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Upregulation of Heat Shock Proteins Rescues Motoneurones from Axotomy-Induced Cell Death in Neonatal Rats

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Heat shock proteins (hsps) are induced in a variety of cells following periods of stress, where they promote cell survival. In this study, we examined the effect of upregulating hsp expression by treatment with BRX-220, a co-inducer of hsps, on the survival of injured motoneurones. Following sciatic nerve crush at birth, rat pups were treated daily with BRX-220. The expression of hsp70 and hsp90, motoneurone survival, and muscle function was examined at various intervals later and the number of functional motor units was assessed by *in vivo* isometric tension recordings. Fourteen days after injury, significantly more motoneurones survived in the BRX-220-treated group (39 ± 2.8%) compared to the saline-treated group (21 ± 1.7%). Moreover, in the BRX-220-treated group no further loss of motoneurones occurred, so that at 10 weeks 42 ± 2.1% of motoneurones survived compared to 15 ± 0.6% in the untreated group. There were also more functional motor units in the hindlimb muscles of BRX-220-treated animals. In addition, treatment with BRX-220 resulted in a significant increase in the expression of hsp70 and hsp90 in glia and neurones. Thus, treatment with BRX-220, a co-inducer of hsps, protects motoneurones from axotomy-induced cell death.

INTRODUCTION

Heat shock proteins (hsps) are widely regarded as ubiquitous members of the defence mechanism on the cellular level against various insults, protecting cellular proteins from damaging insults. Initially, stress-induced hsp accumulation was associated with thermotolerance and later with tolerance to various other forms of stress including UV radiation, oxidative stress, excitotoxicity, trauma, and injury (22, 35, 39). In neurones, stressful insults have been shown to induce the expression of members of various hsp families, including hsp70. Members of the hsp70 family have been shown to play an important role in cytoprotection (6, 24, 51). Hsps can also play a role in normal housekeeping activities of a variety of cells. For example, hsp90 plays a role in muscle development and the signal transduction of steroid receptors (11). Although hsp90 is upregulated *in vitro* following stressful insults, such as heat or ischemia, there is little *in vivo* evidence for injury-induced upregulation of this hsp. Interestingly, overexpression of hsp70 has been found to result in increased resistance to apoptotic stimuli (1, 28, 42). Thus, it is possible that enhancing the endogenous expression of certain hsps within injured cells *in vivo* may protect them from cell death.

Recent results have indicated that such an approach may indeed be successful. Bimoclomol (Biorex R&D Co., Hungary) is a hydroxylamine derivative that enhances the expression of hsps such as hsp70 and hsp90 in response to stressful stimuli (49). Bimoclomol acts by elevating the synthesis of these cellular chaperones induced by environmental stress (49). Bimoclomol has been shown to be beneficial in both *in vitro* and *in vivo* models of ischemic disease and diabetic complications (4, 5, 26).

In this study, we examined the effect of BRX-220 (Biorex R&D Co.), a potent analogue of Bimoclomol, on neuronal death using an *in vivo* model of injury-induced neurodegeneration. We examined whether treatment with this co-inducer of hsps would protect injured motoneurones from cell death.

Developing motoneurones are critically dependent on interaction with their target muscle during both embryonic and early postnatal development. Following injury to the peripheral nerve in neonatal rats, as many as 90% of the affected spinal motoneurones die (33, 43). A large proportion of these motoneurones have been shown to die rapidly after injury, by a process of

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apoptosis (29, 32). Injured motoneurones have also been found to show an increased susceptibility to the excitotoxic effects of glutamate (20). Thus, injury to the peripheral nerve in newborn rats results in a massive motoneurone death.

In this study, following sciatic nerve crush at birth, rat pups were treated with BRX-220 and the effect on (a) motoneurone survival, (b) functional motor unit numbers, and (c) hsp expression were examined at various intervals later.

MATERIALS AND METHODS

Nerve Injury

Sprague–Dawley rats of both sexes were used in these experiments (Biological Services, University College London, UK). All experimental animals were cared for in accordance with guidelines issued by the Institute of Neurology and the British Government. Under halothane anesthesia and sterile conditions, the right sciatic nerve was crushed in the mid-thigh region on the day of birth (P0). Following recovery from the anesthesia, the pups were returned to their mother. In each litter, some pups were left unoperated to act as controls.

BRX-220 Treatment

The pups were divided into four different groups. In the first experimental group, following nerve crush, the pups were treated daily with BRX-220 (2 or 10 mg/kg, ip.) up to a maximum of 3 weeks. In the second group, following nerve injury, the pups were treated with saline (ip., daily). Unoperated littermates either received BRX-220 or remained as untreated controls.

Motoneurone Survival

The effect of treatment with BRX-220 on the survival of injured motoneurones was assessed at 1, 7, 14, and 21 days and 10 weeks following nerve crush, by counting the number of Nissl-stained motoneurones present in the sciatic motor pool within the lumbar enlargement of the spinal cord (L3–L6). The animals were deeply anesthetized (4% chloral hydrate, 1 ml/100 g body weight, ip.) and perfused transcardially with a fixative containing glutaraldehyde (2.5% in Millonings phosphate buffer (MPB), pH 7.3). The spinal cords were removed, the contralateral control dorsal horns were marked using a fine micropin, and the lumbar region was postfixed for 2 h in the same fixative. The spinal cords were then cryoprotected in sucrose (30% in MPB) and frozen transverse sections cut at 30 μm. The free-floating sections were then stained with a Nissl stain (gallocyanin) (12). The number of Nissl-stained motoneurones in both the operated and the control ventral horn between lumbar sections L3 and L6 was counted under a light microscope. To avoid counting the same cell twice in consecutive sections, only large polygonal neurons in which the nucleolus was clearly visible at high magnification were included in the counts. This method of counting has been previously used to assess motoneurone survival (50). As an index of motoneurone survival, the number of motoneurones on the operated side of each spinal cord was expressed as a percentage of the number on the contralateral side.

Immunohistochemistry

At various time points after injury, normal and experimental animals were deeply anesthetized (4% chloral hydrate, 1 ml/100 g body weight, ip.) and perfused transcardially with a fixative containing paraformaldehyde (4% in phosphate buffer; PBS). The lumbar spinal cords were removed and postfixed for 4 h in the same fixative and cryoprotected in sucrose (30% in PBS). Serial sections were then cut on cryostat at 10 μm.

The sections were processed for hsp70 or hsp90 immunohistochemistry. For hsp70 immunostaining an antibody that recognizes both constitutive hsc70 and inducible hsp72 was used (Santa Cruz Biotechnology; mouse monoclonal, Cat. No. sc-24). For hsp90 immunostaining we used an antibody that recognizes both hsp90α and hsp90β proteins (Santa Cruz Biotechnology; rabbit polyclonal Cat. No. sc-7947). In some cases an antibody for glial fibrillary acidic protein GFAP; (SIGMA; mouse monoclonal) was used for identification of astroglial cells.

