Reduction of Lower Motor Neuron Degeneration in *wobbler* Mice by *N*-Acetyl-L-Cysteine

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The murine mutant *wobbler* is a model of lower motoneuron degeneration with associated skeletal muscle atrophy. This mutation most closely resembles Werdnig–Hofmann disease in humans and shares some of the clinical features of amyotrophic lateral sclerosis (ALS). It has been suggested that reactive oxygen species (ROS) may play a role in the pathogenesis of disorders such as ALS. To examine the relationship between ROS and neural degeneration, we have studied the effects of agents such as *N*-acetyl-L-cysteine (NAC), which reduce free radical damage. Litters of *wobbler* mice were given a 1% solution of the glutathione precursor NAC in their drinking water for a period of 9 weeks. Functional and neuroanatomical examination of these animals revealed that *wobbler* mice treated with NAC exhibited (1) a significant reduction in motor neuron

loss and elevated glutathione peroxidase levels within the cervical spinal cord, (2) increased axon caliber in the medial facial nerve, (3) increased muscle mass and muscle fiber area in the triceps and flexor carpi ulnaris muscles, and (4) increased functional efficiency of the forelimbs, as compared with untreated *wobbler* littermates. These data suggest that reactive oxygen species may be involved in the degeneration of motor neurons in *wobbler* mice and demonstrate that oral administration of NAC effectively reduces the degree of motor degeneration in *wobbler* mice. This treatment thus may be applicable in the treatment of other lower motor neuropathies.

Key words: spinal cord; murine; antioxidant; mutant; neurodegenerative; lower motoneuron

A variety of stimuli have been shown to elevate intracellular levels of reactive oxygen species (ROS), which in turn enhance the rate of programmed cell death in different neural cell types (Sheen and Macklis, 1994; Greenlund et al., 1995; Kroemer et al., 1995; Verity et al., 1995). These stimuli include growth factor withdrawal (Mayer and Noble, 1994; Ferrari et al., 1995; Greenlund et al., 1995), ischemia (Folergrova et al., 1995; Knuckey et al., 1995), and glutamate-mediated excitotoxicity (Dugan et al., 1995; Mattson and Goodman, 1995; Schulz et al., 1995). The importance of ROS in programmed cell death also has been suggested from the neuroprotective effects of bcl-2 family proteins, which seem to act by inhibiting ROS-induced cell damage (Kane et al., 1993; Zhong et al., 1993; Myers et al., 1995; Lawrence et al., 1996). In humans, elevated ROS levels also have been linked to neuropathies such as Parkinson's disease, Huntington's chorea, Alzheimer's disease, infantile spinal muscular atrophy, and amyotrophic lateral sclerosis (ALS; Olanow and Arendash, 1994; Beal, 1995; Eisen, 1995; Mattson and Goodman, 1995).

The compound *N*-acetyl-L-cysteine (NAC) has been shown to inhibit ROS, thus increasing the viability of cells in culture, including spinal motor neurons (Rothstein et al., 1994), oligoden-

drocytes (Mayer and Noble, 1994), cortical neurons (Rattan et al., 1994), superior cervical ganglion neurons (Yan et al., 1995), and PC-12 cells (Ferrari et al., 1995; Yan et al., 1995). *In vivo*, NAC also has been shown to reduce the toxicity of compounds such as methylmercury, which induce oxidative stress (Ornaghi et al., 1993). In humans, extensive clinical experience with NAC suggests that it is a compound of low toxicity most commonly used to counteract the oxidative damage caused by acetaminophen overdose, used as a mucolytic, or used in the treatment of AIDS (Berkow and Fletcher, 1992; Brennan and O'Neil, 1995). NAC seems to exert these effects by acting directly as an antioxidant, as a precursor of glutathione synthesis, or via the induction of specific genes (Brennan and O'Neil, 1995; Staal et al., 1995).

Because of the link between oxidative damage and neural degeneration *in vitro*, we examined the possibility that agents that inhibit the formation of ROS may be useful as therapeutic agents *in vivo* for certain forms of neural degeneration. We have used the murine mutant *wobbler* (*wr*), a recessive mutation that induces lower motor neuron degeneration, primarily within the cervical spinal cord and the cranial motor nuclei (Duchen and Strich, 1968; Bird et al., 1971; Andrews, 1975), as a model system to address these issues. Homozygous *wr/wr* animals are distinguishable by 3–4 weeks of age by their unsteady gait and fine head tremor. Homozygous *wr* animals also exhibit skeletal muscle atrophy, infertility, and increased mortality (Bird et al., 1971; Baulac et al., 1983).

