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Parvalbumin overexpression alters immune-mediated increases in intracellular calcium, and delays disease onset in a transgenic model of familial amyotrophic lateral sclerosis

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Abstract

Intracellular calcium is increased in vulnerable spinal motoneurons in immune-mediated as well as transgenic models of amyotrophic lateral sclerosis (ALS). To determine whether intracellular calcium levels are influenced by the calcium-binding protein parvalbumin, we developed transgenic mice overexpressing parvalbumin in spinal motoneurons. ALS immunoglobulins increased intracellular calcium and spontaneous transmitter release at motoneuron terminals in control animals, but not in parvalbumin overexpressing transgenic mice. Parvalbumin transgenic mice interbred with mutant SOD1 (mSOD1) transgenic mice, an animal model of familial ALS, had significantly reduced motoneuron loss, and had delayed disease onset (17%) and prolonged survival (11%) when compared with mice with only the mSOD1 transgene. These results affirm the importance of the calcium binding protein parvalbumin in altering calcium homeostasis in motoneurons. The increased motoneuron parvalbumin can significantly attenuate the immune-mediated increases in calcium and to a lesser extent compensate for the mSOD1-mediated 'toxic-gain-of-function' in transgenic mice.

Keywords: calcium-binding proteins, motoneurons, superoxide dismutase.

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Amyotrophic lateral sclerosis (ALS) is characterized by extensive loss of lower motoneurons in the spinal cord and brain stem, atrophy of the ventral roots, and degeneration of upper cortical motoneurons and the corticospinal tract (Hirano 1991; Leigh and Swash 1991). The specific etiologies and mechanisms leading to this pathological motoneuron injury and cell loss in ALS are still being resolved. Among the various proposals, increased intracellular calcium, increased glutamate excitotoxicity, and increased free radicals have received the most attention. Such perturbations could critically impair motoneuron structures such as mitochondria and/or neurofilaments, and compromise energy production and axoplasmic flow, impairing synaptic function. These mechanisms are not mutually exclusive, and increased intracellular calcium could be a common denominator.

In neurons, intracellular calcium is tightly regulated, and marked increases are associated with cell degeneration (Choi 1992). In ALS, calcium is increased within motor nerve terminals of biopsied human muscle specimens, as well as in motor nerve terminals of mice following passive transfer of ALS immunoglobulin G (IgG) (Engelhardt *et al*. 1995; Siklós *et al*. 1996; Pullen and Humphrey 2000). Calcium homeostasis is impaired in an immune-mediated model of motoneuron cell loss in guinea pigs (Engelhardt *et al*. 1991; Alexianu *et al*. 2000). Calcium is also increased in vesicular structures within spinal motoneurons of transgenic mice expressing mutant human Cu^{2+}/Zn^{2+} superoxide dismutase (mSOD1), an enzyme involved in oxygen free radical metabolism (Deng *et al*. 1993; Rosen *et al*. 1993;

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Abbreviations used: ALS, amyotrophic lateral sclerosis; CaMII, calmodulin II; IgG, immunoglobulin G; mSOD1, mutant human Cu^{2+}/Zn^{2+} superoxide dismutase; RT-PCR, reverse transcriptase polymerase chain reaction protocol.

Gurney *et al*. 1994; Siklós *et al*. 1998). Decreased function of a glial glutamate transporter in human ALS tissue and transgenic mSOD1 mice has suggested the possibility of increased glutamate excitotoxicity, a process believed to be mediated by increased intracellular calcium (Lin *et al*. 1998; Rothstein *et al*. 1992; Ferrante *et al*. 1997). Thus, regardless what may have initiated the process, increased intracellular calcium is present in motoneurons vulnerable to degeneration.

As increased intracellular calcium may be associated with degeneration in diverse neurodegenerative disorders, a key question is why motoneurons in ALS should be selectively vulnerable to an increased calcium load. A possible explanation may be related to their low expression of the calcium-binding proteins calbindin-D_{28K} and/or parvalbumin (Celio 1990). Motoneurons relatively deficient in the calcium-binding proteins calbindin-D_{28K} and/or parvalbumin (e.g. spinal and hypoglossal motoneurons) are lost early in ALS; whereas motoneurons expressing high levels of these proteins (cranial nerves III, IV, VI, and Onuf's nucleus motoneurons) are relatively spared (Ince *et al*. 1993; Alexianu *et al*. 1994; Elliott and Snider 1995). Extraocular motoneurons from mSOD1 transgenic mice, which express abundant levels of calbindin-D_{28K} and/or parvalbumin, are less likely to degenerate as motoneuron disease develops (Siklós *et al*. 1998; Nimchinsky *et al*. 2000). Further, oculomotor neurons which possess ample parvalbumin *in vivo*, have five- to sixfold larger calcium 'buffering' capacity (Vanselow and Keller 2000) and specialized mechanisms to maintain calcium homeostasis compared with vulnerable spinal and hypoglossal motoneurons (Siklós *et al*. 1999). Thus, the inability to handle an increased Ca^{2+} load, associated with low levels of calbindin-D_{28K} and/or parvalbumin may contribute to selective vulnerability of motoneurons in ALS.

To determine whether increased expression of parvalbumin in motoneurons can modulate calcium perturbations and influence calcium-dependent processes *in vivo,* we generated transgenic mice expressing rat parvalbumin within motoneurons under control of the rat calmodulin II (CaMII) promoter. We then determined the morphological and physiological effects in motoneurons of increased expression of parvalbumin on calcium increased by the intraperitoneal injection of ALS immunoglobulins. To test the functional effects of increased parvalbumin on mutant SOD1-mediated disease, parvalbumin transgenic mice were bred with mutant SOD1 (G93A) transgenic mice; and both disease onset and survival were monitored in progeny expressing both transgenes compared with progeny expressing only the mSOD1 transgene.

Materials and methods

Generation of parvalbumin transgenic mice

Transgenic mice overexpressing parvalbumin were developed by fusing the cDNA of rat parvalbumin behind the rat CaMII promoter. Dr Anthony Means kindly provided the promoter and first exon of the rat CaMII gene. A 726-bp fragment containing CaMII promoter was cloned into pGEM3Z. Rat parvalbumin cDNA and the bovine growth hormone polyadenylation signal was subcloned downstream of the CaMII promoter. The complete expression cassette was sequenced. Linear fragments containing the 4.0 kb CaMII-parvalbumin cassette were microinjected into B6/SJL pronuclei at the NICHD Transgenic Mouse Development Facility (University of Alabama at Birmingham, AL, USA). Transgenic parvalbumin mice, housed in micro-isolator cages within a modified pathogen-free barrier facility, had access to food and water *ad libitum*.

Reverse transcriptase polymerase chain reaction protocol

Reverse transcriptase polymerase chain reaction protocol (RT-PCR) was used to determine tissue-specific mRNA expression of parvalbumin. Total RNA from spinal cord, liver, heart kidney, lung, intestine and muscle of parvalbumin transgenic and B6/SJL background mice was prepared using RNeasy kit (Qiagen Inc., Valencia, CA, USA). First-strand cDNA was synthesized from total RNA using SuperScript Preamplification system with SuperScript II RNase H-reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA). One microliter of cDNA template was amplified by PCR using the rat CaMII/parvalbumin-specific primers: 5′-GCGAGC GAGTCGAGTGGTTGTCTG-3′ and 5′-TTAGCTTTCGGCCAC CAGAGTGGAGAATTC-3′ as the forward and reverse primers, respectively. PCR protocol was as follows: 1 cycle at 94°C for 4 min, 20 cycles at 94°C for 45 s, 67°C for 60 s, 72°C for 90 s, 20 cycles at 94°C for 45 s, 57°C for 60 s, 72°C for 90 s, 1 cycle at 72°C for 10 min, then held at 2°C.