The sections were incubated for 1 h in PBS containing 5% milk proteins, 0.1% Triton X-100, and either 3% normal horse serum (Vector) for staining for hsp70 or 3% normal goat serum (Vector) in the case of hsp90 and GFAP staining. The sections were then incubated overnight at 4°C in the primary antibody to hsp70, hsp90, or GFAP used at a dilution of 1:100, 1:300, and 1:1000, respectively. Negative control sections, in which the primary antibody was omitted, were also prepared. After thorough washing in phosphate-buffered saline with 0.1% Triton X-100 (Sigma), the sections were incubated for 2 h at room temperature in biotinylated horse anti-mouse (dilution 1:100; Vector Labs Cat. No. BA-2001) and biotinylated goat anti-rabbit (dilution 1:100; Vector Labs Cat. No. BA-1000) antibodies, respectively, and then incubated for 1 h in avidin–biotin complex (Vector Cat. No. PK6100). They were processed using horseradish peroxidase as the chromagen and diaminobenzidine as the substrate. After drying, the sections were lightly counterstained with a Nissl stain (Gallocyanin).

In some double-labeling experiments, antibody staining was assessed using fluorescent markers. In double-labeling experiments for hsp70 and GFAP, the

sections were first processed for hsp70 immunoreactivity using the monoclonal mouse antibody and the same secondary antibody as described above. Texas Red Avidin D (Vector; dilution 1:100) was used as the fluorescent layer. The same sections were subsequently immunostained for GFAP using a fluorescein-labeled anti-mouse IgG antibody made in horse (Vector; dilution 1:100). Negative control sections omitting one of the two antibodies and applying both secondary antibodies were also prepared to establish cross-reactivity between antibodies. Sections were examined under a fluorescent microscope and the presence of hsp70 was assessed using a blue filter for the detection of Texas red, while the presence of GFAP-positive cells was assessed using a green filter for fluorescein.

Western Blots

At P7, Western blot analysis of hsp70 and hsp90 was carried out in spinal cords from unoperated, untreated rats ($n = 12$), operated, saline-treated rats ($n = 12$), and operated, BRX-220-treated rats ($n = 12$). Each experimental group was divided into four sets of samples containing tissue from 3 animals. Each blot was repeated at least twice, and semiquantitative analysis using an image analysis system (KS300; Imaging Associates, UK) was carried out.

The rats were deeply anesthetised (4% chloral hydrate, ip.) and the lumbar spinal cords quickly removed. The spinal cords were hemisected so that the operated and control sides could be collected separately. The tissue was homogenized in homogenizing buffer (5 mM Tris, 2% SDS, 2 mM EDTA, 2 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, pH 6.8). Samples were spun at 14,000 rpm for 10 min and the supernatant was collected. Protein concentration was determined using a Bio-Rad assay system (Cat. No. 500-0116) and a series of bovine serum albumin standards. Samples were boiled 5 min prior to SDS–polyacrylamide gel electrophoresis. Equal amounts of 10 μg protein were loaded and separated at 200 V for 55 min. Proteins were electrophoretically transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were blocked with 5% nonfat dried milk and 0.1% Triton X-100 in PBS for 1 h at room temperature. The same primary antibodies as described for immunostaining (hsp70 and hsp90) were used at a dilution of 1:1000 in PBS containing 5% dried milk, 0.1% Triton X-100 for 1 h at room temperature. After three washes in PBS containing 0.1% Triton X-100 for 15 min each, membranes were incubated in HRP-conjugated anti-mouse (Dako Cat. No. P0260) and anti-rabbit (Dako Cat. No. P0217) immunoglobulines for staining for hsp70 and hsp90, respectively. These antibodies were used at a dilution of 1:1000 in PBS containing 5% dried milk and 0.1% Triton X-100 for 1 h. After three washes in PBS containing 0.1% Triton X-100 for 15 min each, membranes were treated with a chemiluminescent detection reagent (ECL; Amersham Pharmacia Biotech). Blots were visualized using Kodak Films and fixation. Each Western blot contained samples from three animals in each group and each sample was run on at least two blots. On each blot, equal protein quantities of brain homogenates were run next to the samples as standards.

The films were scanned and the pixel densities of each line were measured. Densities for the samples were normalized against densities of the brain standard in each blot. Results are presented as the mean and standard error of the mean of arbitrary units of line densities.

Assessment of Motor Unit Number

The effect of BRX-220 treatment (10 mg/kg body weight, ip., daily for 3 weeks) on motor unit number in soleus and extensor digitorum longus (EDL) muscles following nerve injury at birth was examined at 10 weeks of age. There were three groups of animals: (1) operated, BRX-220 treated, (2) operated, saline treated, and (3) unoperated, BRX-220 treated. The animals were anesthetized with chloral hydrate (4.5%, 1 ml/100 g body weight, ip.) and prepared for tension recordings of their soleus and EDL hindlimb muscles. The distal tendon of the EDL muscle in both legs was dissected free. The soleus muscle was separated from the surrounding musculature and its nerve exposed. Both hindlimbs were securely fixed to a table with stainless steel pins and the distal tendons attached to isometric force transducers (Dynamometer UFI Devices) using a silk thread. The soleus motor nerve and the deep branch of the common peroneal nerve were dissected and prepared for stimulation. All nerves to other muscles of the leg were cut. The length of the muscle was adjusted so that it developed maximal twitch tension and isometric contractions were elicited by stimulating the cut end of the motor nerve using a pulse width of 0.02 ms.

To estimate the number of motor units in each muscle, the motor nerves of the operated and in some experimental animals contralateral soleus and EDL muscles were stimulated every 4 s. The stimulus strength was gradually increased to obtain stepwise increments of twitch tensions, as individual motor axons were recruited. The number of stepwise increments was counted to give an estimate of the number of motor units present in each muscle (13).

Statistical Analysis

For motoneurone survival, the results were analyzed using the Mann–Whitney U test for comparison of independent samples. Two-tailed tests were used in all instances, and significance level was set at $P < 0.05$.

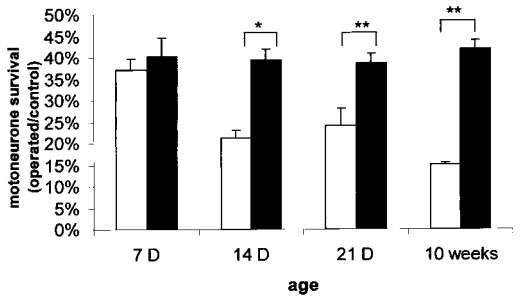

FIG. 1. Motoneurone survival following nerve crush at birth and treatment with BRX-220. The sciatic nerve was crushed in one hindlimb of newborn rats and the animals were treated daily with either saline or BRX-220. One, 2, 3, and 10 weeks later the effect of the treatment on motoneurone survival was assessed by counting the number of Nissl-stained motoneurones within the sciatic motor pool, in both the operated and the contralateral control ventral horns. The bar diagram shows the mean number of motoneurones in the operated ventral horn expressed as a percentage of control (open bars, saline-treated group; filled bars, BRX-220-treated group). It can be seen that a similar proportion of motoneurones have died 1 week after injury in both the saline-and the BRX-220-treated groups. However, in the saline-treated group, motoneurone death continues over the following weeks, while no further death of BRX-220-treated motoneurones takes place (*$P < 0.01$; **$P < 0.02$).