Histological analysis of the cervical spinal cord of *wobbler* mice demonstrates Wallerian degeneration, reductions in axon caliber, and decreases in choline acetyltransferase (ChAT) activity within the anterior horn by 8 weeks of age (Bird et al., 1971; Mitsumoto and Bradley, 1982; Baulac et al., 1983). Affected neurons may exhibit signs of sprouting and eventually undergo vacuolar dila-

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tion of the Golgi complex and endoplasmic reticulum (Mitumoto and Bradley, 1982; Vacca-Galloway and Steinberger, 1986). The morphological properties of degenerating *wobbler* neurons, together with the lack of inflammation at these sites, suggest that *wobbler* motor neurons do not die by necrosis (Andrews, 1975; Mitumoto and Bradley, 1982; Ma et al., 1991).

To examine the effects of NAC on motor neuron degeneration *in vivo*, we treated *wobbler* mice *per os* with NAC over a period of 9 weeks. The results demonstrate that NAC treatment reduces the decline in several important morphological and functional parameters of neurodegeneration.

MATERIALS AND METHODS

Animals. *wobbler* mice were obtained from the mating of confirmed heterozygous *wr/+* mice. All animals received food and fluids *ad libitum*. Animals received drinking water supplemented with either 1% *N*-acetyl-L-cysteine, L-alanine, or D-glucose adjusted to the same pH (pH 3.5) and osmolarity. Fluids were changed every 48 hr. *wobbler* mice were sacrificed for examination at postnatal days 63–66. All animals were raised under identical conditions in the same room of our gnotobiotic animal facility. All experimental protocols conformed to Mount Sinai Hospital's and University of Toronto's animal colony care guidelines.

Sample preparation. Animals were anesthetized deeply with sodium pentobarbital (Somnitol 80 mg/kg). After the absence of twitch responses, animals were perfused transcardially with 15 ml of 100 mM PBS, pH 7.4, followed immediately by 50 ml of freshly prepared 4% paraformaldehyde in PBS at 4°C. At this point, a 2 ml segment representing the major and minor medial branches of the left facial nerve were dissected, along with the c2–c7 region of the spinal cord and several muscles of the left forelimb of each animal. Then samples were post-fixed for a further 2 hr in 4% paraformaldehyde in PBS at 4°C. At this point the spinal cords were dissected further to the c4–c7 region. Samples were processed subsequently for either cryostat, paraffin, or thin sections. At this point each sample was given a coded identification number so that data derived from the samples could be analyzed in a “blind” manner.

Paraffin sections. Samples were dehydrated by being placed in an ascending series of ethanol/water and ethanol baths and embedded into paraffin blocks according to standard procedures (Ausubel et al., 1994). Then 10 μ sections were cut on a Reichert–Jung microtome, mounted on 2× gelatin-coated slides, and heated at 60°C for 1.5 hr. Slides subsequently were de-waxed and stained in thionine as described previously (Culling, 1974).

Thin sections. Specimens were post-fixed in a solution of freshly prepared 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4, for 4 hr at 4°C and then rinsed free of glutaraldehyde and fixed in 1% osmium tetroxide buffered in PBS for 1 hr. Samples were dehydrated in a series of water/ethanol and ethanol/propylene oxide baths. After removal of propylene oxide, samples were embedded in Spurr resin and baked at 50°C for 36 hr. A series of 1- μ -thick cross sections then was cut through the entire nerve and stained with 1% toluidine blue according to standard procedures (Culling, 1974).

Cryostat sections/immunohistochemistry. Freshly post-fixed spinal cords were placed in 30% sucrose and 0.1 M PBS, pH 7.4, at 4°C until sunk (12–15 hr) and then frozen in 2-methyl butane at –20°C. Serial cross sections (10 μ) subsequently were obtained via a Reichardt–Jung Fridgocut cryostat. These were thaw-mounted onto 2× gelatin-stubbed slides and either stained with thionine or processed for ChAT immunoreactivity with a Chemicon (Temecula, CA) goat anti-ChAT antibody as described previously (Henderson et al., 1994).