In situ hybridization

Under deep methoxyflurane anesthesia, mice were perfused with 2% paraformaldehyde containing 0.15% picric acid, their spinal cords removed, post-fixed overnight in the same fixative, placed in 30% sucrose, then cut into 40-μm sections. One hundred microliters of a 1-μg/mL solution of antisense and sense 36-mer fluorescein-labeled oligonucleotides of rat parvalbumin were hybridized to spinal cord sections of parvalbumin transgenic and background B6/SJL mice. The antisense and sense oligonucleotides were 5′-FTCTGTCATCGACATAAGCTGATCCCCGGGCTAC CG-3′ and 5′-FCGGTAGCCCGGGGATCAGCTTATGTCGAT GACAGA-3′, respectively. Following hybridization, fluorescein-labeled oligonucleotides were detected using a biotin-conjugated antifluorescein antibody (Oligo Colour Kit, Amersham, Piscataway, NJ, USA). Signal was developed with 0.05% DAB/H_2O_2 under visual control.

Western blot assay

Animals used for western blot analyses were anesthetized, and the cerebrum, cerebellum, and spinal cord were removed. The tissues were disrupted in a buffer containing 50 mM Tris–HCl (pH 6.8), 4 mM EDTA, and 5 mM benzamidine. To partially purify parvalbumin, the total protein extract was heated to 85°C for 30 min, centrifuged, and the protein concentration of the supernatant determined. Parvalbumin is heat-stable under these conditions and remains in the supernatant. Twenty micrograms of the protein extracts were electrophoresed, transferred on to nitrocellulose, and blocked for 90 min with 1% bovine serum albumin. The membrane was washed and mouse antiparvalbumin primary antibody (1 : 1000) was added

overnight at room temperature. The membrane was washed and goat anti-mouse secondary antibody conjugated with horseradish peroxidase was added (ECL kit, Amersham, Piscataway, NJ, USA). Purified rat muscle parvalbumin was used as a positive control. Band intensities were determined using Bandscan. Western analyses for doubly transgenic mice were performed essentially as described above except total spinal cord protein extract was loaded on the gel and probed. SOD1 protein was detected using a primary antibody from BioDesign (New York, NY, USA; 1 : 000) and a goat anti-mouse secondary antibody conjugated with horseradish peroxidase added (ECL kit, Amersham).

Electron microscopy

The presence of calcium (oxalate–pyroantimonate reaction) was determined using a novel ultrastructural technique with electron spectroscopic imaging (Bauer 1988). With this method, the distribution of calcium in selected ultrastructural fields of the specimen was determined by subtracting images obtained above (dE = 355 eV) and below (dE = 315 eV) the absorption edge for calcium. No significant calcium signal was detected outside of the visualized reaction product. Consequently, the precipitate adequately characterizes the ultrastructural distribution of calcium. Calcium volume fractions were determined by point counting in electron micrographs. Only terminals containing mitochondrial profiles were evaluated. Data are presented as arithmetic mean ± standard deviation and the level of significance determined by Mann–Whitney non-parametric tests. Cross-sectional and surface area fraction values were determined by standard point and intersection counting in electron micrograph and are presented as arithmetic mean ± standard deviation.

Miniature end-plate potential frequency analysis

Parvalbumin transgenic and background B6/SJL mice, 8–11 weeks old, were injected (i.p) with 40 mg total serum protein from a patient with sporadic ALS (s-ALS), as previously described (Mosier *et al*. 2000). After 24 h, mice were anesthetized and the EDL transferred to a chamber perfused at 3 mL/min with oxygenated (95% O2/5% CO_2) saline containing NaCl 145 mM, KCl 2 mM, $MgSO_4$ 1 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 10 mM, and glucose 1 mM (pH 7.2, 21–23°C). Miniature end-plate potential (MEPP) frequencies were recorded with 3 M KCl-filled pipettes (15–50 MΩ), amplified (Axoclamp-2 A, Axon), displayed on a Hitachi VC-6020 oscilloscope, digitized at 100 μs, and stored (pClamp, Axon). Stably penetrated fibers with resting membrane potentials of at least − 70 mV and adequate signal-to-noise ratios were analyzed. Amplitude data were binned at 0.1 mV.

Generation of doubly transgenic mice for parvalbumin and mSOD1

Parvalbumin overexpressing transgenic mice were interbred with transgenic mice expressing mutant human SOD1 in which glycine is substituted for alanine at position 93 (G93A). The G1H line expressing mutant human SOD1 was purchased from the Jackson Laboratories [Bar Harbor, ME, USA; strain designation TgN(SOD1-G93A)1Gur]. Double transgenic animals were identified by PCR using the above parvalbumin primers. The antisense and sense SOD1 oligonucleotides were 5′-AATTTGTGTCTACTCAGTCAA-3′ and 5′-TCACTTTGATTGTTAGTCGCG-3′, respectively.

Determination of ALS-like disease onset

Two blinded investigators examined SOD1 and SOD1/Parv transgenic mice for the onset of ALS-like signs. Four tests performed weekly in a temperature-controlled environment were used to determine the onset of tremor and neuromuscular weakness. Testing began when the animals were 7–8 weeks old. The first test measured hindlimb and trunk functions. Mice were suspended by their hindlimbs on the edge of a 50 × 300 mm cylinder and the time to pull themselves upright was measured. Slow times indicate trunk, proximal lower limb, and/or distal upper limb weakness. The second test measured forelimb strength. The animals were hung by their forelimbs on a 100 × 200 mm cylinder and the time to drop or pull themselves up was measured. This test also is influenced to a lesser extent by hindlimb weakness. The third test consisted of measuring the time for the animals to climb a 170-mm vertical Styrofoam wall. This test is sensitive to reductions in distal extremity strength. The final test was the time it took the animal to travel 60 mm on a 45° incline plane. This test measures balance and is influenced to a lesser extent by spasticity. The average of three measurements was determined for tests 1, 2, and 4. The best of three measurements was recorded for test 3. All animals received 2 weeks of training before actual data were recorded. Weekly scores were totaled for each animal and a weekly group mean ± standard deviation determined. Onset of disease was determined when there was a 2 standard deviation drop below the previous three weekly means.

Stereological analysis of motoneuron number in lumbar spinal cords

Under deep methoxyflurane anesthesia, transgenic and control mice were perfused with 2% paraformaldehyde containing 0.15% picric acid. The spinal cords were removed, post-fixed overnight in the same fixative, placed in 30% sucrose, stained with cresyl violet, then cut into 50-μm sections. The number of lumbar motoneurons, defined as anterior horn cells with diameter > 25 μm containing a distinct nucleus, were obtained using an optical fractionator (MicroBrightField, Colchester, VT, USA), providing an unbiased estimate of cell number (West *et al*. 1991, 1996; Vedel-Jensen and Gunderson 1996). In the Stereo Investigator software (MicroBrightField), the error in the estimate of total number of motoneurons is represented by the Gundersen (1987) coefficient of error (CE), which was treated as a percentage. A CE of 0.05 indicates that the population estimate is within 5% of the true population count.