RESULTS

Motoneurone Survival

Following nerve crush at birth motoneurone survival was assessed 1, 2, 3, and 10 weeks later. The results summarized in Fig. 1 show that 7 days after nerve crush and treatment with saline, only 37.2 ± 2.6% (n = 7) of motoneurones survive within the sciatic motor pool. In the group that was treated with BRX-220, 7 days after injury 40.3 ± 4.3% (n = 7) of sciatic motoneurones survive. Thus, 1 week after neonatal nerve injury there is no significant difference in the survival of motoneurones in the saline- and BRX-220-treated groups. However, by 14 days post injury, although motoneurones had continued to die in the saline-treated group, no further motoneurone death occurred in the BRX-220-treated group. Thus, 14 days after injury only 21.4 ± 1.7% (n = 7) of motoneurones survived in the saline-treated group, whereas 39.4 ± 2.8% (n = 7) survived in the BRX-treated group ($P < 0.01$). At 21 days after injury 38.7 ± 2.4% (n = 6) of motoneurones survived in the BRX-220-treated group compared to 24.2 ± 3.9% (n = 6) in the saline-treated group. This improvement in the survival of injured motoneurones was maintained, since 10 weeks after injury 42.1 ± 2.1% (n = 6) of motoneurones survived in the BRX-220-treated group compared to only 15.0 ± 0.6% (n = 6) in the saline-treated group. This increase in motoneurone survival is statistically significant ($P < 0.02$). Some of these experiments were repeated with a lower dose of BRX-220 (2 mg/kg), and the results showed a similar improvement in motoneurone survival. For example, 2 weeks after injury 41.5 ± 1.7% (n = 7) of injured motoneurones survived in the BRX-220-treated group, which is significantly greater than that in the saline-treated group (21.4 ± 1.7%, n = 7; $P < 0.01$).

Motor Unit Number

To establish whether injured motoneurones rescued by treatment with BRX-220 were able to make and maintain functional synaptic contacts with their target muscle fibers, we carried out a series of experiments where the number of motor units in the soleus and EDL muscles was determined using physiological criteria. Following sciatic nerve crush at birth, the animals were treated daily with either BRX-220 or saline for 3 weeks. When the animals were at least 10 weeks old, the number of functional motor units was established by following the stepwise increments of twitch tension in response to stimulation of the motor nerve with stimuli of increasing intensity. Examples of such recordings from soleus and EDL muscles in normal, saline-treated, and BRX-220-treated animals in which the sciatic nerve was crushed are shown in Fig. 2A. The results are summarized in Fig. 2B, which shows that the number of motor units in both soleus and EDL was greater in the group treated with BRX-220 after nerve crush at birth than in the saline-treated operated group. In normal, uninjured soleus and EDL muscles there were 28.3 ± 0.2 (n = 8) and 38.2 ± 0.4 (n = 8) motor units, respectively. These values compare favorably with previously published data of motor unit numbers in these rat muscles (9, 10, 52). Following nerve injury and treatment with saline, only 3.4 ± 0.4 (n = 5) motor units were present in soleus and 7.9 ± 1.2 (n = 5) in EDL. However, in animals treated with BRX-220 following nerve injury, more motor units remained, so that there were 7.5 ± 1.2 (n = 5) in soleus and 15.2 ± 1.0 (n = 5) in EDL. This improvement in motor unit number promoted by treatment with BRX-220 was significant in both soleus and EDL muscles ($P < 0.04$ and $P < 0.02$, respectively). These results reflect the improvement observed in motoneurone survival in BRX-220-treated, operated animals using morphological criteria. Thus, using both morphological and physiological criteria, the results show that treatment with BRX-220 rescues motoneurones from cell death following neonatal nerve injury.

Immunohistochemistry and Western Blot Analysis

The expression of hsp70 and hsp90 following nerve crush at birth (P0) and treatment with either saline or BRX-220 was examined in spinal cords 2, 7, and 21 days after injury at P0 using immunohistochemistry. In addition, the level of expression of hsp70 and hsp90 was examined at P7 in normal, in operated saline-

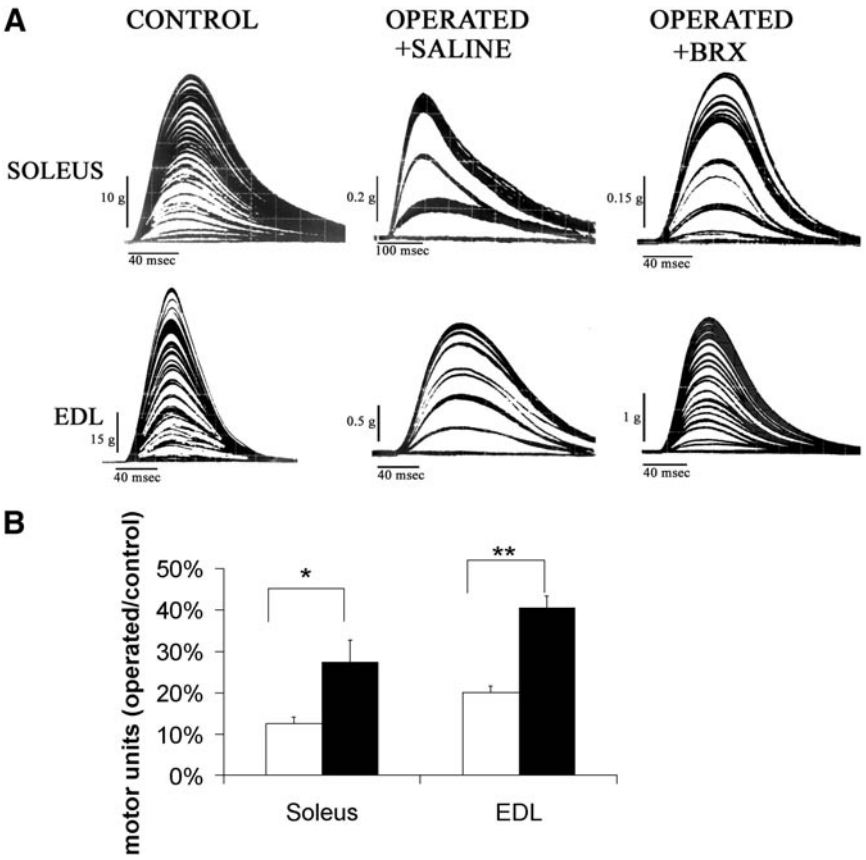

FIG. 2. Effect of treatment with BRX-220 on motor unit number following neonatal nerve injury. Motor unit numbers were established from records of stepwise increments of twitch tension in response to stimulation of the motor nerve by stimuli of increasing intensity. (A) Representative recordings from soleus and extensor digitorum longus (EDL) muscles of control (unoperated), operated saline-treated, and operated BRX-220-treated animals. Note the different scale for force production for each muscle. The block diagram in (B) summarizes the results obtained from at least five animals in each group (open bars, saline-treated group; filled bars, BRX-220-treated group). The results show the number of motoneurones in the operated muscle expressed as a percentage of the number of motor units in the contralateral control muscle. (*$P < 0.04$; **$P < 0.02$).

treated, and in operated BRX-220-treated spinal cords using Western blot analysis.