Axon/nerve morphometry. The morphometry of nerve and muscle cross sections was analyzed by a Leitz Wetzlar scope equipped with 25, 54, and 100× objectives, a JVC model TK-1280U color video camera, and a 360° rotating slide platform with x- and y-axis controllers. Nerve areas were measured by a Leica Quantimet Q500MC system (Leica Canada, Willowdale, Ontario). The system was calibrated before and verified after each use with a Leica 10- μ -ruled calibration slide. Before each cross section was analyzed, a low resolution (25×) “map” was generated first, and then a hard copy printed. This was used as a reference to place each of the individual nerve (analyzed at 100×) or muscle (analyzed at 40×) sectors in a given morphometric analysis. In each case, data were gathered for the nerve cross section in its entirety, and data were analyzed in double-blind manner.

Glutathione peroxidase assay. Animals were sacrificed and flushed with

15 ml of 100 mM PBS, pH 7.4, at 4°C. Selected tissues were dissected and washed in PBS at 4°C; their weights were obtained, and the tissues were frozen immediately in 2-methyl butane at –50°C and stored at –70°C until used (72 hr). Glutathione peroxidase (GPx) activity was determined by monitoring the rate of NADPH conversion to NADP⁺ in the presence of hydrogen peroxide, as described previously (Doroshov et al., 1980). Assays were corrected for the nonenzymatic conversions of NADPH (consistently <11% of the enzymatic rates), and each sample was assayed in triplicate. Rates were expressed as nanomoles of NADPH oxidized to NADP⁺/min per milligram of protein, assuming an extinction coefficient for NADPH of $6.22 \times 10^3 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, and plotted as a percentage of NW control values.

Statistics. Three analyses were performed on each group of data with one-way ANOVA, followed by Student's *t* test, with Bonferroni adjustment for multiple comparisons (Howell, 1992). For each of the treatment groups [NW, NW(N), *wr/wr*, *wr/wr*(N)], three types of preplanned pairwise statistical comparisons were made: *wr/wr* versus NW, *wr/wr* versus *wr/wr*(N), and *wr/wr*(N) versus NW. The α level for all comparisons was set to 0.05, and all *t* values were compared with two-tailed critical *t* values. For each measure in which significant pairwise comparisons are reported, ANOVA yielded significant results ($\alpha = 0.05$).

Analysis of motor function. Mice were scored at 7 weeks postnatal in a blind manner for forelimb motor performance. Each limb was scored separately for each animal. Criteria were as follows: (1) no atrophy, limbs outstretched when suspended, continuous grasping at proximal surface, normal inclination of the phalanges; (2) visible atrophy of limbs, limbs outstretched when suspended, continuous grasping at proximal surface, normal inclination of phalanges; (3) significant atrophy of limbs, limbs not outstretched when suspended, tentative grasping at proximal surface, abnormal ($\leq 60^\circ$) inclination of phalanges; (4) severe limb atrophy, limbs held closely to body when suspended, no grasping at proximal surface, abnormal ($\geq 90^\circ$) inclination of phalanges.

RESULTS

Treatment groups and conditions

wobbler mice were maintained by taking litters derived from *wr/+* × *wr/+* crosses and mating these pups to mice confirmed previously to be heterozygous for the *wobbler* gene. This method was used because *wr/wr* mice are infertile, and the genetic basis of the *wobbler* mutation is unknown (Kaupmann et al., 1992). In addition, there are no genetic loci that can definitively be used to determine the genotype of *wr* animals. After the identification of sufficient numbers of *wr/+* mice, three *wr/+* × *wr/+* breeding pairs were set up from our sibling stock. Pups subsequently born to these breeding pairs were used for analysis.