Results

Characterization of transgenic mice over-expressing parvalbumin

Transgenic mice expressing rat parvalbumin were developed using the rat CaMII promoter. The rat CaMII promoter has previously been shown to promote β-galactosidase expression in embryonic and adult neurons of the mouse CNS (Matsuo *et al*. 1993). Southern analyses identified four transgenic founder mice: two male (lines 11 and 12) and two female (lines 14 and 15) (Fig. 1a). RT-PCR assays of total RNA detected rat parvalbumin expression in spinal cords of transgenic animals but not in total RNA isolated from

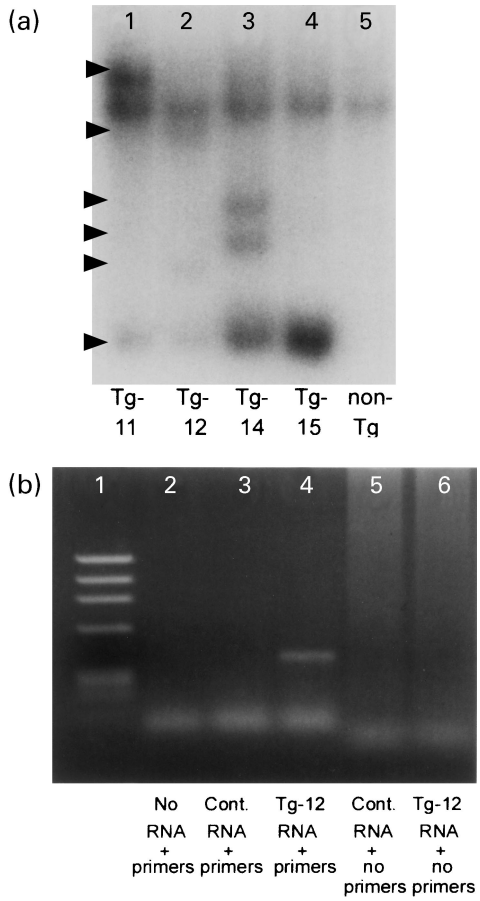

Fig. 1 (a) Southern analyses of tail DNA from control and parvalbumin transgenic (Tg) founder mice. DNA was probed with a ^{32}P-labeled 333 bp CaMII-rat parvalbumin fragment. Arrowheads indicate parvalbumin transgenic signal. (b) Rat parvalbumin transcripts were analyzed by RT-PCR using total RNA isolated from spinal cords of control and Tg-mice. Primers were designed to hybridize to the 5′-untranslated and 3′-coding regions of the CaMII-rat parvalbumin mRNA transcript.

control mice (Fig. 1b). RT-PCR analyses also detected rat parvalbumin transcripts in liver, kidney, and muscle (data not shown). These transcripts were not detected in non-transgenic mice, or in the intestine and lung of transgenic mice. Lines 12 and 14 founder mice were bred to homozygosity and used for subsequent analyses. Both homozygotic transgenic lines were determined to contain 8–10 integrated copies of the CaMII/parvalbumin construct. The transgenic mice were similar in size, weight, gross morphology, behavior, and propagated similarly to their background B6/SJL parents. None of the parvalbumin transgenic mice ($n > 500$) exhibited ALS-like signs of weakness or paralysis, or died prematurely.

Using an antisense probe to the 5′-untranslated region of the rat CaMII promoter and the parvalbumin transgene, *in situ* hybridization confirmed the presence of rat parvalbumin

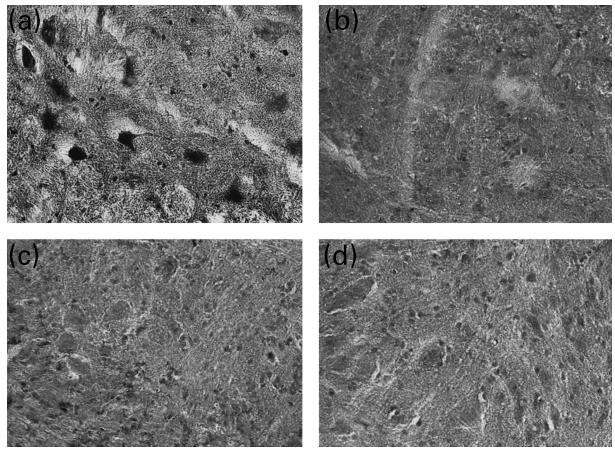

Fig. 2 Localization of rat parvalbumin mRNA in the spinal cords of control and Tg-mice by *in situ* hybridization. Parvalbumin *in situ* hybridization signal is seen lumbar spinal cord neurons of parvalbumin transgenic mice using an antisense parvalbumin probe (a). No *in situ* hybridization signal for parvalbumin mRNA was observed in lumbar spinal cord neurons of control mice using an antisense parvalbumin probe (b). Using a sense parvalbumin probe, *in situ* hybridization signal for parvalbumin mRNA was not observed in lumbar spinal cord neurons of either parvalbumin transgenic (c) or control mice (d).

mRNA in neurons of the lumbar spinal cord ventral horns in both parvalbumin transgenic lines (Figs 2a and b). Rat parvalbumin mRNA was also detected in neurons of the hypoglossal nucleus and in the ventral horn of the cervical spinal cord (data not shown). Hybridization signal was not observed in nearby glial cells and was never observed in motoneurons of the hypoglossal nucleus or spinal cord of control B6/SJL mice. Specific rat parvalbumin signal was not detected with the sense probe (Figs 2c and d).

Western analysis confirmed the overexpression of parvalbumin protein in CNS tissue of transgenic mice. Partially purified parvalbumin isolated from cerebral cortex, cerebellum, and spinal cord of parvalbumin transgenic and control mice demonstrated a single band at 12 kDa (Fig. 3). No signal was observed when the blots were probed with secondary antibody alone. Mice from transgenic line 14 expressed approximately five times the control amount of parvalbumin from spinal cord and cerebrum (Fig. 4). Mice from transgenic line 12 also overexpressed parvalbumin in the spinal cord and cerebrum compared with controls, although slightly less than mice from line 14 (Fig. 4). As parvalbumin is normally amply expressed in the cerebellum compared with the spinal cord, the increase of parvalbumin expression in cerebellum was much less apparent in both transgenic lines compared with controls.

Immunohistochemistry was used to localize parvalbumin protein to specific cells within distinct CNS regions of parvalbumin transgenic mice (Siklós *et al.* 1998). Motoneurons immunoreactive for parvalbumin were infrequent in

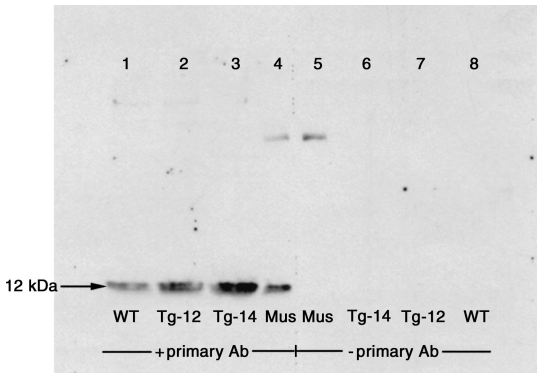

Fig. 3 Western analyses of parvalbumin protein expression in mice. Immunodetection of parvalbumin protein in spinal cord extracts of wild-type (lane 1) and parvalbumin transgenic mice (lanes 2 and 3). Parvalbumin protein expression is increased in both parvalbumin transgenic mouse lines. Lane 4 contains protein extracted from muscle as a positive control for parvalbumin. To ensure equal loading, 20 μg of sample protein, as determined by the Laemmli method, was placed into each well.

age-matched B6/SJL background mice (Fig. 5a). Parvalbumin immunoreactivity was detected in large motoneurons of the hypoglossal nucleus, and of the ventral horns in cervical and lumbar spinal cord sections of transgenic mice (Fig. 5b). These data clearly demonstrate that parvalbumin is overexpressed in neurons of transgenic mice, including motoneurons vulnerable to injury in ALS.