Expression of hsp70 following neonatal nerve injury. The expression of hsp70 in the ventral horn on the operated and control side of spinal cords from rats in which the sciatic nerve was crushed on one side at birth and which were treated with either BRX-220 or saline was established by immunohistochemistry. Examples of the pattern of hsp70 immunoreactivity are shown in Fig. 3. Hsp70 is constitutively expressed in normal motoneurones at all ages studied and in very few glial cells. An example of the pattern of immunoreactivity at P7 is shown in Fig. 3A. Background staining (negative control) where the primary antibody was omitted was also established. The staining intensity in these negatively stained sections was negligible. The intensity of staining does not change with age (not shown). Following neonatal nerve injury, there is little change in the intensity of hsp70 immunoreactivity within motoneurone cell bodies as can be seen in Fig. 3B at P7. However, other cell populations surrounding the injured sciatic motoneurones became hsp70 immunoreactive (Fig. 3B). This pattern of hsp70 immunoreactivity within cells surrounding motoneurones was observed only in the operated ventral horn and by P21 had returned to normal levels (not shown). Using Western blots, at P7, the levels of hsp70 in normal, unoperated hemisected spinal cords were compared to the levels of hsp70 in the operated, hemisected spinal cords by semiquantitative analyses. An example of such a blot is shown in Fig. 4A. Using an image analysis system, the mean pixel density of each band from at least eight Western blots was normalized against hsp70 levels in normal neonatal brain tissue. The results are summarized in Fig. 4B. It can be seen that there is a slight increase in hsp70 levels in operated spinal cords compared to normal controls. The effect of BRX-220 treatment on hsp70 immunoreactivity was also examined and an example of the pattern of immunostaining is shown in Fig. 3C. There was a clear increase in the intensity of hsp70 immunoreactivity within cells surrounding motoneurones on the injured

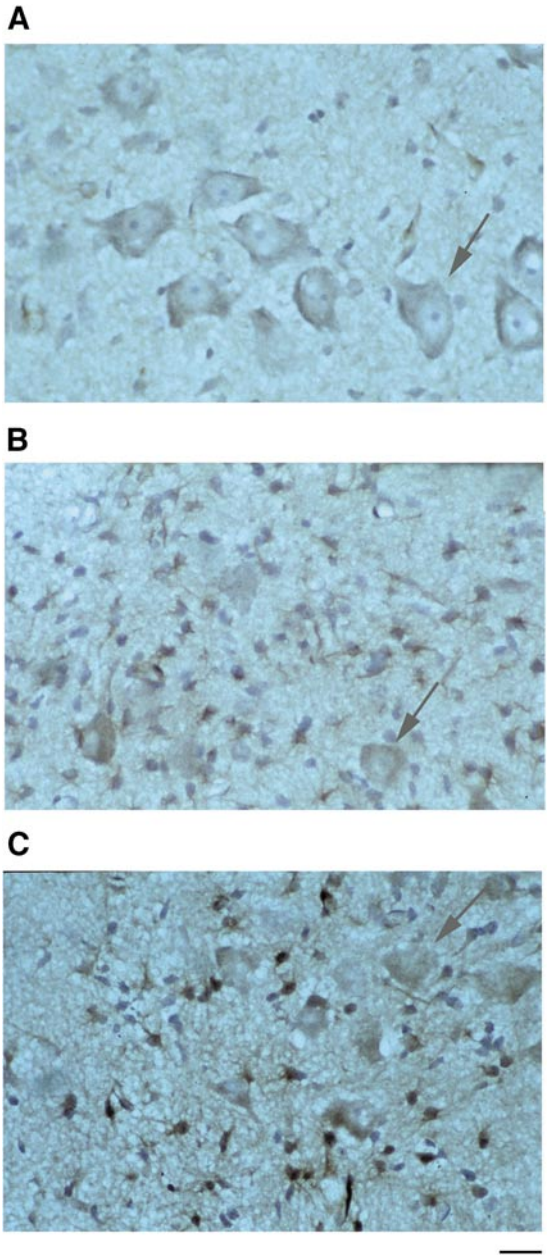

FIG. 3. Pattern of hsp70 immunoreactivity in spinal cords following neonatal nerve injury and treatment with BRX-220. Photomicrographs of cross sections of the ventral horn of the lumbar spinal cord are shown. Sections were first immunostained for hsp70 and counterstained with a Nissl stain. In each section hsp70 immunoreactive motoneurones could be identified (see arrows). (A) The pattern of immunostaining for hsp70 in a control (unoperated) spinal cord at P7. Motoneurones show a specific, but weak immunoreactivity for hsp70 (arrow). In addition, a few small glial cells also show weak staining for hsp70. (B) hsp70 staining in the operated spinal cord 7 days after neonatal nerve crush. There is no apparent change in the immunoreactivity of motoneurones for hsp70 (arrow) but there is a clear increase in the number of hsp70 immunoreactive cells surrounding injured motoneurones. (C) The effect of daily treatment with BRX-220 on hsp70 immunoreactivity in the spinal cord 7 days after nerve injury. In motoneurones, there is no apparent change in hsp70 immunoreactivity (arrow) compared to either control or saline-treated sections. However, the intensity of hsp70 immunostaining increases dramatically in the small cells surrounding the injured motoneurones. Scale bar = 20 μm.

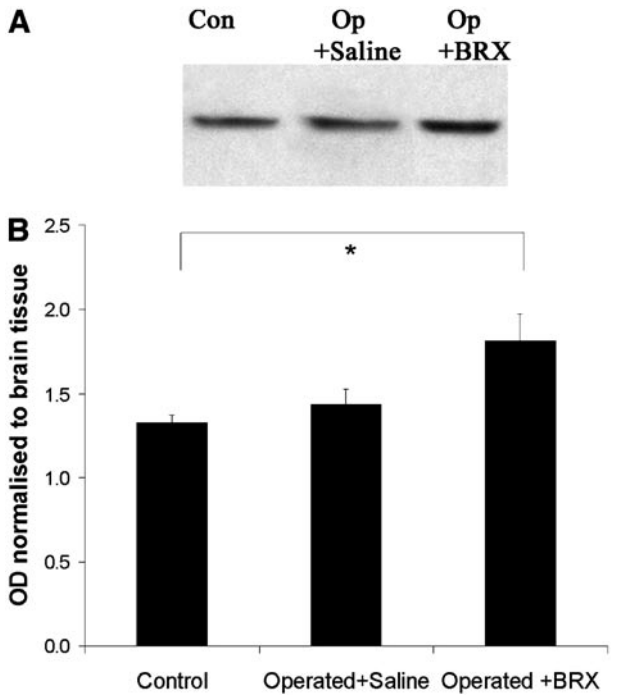

FIG. 4. Western blot analysis for Hsp70 levels in spinal cords following neonatal nerve injury and treatment with BRX-220. Western blot analysis of hsp70 levels in spinal cords from control (unoperated), operated saline-treated, and operated BRX-220-treated animals was carried out 7 days after sciatic nerve crush at birth. (A) An example of such a blot where several spinal cords were pooled (n = 3). Eight such Western blots were run from each group and the mean band density of each blot was measured. The results are summarized in (B). There was a slight but nonsignificant increase in the mean band density of the operated saline-treated spinal cords compared to controls, whereas the operated BRX-220-treated spinal cords contained significantly higher levels of hsp70 (*$P < 0.01$).

side of each spinal cord. The intensity of staining appeared greater than that seen in injured, saline-treated spinal cords (compare Figs. 3B and 3C). Furthermore, Western blot analysis at P7 confirmed that treatment with BRX-220 following nerve injury significantly increased hsp70 levels compared to normal controls (see Fig. 4B; $P < 0.01$).