Each of the breeding pairs received normal acidified (pH 3.5) drinking water supplemented with either 1% *N*-acetyl-L-cysteine (B24001-30, BDH Chemicals, Poole, UK), L-alanine (A5824, Sigma, St. Louis, MO), or (+)D-glucose (G7528, Sigma). Alanine and glucose solutions were adjusted to the same pH and molarity as NAC-treated animals (61.2 mM) and served as control agents. Thus, for a given litter, all animals were exposed to the same agent. Breeding pairs and the mice derived from them were treated continuously with a given factor, which was replenished every 48 hr, to ensure that pups would ingest the agent at the earliest possible time point. Four-week-old NAC-treated mice drank an average of 3.4 ± 0.6 ml per 24 hr period and had an average weight of 14.6 ± 0.8 gm ($n = 20$). The mean quantity of NAC ingested was ~ 2.3 mg/gm body weight. For 9-week-old *wr/wr* mice, the amount of water consumed was 4.5 ± 0.9 ml per 24 hr, and mice had an average weight of 21 ± 1.3 gm ($n = 16$; *wr/wr* weights were $\sim 73\%$ of NW controls). The mean quantity of NAC received was 2.1 mg/gm body weight. Mice born from each breeding pair were treated for a period of 9 weeks, at which time they were killed for analysis. At this time, mice within each treatment group were designated as either *wobbler* (*wr/wr*) or “non-*wobbler*” (NW), and the tissues were coded for analysis. Even within the

Figure 1. NAC reduces axonal loss in *wobbler* facial nerve. *A*, Analysis of the superior medial branch of the facial nerve. *B*, Plot of the total cross-sectional area of the medial facial nerve for each group. Values represent the mean $\pm$ SEM; Asterisk signifies a significant difference ($p < 0.05$), as compared with the *wr/wr* group. Control-treated *wr/wr* mice, $n = 17$; NAC-treated *wr/wr* mice, $n = 11$; NW mice, $n = 11$; NW(N) mice, $n = 10$. *wr/wr* versus *wr/wr*(N), $p < 0.004$; *wr/wr* versus NW, $p < 0.005$; *wr/wr* versus NW(N), $p < 0.006$. *C*, Number of axons in nerve cross sections. For all groups $n = 10$. *wr/wr* versus *wr/wr*(N), $p < 0.027$; *wr/wr* versus NW, $p < 0.030$.

NAC-treated group the distinction between *wr/wr* and NW mice was apparent at this time point. The “NW” groups are thus composed of both *wr/+* and *+/+* genotypes. For all of the analyses presented below, no significant differences in any experimental parameter were observed between NW animals from either the L-alanine or (+)D-glucose groups. Thus data for these animals are all presented under the NW groups. Similarly, no significant differences were observed between *wr/wr* animals from either the L-alanine or (+)D-glucose treatment groups. Data for these animals are presented as the *wr/wr* group.

The litter size and numbers of *wr/wr* mice observed per litter did not differ significantly from that expected by Mendelian segregation (22%), suggesting that *wr/wr* mice do not exhibit elevated mortality rates, as compared with pups from *wr/+* $\times$ *+/+* breeding pairs from 0–9 weeks. This also suggests that the ratio of *wr/+* to *+/+* in the non-*wobbler* group is 2:1.

Two distinct populations of lower motor neurons were examined: cervical spinal motor neurons and motor neurons of the

facial nerve. Both of these populations have been shown to undergo progressive degenerative changes in *wr/wr* animal. These degenerative changes result in overt muscular weakness that is first apparent at 3 weeks of age (Bird et al., 1971). For this study, changes in axon, nerve, and muscle caliber were determined as a function of cross-sectional area rather than diameter because of the intrinsically higher reliability of this measure.

Analysis of the facial nerve

To assess changes occurring within the facial nerve of *wobbler* mice, we examined the major medial branch of the facial nerve in cross section at a point 2 mm distal to the stylomastoid foramen, as shown in Figure 1*A*. Measurement of the cross-sectional nerve area of this branch of the facial nerve for *wr/wr*, NW, and NAC-treated *wr/wr* and NW mice is shown in Figure 1*B*. These data demonstrate that *wr/wr* mice undergo a substantial reduction in nerve caliber, as compared with NW controls, by 9 weeks of age. In NAC-treated *wr/wr* mice [*wr/wr*(N)] this reduction was elimi-

Figure 2. NAC prevents losses in axon caliber in *wobbler* mice. Shown is the distribution of axon areas in medial branch of the facial nerve. *A*, Axon distributions of NW and NW(N) mice, $n = 7$ per group. *B*, Axon distribution of *wr/wr* mice, $n = 6$. *C*, Axon distribution of NAC-treated *wr/wr* mice, $n = 6$. *D*, Comparison of axon distributions in each treatment group. Values represent the mean $\pm$ SEM; the asterisk signifies a significant difference ($p < 0.05$) between *wr/wr(N)* and *wr/wr* groups at the indicated axon area; (+) signifies a significant difference between NW and *wr/wr* groups at the indicated axon area. Within a given group, individual percentage values did not vary by more than $\pm 2\%$ of the mean.