Parvalbumin overexpression *in vivo* attenuates intracellular calcium increases

We have previously demonstrated that i.p. injection of ALS sera or IgG increases calcium in control motor axon terminals *in vivo* (Engelhardt *et al*. 1995, 1997). To determine whether increased expression of parvalbumin reduces the ALS sera- or IgG-initiated calcium increases in spinal motoneurons, we injected the parvalbumin transgenic mice with ALS patient IgG. Intraperitoneal injection of control

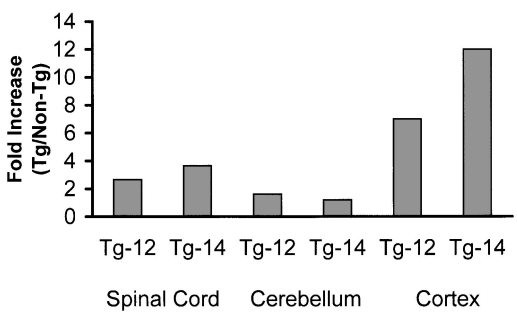

Fig. 4 Column graph displaying the fold increase of parvalbumin protein expression in parvalbumin transgenic lines compared with background control mice. Parvalbumin was detected by western analyses and band intensities were determined using Bandscan.

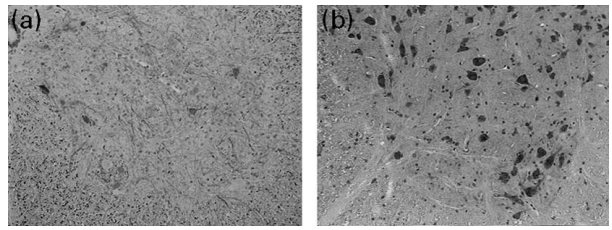

Fig. 5 Immunohistochemical detection of parvalbumin in the spinal cord. (a) Parvalbumin protein immunostaining in the lumbar spinal cords of control mice. (b) Increased parvalbumin protein immunostaining in ventral horn lumbar spinal cord neurons of parvalbumin transgenic mice.

mice with ALS IgG increased calcium precipitates in cytoplasmic and mitochondrial compartments of motor axon terminals of the interosseus muscle (16.58 ± 3.07%, expressed as volume percentage, Fig. 6a and Table 1) compared with injection of disease control IgG (2.21 ± 2.74%, $p < 0.05$, Table 1) (Siklós *et al*. 1996; Engelhardt *et al*. 1997). However, after i.p. injection of the same ALS IgG into parvalbumin transgenic mice, there was no significant difference in total calcium in cytoplasmic and mitochondrial compartments of axon terminals (2.14 ± 0.46%, Fig. 6b and Table 1) compared with values found following injection of disease control IgG (4.19 ± 1.17%, $p > 0.1$; Table 1). Thus, parvalbumin attenuates the total calcium increase within the axon terminal following an immune-mediated perturbation that normally increases motoneuron intracellular calcium *in vivo*.

We next examined the physiological effects of ALS immunoglobulins on another calcium-dependent process, the spontaneous release of acetylcholine (ACh) from motor axon terminals. We have previously demonstrated that i.p. injections of ALS sera increases mean spontaneous

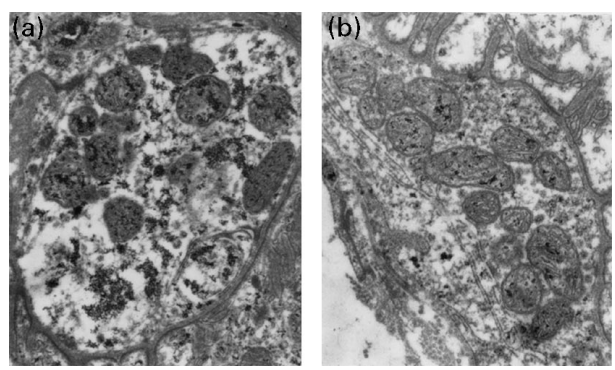

Fig. 6 Calcium precipitates in motor axon terminals of interosseus muscles. There is noticeable calcium precipitate in the axon terminal of a control mouse interosseus muscle following an intraperitoneal injection ALS serum (a). There is much less precipitate in the axon terminal of a transgenic parvalbumin mouse interosseus muscle following ALS serum treatment (b).

Table 1 Total calcium within motor axon terminals

	Disease control IgG ($n = 3$)	ALS IgG ($n = 3$)	p-value – Students t-test (two-tailed
Wild-type mice	2.21 ± 2.74*	16.58 ± 3.07	< 0.05
Parvalbumin transgenic mice	4.19 ± 1.17	2.14 ± 0.46	> 0.10

*Expressed as vol%, mean ± SD. Values are expressed as the mean volume percentages ± standard deviations.

MEPP frequency (Mosier *et al*. 2000). Therefore, we tested the effect of parvalbumin overexpression on spontaneous MEPP frequencies in transgenic lines 12 and 14 as well as background B6/SJL mice following i.p. injection of ALS serum. In control mice, ALS serum enhanced the mean frequency of MEPPs recorded from fibers of the extensor digitorum longus (EDL) compared with uninjected animals (2.04 ± 0.50, $n = 6$ experiments, and 1.02 ± 0.22, $n = 6$ experiments, respectively, $p = 0.006$, Fig. 7). However, no enhancement of EDL MEPP frequencies was observed in line 12 parvalbumin transgenic mice injected with ALS serum (1.40 ± 0.20, 43 fibers, $n = 5$ experiments) compared with uninjected animals (1.20 ± 0.14, 45 fibers, $n = 5$ experiments; $p < 0.28$). Previous studies from our lab demonstrated no MEPP frequency differences between uninjected mice and mice injected with disease control sera (Mosier *et al*. 2000). Injection of parvalbumin line 14 transgenic animals with ALS serum also produced no differences in EDL MEPP frequency (1.34 ± 0.13, 38 fibers, $n = 4$ experiments and 1.19 ± 0.19, 43 fibers, $n = 4$ experiments, respectively; $p = 0.27$). These results suggest that overexpression of parvalbumin attenuates the *in vivo* enhancement of intraterminal calcium and the calcium-mediated increase in ACh release from motor axon terminals induced by passive transfer of ALS sera.

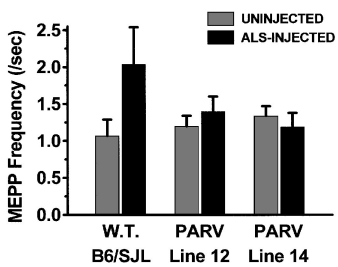

Fig. 7 Mean MEPP frequencies recorded from fibers of the EDL following ALS or control serum treatment. Each column represents the grand mean + 95% confidence interval of data from four to six experiments. MEPP frequency in EDL muscle fibers of ALS-serum injected wild-type mice is greater than in controls ($p = 0.006$). MEPP frequency of ALS-serum injected parvalbumin transgenic mice (lines 12 and 14) did not differ from controls.