Since it is well established that an extensive astrogliosis takes place around the injured motoneurones following peripheral nerve injury (17), we next examined whether those cells in which hsp70 immunoreactivity was increased following injury in the BRX-220-treated group were indeed astrocytes. Sections were immunostained with a monoclonal antibody to GFAP, an astroglial marker. Examples of the pattern of immunoreactivity are shown in Fig. 5. In both the saline- and the BRX-treated groups, 7 days after neonatal nerve injury there is a distinct pattern of GFAP immunoreactivity in cells surrounding the injured motoneurones (Fig. 5A). Double-immunofluorescence studies, where sections were stained for GFAP and hsp70, established that in the operated BRX-treated rats those

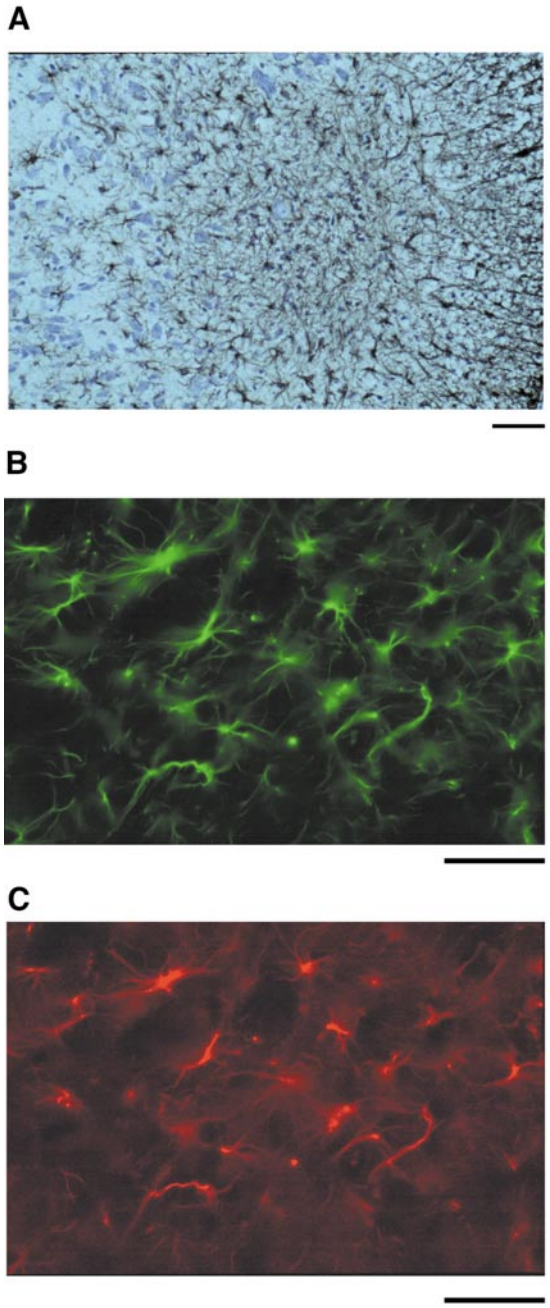

FIG. 5. Expression of hsp70 in GFAP-positive astroglia following neonatal nerve injury and treatment with BRX-220. Seven days after sciatic nerve crush at birth and treatment with BRX-220, spinal cord sections were double-stained for GFAP and hsp70 using immunoflu- orescence. (A) An example of the pattern of GFAP immunoreactivity in astroglia in the ventral horn of the operated side of the spinal cord from a saline-treated animal. Treatment with BRX-220 did not change the pattern or intensity of GFAP staining in the spinal cord. (B) An example of a spinal cord section from an operated BRX-220-treated animal, stained for GFAP using immunofluorescence. (C) hsp70 immunoreactivity in the same section. It can be seen that the hsp70-positive cells seen in (C) are also immunoreactive for GFAP and are therefore likely to be astroglia. Scale bars, 50 μm.

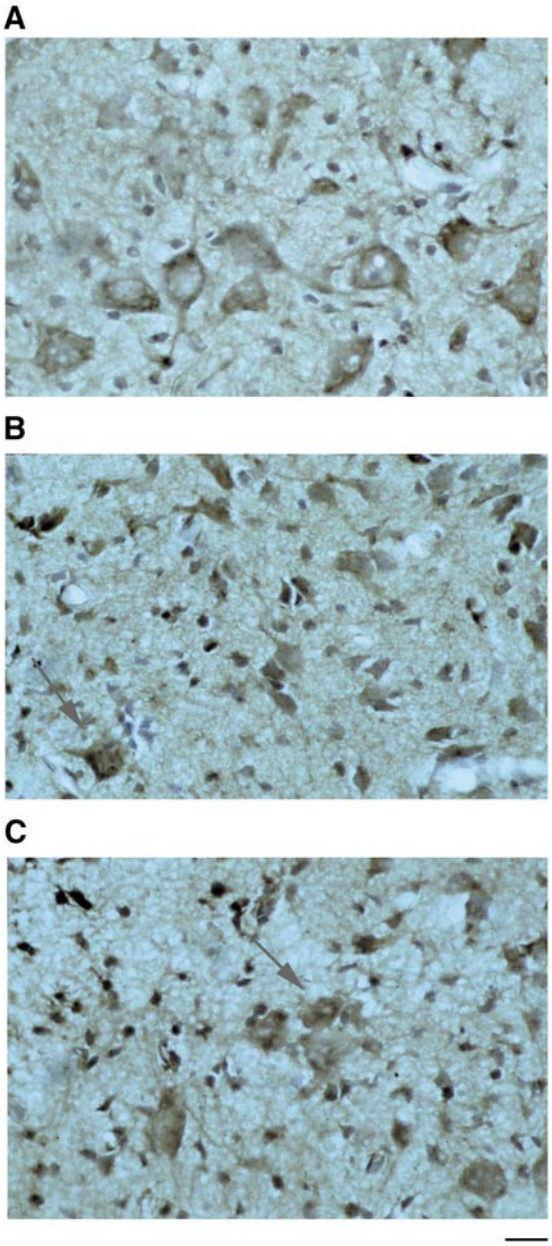

FIG. 6. Pattern of hsp90 immunoreactivity in spinal cords following neonatal nerve injury and treatment with BRX-220. Photomicrographs of cross sections of the ventral horn of the lumbar spinal cord stained for hsp90 are shown. (A) The pattern of immunostaining for hsp90 at P7 in a control (unoperated) spinal cord. There is a specific immunoreactivity in motoneurones and some surrounding small-diameter cells, presumably glia. (B) hsp90 staining in theoperated spinal cord 7 days after neonatal nerve crush. There are fewer motoneurones present than in (A) and some stain more intensely for hsp90 (see arrow). In addition, in some smaller glial cells surrounding injured motoneurones hsp90 immunoreactivity becomes more intense. (C) The effect of daily treatment with BRX-220 on hsp90 immunoreactivity in the operated spinal cord 7 days after nerve injury. There are more motoneurones present than in (B), but there is no apparent increase in hsp90 immunoreactivity in these injured motoneurones compared to saline-treated motoneurones (arrow). However, there is a clear increase in the number of glial cells within the ventral horn and these cells stain intensely for hsp90. Scale bar = 20 μm.

cells which showed increased immunoreactivity for hsp70 were also GFAP-positive (see Figs. 5B and 5C). This double-labeling was not observed in astroglia surrounding the injured motoneurones in the saline-treated group (not shown).

Expression of hsp90 following neonatal nerve injury. The effect of nerve injury and treatment with BRX-220 on the expression of hsp90 in the spinal cord was examined at various intervals in normal uninjured motoneurones. Hsp90 is constitutively expressed in motoneurones and a few glial cells in the spinal cord (see Fig. 6A). Following nerve crush at birth, there was some evidence that 7 days after injury immunostaining for hsp90 was slightly increased in motoneurones and in some glial cells (see Figs. 6A and 6B). However, since hsp90 immunoreactivity was relatively high in normal motoneurones, it was very difficult to establish any significant change after injury. In those animals treated with BRX-220 after nerve injury, injured motoneurones stained as intensely as untreated operated motoneurones for hsp90, although there was a marked increase not only in the number of glial cells but also in the intensity of hsp90 staining within these cells (Fig. 6C). To confirm this effect, 7 days after injury, Western blot analysis of normal, operated saline-treated, and operated BRX-220-treated spinal cords was carried out. An example of such a blot is shown in Fig. 7A, and the mean pixel density from at least eight such blots are summarized in Fig. 7B. The results show that there is no significant increase in hsp90 levels in the operated saline-treated spinal cords compared to normals. However, in the operated BRX-220-treated group there is a clear increase in the levels of hsp90, which is significantly greater than in normal unoperated spinal cords ($P < 0.01$).