nated, and mice exhibited nerve areas that were not significantly different from NW controls. To further define the nature of the reduction in nerve caliber, we collected complete axon counts throughout the entire nerve segment for animals of each treatment group, as shown in Figure 1C. These data indicate that *wr/wr* mice exhibit a small, but significant, reduction in the number of axons that project throughout this branch of the facial nerve, as compared with NW controls. *wobbler* animals treated with NAC do not show this loss of motor axons, instead giving values that are similar to those of the NW group.

The relatively small reduction in the number of axons in *wr/wr* mice, as compared with NW controls, indicates that only a portion of facial motor axons has undergone complete degeneration by 9 weeks of age. The comparatively larger magnitude of the reduction in gross nerve caliber suggests that the predominant cause of this reduction is one of axonal atrophy rather than of axonal loss. To examine this possibility, we determined the area of each axon within the superior median branch of the facial nerve for each treatment group. Axon areas, rather than axon diameters, were determined because of their inherently greater accuracy. Measured axon areas represent the intraluminal area of each axon and

do not include components of the myelin sheath. After their collection, axon areas were rounded to the nearest whole integer (μm^2), and the resulting distribution was plotted as a percentage of the total axons in a given nerve, as shown in Figure 2. Displaying the data in this format eliminates the compounding influence of a change in total axon number within a given group. Thus, if a given treatment group were to lose a subset of axons, but the axons that remained were of normal caliber (in normal proportions) with respect to the control group, no difference in the axon distribution between the two groups would be observed. We have observed that the frequency and size distributions of axon areas (when measured for an entire nerve cross section) are highly reproducible and sensitive measures of the changes that occur within a nerve fiber.

Figure 2A shows the normal distribution of axon areas for the NW control groups. These distributions are similar to those observed for this branch of the facial nerve in other genetic backgrounds at the same location and age (J.T. Henderson, unpublished observations). Figure 2B,C shows the distribution of axon areas in *wr/wr* and NAC-treated *wr/wr* mice, respectively. Comparison of these values, as shown in Figure 2D, indicates that

Figure 3. NAC reduces the loss of ChAT-positive neurons in the cervical spinal cord of *wobbler* mice. Total numbers of choline acetyltransferase-positive neurons are indicated for each group ($n = 4$). Numbers represent counts from every fifth section in a serial series of 10 μ transverse sections through the entire c4–c7 region. Values represent the mean $\pm$ SEM; asterisk signifies a significant difference ($p < 0.05$), as compared with the *wr/wr* group.

wr/wr mice do exhibit a reduction in axon caliber consistent with the overall reduction in nerve area, as compared with NW controls. This seems to be a generalized atrophy that affects axons of all caliber and is marked by a shift in the mean distribution toward smaller axon areas, as compared with the NW group. In contrast, NAC-treated *wobbler* mice do not show a marked reduction in axon caliber by 9 weeks of age, exhibiting an axon distribution that is not substantially different from NW control animals. If one assumes that the *wobbler* mutation exerts a generalized effect on all axons of this population equally, the data can be interpreted in terms of an effect on the true distribution. In this case, the *wobbler* controls are significantly different from both the *wr* NAC treatment and NW groups [pair-wise comparisons: *wr/wr* vs *wr/wr*(N), $p < 0.007$; *wr/wr* versus NW, $p < 0.030$; *wr/wr* versus NW(N), $p < 0.028$; *wr/wr*(N) versus NW, $p < 0.43$; two-tailed distribution]. Even if one takes the more conservative view (as we have in Fig. 2D) that one cannot be assured that the *wobbler* mutation affects all axons of this population equally and that the data from different groups can be analyzed only in terms of their absolute area, significant differences between these groups still exist at several discrete axon calibers.

The data given above demonstrate that oral treatment of *wobbler* mice with NAC can reduce significantly the degree of axonal atrophy and loss that normally occur in this murine mutant.