Effects of parvalbumin on disease onset and survival in a mouse model of ALS

Human mSOD1, expressed in transgenic mice, leads to motoneuron degeneration associated with early increases in intracellular calcium (Siklós *et al*. 1998). In this model of ALS, we assessed whether parvalbumin overexpression in spinal motoneurons delays the clinical progression of disease. To generate transgenic mice expressing rat parvalbumin and human mSOD1, homozygotic parvalbumin transgenic mice were bred with hemizygotic mSOD1 transgenic mice possessing 25 copies of the gene encoding human mSOD1 (G93A; Gurney *et al*. 1994). Both transgenic lines were on B6/SJL genetic backgrounds. Doubly transgenic progeny, now hemizygous for parvalbumin (i.e. 4–5 copies of the transgene) and remaining hemizygous for mSOD1 (i.e. 25 copies of the mSOD1 transgene), were determined by PCR.

To avoid any potential transgene integration effects, parvalbumin lines 12 and 14 were interbred with mSOD mice, then grouped together to study any change in the onset of ALS-like signs as well as survival. Motoneurons in the spinal cord of 2–3-month-old mSOD1/Parv transgenic mice showed strong immunoreactivity for rat parvalbumin (data not shown). Parvalbumin immunostaining was minimal in hypoglossal motoneurons or in cervical and lumbar spinal motoneurons of mice bearing only the mSOD1 transgene. The presence of the parvalbumin transgene did not influence the expression of human mSOD1, as immunoreactivity for human mSOD1 protein was similar in spinal cord homogenates of mSOD1 and mSOD1/Parv transgenic mice (data not shown). Endogenous mouse SOD1 was also detected in control and parvalbumin transgenic mice (data not shown).

A detailed analysis of disease onset was performed in both mSOD1/Parv ($n = 18$) and mSOD1 ($n = 15$) transgenic mice. In mSOD1 transgenic mice, the onset of weakness occurred at age 96 ± 4 days (mean ± SEM), whereas in the mSOD1/Parv transgenic mice, onset of ALS-like signs occurred substantially later ($p < 0.02$), at age 112 ± 5 days (Fig. 8). At end-stage, paralysis was so severe and generalized that animals in both transgenic groups were euthanized. mSOD1/Parv transgenic mice reached terminal stages significantly later (146 ± 5 days, $p = 0.012$) than mSOD1 transgenic mice (132 ± 3 days) (Table 2). Survival was unchanged for mSOD1 transgenic mice bred in our

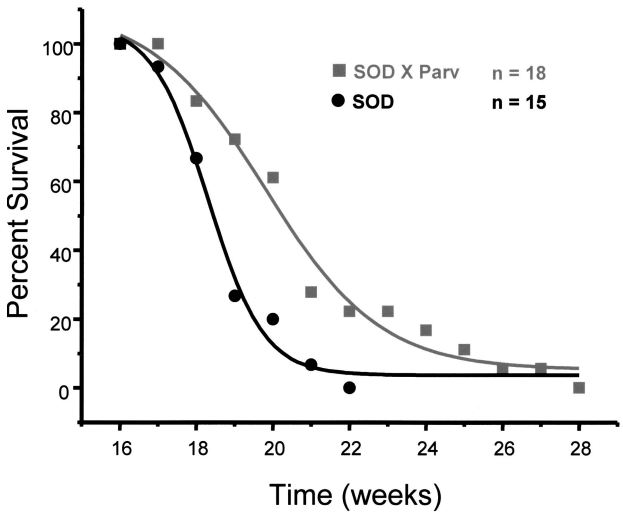

Fig. 8 Comparison of age of end-stage disease in mutant human SOD1 (G93A) transgenic mice bred with parvalbumin transgenic mice (SOD1/parv lines 12 and 14 group together, $n = 18$, ■) compared with mice transgenic for only mutant human SOD1 gene ($n = 15$, ●).

facility, compared with mice obtained directly from Jackson Laboratory (Bar Harbor, MA, USA). Duration of disease, determined by the number of days that elapsed from onset to death, did not differ between mSOD1/Parv (34 ± 7 days) and mSOD1 (35 ± 5 days, $p < 0.98$) transgenic mice. Thus, transgenic expression of parvalbumin delayed the onset of disease in human mSOD1 transgenic mice by 17% and prolonged survival by 11%.

Effects of parvalbumin on motoneuron survival in transgenic mice

To determine if parvalbumin has a neuroprotective effect, we stereologically counted motoneurons in wild-type ($n = 3$), mSOD1 ($n = 3$), and mSOD1/Parv ($n = 3$) transgenic mice that were killed between 110 and 115 days of age. At this age, mSOD1 transgenic mice showed signs of moderate hindlimb weakness. In contrast, mSOD1/Parv animals had little clinical signs of weakness. Motoneuron counting data was analyzed after the CE was < 0.05 for each group.

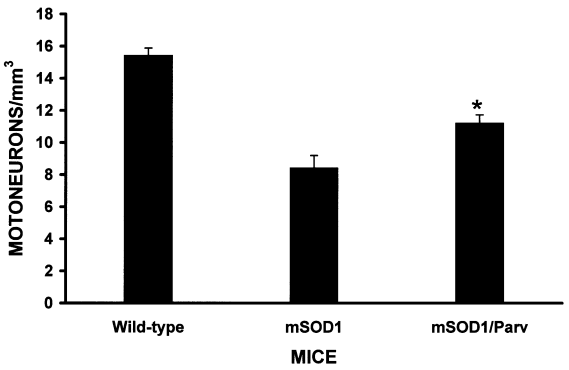

Fig. 9 Effects of parvalbumin on motoneuron survival in 110–115-day-old-mice. Although there was a loss of motoneurons in mSOD1 (8.41 ± 0.45, $n = 3$) and mSOD1/Parv (11.19 ± 0.51, $n = 3$) transgenic mice compared with wild-type (15.41 ± 0.47, $n = 3$), there was significantly more motoneurons in the mSOD1/Parv transgenic mice (33.1%) compared with mSOD1 at this age (*$p = 0.005$).

Between 110 and 115 days of age, there was a significant neuronal loss in both mSOD1 (8.41 ± 0.45) and mSOD1/Parv (11.19 ± 0.51) transgenic mice compared with wild-type mice (15.41 ± 0.47, $p = 0.0002$ and 0.002, respectively) (Fig. 9). mSOD1 transgenic animals had a 45.4% reduction in the number of motoneurons compared with controls, whereas mSOD1/Parv transgenic animals had lost only 27.4% of their motoneurons. However, when mSOD1 and mSOD1/Parv transgenic mice were compared, mSOD1/Parv transgenic mice had significantly more motoneurons (33.1%, $p = 0.005$) at 110–115 days of age than mSOD1 transgenic mice at a comparable age.