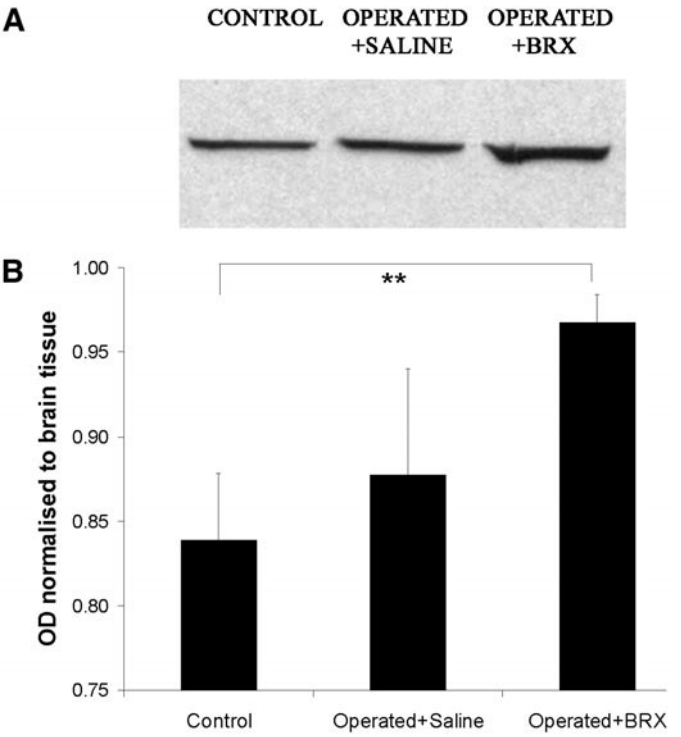

FIG. 7. Hsp90 levels in spinal cords following neonatal nerve injury and treatment with BRX-220. Western blot analysis of hsp90 levels in spinal cord samples from control (unoperated), operated saline-treated, and operated BRX-220-treated animals was carried out 7 days following sciatic nerve crush at birth. (A) An example of such a blot where several spinal cords were pooled ($n = 3$). Eight such Western blots were run from each group and the mean band density of each blot was measured. The results are summarized in (B). There was a slight, but nonsignificant increase in the mean band density of the operated samples compared to controls. However, the BRX-220-treated spinal cords contained significantly higher levels of hsp90 (**$P < 0.01$).

DISCUSSION

In this study, we examined the effect of treatment with BRX-220, a co-inducer of hsps, on motoneurone survival and function in a model of motoneurone degeneration. In addition, we studied the effect of BRX-220 on the expression of hsp70 and hsp90 in injured spinal cords. The results show that, following neonatal nerve injury, treatment with BRX-220 rescues approximately 50% of motoneurones from cell death 10 weeks post injury. This long-term improvement in motoneurone survival in BRX-220-treated axotomized animals is reflected in a corresponding increase in the number of functionally active motor units. In addition, our results show that there is a significant increase in the levels of both hsp70 and hsp90 in the spinal cord following peripheral nerve injury and treatment with BRX-220, as assessed using both immunohistochemistry and Western blot analysis. Double-labeling immunostaining reveals that the cells that upregulate hsp70 expression in the spinal cord in response to injury and treatment with BRX-220 are astroglia.

BRX-220 is a nontoxic hydroxylamine derivative that belongs to a group of compounds including Bimoclomol (Biorex R&D Co). Bimoclomol has been shown to act as a co-inducer of different subsets of heat shock proteins, including hsp70 and hsp90, under conditions of cellular stress (49). These compounds can interact with the cellular defence mechanisms of stressed cells to enhance their capacity to cope with various pathological conditions (4, 5, 26).

There is increasing evidence to show that heat shock proteins play an important role in the resistance of cells to stressful stimuli. Indeed, hsps have recently been shown to be capable of interacting with members of the apoptotic cascade. For example, results show that hsp27 can bind to cytochrome *c* released from mitochondria, thereby inhibiting the interaction of Apaf-1 with procaspase-9, which is the initial step in the apoptotic cascade (7). A similar mechanism of action has also been suggested for hsp70 and hsp90, since

both of these hsps can bind to Apaf-1, thereby inhibiting the activation of procaspase-9 (2, 40). *In vivo* experiments have also indicated that hsps can prevent cell death. For example, exogenously applied hsp70 can rescue dorsal root ganglia neurones from injury-induced cell death (24). In addition, neurones that have the ability to endogenously upregulate the expression of hsp27 in response to stress are more likely to survive (27, 31). Unlike hsp27 and hsp70, the role of hsp90 in neuroprotection is less well established. However, it has been shown that upregulation of hsp90 in stressed neurones provides protection against apoptosis through inhibition of caspase activation (30). Thus, when hsp90 function was inhibited by treatment with an antisense oligonucleotide, there was an extensive death of stressed neurones. Moreover, hsp90 has also been shown to promote neurite outgrowth of dissociated neurones, implying a possible role in neuroregeneration (25). Thus, upregulating the expression of these hsps in response to stress may increase the ability of neurones to survive following nerve injury.

It is well established that developing motoneurones are particularly susceptible to injury to their axons during a critical period of postnatal development. Injury to a peripheral nerve during the first few days of postnatal development results in the death of up to 90% of motoneurones (18, 32). Many of these motoneurones die rapidly within the first 2 days following injury, by the process of apoptosis (29). However, we have recently found that motoneurones that survive this initial period of apoptotic cell death immediately after neonatal axotomy have the ability to upregulate their expression of hsp27. Our results indicate that motoneurones which undergo apoptosis are unable to increase the expression of hsp27 following nerve injury and therefore enter a cascade that results in cell death (27). However, a significant proportion of those motoneurones that are able to increase hsp27 levels in response to injury will nevertheless eventually die (27). It is likely that these target-deprived motoneurones die because they show an increased vulnerability to the excitotoxic effects of glutamate, the main excitatory neurotransmitter in the spinal cord (19, 20, 48). It is clear that injured motoneurones are indeed more susceptible to glutamate agonists and there is some indication that motoneurones in conditions such as amyotropic lateral sclerosis (ALS) are also more susceptible to the toxic effects of glutamate (16, 34, 45). In addition, treatment with glutamate receptor antagonists or glutamate release inhibitors has been found to provide effective protection against motoneurone death both in animal models of motoneurone degeneration and in ALS (3, 13, 38, 41). One possible explanation for the selective vulnerability of motoneurones to the toxic effects of glutamate is that they lack certain calcium-binding proteins such as parvalbumin and calmodulin (44, 48). However, it is also possible that the increased susceptibility of injured or diseased motoneurones to glutamate may be a consequence of the reduced capacity of astrocytes adjacent to injured motoneurones to clear glutamate from the synaptic cleft. It has been found that in patients with ALS, the expression of a specific glutamate transporter, EAAT2 (also referred to as GLT1), is decreased, indicating its lack of astrocytic function (16, 46). This suggests that the control of glutamate metabolism may be disturbed in ALS. Other lines of evidence also indicate that astrocytes may play a role in the pathogenesis of ALS. For example reactive astrocytes and increased levels of markers for oxidative stress within astrocytes have been found in samples from ALS patients (15, 36).

