12 weeks postoperatively (Novikova et al., 1997).

Whether NOS/NO is neuroprotective or neurotoxic may also depend on the amount of NO produced and its colocalization with other antioxidant molecules. Oxidative stress and massive production of NO have been implicated in the neuropathogenesis following hypoglossal nerve transaction (Chang et al., 2000).

The activity of nNOS is thought to be regulated by postsynaptic density-95 (PSD-95), which plays a pivotal role in regulating synaptic plasticity and synaptogenesis. Following facial nerve axotomy, mRNA signals for PSD-95 transiently decrease and gradually recover to the control level. Just prior to the recovery of PSD-95 mRNA expression, mRNA and protein expression of nNOS starts to increase in the axotomized facial motoneurons (Che et al., 2000).

Persistent up-regulation of HSP27 co-localized with nNOS in surviving motoneurons following root avulsion injury is believed to imply competition in motoneuron survival between cytotoxic and cytoprotective systems (He et al., 2003). It was also suggested that axotomized neurons expressing neuronal nNOS may use NO, known for its antioxidant activities and ability to scavenge free radicals, to protect against oxidative stress. In the hypoglossal nucleus, emergence of nNOS coincides temporally with reduction of CuZn–SOD immuno-reactivity (ir). The two nerve lesion models further revealed the concurrence of nNOS abatement with recovery of CuZn–SOD ir and the absence of nNOS abatement with persistent low CuZn–SOD ir. In the neurons of the anterior horn, reduced CuZn–SOD ir was localized in segments containing nNOS-positive neurons as a result of sciatic nerve avulsion. CuZn–SOD ir was unchanged in the absence of nNOS induction following sciatic nerve crush. The dorsal motor nucleus of the vagus nerve lacks both nNOS abatement and CuZn–SOD ir. These data suggest that axotomy-induced nNOS expression is causally linked to oxidative stress and that NO is neuroprotective but can become neurodestructive when produced in excess (Yu, 2002).

A more direct means of elucidating the role of injury-induced nNOS in motoneuron survival after root avulsion is to prevent the production of the nNOS enzyme by knockdown of nNOS expression in the injured neurons. In adult spinal motoneurons, de novo expression of nNOS mRNA is first detected 3 days following root avulsion (Wu et al., 1994a,b,c), and the nNOS enzyme is produced from 1 week post-injury. The nNOS enzyme accumulates within the cytoplasm of injured motoneurons and reaches a maximum by 3–4 weeks post-injury (Wu, 1993), following which significant motoneuron loss occurs (Wu, 1993). Based on these observations, in the present study, nNOS AS-ODN was used to prevent the production of nNOS enzyme at the time point 2 weeks post-avulsion when the enzyme is produced but no significant motoneuron death is observed. Then, motoneuron survival was studied at 3 and 4 weeks after root avulsion.

The present results demonstrated that knockdown of the nNOS gene by nNOS AS-ODN aggravates the death of injured motoneurons. Such an increase of motoneuron death did not result from the toxicity of repeated doses of phosphorothioated nNOS AS-ODN since the same doses of phosphorothioated R-ODN, which had the same phosphorothioate modification, did not change the survival rate of injured motoneurons from that of buffer control. The results indicate that injury-induced nNOS either plays a role in a survival signaling pathway or represents a response of injured motoneurons to an unknown lethal molecule. This hypothesis seems supported by the results of nNOS knockout experiments. Knockout of the nNOS gene in mice induces more severe motoneuron death in the lumbar spinal cord following sciatic nerve transection than in the wild-type, suggesting that deficiency in the nNOS gene enhances the competence-to-die and leads to substantial cell death of spinal cord motoneurons (Keilhoff et al., 2004).

Conclusion

The present study demonstrates that knockdown of de novo nNOS gene expression by nNOS AS-ODN aggravates the motoneuron loss induced by spinal root avulsion. Although the mechanistic relationship between nNOS and the fate of injured motoneurons remains unclear, injury-induced nNOS does not seem to be involved in adult motoneuron death following root avulsion. Expression of injury-induced nNOS in spinal motoneurons, instead, may play a favorable role for spinal motoneurons after root avulsion. There must be crosstalk between nNOS and key molecules that are responsible for the
death of injured motoneurons. Further studies are needed to understand the precise signaling pathways of avulsion-induced motoneuron death and the crossstalk between nNOS and the molecules involved in these pathways.

Acknowledgments

This study was supported by grant from the Hong Kong Research Grants Council (RGC) and the National Key Basic Research Programme of China (2003CB515303). The authors would like to thank Dr. Iain C Bruce for reading and commenting on the manuscript.

References