Discussion

The calcium-binding proteins calbindin-D_{28K} and parvalbumin have been demonstrated to protect motoneurons against human mSOD1-mediated and ALS IgG-induced cell death *in vitro* (Ho *et al*. 1996; Roy *et al*. 1998), but effects *in vivo* and possible mechanism(s) of protection have not been characterized. In the present study, we demonstrated that parvalbumin is transcriptionally and translationally expressed in the spinal cords of mice possessing a parvalbumin transgene coupled with a CaMII promoter by *in situ* hybridization

Table 2 Comparison of disease onset and survival between SOD1/Parv and SOD1 transgenic mice

	Human mutant SOD1 (G93A) X parvalbumin transgenic mice ($n = 18$)	Human mutant SOD1 (G93A) transgenic mice ($n = 15$)	p-value – Student's t-test (two-tailed)
Disease onset (days)	112 ± 5	96 ± 4	0.02
Survival (days)	146 ± 5	132 ± 2	0.01
Disease duration (days)	34 ± 7	35 ± 5	0.98

Values are expressed as the mean ± SEM.

and immunohistochemistry, respectively. Furthermore, parvalbumin mRNA and protein were expressed in large spinal motoneurons that are particularly vulnerable to increased calcium concentrations and degeneration in ALS. This increased expression of parvalbumin in motoneurons completely blocked the ability of ALS immunoglobulins to increase calcium within motor axon terminals, as assayed by the oxalate–pyroantimonate morphological technique. Increased expression of parvalbumin also blocked the ability of ALS immunoglobulins to increase spontaneous acetylcholine release, a calcium-mediated physiological process monitored as MEPP frequency. Thus, spinal motoneurons in parvalbumin transgenic animals are able to prevent the increases in intracellular calcium induced by ALS immunoglobulins.

ALS immunoglobulins were used to increase intracellular calcium based on our demonstration that they could increase calcium currents in a differentiated motoneuron cell line (VSC4.1) *in vitro,* as assayed by whole-cell patch-clamp techniques (Mosier *et al*. 1995) as well as by fluo-3 fluorescence imaging (Colom *et al*. 1997). Transfection of this cell line with calbindin-D_{28K} cDNA under control of a phosphoglycerate kinase promoter protected against Ca^{2+}-dependent cytotoxicity (Ho *et al*. 1996). ALS immunoglobulins can also increase motoneuron calcium in axon terminals *in vivo* as monitored with the oxalate–pyroantimonate technique (Engelhardt *et al*. 1995; Pullen and Humphreys 2000). Further, mice injected with ALS sera or immunoglobulins demonstrate increased MEPP frequency with normal amplitude, time course, and resting membrane potential, indicating an increased resting quantal release of acetylcholine from the nerve terminal (Appel *et al*. 1991). These results have been replicated in our own laboratory (Mosier *et al*. 2000) as well as two other laboratories (O'Shaughnessy *et al*. 1998; Fratantoni *et al*. 2000). The oculomotor neuron, which is relatively resistant to degeneration in ALS and has ample parvalbumin expression, is also resistant to the calcium-increasing effects of ALS immunoglobulins (Mosier *et al*. 2000).

Calbindin-D_{28K} and parvalbumin appear to exert their neuronal effects by altering intracellular calcium homeostasis, but their precise physiological functions remain to be clarified. In a transfected neuroblastoma cell line, parvalbumin overexpression suppressed large depolarization-induced increases in calcium by effectively buffering calcium in the intracellular environment (Dreessen *et al*. 1996). When transiently injected into primary rat dorsal root ganglion neurons, both calbindin-D_{28K} and parvalbumin significantly attenuate depolarization-induced peak intracellular calcium concentrations, but neither protein changed the basal calcium level, or altered the inactivation or rate of run-down of the calcium current (Chard *et al*. 1993). However, calbindin-D_{28K}, in addition to buffering calcium, can increase resting intracellular calcium levels, modulate both T- and L-type calcium currents, and influence calcium/ATPase pumps (Lledo *et al*. 1992).

In oculomotor neurons *in vivo,* endogenous calcium-binding proteins reduce the volume of local calcium elevations around open calcium channels, lower peak amplitudes of global calcium transients for a given influx, and prolong calcium recovery times for a given set of uptake and extrusion mechanisms (Vanselow and Keller 2000). These neurons, which possess ample parvalbumin, have a five- to sixfold larger calcium 'buffering' capacity (Vanselow and Keller 2000). They also possess specialized mechanisms to maintain calcium homeostasis compared with vulnerable spinal cord and hypoglossal motoneurons (Siklós *et al*. 1999).

We next examined whether increasing parvalbumin expression in spinal motoneurons could attenuate motoneuron dysfunction in mutant SOD1 transgenic mice (Gurney *et al*. 1994). We had previously demonstrated that calcium is increased in motor axon terminals of sporadic ALS patients as well as in spinal motoneurons of G93A mSOD1 mice (Siklós *et al*. 1996, 1998). In contrast, oculomotor neurons, with ample parvalbumin expression, did not have increased intracellular calcium and were relatively spared in mSOD1 mice (Siklós *et al*. 1998, 2000). Furthermore, using another mSOD1 ALS model, Nimchinsky *et al*. (2000) recently demonstrated that calcium-binding protein-containing neurons of the oculomotor nucleus are spared in mSOD1^{G86R} transgenic mice, whereas calcium-binding protein deficient neurons, i.e. those of the facial nucleus, are severely affected.

In the present studies, transgenic expression of parvalbumin delayed the onset of disease in human mSOD1 transgenic mice by 17% and prolonged survival by 11%. This delay in the onset of disease is similar to the delay in onset noted with increased motoneuron expression of Bcl-2, or following the administration of vitamin E (a free radical scavenger), creatine, or a caspase inhibitor (Gurney *et al*. 1996; Kostic *et al*. 1997; Klivenyi *et al*. 1999; Li *et al*. 2000). These data contrast with the effects of riluzole (an inhibitor of glutamate release) or a dominant-negative inhibitor of interleukin-1β-converting enzyme (ICE), both of which prolonged survival without affecting onset (Friedlander *et al*. 1997; Kostic *et al*. 1997).

We also confirmed the observation that there is a significant loss of motoneurons in the mSOD1 (G93A) at end-stage disease. At 110–115 days of age, 45.4% of motoneurons were lost in mSOD1 transgenic mice compared with their wild-type controls. In our laboratory, mSOD1 animals at this age are moderately weakened. However, mSOD1/Parv animals showed no clinical signs of weakness and had 33.1% more motoneurons ($p = 0.005$) at this age when compared with mSOD1 transgenic mice. Thus, at our current level of parvalbumin over-expression, motoneurons continue to die in mSOD1/Parv transgenic mice, but do so at

a slower rate. These data are in agreement with the observed onset/survival data.

The key finding in our study is the robust effect of parvalbumin expression within motoneurons in preventing the sustained increase in intracellular calcium induced by ALS immunoglobulins, and the more modest effects of parvalbumin expression on delaying onset of disease in mSOD1 transgenic mice. One possible explanation could be the higher PV transgene copy number in the ALS immunoglobulin experiments and the lower copy number in the mSOD1/Parv progeny. The former had 8–10 copies of the parvalbumin transgene, while the latter (hemizygous parvalbumin) mice had only 4–5 copies of the parvalbumin transgene. Studies are in progress to develop mSOD1/Parv mice with 8–10, and 16–20 copies of the PV transgene to test this hypothesis.