Interestingly, *in vitro* studies have shown that preconditioning of astrocytes with a mild heat shock significantly increases their uptake of glutamate from the culture medium and results in an increase in neuronal survival (14). Although these authors did not examine hsp expression in their model, it has been established that elevated temperatures induce hsps in cultured astrocytes (37). Taken together, these results may therefore explain the observations of the present study that show that although BRX-220 has a remarkable effect on motoneurone survival, it does not appear to enhance hsp70 expression within these injured motoneurones. However, there is a clear increase in the levels of hsp70 and hsp90 in the astroglia which surround the injured motoneurones. This increase in hsp expression in astroglia may be important for astrocytic regulation and uptake of glutamate around the injured motoneurones, thereby decreasing the excitotoxic effects of glutamate. The importance of glial function in models of nerve injury has been previously demonstrated in studies in which a reactive astrogliosis was induced by treatment with an I_2–imidazoline receptor ligand, which resulted in an increase in motoneurone survival after axotomy, accompanied by an increase in the expression of EAAT2 (GLT1) in astrocytes (8). Apart from improvement in physiological function, there are also other ways by which astroglial hsp70 upregulation may be beneficial to neurones. It has been shown that hsp70 can be released from glial cells (23), and there are also reports demonstrating the transfer of hsp70 protein from glial cells to neurones (21, 47). Taken together with the findings of the present study these results indicate that astroglial cells may play a crucial role in the survival of injured motoneurones.

In this study, we have shown that treatment with BRX-220, a co-inducer of hsps, rescues neonatal motoneurones from injury-induced cell death, possibly by enhancing cellular defence mechanisms acting in both injured motoneurones and those cells that surround them within the spinal cord.

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REFERENCES

1. Beere, H. M., and D. R. Green. 2001. Stress management—Heat shock protein-70 and the regulation of apoptosis. *Trends Cell Biol.* **11:** 6–10.
2. Beere, H. M., B. B. Wolf, K. Cain, D. D. Mosser, A. Mahboubi, T. Kuwana, P. Tailor, R. I. Morimoto, G. M. Cohen, and D. R. Green. 2000. Hsp 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nat. Cell Biol.* **2:** 469–475.
3. Bensimon, G., L. Lacomblez, V. Meininger, and the ALS/Riluzole study group. 1994. A controlled trial of riluzole in amyotropic lateral sclerosis. *N. Engl. J. Med.* **330:** 585–591.
4. Biro, K., A. Jednakovits, T. Kukorelli, E. Hegedus, and L. Koranyi. 1997. Bimoclomol (BRLP-42) ameliorates peripheral neuropathy in streptozotocin-induced diabetic rats. *Brain Res. Bull.* **44:** 259–263.
5. Biro, K., J. Palhalmi, A. J. Toth, T. Kukorelli, and G. Juhasz. 1998. Bimoclomol improves early electrophysiological signs of retinopathy in diabetic rats. *Neuroreport* **9:** 2029–2033.
6. Brar, B. K., A. Stephanou, M. J. D. Wagstaff, R. S. Coffin, M. S. Marber, G. Engelmann, and D. S. Latchman. 1999. Heat shock proteins delivered with a virus vector can protect cardiac cells against apoptosis as well as against thermal and hypoxic stress. *J. Mol. Cell Cardiol.* **31:** 135–146.
7. Bruey, J. M., C. Ducasse, P. Bonniaud, L. Ravagnan, A. S. Susin, C. Diaz-Latoud, S. Gurbuxani, A. P. Arrigo, G. Kroemer, E. Solary, and C. Garrido. 2000. Hsp27 negatively regulates cell death by interacting with cytochrome c. *Nat. Cell Biol.* **2:** 645–652.
8. Casanovas, A., G. Olmos, J. Ribera, M. A. Boronat, J. E. Esquerda, and J. A. Garcia-Sevilla. 2000. Induction of reactive astrogliosis and prevention of motoneuron cell death by the I2-imidazoline receptor ligand LSL 60101. *Br. J. Pharmacol.* **130:** 1767–1776.
9. Close, R. I. 1967. Properties of motor units in fast and slow skeletal muscles in the rat. *J. Physiol.* **193:** 44–55.
10. Connold A. L., T. J. Fisher, S. Maudarbocus, and G. Vrbova. 1992. Response of developing rat fast muscles to partial denervation. *Neuroscience* **46:** 981–988.
11. Csermely, P., T. Schnaider, Cs. Soti, Z. Prohaszka, and G. Nardai. 1998. The 90-kDa molecular chaperone family: Structure, function and clinical applications. A comprehensive review. *Pharmacol. Ther.* **79:** 129–168.
12. Culling, C. F. A. 1963. *Handbook of Histopathological Techniques,* 2nd ed. Butterworth, London.
13. Dick, J., L. Greensmith, and G. Vrbova. 1995. Blocking of NMDA receptors during a critical stage of development reduces the effects of nerve injury at birth on muscles and motoneurones. *Neuromusc. Disord.* **5:** 371–382.
14. Dwyer, B. E., and R. N. Nishimura. 1994. Heat shock proteins and neuroprotection in CNS culture. In *Heat Shock Proteins in the Nervous System* (J. Mayer and I. Brown, Eds.), pp. 101–121. Academic Press, London.
15. Engel, W. K., V. Askanas, R. B. Alvarez, and E. Sarkozi. 1998. Oxydative stress demonstrated histologically in ALS astrocytes harmonizing with the hypothesis that malfunction of astrocytes may underlie sporadic ALS. *Neurology* **50:** A430.
16. Fray, A. E., P. G. Ince, S. J. Banner, I. D. Milton, P. A. Usher, M. R. Cookson, and P. J. Shaw. 1998. The expression of the glial glutamate transporter protein EAAT2 in motor neuron disease: An immunohystochemical study. *Eur. J. Neurochem.* **10:** 2481–2489.
17. Gilmore, S. A., T. J. Sims, and J. E. Leiting. 1990. Astrocitic reactions in spinal gray matter following sciatic axotomy. *Glia* **3:** 342–349.
18. Greensmith, L., and G. Vrbová. 1992. Alterations of nerve–muscle interaction during postnatal development influence motoneurone survival in rats. *Brain Res. Dev. Brain Res.* **81:** 125–131.
19. Greensmith, L., and G. Vrbová. 1996. Motoneurone survival: A functional approach. *Trends Neurosci.* **19:** 450–455.
20. Greensmith, L., H. Hasan, and G. Vrbová. 1994. Nerve injury induces the susceptibility of motoneurones to *N*-methyl-D-aspartate-induced neurotoxicity in the developing rat. *Neuroscience* **58:** 727–733.
21. Guzhova I., K. Kislyakova, O. Moskaliova, I. Fridlanskaya, M. Tytell, M. Cheetham, and B. Margulis. 2001. In vitro studies show that hsp70 can be released by glia and that exogenous hsp70 can enhance neuronal stress tolerance. *Brain Res.* **914:** 66–73.
22. Heikkila, J. J. 1993. Heat shock gene expression and development II. An overview of mammalian and avian developmental systems. *Dev. Genet.* **14:** 87–93.
23. Hightower, L. E., and P. T. Guidon, Jr. 1989. Selective release from cultured mammalian cells of heat shock (stress) proteins that resemble glia-axon transfer proteins. *J. Cell. Physiol.* **138:** 257–266.
24. Houenou, L. J., L. Li, M. Lei, C. R. Kent, and M. Tytell. 1996. Exogenous heat shock cognate protein Hsc 70 prevents axotomy-induced death of spinal sensory neurones. *Cell Stress Chaperones* **1:** 161–166.
25. Ishimoto T., A. Kamei, S. Koyanagi, N. Nishide, Uyeda, M. Kasai, and T. Taguchi. 1998. Hsp90 has neurite-promoting activity *in vitro* for telencephalic and spinal neurons of chick embryos. *Biochem. Biophys. Res. Commun.* **253:** 283–287.
26. Jednakovits, A., I. Kurucz, and P. Nanasi. 2000. Effect of subchronic Bimoclomol treatment on vascular responsiveness and heat shock protein production in spontaneously hypertensive rats. *Life Sci.* **67:** 1791–1797.
27. Kalmar, B., G. Burnstock, G. Vrbová, and L. Greensmith. 2002. The effect of neonatal nerve injury on the expression of heat shock proteins in developing rat motoneurones. *J. Neurotrauma,* in press.
28. Kobayashi, Y., A. Kume, M. Li, M. Doyu, M. Hata, K. Ohtsuka, and G. Sobue. 2000. Chaperones Hsp70 and Hsp40 suppress aggregate formation and apoptosis in cultured neuronal cells expressing trunkated androgen receptor protein with expanded polyglutamine tract. *J. Biol. Chem.* **275:** 8772–8778.
29. Lawson, S. J., and M. B. Lowrie. 1998. The role of apoptosis and excitotoxicity in the death of spinal motoneurons and interneurons after neonatal nerve injury. *Neuroscience* **87:** 337–348.
30. Lee M-W., S. C. Park, H.-S. Chae, J.-H. Bach, H.-J. Lee, S. H. Lee, Y. K. Kang, K. Y. Kim, W. B. Lee, and S. S. Kim. 2001. The protective role of hsp90 against 3-hydroxykynurenine-induced neuronal apoptosis. *Biochem. Biophys. Res. Commun.* **284:** 261–267.
31. Lewis, S. E., R. J. Mannion, F. A. White, R. E. Coggeshall, S. Breggs, M. Costigan, J. Martin, W. H. Dillmann, and C. J. Woolf. 1999. A role of hsp27 in sensory neuron survival. *J. Neurosci.* **19:** 8945–8953.