Analysis of cervical motor neurons

Atrophy was apparent in the forelimb of *wr/wr* animals by 9 weeks of age. To examine the effects of NAC on neuromuscular aspects of the forelimb, we took 10 μ serial frozen sections through the c4–c7 level of the spinal cord. Then every fifth section was stained for choline acetyltransferase. The number of ChAT-positive neurons shown in Figure 3 represents the number of ChAT-positive neurons counted for every fifth section. As shown in the Figure 3, *wr/wr* mice exhibit a substantial reduction in the number of ChAT-

positive neurons by 9 weeks of age. *wobbler* mice treated with NAC exhibited significantly greater numbers of ChAT-positive neurons than *wr/wr* mice (58 vs 44% of control values, respectively). However, NAC-*wobbler* mice still exhibited a substantial loss of ChAT-positive neurons, as compared with the NW mice of the same age.

Structure and function of forelimb muscles

To determine the effects of NAC treatment on the innervation targets of these spinal motor neurons, we examined proximal and distal muscles of the forelimb. As shown in Figure 4A,B, animals treated with NAC show a significant increase in the overall mass of the triceps and flexor carpi ulnaris, respectively, in comparison with animals in the *wr/wr* group. Thus, treatment with NAC reduces the degree of overt muscular atrophy that is induced by the *wobbler* mutation. To delineate more clearly the effects of NAC on muscle morphology within the distal forelimb, we determined cross-sectional areas of muscle fibers within the flexor carpi ulnaris for each group. The results, shown in Figure 4C, indicate that animals treated with NAC show a marked increase in mean fiber area ($\sim 603 \mu\text{m}^2$), as compared with *wobbler* controls ($\sim 403 \mu\text{m}^2$). As indicated in Figure 4, each group exhibits a distribution of muscle fiber areas that were significantly different [for each comparison *wr/wr*:*wr/wr*(N), *wr/wr*:NW, *wr/wr*(N):NW; $p < 0.001$].

These data demonstrate that oral administration of NAC can reduce significantly motor neuron loss and axonal atrophy in *wobbler* mice as well as retard muscle atrophy within the forelimbs of these animals. However, it is also important to determine what the functional consequences, if any, are of this treatment. To determine this, we scored animals in a blind manner for several overt properties of forelimb function. As shown in Figure 5, the distribution of forelimb function was significantly different for each of the pair-wise comparisons [*wr/wr* vs *wr/wr*(N), *wr/wr* versus NW or NW(N), *wr/wr*(N) versus NW or NW(N); $p < 0.001$]. These results demonstrate that, although *wobbler* mice receiving NAC do exhibit a significant reduction in forelimb function, they perform better on average than animals from the control *wr/wr* group. These data suggest that treatment with NAC does result in some reduction in the functional losses that occur within the forelimbs of *wobbler* mice.

Glutathione peroxidase activity

Previous work has suggested that NAC acts in part by increasing intracellular supplies of cysteine, the rate-limiting step in glutathione synthesis (Ferrari et al., 1995). Glutathione is an important component of the free-radical scavenging system of the body, because it serves as a substrate for glutathione-dependent enzymes such as glutathione peroxidase (GPx; Bergelson et al., 1994). The importance of GPx in the protection of neural cells from programmed cell death and its regulation by neurotrophins has been demonstrated in several studies (Pan and Perez-Pollo, 1993; Sampath et al., 1994; Kroemer et al., 1995; Mattson et al., 1995). To determine the ability of NAC to enhance the free-radical scavenging ability of the GPx system, we directly assessed the level of GPx activity in *wobbler* mice. Segments of c3–c7 spinal cord were dissected from 9-week-old mice in each of the treatment groups and assayed in triplicate for GPx activity. As shown in Figure 6, animals that received NAC in their drinking water exhibited a substantial increase in GPx activity within the cervical spinal cord. The level of GPx activity was similar to that of NW mice that did not receive NAC supplementation. To further assess the ability of NAC to effect GPx activity levels in non-*wobbler*