Alternatively, regardless of the extent of parvalbumin expression, increases in calcium in motoneurons in mSOD1 transgenic mice may be less sensitive to the level of parvalbumin expression than immune-mediated increases in calcium. In mSOD1 transgenic mice, the source of increased motoneuron calcium is not known, and increased intracellular calcium may be a later consequence of the toxic gain-of-function. Whether increased calcium is an early or a late event, the increases may have adverse effects on mitochondria, and may amplify neuronal injury. Mitochondria may act as a sink by sequestering calcium and effectively reduce cytosolic calcium concentrations (Nicholls and Ward 2000). Increased intramitochondrial calcium may in turn increase the generation of reactive oxygen species and alter the synthesis of many mitochondrial polypeptides (Borthwick *et al*. 1999).

Such mitochondrial alterations have been described as an early event in human mSOD1 transgenic models of ALS and could be the cause rather than the consequence of the increased intracellular calcium (Dal Canto and Gurney 1994; Wong *et al*. 1995; Carri *et al*. 1997; Kong and Xu 1998). At a critical mitochondrial calcium load, the mitochondria depolarize, leading to irreversible mitochondrial dysfunction and decreased ATP synthesis. By modulating calcium perturbations with calcium-binding proteins at an early stage, increased cytosolic calcium loads may be attenuated, and irreversible mitochondrial dysfunction may be delayed. Once mitochondria become sufficiently damaged and can no longer modulate intracellular calcium levels, it is unlikely that increased parvalbumin expression could significantly alter disease onset. This progression of mitochondrial injury to a stage incapable of modulating intracellular calcium may possibly explain the only modest delay in disease onset and survival. Nevertheless, the fact that increased parvalbumin expression did delay disease onset in the mSOD1 transgenic mice does suggest that, by decreasing voltage-dependent calcium channel current, enhancing Na^+/Ca^{2+} ATPase activity, and/or by increasing Ca^{2+} buffering, parvalbumin may have a protective effect on motoneuron function and subsequent injury.

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References

Alexianu M. E., Ho B.-K., Mohamed A. H., La Bella V., Smith R. G. and Appel S. H. (1994) The role of calcium-binding proteins in selective motoneuron vulnerability in amyotrophic lateral sclerosis. *Ann. Neurol.* **36**, 846–858.

Alexianu M. E., Manole E., Engelhardt J. I. and Appel S. H. (2000) Ultrastructural evidence of calcium involvement in experimental autoimmune gray matter disease. *J. Neurosci. Res.* **60**, 98–105.

Appel S. H., Engelhardt J. I., Garcia J. and Stefani E. (1991) Immunoglobulins from animal models of motor neuron disease and from human amyotrophic lateral sclerosis patients passively transfer physiological abnormalities to the neuromuscular junction. *Proc. Natl. Acad. Sci. USA* **88**, 647–651.

Bauer R. (1988) Electron spectroscopic imaging: An advanced technique for imaging and analysis in transmission electron microscopy. *Methods Microbiol.* **20**, 113–146.

Borthwick G. M., Johnson M. A., Ince P. G., Shaw P. J. and Turnbull D. M. (1999) Mitochondrial enzyme activity in amyotrophic lateral sclerosis: implications for the role of mitochondria in neuronal cell death. *Ann. Neurol.* **46**, 787–790.

Carri M. T., Ferri A., Battistoni A., Famhy L., Cabbianelli R., Poccia F. and Rotilio G. (1997) Expression of Zn,Cu superoxide dismutase typical of familial amyotrophic lateral sclerosis induces mitochondrial alteration and increase in cytosolic Ca^{2+} concentration in transfected neuroblastoma SH-SY5Y cells. *FEBS Lett.* **414**, 365–368.

Celio M. R. (1990) Calbindin D-28k and parvalbumin in the rat nervous system. *Neuroscience* **35**, 375–475.

Chard P. S., Bleakman D., Christakos S., Fullmer C. S. and Miller R. J. (1993) Calcium buffering properties of calbindin D_{28k} and parvalbumin in rat sensory neurones. *J. Physiol.* **472**, 341–357.

Choi D. W. (1992) Excitotoxic cell death. *J. Neurobiol.* **93**, 1261–1276.

Colom L. V., Alexianu M. E., Mosier D. R., Smith R. G. and Appel S. H. (1997) Amyotrophic lateral sclerosis immunoglobulins increase intracellular calcium in a motoneuron cell line. *Exp. Neurol.* **146**, 354–360.

Dal Canto M. C. and Gurney M. E. (1994) Development of central nervous system pathology in a murine transgenic model of human amyotrophic lateral sclerosis. *Am. J. Pathol.* **145**, 1271–1279.

Deng H. X., Hentati A., Tainer J. A., Iqbal Z., Cayabyab A., Hung W. Y., Getzoff E. D., Hu P., Herzfeldt B., Roos R. P. *et al.* (1993) Amyotrophic lateral sclerosis and structural defects in Cu,Zn superoxide dismutase. *Science* **261**, 1047–1051.

Dreessen J., Lutum C., Schafer B. W., Heizmann C. W. and Knopfel T. (1996) Alpha-parvalbumin reduces depolarization-induced elevations of cytosolic free calcium in human neuroblastoma cells. *Cell Calcium* **19**, 527–533.

Elliott J. L. and Snider W. D. (1995) Parvalbumin is a marker of ALS-resistant motor neurons. *Neuroreport* **6**, 449–452.

Engelhardt J. I., Appel S. H., Jakab K., Garcia J. and Stefani E. (1991) Immune-mediated models of motor neuron destruction in the guinea pig. *Adv. Neurol.* **56**, 369–379.

Engelhardt J. I., Siklós L., Komuves L., Smith R. G. and Appel S. H. (1995) Antibodies to calcium channels from ALS patients passively transferred to mice selectively increase intracellular calcium and induce ultrastructural changes in motoneurons. *Synapse* **20**, 185–199.

Engelhardt J. I., Siklós L. and Appel S. H. (1997) Altered calcium homeostasis and ultrastructure in motoneurons of mice caused by passively transferred anti-motoneuronal IgG. *J. Neuropathol. Exp. Neurol.* **56**, 21–39.

Ferrante R. J., Browne S. E., Shinobu L. A., Bowling A. C., Baik M. J., MacGarvey U., Kowall N. W., Brown R. H. Jr and Beal M. F. (1997) Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. *J. Neurochem.* **69**, 2064–2074.

Fratantoni S. A., Weisz G., Pardal A. M., Reisin R. C. and Uchitel O. D. (2000) Amyotrophic lateral sclerosis IgG-treated neuromuscular junctions develop sensitivity to 1-type calcium channel blocker. *Muscle Nerve* **23**, 543–550.

Friedlander R. M., Brown R. H., Gagliardini V., Wang J. and Yuan J. (1997) Inhibition of ICE slows ALS in mice. *Nature* **388**, 31.

Gundersen H. J. G. and Jensen E. B. (1987) The efficiency of systematic sampling in stereology and its prediction. *J. Microsc.* **147**, 229–263.

Gurney M. E., Pu H., Chiu A. Y., Dal Canto M. C., Polchow C. Y., Alexander D. D., Caliendo J., Hentati A., Kwon Y. W., Deng H. X. *et al.* (1994) Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* **264**, 1772–1775.

Gurney M. E., Cutting F. B., Zhai P., Doble A., Taylor C. P., Andrus P. K. and Hall E. D. (1996) Benefit of vitamin E, riluzole, and gabapentin in a transgenic model of familial amyotrophic lateral sclerosis. *Ann. Neurol.* **39**, 147–157.