32. Li, L., L. J. Houenou, W. Wu, M. Lei, D. M. Prevette, and R. W. Oppenheim. 1998. Characterization of spinal motoneuron degeneration following different types of peripheral nerve injury in neonatal and adult mice. *J. Comp. Neurol.* **396:** 158–168.

33. Lowrie, M. B., S. Krishnan, and G. Vrbová. 1987. Permanent changes in muscle and motoneurones induced by nerve injury during a critical period of development of the rat. *Dev. Brain Res.* **31:** 91–101.

34. Ludolph, A. C., T. Meyer, and M. W. Riepe. 2000. The role of excitotoxicity in ALS—What is the evidence? *J. Neurol.* **247** (Suppl 1): I7–I16.

35. Moseley, P. L. 1997. Heat shock proteins and heat shock adaptation of the whole organism. *J. Appl. Physiol.* **83:** 1413–1417.

36. Nagy, D., T. Kato, and P. D. Kushner. 1994. Reactive astrocytes are widespread in the cortical gray matter of amyotropic lateral sclerosis. *J. Neurosci. Res.* **38:** 336–347.

37. Narashimhan, P., R. A. Swanson, S. M. Sagar, and F. R. Sharp. 1996. Astrocyte survival and hsp70 heat shock protein induction following heat shock and acidosis. *Glia* **17:** 147–159.

38. Nogradi, A., and G. Vrbová. 2001. The effect of riluzole treatment in rats on the survival of injured adult and grafted embryonic motoneurones. *Eur. J. Neurosci.* **13:** 113–118.

39. Nowak, T. S., Jr. 1993. Synthesis of heat shock/stress proteins during cellular injury. In *Markers of Neuronal Injury and Degeneration* (J. N. Johannessen, Ed.) Ann. N. Y. Acad. Sci. **679:** 142–156.

40. Pandey, P., A. Saleh, A. Nakazava, S. Kumar, S. M. Srinivasula, V. Kumar, R. Weichselbaum, C. Nalin, E. S. Alnemri, D. Kufe, and S. Kharbanda. 2000. Negative regulation of cytochrome c- mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *EMBO J.* **19:** 4310–4322.

41. Rothstein, J. D. 1995. Excitotoxic mechanisms in the pathogenesis of amyotropic lateral sclerosis. *Adv. Neurol.* **68:** 7–20.

42. Samali, A., and T. G. Cotter. 1996. Heat shock proteins increase resistance to apoptosis. *Exp. Cell Res.* **223:** 163–170.

43. Schmalbruch, H. 1984. Motoneurone death after sciatic nerve section in newborn rats. *J. Comp. Neurol.* **224:** 252–258.

44. Shaw, P. J., and C. J. Eggett. 2000. Molecular factors underlying selective vulnerability of motor neurons to neurodegeneration in amyotropic lateral sclerosis. *J. Neurol.* **247:** 117–127.

45. Shaw, P. J., and P. G. Ince. 1997. Glutamate, excitotoxicity and amyotrophic lateral sclerosis. *J. Neurol.* **244:** 3–14.

46. Trotti, D., A. Rolfs, N. C. Danbolt, R. H. Brown, Jr., and M. A. Hediger. 1999. SOD1 mutants linked to amyotropic lateral sclerosis selectively inactivate a glial glutamate transporter. *Nat. Neurosci.* **2:** 427–433.

47. Tytell M., S. G. Greenberg, and R. J. Lasek. 1986. Heat shock-like protein is transferred from glia to axon. *Brain Res.* **363:** 161–164.

48. Van Den Bosh, L., W. Vanderberghe, H. Klaassen, E. Van Houtte, and W. Robberecht. 2000. Ca (2+)-permeable AMPA receptors and selective vulnerability of motor neurons. *J. Neurol. Sci.* **180:** 29–34.

49. Vigh, L., P. N. Literati, I. Horvath, Zs. Torok, G. Balogh, A. Glatz, E. Kovacs, I. Boros, P. Ferdinandy, B. Farkas, L. Jaszlits, A. Jednakovits, L. Koranyi, and B. Maresca. 1997. Bimoclomol: A nontoxic, hydroxylamine derivative with stress protein-inducing activity and cytoprotective effects. *Nat. Med.* **3:** 1150–1154.

50. White, C. M., L. Greensmith, and G. Vrbová. 2000. Repeated stimuli for axonal growth causes motoneuron death in adult rats: The effect of botulinum toxin followed by partial denervation. *Neuroscience* **95:** 1101–1109.

51. Xu, L., and R. G. Giffard. 1997. Hsp70 protects murine astrocytes from glucose deprivation injury. *Neurosci. Lett* **224:** 9–12.

52. Zelena, J., and P. Hnik. 1963. Motor and receptor units in the soleus muscle after nerve regeneration in very young rats. *Physiol. Bohemoslov* **12:** 227–289.