Figure 4. NAC reduces the atrophy of forelimb muscles in *wobbler* mice. *A*, Plot of triceps muscle mass for each treatment group ($n = 12$). Values represent the mean $\pm$ SEM; (*) signifies a significant difference ($p < 0.05$), as compared with the *wr/wr* group. For *wr/wr* versus *wr/wr(N)*, $p < 0.003$. *B*, Plot of flexor carpi ulnaris muscle mass for each treatment group. $n \geq 12$ animals per group; values represent the mean $\pm$ SEM; asterisk signifies a significant difference ($p < 0.05$), as compared with the *wr/wr* group. For *wr/wr* versus *wr/wr(N)*, $p < 0.021$. *C*, Percentage of distribution of muscle fiber areas. For each treatment group, mean cross-sectional areas were determined for 70 muscle fibers in each of four separate individuals. Muscle cross sections were taken at the midpoint of the main body of the flexor carpi ulnaris. Then areas were determined for the medial fibers within each cross section. All fiber areas were determined in a blind manner from coded 1μ thin sections. For each pair-wise comparison of the treatment groups [*wr/wr*:*wr/wr(N)*, *wr/wr*:*NW*, *wr/wr(N)*:*NW*], $p < 0.001$.

mice, we injected several NW mice (glucose treatment group) daily with 1 mg/gm NAC for 3 d before the analysis. As shown in Figure 6, this treatment resulted in a marked increase in GPx activity. These results demonstrate the ability of NAC to enhance antioxidant activity in tissues affected by the *wobbler* mutation. This effect was not specific to *wr/wr* mice but, rather, reflected a generalized enhancement of GPx activity.

DISCUSSION

The results demonstrate that daily oral administration of NAC significantly reduces the degeneration of lower motor neurons in *wobbler* mice. For motor axons of the facial nerve, application of NAC resulted in a generalized increase in axon number and caliber. Within the cervical spinal cord, NAC reduced losses of choline acetyltransferase-positive neurons. Examination of both

proximal (triceps) and distal (flexor carpi ulnaris) forelimb muscles also revealed significant increases in both muscle mass and mean fiber area. Although these increases were nominal, they were sufficient to promote a substantial increase in the functional activity of *wobbler* forelimbs, as compared with littermates that received D-glucose or L-alanine supplementation.

The mechanism by which lower motor neurons degenerate in *wobbler* mice presently is unknown. However, ROS have been suggested to play an important role in several human neurodegenerative diseases, including ALS, a disorder that shares some of the features of the *wobbler* mutation (Wakai et al., 1994; Abe et al., 1995; Beal, 1995; Busciglio and Yanker, 1995; Eisen, 1995). Consistent with this, agents that inhibit ROS have been shown to be neuroprotective, both *in vitro* and *in vivo*, under conditions of

Figure 5. NAC reduces functional losses in the forelimb of *wobbler* mice. Seven-week-old mice from each of the treatment groups were examined by an observer blinded with respect to the animals' treatment status. Functional classes were divided as follows: 1, no apparent atrophy, limbs outstretched when suspended, continuous grasping at proximal surface, normal inclination of the phalanges; 2, visible atrophy of limbs, limbs outstretched when suspended, continuous grasping at proximal surface, normal inclination of phalanges; 3, significant atrophy of limbs, limbs not outstretched when suspended, tentative grasping at proximal surface, abnormal ($\leq 60^\circ$) inclination of phalanges; 4, severe limb atrophy, limbs held closely to body when suspended, no grasping at proximal surface, abnormal ($\geq 90^\circ$) inclination of phalanges. Forelimbs were scored separately for each animal. For the NW and NW(N) groups, $n = 6$, with all animals scoring "1." For wr/wr, wr/wr(N) groups, $n = 8$ animals per group. For each pair-wise comparison of the treatment groups [wr/wr vs wr/wr(N), wr/wr vs NW or NW(N), wr/wr(N) vs NW or NW(N)], $p < 0.001$.

acute neural injury (Pan and Perez-Pollo, 1993; Sampath et al., 1994; Sato et al., 1995). NAC has been shown previously in several of these paradigms to be particularly effective in enhancing cell survival and reducing free radical damage in neural cells (Mayer and Noble, 1994; Rothstein et al., 1994; Ferrari et al., 1995; Knuckey et al., 1995; Talley et al., 1995).

The ability of NAC to reduce the loss of these lower motor neurons in *wobbler* mice, together with the ability of this treatment to elevate levels of GPx activity in the cervical spinal cord, suggests a means by which NAC may act to reduce local ROS levels. Previous work also demonstrates that NAC directly supports glutathione synthesis in neural cells, thus reducing intracellular ROS levels (Mayer and Noble, 1994; Rattan et al., 1994; Rothstein et al., 1994). However, it is important to note that Yan et al. (1995) have demonstrated that both the D and L isomers of NAC are capable of promoting the survival of PC12 cells in culture. In addition, NAC has been shown to induce genes such as NF- κ b (Brennan and O'Neil, 1995; Staal et al., 1995). These data suggest that NAC may exert survival-promoting effects in a manner independent of its actions as a direct antioxidant.