Hirano A. (1991) Cytopathology of amyotrophic lateral sclerosis. In: *Amyotrophic Lateral Sclerosis and Other Motor Neuron Diseases*, **Vol. 56**. (Rowland, L. P., ed.), pp. 91–101. Raven Press, New York.

Ho B. K., Alexianu M. E., Colom L. V., Mohamed A. H., Serrano F. and Appel S. H. (1996) Expression of calbindin-D_{28K} in motoneuron hybrid cells after retroviral infection with calbindin-D_{28K} cDNA prevents amyotrophic lateral sclerosis IgG-mediated cytotoxicity. *Proc. Natl. Acad. Sci. USA* **93**, 6796–6801.

Ince P., Stout N., Shaw P., Slade J., Hunziker W., Heizmann C. W. and Baimbridge K. G. (1993) Parvalbumin and calbindin D_{28k} in the human motor system and in motor neuron disease. *Neuropathol. Appl. Neurobiol.* **19**, 291–299.

Klivenyi P., Ferrante R. J., Matthews R. T., Bogdanov M. B., Klein A. M., Andreassen O. A., Mueller G., Wermer M., Kaddurah-Daouk R. and Beal M. F. (1999) Neuroprotective effects of creatine in a transgenic animal model of amyotrophic lateral sclerosis. *Nature Med.* **5**, 347–350.

Kong J. and Xu Z. (1998) Massive mitochondrial degeneration in motor neurons triggers the onset of amyotrophic lateral sclerosis in mice expressing a mutant SOD1. *J. Neurosci.* **18**, 3241–3250.

Kostic V., Jackson-Lewis V., de Bilbao F., Dubois-Dauphin M. and Przedborski S. (1997) Bcl-2: prolonging life in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Science* **277**, 559–562.

Leigh P. N. and Swash M. (1991) Cytoskeletal pathology in motor neuron disease. In: *Amyotrophic Lateral Sclerosis and Other Motor Neuron Diseases*, **Vol. 56**. (Rowland, L. P., ed.), pp. 115–124. Raven Press, New York.

Li M., Ona V. O., Guegan C., Chen M., Jackson-Lewis V., Andrews L. J., Olszewski A. J., Stieg P. E., Lee J. P., Przedborski S. and Friedlander R. M. (2000) Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model. *Science* **288**, 335–339.

Lin C. L., Bristol L. A., Jin L., Dykes-Hoberg M., Crawford T., Clawson L. and Rothstein J. D. (1998) Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. *Neuron* **20**, 589–602.

Lledo P. M., Somasundaram B., Morton A. J., Emson P. C. and Mason W. T. (1992) Stable transfection of calbindin-D_{28k} into the GH3 cell line alters calcium currents and intracellular calcium homeostasis. *Neuron* **9**, 943–954.

Matsuo K., Ikeshima H., Shimoda K., Umezawa A., Hata J., Maejima K., Nojima H. and Takano T. (1993) Expression of the rat calmodulin gene II in the central nervous system: a 294-base promoter and 68-base leader segment mediates neuron-specific gene expression in transgenic mice. *Brain Res. Mol. Brain Res.* **20**, 9–20.

Mosier D. R., Baldelli P., Delbono O., Smith R. G., Alexianu M. E., Appel S. H. and Stefani E. (1995) Amyotrophic lateral sclerosis immunoglobulins increase Ca^{2+} currents in a motoneuron cell line. *Ann. Neurol.* **37**, 102–109.

Mosier D. R., Siklós L. and Appel S. H. (2000) Resistance of extraocular motoneuron terminals to effects of amyotrophic lateral sclerosis sera. *Neurology* **54**, 252–255.

Nicholls D. G. and Ward M. W. (2000) Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts. *Trends Neurosci.* **23**, 166–174.

Nimchinsky E. A., Young W. G., Yeung G., Shah R. A., Gordon J. W., Bloom F. E., Morrison J. H. and Hof P. R. (2000) Differential vulnerability of oculomotor, facial, and hypoglossal nuclei in G86R superoxide dismutase transgenic mice. *J. Comp. Neurol.* **416**, 112–125.

O'Shaughnessy T. J., Yan H., Kim J., Middlekauff E. H., Lee K. W., Phillips L. H., Kim J. and Kim Y. I. (1998) Amyotrophic lateral sclerosis: serum factors enhance spontaneous and evoked transmitter release at the neuromuscular junction. *Muscle Nerve* **21**, 81–90.

Pullen A. H. and Humphreys P. (2000) Ultrastructural analysis of spinal motoneurones from mice treated with IgG from ALS patients, healthy individuals, or disease controls. *J. Neurol. Sci.* **180**, 35–45.

Rosen D. R., Siddique T., Patterson D., Figlewicz D. A., Sapp P., Hentati A., Donaldson D., Goto J., O'Regan J. P., Deng H. X. *et al.* (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **362**, 59–62.

Rothstein J. D., Martin L. J. and Kuncl R. W. (1992) Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis. *N. Engl. J. Med.* **326**, 1464–1468.

Roy J., Minotti S., Dong L., Figlewicz D. A. and Durham H. D. (1998) Glutamate potentiates the toxicity of mutant Cu/Zn-superoxide dismutase in motor neurons by post-synaptic calcium-dependent mechanisms. *J. Neurosci.* **18**, 9673–9684.

Siklós L., Engelhardt J. I., Harati Y., Smith R. G., Joo F. and Appel S. H. (1996) Ultrastructural evidence for altered calcium in motor nerve erminals in amyotrophic lateral sclerosis. *Ann. Neurol.* **39**, 203–216.

Siklós L., Engelhardt J. I., Alexianu M. E., Gurney M. E., Siddique T. and Appel S. H. (1998) Intracellular calcium parallels motoneuron degeneration in SOD-1 mutant mice. *J. Neuropathol. Exp. Neurol.* **57**, 571–587.

Siklós L., Engelhardt J. I., Adalbert R. and Appel S. H. (1999) Calcium-containing endosomes at oculomotor terminals in animal models of ALS. *Neuroreport* **10**, 2539–2545.

Siklós L., Engelhardt J. I., Reaume A. G., Scott R. W., Adalbert R., Obal I. and Appel S. H. (2000) Altered calcium homeostasis in spinal motoneurons but not in oculomotor neurons of SOD-1 knockout mice. *Acta Neuropathol.* **99**, 517–524.

Vanselow B. K. and Keller B. U. (2000) Calcium dynamics and buffering in oculomotor neurones from mouse that are particularly resistant during amyotrophic lateral sclerosis (ALS)-related motoneurone disease. *J. Physiol.* **525**, 433–445.

Vedel-Jensen E. B. and Gundersen H. J. G. (1996) The rotator. *J. Microsc.* **170**, 35–44.

West M. J., Slomianka L. and Gunderesen H. J. (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat. Rec.* **231**, 482–497.

West M. J., Ostergaard K., Andreassen O. A. and Finsen B. (1996) Estimation of the number of somatostatin neurons in the striatum: an in situ hybridization study using the optical fractionator method. *J. Comp. Neurol.* **370**, 11–22.

Wong P. C., Pardo C. A., Borchelt D. R., Lee M. K., Copeland N. G., Jenkins N. A., Sisodia S. S., Cleveland D. W. and Price D. L. (1995) An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron* **14**, 1105–1116.