NAC has been used previously in a limited trial over a 12 month period with both the bulbar and limb onset isoforms (50 mg/kg; de Jong et al., 1987; Louwerse et al., 1995). Although no net clinical improvement was observed in patients treated with NAC, segregation of ALS cases into bulbar and limb onset groups is informative. In ALS cases of bulbar onset, NAC did not improve survival. However, in patients with the limb onset form, NAC does seem to improve survival (NAC 74%, 28/38; vehicle 51%, 22/43). However, these results were not quite significant ($p < 0.06$) because of the relatively small trial size and heterogeneous state

Figure 6. NAC treatment augments glutathione peroxidase (GPx) levels in the cervical spinal cord of *wobbler* mice. Segments of the cervical spinal cord (c3–c7) were dissected and examined for GPx activity in 9-week-old *wobbler* mice. GPx values are plotted as a percentage of the NW group. The NW(N+) group represents a positive control consisting of NW animals (glucose supplement) given daily intraperitoneal injections of *N*-acetyl-L-cysteine ($1 \text{ mg} \cdot \text{gm}^{-1} \cdot \text{d}^{-1}$) for 3 d before killing. For NW and NW(N) groups, $n = 4$; for wr/wr and wr/wr(N) groups, $n = 6$. Values represent the mean $\pm$ SEM; asterisk signifies $p < 0.05$ for a given group versus the wr/wr group. wr/wr versus wr/wr(N), $p < 0.003$; wr/wr versus NW or NW(N), $p < 0.01$.

of presenting patients and disease progression. It is interesting to speculate that the differential availability of NAC to these two neural populations may underlie the differences in their response. For comparison, one current therapy that shows promise for ALS patients is studies with the glutamate antagonist riluzole (100 mg/d). When riluzole is given over a 12 month period, there are some indications of clinical improvements in ALS patients, suggesting that aberrant glutamate metabolism (and perhaps ROS) may play a role in this disorder (Bensimon et al., 1994).

The neuropathy present in *wobbler* mice has been treated previously with a combination of CNTF (1 mg/kg) and BDNF (5 mg/kg) by subcutaneous injection three times per week over a 4 week period (postnatal weeks 4–8; Mitsumoto et al., 1994). Treated animals exhibited substantial improvements in the number of ChAT-positive neurons within the cervical spinal cord, together with improvements in muscle mass, fiber size, and forelimb function. Although the measures used in these studies differ somewhat from our own, these results demonstrate that wr mice treated with neurotrophic factors retain greater function over a similar time frame than those treated with NAC. These differences may reflect differences in the efficiency of the treatment agent to act on the affected population. For example, to be accessible to motor neurons, NAC must be taken up via contact with somatic tissue through its motor (and sensory) innervation. Oral administration of NAC does result in enhanced GPx activity in the cervical spinal cord, suggesting that there is significant accumulation from the periphery. However, motor neurons that have already begun to degenerate at their terminals or to show impaired axonal transport may be impaired in their ability to accumulate NAC and its metabolites, thus limiting its potential

therapeutic effects. Because of this, this form of antioxidant treatment is likely to be more beneficial in the early stages of motor degeneration rather than later, when axon transport has already been substantially impaired. Clearly this issue can be addressed by altering the administration route. In addition, it is important to realize that NAC may be affecting only a portion of the pathways involved in the control of programmed cell death. The coordinated support of some, or all, of these pathways may be necessary to achieve optimal results with respect to cell viability. Despite this, the use of agents such as NAC would seem to be a practical approach to enhancing cell survival for several reasons. Unlike current neurotrophic therapies that involve the subcutaneous or intrathecal implantation of proteins, which are quite labile *in vivo*, NAC can be given *per os* over dispersed intervals. NAC is also an inexpensive drug, possessing few side effects and having extensive clinical experience, in contrast to current neurotrophic therapies (Berkow and Fletcher, 1992; Brennan and O'Neil, 1995). Given the similarities in the neuropathy observed in *wobbler* to that of several human neurodegenerative disorders, the application of NAC may prove useful in treating other mammalian neurodegenerative diseases.

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